Antiangiogenic Activity of rPAI-123 Promotes Vasa Vasorum Regression in Hypercholesterolemic Mice Through a Plasmin-Dependent Mechanism

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Rationale: The antiangiogenic activity of rPAI-123, a truncated plasminogen activator inhibitor-1 (PAI-1) protein, induces vasa vasorum collapse and significantly reduces plaque area and plaque cholesterol in hypercholesterolemic low-density lipoprotein receptor–deficient/apolipoprotein B48–deficient mice.

Objective: The objective of this study was to examine rPAI-123–stimulated mechanisms that cause vasa vasorum collapse.

Methods and Results: The rPAI-123 protein opposed PAI-1 antiproteolytic function by stimulating a 1.6-fold increase in plasmin activity compared with the saline-treated counterpart. The increased proteolytic activity corresponded to increased activity of matrix metalloproteinase-3 and degradation of fibrin(ogen), nidogen, and perlecan in the adventitia of descending aortas. PAI-1 activity was reduced by 48% in response to rPAI-123; however, PAI-1 protein expression levels were similar in the rPAI-123– and saline-treated hypercholesterolemic mice. Coimmunoprecipitation assays demonstrated a novel PAI-1–plasminogen complex in protein from the descending aorta of rPAI-123– and saline-treated mice, but complexed PAI-1 was 1.6-fold greater in rPAI-123–treated mice. Biochemical analyses demonstrated that rPAI-123 and PAI-1 binding interactions with plasminogen increased plasmin activity and reduced PAI-1 antiproteolytic activity.

Conclusions: We conclude that rPAI-123 causes regression or collapse of adventitial vasa vasorum in hypercholesterolemic mice through a novel mechanism by which rPAI-123 and PAI-1 bound plasminogen in a cooperative manner to increase plasmin activity and reduce PAI-1 activity. (Circ Res. 2011;108:00-00.)

Key Words: angiogenesis • atherosclerosis • vasa vasorum • plasminogen activator inhibitor-1 • proteolysis
Plasmin also contributes to ECM remodeling by degrading fibrin,15,20,21 an ECM protein that forms a supportive scaffold for angiogenic vessels,22,23. Fibrin, the major constituent of provisional matrix,24 enables ECs to adhere, spread, and proliferate.25,26 Fibrin or accumulated fibrinogen can be broken down by plasmin to negatively regulate angiogenesis.27 Plasmin also degrades nidogen28 and perlecan,29 2 of the 4 key components of the ECM/BM that are important in blood vessel formation (see Rhodes and Simons for review12).

Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of plasmin production. It functions in this capacity by exposing a reactive center loop that binds tissue-type plasminogen activator (tPA) or urokinase plasminogen activator (uPA). This interaction prevents tPA and uPA from converting plasminogen to plasmin (see Dellas and Loskutov for review30). The binding reaction is detected with a horseradish peroxidase– linked anti-human PAI-1 antibody (Dako, Carpinteria, CA). Confocal images at 63× magnification were acquired.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Mouse Strain, Diet, and Treatment
Female LDLR−/−/ApobE−/− mice (B6; 129S-Apobtm1Hsd) mouse strain [double-knockout, or DKO]; The Jackson Laboratory, Bar Harbor, ME; stock No. 003000) were fed either a Paigen diet without cholate (PD) or a normal chow diet (CH) for 20 weeks and received either rPAI-123 (5.4 μg·kg−1·d−1) or saline treatment for the last 6 weeks of the diet, as described previously1 (Online Data Supplement).

Zymographic Detection of Proteolytic Activity in Mouse Plasma
Blood from fasted DKO mice treated with either rPAI-123 or saline, as described previously,1 was collected into either sodium citrate or lithium heparin tubes (Thermo Scientific, Waltham, MA). Plasma was separated by centrifugation. Equivalent amounts of plasma protein were resolved on a casein zymogram as described previously33,34 or on a polyacrylamide gel that contained gelatin 1 mg/mL. Detection of proteolytic and gelatinolytic proteins were as described previously for casein zymograms.33,34

Plasmin Activity Measurement
Plasmin activity, in 168 μg of plasma protein (sodium citrated collected), was measured in a fluorometric mouse plasmin activity assay kit (Molecular Innovations, Novi, MI) and in a chromzym PL assay (Roche, Basel, Switzerland) with and without active α1-antiplasmin (3.2 μg/well; Abcam, Cambridge, United Kingdom). The assay was performed according to the manufacturer’s instructions (Online Data Supplement).

Measurement of MMP Activity
MMP-3 activity in 600 μg of mouse plasma protein collected into lithium heparin tubes was measured with and without 3.2 μmol/L N-[[(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-yl)carbonyl]l-phenylalanine, an MMP-3–specific inhibitor (EMD Biosciences, Gibbstown, NJ), in a fluorometric MMP-3 activity assay kit according to the manufacturer’s instructions (ENZO Life Sciences; Online Data Supplement, Method 4).

Western Blot Analysis of Fibrinogen
Equivalent amounts of either adventitial or plasma protein from saline- and rPAI-123–treated mice were gel resolved and transferred to nitrocellulose. The membrane was probed for fibrinogen (Dako, Carpinteria, CA) on a Western blot (Online Data Supplement).

D-Dimer Assay
Plasma protein (sodium citrated collected) was analyzed for fibrinogen breakdown products in a quantitative D-dimer ELISA kit, according to the manufacturer’s instructions (Diagnostica Stago, Asnieres, France; n=5 per group).

Immunohistochemical Analysis of Fibrinogen
Ten-micrometer frozen sections of the DA were probed for fibrinogen (Dako) with a rabbit antihuman polyclonal primary antibody (Dako). The binding reaction was detected with a horseradish peroxidase– conjugated secondary antibody (GE Healthcare, Piscataway, NJ) as described previously1 (Online Data Supplement).

Confocal Imaging of Nidogen-Probed BM
Mice were perfused with fluorescein-labeled Lycopersicon esculentum lectin (FITC-lectin). The DA adventitia was probed for nidogen (Millipore, Billerica, MA). DA whole mounts were examined by confocal microscopy at 20× and 63× magnification as described previously1 (Online Data Supplement).

Detection of Perlecan in the Adventitia of DAs
Sequential 10-μm frozen sections of DA from rPAI-123– or saline-treated PD-fed DKO mice were incubated with perlecan (139 nmol/mL; Molecular Innovations), APMA-activated MMP-3 (185 nmol/mL; EMD Biosciences), or plasmin plus MMP-3 in a 15-hour, 37°C incubation. Next, the sections were incubated overnight at 4°C with an antibody specific for perlecan (Santa Cruz Biotechnology, Santa Cruz, CA). Confocal images at 63× magnification were acquired.

Quantitative Analysis of Plasminogen Activator Activities and Expression Levels
tPA and uPA activities in 160 μg of plasma protein collected in sodium citrate tubes were measured in chromozym activity assays (Roche). Protein expression levels were determined by ELISA in 600 μg of plasma protein (American Diagnostica, Stamford, CT). The experiments were performed in triplicate wells per sample (n=8 per test group) according to the manufacturer’s instructions.
PAI-1 Activity Measurements
PAI-1 activity was measured in plasma protein (600 μg) by 2 methods. One method measured activity by ELISA (AssayPro, St Charles, MO) and the other in a chromozym assay (American Diagnostica). The experiment was performed on plasma collected into sodium citrate tubes from 5 mice per treatment group and analyzed according to the manufacturer’s protocol. Each assay was repeated twice in triplicate wells per sample (n=5 per group).

Measurement of PAI-1 Protein Expression
PAI-1 protein in 600 μg of mouse plasma (sodium citrate collected) was measured in a mouse PAI-1 total antigen kit (Molecular Innovations) according to the manufacturer’s instructions. The experiment was performed twice in triplicate wells per sample (n=8 per test group). Control wells for each sample included all assay components except the detection substrate.

Measurement of Plasminogen Protein Expression
Plasma protein (600 μg; sodium citrated collected) from PD DKO mice was assayed for plasminogen expression levels with a mouse plasminogen ELISA kit (Molecular Innovations). The experiment was performed according to the manufacturer’s instructions. Each sample was tested in triplicate (n=8 per group).

Detection of PAI-1 in DA Protein
The thoracic DA was removed from PD-fed mice treated with rPAI-123 or saline. Equivalent amounts of protein from 5 mice per group were probed for PAI-1 on a Western blot with a monoclonal antibody to latent and active forms of PAI-1 (EMD Biosciences).

Coimmunoprecipitation of rPAI-123 and PAI-1 With Plasminogen
PAI-1 in pooled thoracic DA protein (320 μg) from 5 PD-fed mice per treatment group was immunoprecipitated with a monoclonal antibody to active and latent forms of PAI-1 (EMD Biosciences). The antibody–PAI-1 complexes were isolated with protein G–coupled magnetic beads.32,35 Isolated protein complexes were probed with an antibody specific for the plasminogen kringles 1 to 3 domain (EMD Biosciences) on Western blots. The binding reaction was amplified and detected as described previously.36 The membrane was cut above 110 kDa and probed with a secondary antibody to PAI-1 to determine lane loading.

Binding Affinity Assays
An antibody specific for plasminogen kringles 5 and the serine protease domain (American Diagnostica) was bound to protein G–coupled magnetic beads. PAI-1 and rPAI-131 (1 μg) were biotinylated with EZ-Link amine-PEG-biotin (Thermo Scientific). Each biotinylated protein was incubated with 1.8 μg of Lys-Plg (EMD Biosciences) followed by a second incubation with the antibody–protein G–bead complex. The final biotinylated rPAI-123 (or PAI-1)–plasminogen–antibody–protein G–bead complex was isolated with a magnet. Horseradish peroxidase–conjugated streptavidin (Thermo Scientific) was incubated with the bead-bound complex. The final complex was isolated with a magnet, then incubated with either unlabeled PAI-1 or rPAI-123 at concentrations ranging from 0 to 0.5 μg. The complex was isolated by magnet. Bound and unbound fractions were incubated with 3,3’,5,5’-tetramethylbenzidine substrate (Thermo Scientific). Colorimetric change was measured at 370 nm on a spectrophotometer (BioTek, Winooski, VT). Readings were obtained every 2 minutes until saturation was reached.

Biochemical Analysis of rPAI-123 Modulation of Plasmin Activity
Biochemical reactions that contained tPA (American Diagnostica) and plasminogen (Molecular Innovations) were incubated for 1 hour with either 25, 50, or 90 nmol/L rPAI-123, 12, 25, or 50 nmol/L glycosylated human PAI-1 (Molecular Innovations); or combinations of the 2. In a second set of reactions, tPA was added at 2 different time points: (1) simultaneously with plasminogen, PAI-1, and/or rPAI-123, or (2) after a 1-hour, 37°C incubation of reactions that contained plasminogen, rPAI-123, and/or PAI-1. A 1-hour incubation at 37°C followed the addition of tPA. Plasmin activity was measured in a plasmin chromozym assay (Roche) in triplicate wells in 3 separate experiments. The 3 sets of experiments were normalized to the tPA plus plasminogen controls.

Results
rPAI-123 Treatment Increases Proteolytic Activity in Plasma From Hypercholesterolemic Mice
The rPAI-123 protein lacks the reactive center loop that contains the tPA and uPA binding site; therefore, we considered the possibility that plasmin activity may be altered in hypercholesterolemic (PD-fed) LDLR−/−/ApoB48−/− (DKO) mice treated with rPAI-123. Proteolysis examined by casein zymography suggested that plasma from PD-fed rPAI-123–treated mice had more plasmin activity than that from PD-fed saline-treated and untreated CH-fed nonhypercholesterolemic mice (controls; Figure 1A). A chromozym assay measured 1.6-fold more plasmin activity in PD-fed rPAI-123–treated DKO mice than in PD-fed saline controls (rPAI-123 0.78±0.07×10−3 versus saline 0.47±0.1×10−3 U/mL; P=0.04; Figure 1B). Plasmin activity in CH-fed mice treated with rPAI-123 was not significantly greater than that in the CH-fed controls. A plasmin-specific inhibitor, α2-antiplasmin, blocked plasmin activity in all conditions. An active plasmin capture immunoassay measured similar differences in the concentration of active plasmin in PD-fed rPAI-123–treated mice as in the saline counterpart (rPAI-123 5.6±0.54 versus saline 3.7±0.57 ng/mL, P=0.03).

Plasmin activates numerous MMPs, and therefore, the potential plasmin effects on plasma MMP activity levels were examined on gelatin zymograms. The rPAI-123–treated mice had significantly more gelatinolytic activity than CH-fed and saline-treated PD-fed DKO mice (Figure 1C). An MMP-3 activity assay measured 1.3-fold more hydrolyzed MMP-3 substrate in the PD-fed rPAI-123–treated mice than in their saline counterpart (rPAI-123, 23±0.6 versus saline 18±0.45 pmol of substrate hydrolyzed per minute; P<0.001) and 1.2-fold more than in the CH-fed rPAI-123–treated DKO mice (CH rPAI-123 19±2 versus saline 17±0.6 pmol of substrate hydrolyzed per minute; P=0.04; Figure 1D). An MMP-3 inhibitor blocked 90% of the activity. Assays for MMP-9 showed residual activity, and no differences were measured in MMP-2 and MMP-10 activities (data not shown).

rPAI-123 Generates Fibrin(ogen) Breakdown Products
Because plasmin degrades fibrin or accumulated fibrinogen,27 their breakdown was examined to further validate the effects of elevated plasmin levels in rPAI-123–treated PD mice (Figure 2). Western blots probed for fibrin(ogen) show a defined fragment at 115 kDa in most saline-treated mice, whereas plasma from the rPAI-123–treated mice was degraded to a smaller fragment near 50 kDa (Figure 2A). Similarly, protein isolated from the DA within the thoracic cavity of each treatment group showed that the amount of fibrin(ogen) degradation products in the DA from rPAI-123–
treated mice was substantially greater than in the saline control (Figure 2B). D-dimer assays measured 1.65-fold more fibrin(ogen) breakdown products in plasma from the rPAI-123 treatment group than from the control group (rPAI-123 229 ± 25 versus saline 139 ± 20 ng/mL, *P = 0.001; Figure 3C), thus adding quantitative evidence for loss of fibrin(ogen) stability in rPAI-123–treated mice.

The effect of rPAI-123–stimulated proteolysis on fibrin(ogen) in plaque and the ECM supporting the DA wall was examined by probing cross sections for fibrin(ogen). Its expression in the ECM and adjacent plaque was barely detectable in rPAI-123–treated mice (Figures 2F and 2G), whereas the saline-treated mice had extensive levels of fibrin(ogen) in both areas (Figures 2D and 2E). Fibrin(ogen) observed in the ECM of saline-treated mice followed a distinct structural pattern that was absent in rPAI-123–treated mice. Plaque fibrin(ogen) was barely detectable in rPAI-123–treated mice, whereas plaque from the saline group contained large areas of dense fibrin(ogen).

**BM Breakdown in rPAI-123–Treated Mice**

The effects of rPAI-123–enhanced proteolytic activity on BM stability were examined in DA adventitia. Whole mounts from mice perfused with FITC-labeled lectin were probed for nidogen, a BM marker that is cleaved by plasmin\(^{28}\) (Figures 3A and 3B). Confocal microscopy images showed that the saline-treated mice had an organized vasa vasorum network colocalized extensively with nidogen (Figure 3A). On the other hand, the adventitia of rPAI-123–treated mice had very low levels of nidogen expression, and the lectin-perfused vessels were discontinuous and collapsing and demonstrated loss of the vascular network (Figure 3B).

Further examination of rPAI-123–stimulated effects on BM breakdown was performed on DA cross sections from saline- and rPAI-123–treated PD-fed mice. Sequential sections were incubated with plasmin, MMP-3, or both. Next, the sections were probed for the perlecacin core protein, which is reported to be degraded by plasmin\(^{29}\) (Figures 3A and 3B). Confocal images showed that rPAI-123–treated mice lacked perlecacin in the matrix surrounding the DA (Figure 3C), and CD-31–probed

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**Figure 1.** rPAI-123 stimulates proteolytic activity in hypercholesterolemic mice. Plasma from hypercholesterolemic (PD fed) and nonhypercholesterolemic (CH fed) LDLR \(^{-/-}\)/ApoB-100 \(^{-/-}\) mice was examined for (A) plasmin activation in PD-fed rPAI-123– and saline-treated or CH-fed untreated mice by casein zymography and (B) plasmin activity in a chromzym assay in PD-fed rPAI-123– and saline-treated, CH-fed rPAI-123– and CH-fed saline-treated, and CH-fed untreated mice. Plasmin activity was inhibited by α-2 antiplasmin (α-2-AP). Data are shown as mean ± SEM; probability values were determined by ANOVA. *P = 0.05, PD rPAI-123–treated vs other treatments. C, Gelatinolytic activity in PD-fed rPAI-123– and saline-treated or CH-fed untreated mice by gelatin zymography; (D) MMP-3 activity in PD-fed rPAI-123– and saline-treated, CH-fed rPAI-123– and saline-treated, and CH-fed untreated mice in a fluorogenic assay. MMP-3 activity was inhibited by N-(4,5-dihydro-5-thioxo-1,3,4-thiadiazol-2-ylcarbonyl)-L-phenylalanine. Data are shown as mean ± SEM; probability values were determined by ANOVA. *P = 0.05, **P = 0.001 vs PD rPAI-123–treated.
vessels lacked an organized structure (Online Figure IA). On the other hand, DA sections from the saline-treated mice had a distinct distribution of perlecan (Figure 3D) that was colocalized with vascular structures (Online Figure IB). Sequential sections incubated with plasmin had a significant loss of perlecan (Figure 3E), which was completely absent after exposure to plasmin and MMP-3 (Figure 3F).

**Plasminogen, tPA, and uPA Expression and Activity Levels Are Not Altered by rPAI-123**

Next, we examined the possibility that elevated protein expression or activity levels of plasminogen, tPA, or uPA in rPAI-123–treated mice could explain the higher plasmin expression or activity levels of plasminogen, tPA, or uPA in rPAI-123–treated mice. The rPAI-123–treated mice had a significant 48% reduction in PAI-1 activity compared with their saline counterpart (rPAI-123 1.5 ± 0.18 versus saline 1.8 ± 0.2, CH 1.3 ± 0.06 U/mL, P = NS; Figure 4A). Similarly, ELISA assays did not detect differences in tPA protein expression (rPAI-123 1.5 ± 0.14 versus saline 1.6 ± 0.17 ng/mL, P = NS). Urokinase activities and protein expression levels were below the lower limits of detection.

**Reduced PAI-1 Activity in rPAI-123–Treated Hypercholesterolemic Mice**

Because PAI-1 is the primary inhibitor of tPA/uPA conversion of plasminogen to plasmin, its activity was measured in plasma protein from rPAI-123– and saline-treated PD-fed mice. The rPAI-123–treated mice had a significant 48% reduction in PAI-1 activity compared with their saline counterpart (rPAI-123 8.5 ± 3.5 versus saline 20.4 ± 2.6 IU/mL, P = 0.001) but activity was comparable to that measured in plasma protein from PD-fed rPAI-123– and saline-treated mice.

**Figure 3. BM breakdown in response to rPAI-123 treatment.** Hypercholesterolemic mice treated with rPAI-123 or saline were perfused with FITC-labeled lectin. DA whole mounts were probed for the BM marker perlecan in either saline-treated (A) or rPAI-123–treated (B) mice. DA cross sections from the same treatment groups were probed with an antibody to the perlecan core protein in sections from rPAI-123–treated mice (C); sections from saline-treated mice (D); sequential sections from saline-treated mice incubated with plasmin (E); and sequential sections from saline-treated mice incubated with plasmin and MMP-3 (F). Confocal images were acquired at either 20× or 63× magnification.

**Figure 4. rPAI-123 effects on PAI-1 in hypercholesterolemic mice.** Equivalent amounts of plasma protein from hypercholesterolemic mice (PD) treated with rPAI-123 or saline and nonhypercholesterolemic mice (CH) were analyzed for IPA activity in a chromozyg assay (A); PAI-1 activity in a chromogenic assay (B); and PAI-1 protein expression in an ELISA (C). Equivalent amounts of protein isolated from the DA of PD-fed mice treated with rPAI-123 or saline were probed for PAI-1 on a Western blot (D) and immunoprecipitated (IP) with an antibody to PAI-1, then immunoblotted (IB) with an antibody to plasminogen kringles 1 to 3 (E). P < 0.05 vs rPAI-123; *P < 0.001 vs rPAI-123 and saline treatment.
Although its activity was reduced significantly in rPAI-123–tolerated mice, plasminogen was complexed with PAI-1 in the DA of untreated, CH-fed control (untreated CH 0.46 ng/mL, saline 0.86 ng/mL, P < 0.001). Interestingly, the concentration of PAI-1 in the 2 treatment groups was significantly higher than in the untreated CH-fed control (untreated CH 0.46 ± 0.06 ng/mL, P < 0.001).

PAI-1 Is Complexed With Plasminogen in the DA

To gain insight into how PAI-1 protein expression levels could be the same in rPAI-123– and saline-treated mice although its activity was reduced significantly in rPAI-123–treated mice, DA protein was probed for PAI-1 on a Western blot (Figure 4D). Surprisingly, PAI-1 at its normal 50-kDa molecular mass was either absent or barely detectable in the protein from rPAI-123–treated mice (Figure 4D, lanes 9 to 13), but it was abundant at 80 to 118 kDa. Protein from 5 saline-treated mice contained PAI-1 at 50 kDa and slightly greater than 50 kDa (Figure 4D, lanes 3 to 7), which was also visible in the control (lane 1); however, it was not detected at 80 to 118 kDa in the saline-treated mice. These data suggest that PAI-1 is complexed with other proteins. That possibility was investigated by immunoprecipitation of PAI-1 from DA protein with an antibody that has affinity for active and inactive PAI-1. The antibody-bound complexes probed for plasminogen kringle 1 to 3 in a Western blot showed that plasminogen was complexed with PAI-1 in rPAI-123– and saline-treated mice (Figure 4E); however, densitometry measurements of the plasminogen bands and the precipitating antibody lane loading control indicated that there was 1.6-fold more plasminogen complexed with PAI-1 in the DA of rPAI-123–treated mice.

**Figure 5. rPAI-123 and PAI-1 bind plasminogen.**

A coimmunoprecipitation assay containing purified plasminogen and PAI-1 validated that PAI-1 binds plasminogen (Figure 5A). The assay was repeated in reactions that contained plasminogen, tPA, PAI-1, and/or rPAI-123 to determine whether tPA or rPAI-123 alters the ability of PAI-1 to bind plasminogen. PAI-1 antibody–precipitated complexes probed for plasminogen kringle 1 to 3 showed that plasminogen forms a complex with PAI-1 (Figure 5B, lane 4), rPAI-123 (Figure 5B, lane 5), or a mixture of both isoforms (Figure 5B, lane 6) in the presence of tPA. Binding affinity studies showed that rPAI-123 had slightly less affinity for plasminogen (Figure 4C) than PAI-1 (Figure 4D; Kd rPAI-123 31.1 x 10^-9 versus Kd PAI-1 8.1 x 10^-9).

**rPAI-123 Enhances Plasmin Activity in Biochemical Reactions**

Biochemical studies were performed to investigate whether rPAI-123 or PAI-1 binding affinity for plasminogen modifies plasmin activity. In one set of reactions, varied concentrations of rPAI-123 and PAI-1 were prebound to plasminogen in a 1-hour, 37°C incubation before the addition of tPA for an additional 1-hour incubation (denoted as late [L] in Figure 6A). In a second set of reactions, tPA, plasminogen, rPAI-123, or PAI-1 was added simultaneously (denoted as [S] in Figure 6B) to a reaction mixture and incubated at 37°C for 1 hour. A third set of reactions contained tPA, plasminogen, rPAI-123, and PAI-1 added simultaneously to a reaction mixture and incubated for 1 hour at 37°C (Figure 6C). Plasmin activity was measured in a chromozym assay.

The first set of reactions revealed several interesting findings. First, plasmin activity was 65% less when plasminogen was incubated for 1 hour before the addition of tPA (Figure 6A, lane 1) than when plasmas were added simulta-
Increased Proteolytic Activity in rPAI-123–Treated ECs Stimulates Collapse of Tubes

Human umbilical vein ECs were treated with rPAI-123 to determine whether it would stimulate plasmin or MMP-3 activity in ECs (Online Figure II) and the effect the proteolytic activities would have on EC tube formation in a 3-dimensional collagen-overlay assay (Online Figure III). Plasmin activity was 2.2-fold greater in culture medium from ECs treated with rPAI-123 than from PAI-1–treated cells (Online Figure II; rPAI-1, 0.6 ± 0.18 versus PAI-1, 0.27 ± 0.01 × 10⁻³ U/mL, P = 0.01). MMP-3 activity was 2-fold higher in the media of rPAI-123–treated cells than in media from controls (Online Figure II; rPAI-123, 0.06 ± 0.006 pmol of substrate per minute, versus untreated, 0.03 ± 0.003 pmol of substrate per minute, P = 0.003).

The second set of reactions, in which plasminogen was not prebound to rPAI-123 or PAI-1, showed that rPAI-123, at all 3 tested concentrations did not alter plasmin levels relative to the plasminogen/tPA simultaneous control (Figure 6B, lanes 6 to 8). PAI-1 efficiently inhibited plasmin activity in a dose-dependent manner (lanes 3 to 5), as expected.

The third set of reactions tested potential opposing effects of rPAI-123 on PAI-1 antiproteolytic activity. The results indicated that combining the 2 PAI-1 isoforms simultaneously with plasminogen and tPA altered the antiproteolytic effects of PAI-1 (Figure 6C, lanes 6 to 14 compared with lanes 3 to 5). The increase in plasmin activity occurred in a stepwise fashion that was dependent on and limited by the concentrations of rPAI-123 and PAI-1.
bind plasminogen. The interaction of PAI-1 with plasminogen has not been demonstrated by others.

EC adhesion to the appropriate matrix enables ECs to undergo migration, proliferation, and morphogenesis, which are necessary for neovascularization.\textsuperscript{12} Degradation of the ECM/BM leads to vessel instability and collapse/regression.\textsuperscript{13,14} The rPAI-1\textsubscript{23} protein has the unique capability of stimulating activation of plasmin and MMP-3 to destabilize fibrin, perlecan, and nidogen in the adventitia of PD-fed mice. Altogether, these 3 proteins have an enormous impact on vessel stability. Fibrin enables ECs to adhere, spread, and proliferate. Nidogen connects laminin and type IV collagen.\textsuperscript{12,28} Perlecan is a heparan sulfate proteoglycan whose core protein binds nidogen, type IV collagen, laminin, and angiogenic growth factors (fibroblast growth factor-2 and vascular endothelial growth factor).\textsuperscript{12,29} In vitro tube formation assays verify the effect of rPAI-1\textsubscript{23}–stimulated plasmin and MMP-3 activities on vessel stability. The correlation between plasmin activity and vessel regression demonstrated in the present study is in keeping with reports of others.\textsuperscript{18}

The significant increase in plasmin activity in hypercholesterolemic rPAI-1\textsubscript{23}–treated mice corresponded to a 48% decline in PAI-1 activity compared with the saline counterpart. The differences in PAI-1 activity were not attributable to reduced PAI-1 expression levels or to increases in plasminogen, tPA, or uPA expression. However, PAI-1 communoprecipitated with plasminogen in DA protein from rPAI-1\textsubscript{23}– and saline-treated mice, but to a 1.6-fold greater extent in those treated with rPAI-1\textsubscript{23}. These data indicate that PAI-1 can bind plasminogen in the absence of rPAI-1\textsubscript{23}, but rPAI-1\textsubscript{23} binding to plasminogen enhances plasmin activity.

Plasmin activity measured in biochemical reactions that contained either rPAI-1\textsubscript{23} or PAI-1, plasminogen, and tPA provided significant insight into the mechanisms by which rPAI-1\textsubscript{23} was able to stimulate elevated plasmin activity in PD-fed mice. Figure 6A indicates that rPAI-1\textsubscript{23} and PAI-1 can independently bind plasminogen to enhance tPA conversion of plasminogen to plasmin. The preformed plasminogen–rPAI-1\textsubscript{23} interaction increased plasmin activity to levels that exceeded those measured in a reaction of plasminogen and tPA added simultaneously (Figure 6A, lane 8 versus lane 2). However, rPAI-1\textsubscript{23} did not elevate plasmin activity when it was not prebound to plasminogen (Figure 6B, lanes 6 to 8). The surprising aspect of the plasminogen–PAI-1 interaction is that it abolished the PAI-1 antiproteolytic function (Figure 6A, lanes 4 and 5) that normally occurs when PAI-1 is not prebound to plasminogen (Figure 6B, lanes 4 and 5).

When plasminogen, tPA, rPAI-1\textsubscript{23}, and PAI-1 were combined simultaneously, rPAI-1\textsubscript{23} reversed the antiproteolytic activity of PAI-1 (Figure 6C). The extent to which this occurred was dependent on the molar ratios of rPAI-1\textsubscript{23} to PAI-1. The greatest increase in plasmin activity occurred when both PAI-1 isoforms were at the highest concentration (Figure 6C, lane 14). As rPAI-1\textsubscript{23} concentration decreased, plasmin activity declined in a stepwise manner, but not to the level measured in the absence of rPAI-1\textsubscript{23} (lanes 4 and 5). Similarly, plasmin activity declined in a stepwise manner when rPAI-1\textsubscript{23} was in excess and PAI-1 concentrations were reduced. These data suggest that rPAI-1\textsubscript{23} and PAI-1 work together to increase plasmin activity through a mechanism that requires their interaction with plasminogen.

On the basis of the combined studies, we predict a model for this novel plasmin regulatory pathway that contributes to collapse of the vasa vasorum in rPAI-1\textsubscript{23}–treated PD mice (Online Figure IV). In this model, rPAI-1\textsubscript{23} and PAI-1 bind plasminogen. In both cases, the interaction with plasminogen increased plasmin activity, but to a much greater extent in response to rPAI-1\textsubscript{23}. We do not know whether the enhanced plasmin activity was due to increased tPA conversion of plasminogen to plasmin or increased plasmin specific activity. One possible mechanism for increasing plasmin activity is that plasminogen-bound rPAI-1\textsubscript{23} alters plasminogen conformation. The altered conformation may then serve as a decoy for PAI-1, thus making it unavailable for binding and inhibiting tPA. Another possibility is that a plasminogen–rPAI-1\textsubscript{23}–PAI-1 complex has novel properties that facilitate enhanced tPA activity. Further studies to determine the precise order of interactions and binding partners will be performed.

Achieving levels of plasmin that exceed control levels, such as those measured in rPAI-1\textsubscript{23}–treated hypercholesterolemic DKO mice, requires elevated PAI-1 expression. Although excess PAI-1 in saline-treated PD-fed mice did bind plasminogen, the plasmin levels were significantly less than those measured in rPAI-1\textsubscript{23}–treated mice. The reason for this is that PAI-1 bound to plasminogen, in the absence of rPAI-1\textsubscript{23}, cannot elevate plasmin activity. The data suggest that the role of rPAI-1\textsubscript{23} in this mechanism is to limit excess PAI-1–induced antiproteolytic activity. Nevertheless, the combined data clearly demonstrate that rPAI-1\textsubscript{23} stimulates increased plasmin activity through a novel pathway that includes PAI-1 binding to plasminogen, an interaction not reported previously.

The proteolytic and fibrinolytic pathways are associated with angiogenesis. PAI-1 has been shown to be proangiogenic and antiangiogenic.\textsuperscript{37–42} Some studies have shown that the functional difference lies in PAI-1 concentration, with low concentrations being proangiogenic and promigratory and high concentrations being inhibitory.\textsuperscript{43} The present study supports those findings. The work of others suggests that the proangiogenic role of PAI-1 is to prevent excessive pericellular proteolysis, which would maintain the integrity of the ECM scaffold required for tube formation\textsuperscript{44,45}. The results of the present study support that concept; the elevated proteolytic activity stimulated by rPAI-1\textsubscript{23} degraded key ECM/BM proteins. Overall, the novel pathway uncovered in the present study provides significant insight into the discrepancies in the role of PAI-1 in angiogenesis.

Others who have studied PAI-1 in atherosclerosis have shown that it is both atheroprone\textsuperscript{46} and atheroprotective.\textsuperscript{47} In the present study, we have shown that PAI-1 expression is elevated in hypercholesterolemic DKO mice, which suggests that it contributes to the disease process. It will be interesting to study the effect of rPAI-1\textsubscript{23} in LDLR/\textsuperscript{−/−}/ApoB48/\textsuperscript{−/−} PAI-1/\textsuperscript{−/−} mice fed a Paigen diet without cholate. The results of the present study suggest that those mice will not develop atherosclerosis. If that is not the case, then the studies will...
help uncover other potential antiangiogenic and atheroprotective mechanisms stimulated by rPAI-123.

Plasmin was initially considered to be angiogenic because it degrades fibrin, the provisional matrix on which angiogenic ECs migrate; however, more recent studies have shown that angiogenic ECs invade fibrin matrices in a plasmin-independent manner.48 This is consistent with our finding that plasmin has a role that is compatible with inhibition of angiogenesis.

Elevated levels of MMPs in atherosclerotic plaques in general have been considered to be atheropane because of their ability to degrade collagen, a stabilizing factor in plaque.49 More recent studies have used mouse models deficient in various MMPs to identify those that are atheropane versus atheroprotective. Studies conducted in ApoE−/−/MMP-3−/− mice have clearly shown that in the absence of MMP-3, plaque area is significantly increased compared with ApoE−/− mice.50

The present study demonstrates a novel rPAI-123–stimulated mechanism whereby plasminogen sequesters PAI-1 to result in increased plasmin activity. The excess plasmin degrades the ECM/BM scaffold, which destabilizes the angiogenic vessels, which leads to their collapse. These data, combined with our previous studies, which showed that rPAI-123 reduces plaque size in hypercholesterolemic LDLR−/−/ApoB48 mice,51 further elaborate the antiangiogenic mechanisms of rPAI-123 related to its atheroprotective effects. These data also provide significant insight into the disparate reports of the role of PAI-1 in angiogenesis and atherosclerosis.

Sources of Funding

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Disclosures

None.

References

A truncated plasminogen activator inhibitor-1 protein induces and inhibits angiostatin. The interactions whereby rPAI-1 23 and PAI-1 bound plasminogen. The interactions whereby rPAI-1 stimulated plasmin activity on plaque stability and regression.

The vasa vasorum in the adventitia of large arteries becomes activated during atherosclerosis in humans and in mouse models of atherosclerosis. A truncated plasminogen activator inhibitor-1 protein, rPAI-1 23, stimulates the collapse of vasa vasorum in hypercholesterolemic low-density lipoprotein receptor-deficient (LDLR −/−) mice. The vasa vasorum appears to collapse in response to rPAI-1 23 treatment. The objective of this study was to identify mechanisms by which rPAI-1 23 stimulates vasa vasorum collapse. We found that rPAI-1 23 causes regression and collapse of the vasa vasorum by stimulating plasmin activity. The increased plasmin activity, accompanied by elevated matrix metalloproteinase-3 activity, degrades major ECM/BM components that provide support and stability to expanding vasa vasorum. The rPAI-1 23 enhanced plasmin activity through a novel mechanism whereby rPAI-1 23 and PAI-1 bind to plasminogen.

We had previously demonstrated that a truncated plasminogen activator inhibitor-1 protein, rPAI-1 23, inhibits angiogenic vasa vasorum, reduces plaque area, and decreases plaque cholesterol in the descending aorta of hypercholesterolemic LDLR −/−/ ApoB48 −/− mice. The vasa vasorum, appears to collapse in response to rPAI-1 23 treatment. The objective of this study was to identify mechanisms by which rPAI-1 23 stimulates vasa vasorum collapse. We found that rPAI-1 23 causes regression and collapse of the vasa vasorum by stimulating plasmin activity. The increased plasmin activity, accompanied by elevated matrix metalloproteinase-3 activity, degrades major ECM/BM components that provide support and stability to expanding vasa vasorum. The rPAI-1 23 enhanced plasmin activity through a novel mechanism whereby rPAI-1 23 and PAI-1 bound plasminogen. The interactions with plasminogen reduced the antiproteolytic activity of PAI-1 and increased plasmin activity. Elevation of plasmin levels to the extent measured in rPAI-1 23-treated hypercholesterolemic mice requires elevated PAI-1 expression. These findings provide novel insight into the antiangiogenic and atheroprotective roles of rPAI-1 23. Additional studies are required to assess the impact of rPAI-1 23-stimulated plasmin activity on plaque stability and regression.
Antiangiogenic Activity of rPAI-123 Promotes Vasa Vasorum Regression in Hypercholesterolemic Mice Through a Plasmin-Dependent Mechanism
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Supplemental Material

Supplemental Methods and Materials

Production and purification of rPAI-123.
The DNA encoding rPAI-123 protein was obtained by deleting the porcine PAI-1 gene (poPAI-1)\textsuperscript{1-3}. Selection of the gene fragment was based on the poPAI-1 sequences that correspond to the human PAI-1 gene (huPAI-1) sequences reported to code for functional domains in human PAI-1\textsuperscript{4,5}. The DNA fragment was isolated from porcine aortic endothelial cells by reverse-transcribing RNA into cDNA. The cDNA was made double-stranded in a PCR reaction containing porcine PAI-1-specific primers\textsuperscript{1,2}. The PCR-amplified rPAI-1\textsubscript{23} DNA was ligated into a Pichia pastoris yeast shuttle vector, pGAPZ-α (Invitrogen, Carlsbad, CA). The TOP 10 strain of Escherichia coli was transformed by electroporation. Positive isolates were verified by sequencing. The recombinant protein was expressed in P. pastoris. The secreted protein is purified on a heparin sepharose column containing 0.02 % sodium azide. The rPAI-1\textsubscript{23} protein is eluted in 400 mM NaCl, and then dialyzed against PBS. The protein is tested at each purification step for potential bacterial, yeast and endotoxin contamination (PyroGene recombinant Factor C endotoxin detection kit, Cambrex, Walkersville, MD).

Diet and treatment.
Twelve week old, weight-matched female LDLR\textsuperscript{-/-}ApoB48 deficient mice were fed Paigen’s hypercholesterolemic diet without cholate\textsuperscript{6} (PD) for a total of 20 weeks. One group received intraperitoneal injections of rPAI-1\textsubscript{23} (5.4 µg/kg/day) beginning at week 14 of PD and another group received saline treatment\textsuperscript{7}. The rPAI-1\textsubscript{23} dose was based on in vivo Matrigel plug assays in C57B6 mice.

Plasmin activity measurement. In order to read multiple samples simultaneously over time in a microtiter plate, sample and reagent volumes were scaled down to 1/10 of those used in a 1 ml reaction. Each sample was measured in triplicate and the experiment was performed three times in n=6 per group. Control samples included all assay components except the substrate. Activity was calculated based on the manufacturer’s formula (using appropriate volumes) after protein background and residual inhibitor readings were subtracted.

Measurement of MMP activity. Each plasma sample (n=9 for rPAI-1\textsubscript{23}, n=7 for saline) was measured in triplicate wells and the experiment was performed twice. Control wells for each sample included all assay components except the substrate. Activity was calculated based on the manufacturer’s formula after protein background and residual inhibitor readings were subtracted.

Western blot analysis of fibrinogen. Equivalent amounts of either descending aorta or plasma protein from saline and rPAI-1\textsubscript{23} treated mice were gel resolved and transferred to nitrocellulose. The membrane was probed for fibrin(ogen) (Dako, Carpinteria, CA) in an overnight incubation at 4º C. The binding reaction was amplified in a1h RT incubation with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody. The binding reaction was detected with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL). Similarly, adventitial protein was probed for fibrin(ogen) following the same protocol. *Antibodies do not differentiate between fibrinogen and fibrin, therefore they are denoted as fibrin(ogen).

Immunohistochemical analysis of fibrin(ogen). Ten micron frozen sections of the descending aorta were probed for fibrin(ogen) with a rabbit anti-human polyclonal primary antibody (Dako) in a 4º C. overnight incubation. A horse radish peroxidase conjugated donkey anti-rabbit secondary antibody (Amersham) amplified the binding reaction in a 2h room temperature incubation. The enzymatic reaction was detected with DAB chromagen (BD Pharmingen).
Confocal imaging of nidogen-probed basement membrane. Atherogenic mice from each treatment group were perfused with fluorescein-labeled *Lycopersicon esculentum* lectin (FITC-lectin). Adventitial basement membrane stability in DA whole mounts was examined by confocal microscopy. The adventitia was probed for nidogen, a basement membrane marker (Millipore, Billerica, MA) in an overnight reaction at 4°C. A goat anti-rat secondary antibody conjugated to Alexa 568 (Molecular Probes Invitrogen, Carlsbad, CA) amplified and detected the binding reaction. Z-stack images were acquired on a Zeiss LSM-510 META point scanning confocal microscope (Thornwood, NY). n=4 per group.

Measurement of rPAI-1<sub>23</sub> stimulated proteolytic activity in endothelial cells. Human umbilical vein endothelial cells (HUVECs) were grown in medium supplemented with 10% fetal bovine serum. Cells were incubated for 18 h at 37°C with rPAI-1<sub>23</sub> (90 nM), PAI-1 (50 nM) or rPAI-1<sub>23</sub> + PAI-1. Each culture was supplemented with 2 nM plasminogen. Untreated HUVECs served as the control. Culture medium was collected after 18h of incubation. Plasmin, MMP-3 and PAI activities, with and without their respective inhibitors, were measured in conditioned medium. N=3 per group.

Endothelial cell tube formation in a collagen overlay assay. Angiogenesis was stimulated by adding fibroblast growth factor (FGF-2) (25 ng/ml)(EMD Biosciences) to the conditioned medium collected from HUVECs treated with rPAI-1<sub>23</sub>, PAI-1 or rPAI-1<sub>23</sub> +PAI-1 for 18h. The mixture was added to HUVECs embedded in a collagen-1 overlay. Complete endothelial cell enclosures were counted in 5 fields per 3 wells after 18h of incubation at 37°C, as previously described.

References

Online Figure I: Perlecan co-localizes with vasa vasorum
Descending aorta cross sections from hypercholesterolemic mice treated with (A)rPAI-1$_{23}$ or (B) saline were probed with an antibody to the perlecan core protein, a basement membrane proteoglycan and for CD-31, a marker for endothelial cells. Note the significantly diminished perlecan and the unstructured vessels in the rPAI-1$_{23}$ group compared to the co-localization of perlecan with structured vessels in the saline treatment group.
Online Figure II. **rPAI-1_{23}** stimulated proteolytic activity in endothelial cells. Human umbilical vein endothelial cells (HUVECs), grown in 10% fetal bovine serum, were incubated for 18 h at 37º C. with rPAI -1_{23} and/or PAI or no treatment. Each culture was supplemented with 2 nM plasminogen. Culture medium was assayed for activated (A) plasmin; (B) MMP-3; and (C) PAI. Data shown as mean ± standard deviation. Probability values were determined by ANOVA. **p≤0.001 vs. PAI control.
Online Figure III: rPAI-1_{23} conditioned medium inhibits tube formation. Conditioned culture medium from HUVECs treated with rPAI-1_{23}, PAI-1 or a combination of both was combined with FGF-2, then added to HUVECs embedded in a collagen 1 overlay. The number of complete enclosures were counted. N= 6 per treatment group. Data shown as mean ± standard deviation. Probability values were determined by ANOVA. **p≤0.001 vs. rPAI-1_{23}. 

Lane: 1 2 3 4 5 6 7

10% FBS: + + + + +
Plasminogen: + + + + +
FGF-2: + + + + +
PAI-1: + + + + +
rPAI-1_{23}: + + + + +