Age-Dependent Spontaneous Coronary Arterial Thrombosis in Transgenic Mice That Express a Stable Form of Human Plasminogen Activator Inhibitor-1

Mesut Eren, PhD; Corrie A. Painter, BS; James B. Atkinson, MD, PhD; Paul J. Declerck, PhD; Douglas E. Vaughan, MD

Background—Plasminogen activator inhibitor-1 (PAI-1) regulates fibrinolysis and has been reported to be an independent risk factor for ischemic cardiovascular events. This study describes the age-dependent development of spontaneous coronary arterial thrombi that are associated with evidence of subendocardial myocardial infarction in mice transgenic for human PAI-1.

Methods and Results—We generated two independent transgenic mice founder lines that express a stable variant of active human PAI-1 under control of the murine preproendothelin-1 (mPPET-1) promoter. Backcrossed homozygous transgenic animals from founder line I had plasma PAI-1 levels of 23 ± 12 ng/mL. PAI-1 transgenic animals younger than 4 months do not exhibit any evidence of arterial or venous thrombosis. Ninety percent of transgenic animals (n = 10) older than 6 months developed spontaneous occlusions of typically multiple, penetrating coronary arteries, with histological evidence of subendocardial infarction identified in 50% of animals.

Conclusions—This study shows that chronically elevated levels of PAI-1 are associated with age-dependent coronary arterial thrombosis in mice transgenic for human PAI-1. This is the first study of a murine model of coronary thrombosis that occurs in the absence of severe hypercholesterolemia or multiple genetic manipulations. These findings provide new evidence to support the hypothesis that PAI-1 excess contributes to the development of coronary arterial thrombosis. (Circulation. 2002;106:491-496.)

Key Words: thrombus ■ coronary disease ■ myocardial infarction ■ plasminogen activators ■ fibrinolysis

The fibrinolytic system is an important endogenous defense mechanism against intravascular thrombosis. Vascular fibrinolytic activity reflects the balance between plasminogen activators (primarily tissue-type plasminogen activator [t-PA]) and plasminogen activator inhibitors (predominantly plasminogen activator inhibitor-1 [PAI-1]). Both of these essential fibrinolytic components are synthesized in the vascular wall, have short half-lives, and circulate in low concentrations in plasma. It has been suggested that t-PA and urokinase-type plasminogen activator (u-PA), in conjunction with thrombomodulin (TM), are critical for prevention of thrombosis in the coronary circulation. If this hypothesis is correct, then an excess of PAI-1 would be expected to increase the risk of coronary thrombosis. There is substantial epidemiological evidence that PAI-1 excess may contribute to the development of ischemic cardiovascular disease. PAI-1 excess has been identified in youthful survivors of acute myocardial infarction (MI), and plasma PAI-1 activity is increased in MI survivors that later develop recurrent MI. A recent study identified plasma PAI-1 levels as an independent determinant of risk factor for MI in middle-aged men and women.

Newly synthesized PAI-1 is released in an active conformation with a circulating half-life of 5 minutes in plasma. The structural characteristics of PAI-1 endow the protein with a strong tendency to assume a noninhibitory and thermodynamically stable latent conformation. The active conformation of human PAI-1 can be stabilized by substitution of specific amino acids, prolonging its functional half-life to >145 hours at 37°C in vitro. In this study, we investigated the cardiovascular effects of long-term elevations in PAI-1 levels in transgenic mice that overexpress this stable variant of human PAI-1.

Generation of Transgenic Mice

The coding-domain sequence for the stable variant of human PAI-1 was amplified by polymerase chain reaction (PCR) from the plasmid pMaPAI-1.stab using the high-fidelity Bio-X-Act DNA polymerase enzyme (Bioline) and was subcloned into a plasmid containing a 5.9-kb fragment of the murine preproendothelin promoter (mPPET-1).
Figure 1. Generation of PAI-1 transgenic mice. A, Human PAI-1 transgene construct. The 8.4-kb Xho I-Not I fragment containing 5.9-kb promoter sequences of mPPET-1 gene, PAI-1-stab gene, SV40 polyadenylation signal, and first exon and intron of mPPET-1 gene was injected into the one-cell embryos. B, Identification of transgenic founder lines. Screening genomic DNA (10 μg) from tail biopsies by Southern blot hybridization using a 32P-labeled DNA probe that detects 1552-bp Eco RI-Cla I fragment containing PAI-1 transgene and SV40 Poly A sequence. Lane 1, genomic DNA from a wild-type mouse as negative control; 2, p5.9-PAI-1-stab plasmid as positive control; 3 through 16, genomic DNA from pups generated by microinjection of transgene construct into one-cell embryos. Lanes 7 and 16 show the transgenic founders line II and line I, respectively. As determined by PhosphorImager quantitation, line I transgenic animals have twice the copy number of the transgene as line II animals do.

1). Subsequently, the PAI-1 signal peptide coding sequence from pUC18-PAI-1-wt plasmid and SV40 polyadenylation signal from pGL3-Basic (Promega) were subcloned into 5′- and 3′-ends of PAI-1 cDNA, respectively, generating the transgene construct in Figure 1A, and this plasmid was designated as p5.9-PAI-1-stab (11.6 kb). DNA sequencing in both directions confirmed the orientation and sequences of cloned inserts in this plasmid. The 8.4-kb transgenic construct containing the 5.9-kb 5′ flanking promoter region of mPPET-1 gene, stable PAI-1 gene, SV40 Poly A signal, and first exon and first intron of mPPET-1 was excised from p5.9-PAI-1 with Xho I and Not I enzymes and then gel-purified from low melting agarose by extraction of DNA over a spin column (QIAGEN). The linearized transgenic construct was injected into one-cell embryos retrieved from B6/D2 F1 hybrids. A 32P-labeled DNA probe made to the SV40 Poly A signal by RediPrime labeling kit (Amersham) was used for screening the Eco RI and Cla I-digested genomic DNA from tail biopsies by Southern blot hybridization to identify transgenic founder lines. The intensity of the transgene signal obtained by Southern blot hybridization was determined by PhosphorImager. After backcrossing, DNA from mice that produced transgene signals were used for screening the Eco RI and Cla I-digested genomic DNA from tail biopsies by Southern blot hybridization to identify transgenic founder lines. The intensity of the transgene signal obtained by Southern blot hybridization was determined by PhosphorImager.

**Histopathological and Molecular Analysis**

Organs from age- and sex-matched transgenic and wild-type animals that were perfused with 20 mL of 1× PBS were harvested and then embedded in paraffin. Tissues were sectioned at 5 μm and stained for fibrosis using Masson’s trichrome. Photomicrographs were captured on an Olympus Provis microscope equipped with an Optronics digital camera. To assess the level of expression of human PAI-1 in the transgenic mice, PAI-1 antigen levels were determined in various tissues frozen in liquid nitrogen within 3 minutes of collection and stored at −80°C. After thawing, tissues were homogenized with a polytron in TGH buffer (20 mmol/L HEPES, pH 7.4, 50 mmol/L NaCl, 10% glycerol, and 1% Triton X-100) containing a cocktail of protease inhibitors (Roche) on ice (3 pulses of 20 seconds each with 2-minute intervals of incubation on ice). The tissue to TGH buffer ratio was 0.1 g to 1.0 mL buffer. Proteins in the homogenized samples were extracted additionally by mixing on a tilt board for 10 minutes at 4°C. These samples were spun at 10 000g for 10 minutes at 4°C, and the supernatants were frozen and stored at −80°C. PAI-1 antigen levels in tissue samples were determined as described above. Tissue-specific PAI-1 expression was determined by reverse transcriptase (RT)-PCR on total RNA samples prepared from tissues homogenized in RNAzol (0.1 g tissue/mL RNAzol) with a polytron. The RNA from aqueous phase was precipitated with equal volume of isopropanol and washed with 70% ethanol and resuspended in DEPC-treated water. Total RNA 1 μg was added into the Access RT-PCR (Promega) mix to detect the transcription of human PAI-1 transgene. The primers used to amplify the 262-bp SV40 Poly A signal were CTAGAGTCGGGGCGGC for the 5′ end and CTTATCGATTTTACCACATTTGTAGAGG for the 3′ end of PAI-1, and this plasmid was designated as p5.9-PAI-1-stab (11.6 kb). DNA sequencing in both directions confirmed the orientation and sequence of cloned inserts in this plasmid. The linearized transgenic construct was injected into one-cell embryos retrieved from B6/D2 F1 hybrids. A 32P-labeled DNA probe made to the SV40 Poly A signal by RediPrime labeling kit (Amersham) was used for screening the Eco RI and Cla I-digested genomic DNA from tail biopsies by Southern blot hybridization to identify transgenic founder lines. The intensity of the transgene signal obtained by Southern blot hybridization was determined by PhosphorImager. After backcrossing, DNA from mice that produced transgene signals were used for screening the Eco RI and Cla I-digested genomic DNA from tail biopsies by Southern blot hybridization to identify transgenic founder lines. The intensity of the transgene signal obtained by Southern blot hybridization was determined by PhosphorImager.

**Immunohistochemical Detection of PAI-1**

Tissue-specific expression and cellular localization of PAI-1 in cardiovascular tissue was additionally assessed by immunohistochemical staining for human PAI-1 in 5-micron paraffin-embedded tissue sections. Rabbit anti-human PAI-1 (Molecular Innovations, Southfield, Mich; dilution 1:400) and rabbit anti-rat PAI-1 (American Diagnostica, Greenwich, Conn; dilution 1:500) antibodies were used for detection of stable PAI-1 antigen. Antigen retrieval was done with Retrievit (pH 8.0) (Inno Genex, Inc) by microwaving the slides 4 times for 5 minutes each. After quenching the endogenous peroxidase activity in 3% H2O2/methanol solution, the sections were blocked with 10% Powerblock solution (Bio Genex, Inc), which was diluted in 1× PBS containing 0.1% BSA and 0.4% Triton X-100 for 30 minutes. Both primary antibodies were diluted in 10% Power-
block solution, added on the sections, and incubated at 4°C for overnight in a humid chamber. The secondary antibody (biotinylated goat anti-rabbit IgG from Bio Genex, Inc) was incubated with the tissue sections for 20 minutes in the humid chamber at the room temperature. The streptavidin-HRP conjugate (Inno Genex) and the chromogenic substrate 3-aminoethyl carbazole were used for visualization of immunoreactivity. The sections were counter-stained with hematoxylin to see the cellular architecture.

Statistics
Data are reported as mean values±SD. Comparisons of PAI-1 antigen, t-PA, and APC activities between wild-type and transgenic animals were performed using Student’s t-test (2-tailed) for unpaired data.

Results
Generation and Characterization of PAI-1 Transgenic Mice
We designed and constructed a PAI-1 transgene using the cDNA for a stable form of active human PAI-1 and mPPET-1 promoter9 to target transgene expression to the vascular wall (Figure 1A). Two independent transgenic founder lines were identified by Southern blot hybridization (Figure 1B). Screening of genomic DNA indicated that transgene copy number in line I was approximately twice that of line II and directly proportional to the plasma PAI-1 levels. Backcrossed homozygous transgenic animals from line I have plasma levels of human PAI-1 of 23±12 ng/mL (Figure 2A), which corresponds to the average physiological PAI-1 concentration in adult humans.10,11 Plasma PAI-1 levels did not vary significantly with age when homozygous transgenic animals were tested at 2, 4, 6, 8, 11, and 19 months. Homozygous transgenic animals displayed a 3-fold reduction in t-PA activity from 2.36±0.46 in wild-type animals to 0.78±0.49 IU/mL (P<0.01). Transgenic animals also exhibited dose-dependent reduction in plasma levels of APC by 68% (P<0.002) in homozygous and 55% (P<0.02) in hemizygous animals compared with wild-type (Figure 2B). Transgenic (hemizygous and homozygous) animals had normal systolic and diastolic blood pressures as well as normal life span and fertility.

The distribution of human PAI-1 was measured in tissue extracts using a specific ELISA for human PAI-1, which detected PAI-1 antigen in lung, trachea, heart, aorta, pancreas, kidney, brain, and skin (Figure 3A) with no significant variation in levels in 4- and 8-month-old transgenic animals. In parallel with these studies, tissue-specific expression of human PAI-1 in transgenic mice was detected by RT-PCR amplification of SV40 polyadenylation signal of stable PAI-1 transcript. Human PAI-1 expression was detected in heart, aorta, trachea, skin, brain, and lung and in low levels in kidney and liver (Figure 3B). Immunohistochemical staining of cardiac tissue from transgenic mice with anti–PAI-1 antibodies localized the PAI-1 expression predominantly to the endothelial cells of coronary arteries (Figures 4A and 4C), with lower levels of expression in veins and capillaries.

Tissue sections from brain, liver, lung, heart, and kidney were systematically examined for the presence of venous or arterial thrombosis. Homozygous and homozygous transgenic animals younger than 4 months did not exhibit any evidence of thrombi in any of these vascular beds. We occasionally observed small coronary arterial thrombi in 4-month-old homozygous transgenic animals. However, 90% (N=10) of line I homozygous animals older than 6 months exhibited spontaneous thrombotic occlusions of multiple medium to large size penetrating coronary arteries (Figures 5A through 5G). In these animals, 6.3±2.3% of the coronary arteries examined showed evidence of thrombosis that was randomly distributed in the coronary tree. In contrast, we have carefully examined multiple cardiac sections from 30 age-matched wild-type mice and have never identified a single coronary thrombus (P<0.0001, χ² analysis). In addition to coronary arterial thrombi, homozygous transgenic mice exhibited other cardiac pathology, including perivascular fibrosis (Figures 5B through 5E), intimal thickening of coronary vessels (Figures 5B, 5C, and 5E), and fibrosis of the mitral valve annulus. Histologic evidence of MI was observed in 5 of 10 (50%) transgenic animals, characterized by focal subendocardial fibrosis, myocyte dropout, and hemosiderin deposition (Figures 6B through 6D).

Discussion
In this study, we describe the development of coronary arterial thrombi in transgenic mice that overexpress a stable form of human PAI-1. Two other groups have previously
reported PAI-1 transgenic mice. Erickson et al.\textsuperscript{12} first described transgenic animals overexpressing the native form of human PAI-1 under the control of the metallothionein promoter. The phenotype of these transgenics included partial hair loss, subcutaneous hemorrhage, and venous occlusions in tail and hind limbs. The thrombi detected in these animals were atypical, consisting primarily of mononuclear leukocytes with lesser amounts of platelets, red blood cells, and fibrin. Furthermore, the phenotypic alterations reported by Erickson et al.\textsuperscript{12} were transient, reflecting a decline in the activity of metallothionein promoter within 4 to 28 days after birth. Subsequently, Eitzman et al.\textsuperscript{13} described a different transgenic line that overexpressed murine PAI-1 under the control of CMV promoter. Although this line of transgenic mice shows an increased tendency toward fibrosis in lung after chemical injury, no information is available regarding coronary or age-dependent thrombosis.

Based on these prior reports, we did not anticipate that transgenic overexpression of PAI-1 would result in a thrombotic phenotype. However, expression of active human PAI-1 in vascular endothelial cells of transgenic mice seems to impair the antithrombotic defense mechanisms and results in development of spontaneous coronary arterial thrombi. The localization and composition of these coronary arterial thrombi are distinctly different from the cellular aggregates described by Erickson et al.\textsuperscript{12} Furthermore, coronary thrombosis has not been reported in plasminogen-deficient\textsuperscript{11} or combined t-PA/u-PA–deficient\textsuperscript{14} mice, whereas both strains exhibit impaired wound healing, an increased tendency to venous thrombosis, growth retardation, and shortened life span. There may be multiple explanations for the thrombotic phenotype seen in our PAI-1 transgenic mice. First, the development of coronary arterial thrombosis in PAI-1 transgenic mice may be explained by the fact that PAI-1 also inhibits APC.\textsuperscript{15} APC is activated by thrombin when bound to TM and specifically inhibits coagulation factors V and VIII. Although APC deficiency is associated with venous, not arterial, thrombosis, mice with a combined deficiency of t-PA and TM exhibit microvascular thrombosis and cardiac fibrin deposition.\textsuperscript{16} In the present study, we found that transgenic overexpression of stable human PAI-1 is associated with dose-dependent reductions in APC activity and t-PA activity in plasma (Figure 2B). Therefore, we hypothesize that occlusion of medium to large coronary arteries in PAI-1 transgenic animals likely reflects simultaneous inhibition of PAs and APC, thus impairing all 3 of the suggested critical antithrombotic mechanisms in the mammalian coronary circulation.\textsuperscript{2} Second, the development of arterial thrombi in PAI-1 transgenic mice may reflect the biology of the promoter used to drive PAI-1 expression in this study. Harats et al.\textsuperscript{9} have previously shown that the identical mPPET-1 promoter selectively promotes transgene expression in the aorta and reported PAI-1 transgenic mice, whereas no antigen was detected in arteries or veins from nontransgenic animal.
endothelial cells of both large and small arteries, with low levels of expression in veins and capillaries. Based on these properties, it has been suggested that this promoter is a reasonable choice for studying the biological significance of overexpression of specific molecules in the vascular wall. As expected, we detected relatively intense immunohistochemical localization of PAI-1 in the vessel walls of coronary arteries (Figures 4A and 4C) from transgenic mice, confirming the expression pattern of mPPET-1 promoter reported by Harats et al.9 However, it is not certain that the mPPET-1 promoter generates increased plasma levels of PAI-1 in the coronary circulation compared with other vascular beds. We have not observed any important differences between arterial and venous PAI-1 concentrations in the transgenic mice described here. Given the small blood volume of mice and their hyperdynamic cardiovascular system, we would not predict the presence of major arterial-venous concentration gradient. Third, there is clearly a temporal aspect to the coronary thrombosis seen in PAI-1 transgenic mice. However, we did not observe significant changes in PAI-1 levels in plasma and other tissues from the transgenic animals over time. This may reflect the requirement of an additional, unidentified factor or factors that contribute to coronary thrombosis in PAI-1 transgenic animals. This may include time-dependent impairment in endothelial function, which has been reported to deteriorate with aging in humans and in rodents.17–19 Finally, it is possible that PAI-1 inhibits other proteases that play a role in antithrombotic defense mechanisms. However, because the reactive site of the (Arg-Met) stable human PAI-1 used in this study is identical with the reactive site of wild-type PAI-1, it seems less likely that the thrombotic phenotype described in this study is a consequence of inhibition of other proteases by PAI-1.

This is the first report of a mouse model with a single gene modification (either deletion or transgenic) that yields spontaneous coronary arterial thrombi and MI in an age-dependent manner in the absence of hypertension or hyperlipidemia. This novel finding provides new experimental evidence to support the hypothesis that PAI-1 excess contributes to the pathogenesis of ischemic cardiovascular events. Indeed, these transgenic mice provide a proof of principle that PAI-1 excess may be sufficient to promote coronary arterial throm-

Figure 5. Perivascular fibrosis and coronary arterial thrombi detected in PAI-1 transgenic mice. A through C, Examples of thrombi detected in 3 mice. D through G, Serial heart sections to detect the extent of coronary thrombi in a line 1/1 mouse. As schematically illustrated below the photomicrographs, sections at 0 (starting point), 30, 90, and 180 μm follow the thrombus in this large-sized coronary artery that bifurcates at the 90-μm-section into 2 branches that are also thrombosed. Additional sectioning showed that this coronary artery was occluded for >500 μm in length. Thrombi detected in these transgenic animals are typically acellular and predominantly fibrin. The insert shows 200 μm at ×40 magnification.

Figure 6. A through D, Ischemic subendocardial fibrosis and vascular pathology in PAI-1 transgenic mice. As a result of the spontaneously occluded coronary arteries, 50% of transgenic animals analyzed were found to display evidence of focal subendocardial myocardial infarction characterized by subendocardial fibrosis, myocyte dropout, and hemosiderin deposition. Wild-type heart tissue (A) and homozygous PAI-1 transgenic mice (B through D).
bosis. Although the transgenic animals described in this study may define a worse case scenario reflecting the combined effects of the choice of promoter and the functional stability of PAI-1 used in these experiments, these animals provide a convenient platform for testing the efficacy of synthetic inhibitors of human PAI-1 in vivo. In light of results presented in this study, the selective inhibition of PAI-1 for the prevention of coronary thrombosis and MI in humans deserves additional investigation.

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