The Antiangiogenic Activity of rPAI-1_23 Inhibits Vasa Vasorum and Growth of Atherosclerotic Plaque

Mary Drinane,* Jessica Mollmark,* Lyubomir Zagorchev, Karen Moodie, Baiming Sun, Amy Hall, Samantha Shipman, Peter Morganelli, Michael Simons, Mary Jo Mulligan-Kehoe

Abstract—Plaque vascularity has been implicated in its growth and stability. However, there is a paucity of information regarding the origin of plaque vasculature and the role of vasa vasorum in plaque growth. To inhibit growth of vasa vasorum in atherogenic mice and assess its effect on plaque growth, we used a truncated plasminogen activator inhibitor (PAI)-1 protein, rPAI-1_23, that has significant antiangiogenic activity. Female LDLR−/−/ApoB-48−/− deficient mice fed Paigen’s diet without cholate for 20 weeks received rPAI-1_23 treatment (n = 21) for the last 6 weeks. Plaque size and vasa vasorum density were compared to 2 controls: mice fed Paigen’s diet and treated with saline for the last 6 weeks (n = 16) and mice fed Paigen’s diet until the onset of treatment (n = 14). The rPAI-1_23 treatment significantly reduced plaque area and plaque cholesterol in the descending aorta and plaque area in the innominate artery. Measurements of reconstructed confocal microscopy images of vasa vasorum demonstrate that rPAI-1_23 treatment decreased vasa vasorum area and length, which was supported by microCT images. Confocal images provide evidence for vascularized plaque in the saline-treated group but not in rPAI-1_23–treated mice. The increased vessel density in saline-treated mice is attributable, in part, to upregulated fibroblast growth factor-2 expression, which is inhibited by rPAI-1_23. In conclusion, rPAI-1_23 inhibits growth of vasa vasorum, as well as vessels within the adjacent plaque and vessel wall, through inhibition of fibroblast growth factor-2, leading to reduced plaque growth in atherogenic female LDLR−/−/ApoB-48−/− deficient mice. (Circ Res. 2009;104:337-345.)

Key Words: atherosclerosis ■ angiogenesis inhibitors ■ FGF-2

Atherosclerosis is a chronic disease of large and medium size arteries1,2 and is the most frequent cause of coronary, peripheral, and carotid artery disease. Neoangiogenesis associated with more advanced stages of human atherosclerosis is found in plaque3 and the vasa vasorum,4 the microvasculature in the adventitial layer of large arteries that provides arterial blood supply to the arterial wall.5 The presence and extent of vasa vasorum correlate with atherosclerotic lesion size and lumen diameter in hypercholesterolemic animal models.5–9 Vasa vasorum are considered to be the conduit for nutrient supplies to atherosclerotic plaque. Studies have demonstrated that inhibition of neovascularization in the vasa vasorum is associated with reduced plaque progression.9,10 At the same time, there is little information about the origin of plaque vasculature and the role of vasa vasorum in plaque growth, nor is there conclusive evidence that angiogenesis in vasa vasorum promotes plaque development.11

Proteases that degrade the extracellular matrix play an important role in angiogenesis and plaque remodeling. The plasminogen activator (PA) system contributes to both processes through the proteolytic activity of plasmin. Plasmin activity is tightly regulated by plasminogen, PAs, and PA inhibitor (PAI)-1. Activation of PAI-1 inhibitory function requires a conformational change to expose the reactive center loop containing a binding site for PA.12 The PA binding interaction with PAI-1 prevents PAs from cleaving plasminogen, thereby limiting plasmin levels and its role in extracellular matrix proteolysis.13

The role of PAI in plaque progression is poorly understood and controversial. It is implicated in promoting plaque progression in the carotid artery,14,15 enhancing thrombosis,15 and increasing neointima formation in atherosclerosis-prone mice.16 It has also been demonstrated that PAI-1 is atheroprotective in ApoE−/− mice, whereas the loss of PAI-1 promotes plaque progression in advanced stages of atherosclerosis by increasing matrix deposition.17 Still others have shown that PAI-1 has no effect on atherosclerosis progression in ApoE−/− and LDLR−/− mice.18

The role of PAI-1 in angiogenesis is also controversial. PAI-1 modulates the functions of plasmin, urokinase recep-
PAI-1 is cleaved by matrix metalloproteinase-3 and plasmin to produce a truncated PAI-1 protein with a molecular mass less than 30 kDa. We hypothesized that cleaved PAI-1 has a function and produces truncated PAI-1 proteins (rPAI-1) that lack a reactive center loop at the carboxyl terminus. In the absence of the reactive center loop, the rPAI-1 proteins could not bind and inactivate PAs, thus making them “inactive” PAI-1 proteins. Partial removal of the heparan sulfate binding domain at the amino terminus produced an rPAI-1 protein, rPAI-123, with significant antiangiogenic activity. It inhibits fibroblast growth factor (FGF)-2–stimulated migration, tube formation, and proliferation, vascular endothelial growth factor (VEGF)-stimulated migration in aortic endothelial cells, and VEGF- and FGF-2–stimulated tubulogenesis in embryonic chick aortic rings. The goal of this study was to determine whether rPAI-123 inhibits angiogenic vasa vasorum that corresponds with reduced plaque growth in mice on an atherogenic diet.

**Materials and Methods**

**Animals**

Female B6: 129S-ApoB<sup>−/−</sup>Ldl<sup>−/−</sup>H/Tm1Her/J mice (LDLR/−/−/ApoB<sup>−/−</sup>) were used for all experiments (see Method 1 in the online data supplement, available at http://circres.ahajournals.org).

**Recombinant rPAI-1<sub>23</sub> Protein Production and Purification**

Recombinant, truncated PAI-1 protein rPAI-1<sub>23</sub> was produced, purified, and tested for bacterial, yeast, and endotoxin contaminants as previously described.

**Diet and Treatment**

Thirty-seven 12-week-old, weight-matched female mice were fed Paigen’s atherogenic diet<sup>19</sup> without cholate (PD) (online data supplement, Method 2) for a total of 20 weeks and fourteen 12-week-old female mice were fed PD for 14 weeks. Twenty-one female mice were fed PD for 14 weeks and treated with either rPAI-1<sub>23</sub> (n=13) or saline (n=11), and mice fed PD for 14 weeks (n=8) were stained with hematoxylin/eosin (H&E), picro-Sirius red, and Masson’s trichrome (Dartmouth Clinical Pathology Laboratory). Mice were perfused with fluorescein-labeled **Lycopersicon esculentum** lectin (tomato) (Vector Labs). Vessels in DA adventitia, wall, and plaque were analyzed for endothelial and smooth muscle cells by imaging lectin-bound endothelium and antibody-bound smooth muscle actin. Confocal Z-stack were collected with a ×63 objective (online data supplement, Method 7).

**Detection of Apoptotic Cells**

DNA strand breaks in apoptotic cells were detected with a TUNEL labeling and enzyme kit following the instructions of the manufacturer for cryopreserved tissue (Roche). Major histocompatibility class (MHC) II cells were detected with a rat I-A/I-E antibody (BD Pharmingen). Sections probed in the absence of the primary antibody served as negative controls (supplemental Figure III).

**Confocal Microscopy and Vessel Reconstruction**

Mice were perfused as described. DAs were removed, and adventitial vessels were probed for CD31 and imaged by confocal microscopy (online data supplement, Method 6).

**Probing for FGF-2**

Mice were perfused with FITC-labeled lectin. DAs were permeabilized and then incubated with an antimouse FGF-2 antibody (Sigma). Amplification and detection of the binding reaction were as described. Confocal images were acquired at ×63, as described.

**Quantitative Real-Time PCR of Growth Factor RNA**

RNA was isolated and purified from the DA of atherogenic mice treated with rPAI-1<sub>23</sub> or saline for 6 weeks and age-matched chow-fed mice. RNA was reverse transcribed into cDNA and quantification of VEGF-A<sub>165</sub>, FGF-2, and β-actin gene expression was performed by the real-time PCR technique. Relative mRNA copy number was calculated by the 2<sup>−ΔΔCT</sup> method (online data supplement, Method 8).

**Vessel Area and Length Measurements**

Projection images of adventitial vessels from confocal microscopy Z-stack reconstruction were preformed with Velocity software and then transformed to black and white images using the thresholding tool in Volocity. Total vessel area and length were measured by MatLAB script. Vessel area is unskeletonized image and is the sum of white pixels in a field. Length is the same measurement on skeletonized image.

**Reconstruction of Vessels in Plaque, Vessel Wall, and Adventitia**

DA cross-sections probed for smooth muscle actin and lectin were imaged by confocal microscopy at ×63 resolution. Z-stacks consisting of 15 slices at physical resolution of 2.54 μm were acquired. Slices were aligned in a 3D volumetric image, and resolution was increased by trilinear interpolation. Reconstructed Z-stacks were manually segmented to represent colocalized probes in consecutive

**Plaque Cholesterol Measurement**

Mice were euthanized, and DAs were removed and weighed. Cholesterol was extracted, evaporated, and rehydrated for measuring plaque cholesterol levels (online data supplement, Method 5).

**Histological Measurements of Plaque Morphology**

Alternating 5-micron frozen sections of innominate arteries from mice fed PD for 20 weeks and treated with either rPAI-1<sub>23</sub> (n=13) or saline (n=11), and mice fed PD for 14 weeks (n=8) were stained with hematoxylin/eosin (H&E), picro-Sirius red, and Masson’s trichrome (Dartmouth Clinical Pathology Laboratory). Vessel circumference and plaque and lumen area were measured using Regions of Interest imaging software (Philips Imaging). Macrophage content was examined by probing DA cross-sections for MOMA-2, a mouse macrophage and monocyte intracellular antigen (Abcam). Major histocompatibility class (MHC) II cells were detected with a rat I-A/I-E antibody (BD Pharmingen). Sections probed in the absence of the primary antibody served as negative controls (supplemental Figure III).

**Preparation of Tissue**

At the end of each treatment period the animals were fasted overnight. The next day blood was drawn and mice were euthanized and perfused. The innominate artery, carotid arteries, aortic arch, and descending aorta (DA) to the iliac bifurcation were surgically removed. Innominate arteries and DAs were placed in OCT tissue embedding compound (Sakura Finetek USA Inc.) in preparation for serial sectioning (online data supplement, Method 4).

**Detection of Apoptotic Cells**

DNA strand breaks in apoptotic cells were detected with a TUNEL labeling and enzyme kit following the instructions of the manufacturer for cryopreserved tissue (Roche). Major histocompatibility class (MHC) II cells were detected with a rat I-A/I-E antibody (BD Pharmingen). Sections probed in the absence of the primary antibody served as negative controls (supplemental Figure III).

**Confocal Microscopy and Vessel Reconstruction**

Mice were perfused as described. DAs were removed, and adventitial vessels were probed for CD31 and imaged by confocal microscopy (online data supplement, Method 6).

**Probing for FGF-2**

Mice were perfused with FITC-labeled lectin. DAs were permeabilized and then incubated with an antimouse FGF-2 antibody (Sigma). Amplification and detection of the binding reaction were as described. Confocal images were acquired at ×63, as described.

**Quantitative Real-Time PCR of Growth Factor RNA**

RNA was isolated and purified from the DA of atherogenic mice treated with rPAI-1<sub>23</sub> or saline for 6 weeks and age-matched chow-fed mice. RNA was reverse transcribed into cDNA and quantification of VEGF-A<sub>165</sub>, FGF-2, and β-actin gene expression was performed by the real-time PCR technique. Relative mRNA copy number was calculated by the 2<sup>−ΔΔCT</sup> method (online data supplement, Method 8).

**Vessel Area and Length Measurements**

Projection images of adventitial vessels from confocal microscopy Z-stack reconstruction were preformed with Velocity software and then transformed to black and white images using the thresholding tool in Volocity. Total vessel area and length were measured by MatLAB script. Vessel area is unskeletonized image and is the sum of white pixels in a field. Length is the same measurement on skeletonized image.

**Reconstruction of Vessels in Plaque, Vessel Wall, and Adventitia**

DA cross-sections probed for smooth muscle actin and lectin were imaged by confocal microscopy at ×63 resolution. Z-stacks consisting of 15 slices at physical resolution of 2.54 μm were acquired. Slices were aligned in a 3D volumetric image, and resolution was increased by trilinear interpolation. Reconstructed Z-stacks were manually segmented to represent colocalized probes in consecutive...
Infusion of Microfil Into Vessels
Mice were anesthetized, heparinized, and perfused. A silicone rubber compound, Microfil Blue, was infused through the aortic cannula. DAs with a wide adventitial margin were removed after polymerization was complete (online data supplement, Method 10). Three mice per group were imaged.

MicroCT Imaging of the Vasa Vasorum
DAs containing Microfil were scanned with a GE eXplore Locus SP microCT scanner at 6.5-μm resolution. Three-dimensional volumetric images were reconstructed from acquired 2D projections without averaging, yielding a final voxel size of 6.5 μm (online data supplement, Method 11).

Statistical Analysis
Statistical analysis was performed with a 2-tailed indirect Student’s t test, 1-way ANOVA with a post hoc least significant difference test with or without repeated measures, or with a χ² test, as appropriate, using the SPSS 12.0.1 statistical software package.

Results
rPAI-123 Inhibits Plaque Size
The effects of rPAI-123 on plaque growth were studied by visualizing the extent of Sudan IV–stained plaque located between the top of the DA and the iliac bifurcation. Plaque area relative to total DA area was calculated. Measurements were taken in mice fed PD for 14 weeks or PD for 20 weeks and received either saline or rPAI-123 treatment for the last 6 weeks of the diet. Plaque area in rPAI-123–treated mice was reduced by 73% (P<0.001) when compared to saline-treated mice fed the same diet and 39% (P=0.02) less than mice fed PD for 14 weeks (Figure 1A through 1D).

Figure 1. Sudan IV–stained lipid in the aortic arch and DA. Twelve-week-old female mice were fed PD for various time periods and received variable treatment in the final 6 weeks of diet. DAs were stained with Sudan IV were cut longitudinally for en face preparations. A, PD for 14 weeks (n=14). B, PD for 20 weeks plus saline from weeks 14 to 20 (n=16). C, PD for 20 weeks plus rPAI-123 from weeks 14 to 20 (n=21). D, Sudan IV–stained plaque area relative to total area of the DA. E, Plaque cholesterol measured in the DA from fasted mice. White bars represent Chow diet in age-matched mice (n=6); gray bars, PD for 20 weeks plus saline from weeks 14 to 20 (n=6); black bars, PD for 20 weeks plus rPAI-123 from weeks 14 to 20 (n=6). Data are shown as means±SEM, and probability values were determined by ANOVA. *P<0.05 vs rPAI-123, **P<0.001 vs rPAI-123.

Statistical analysis was performed with a 2-tailed indirect Student’s t test, 1-way ANOVA with a post hoc least significant difference test with or without repeated measures, or with a χ² test, as appropriate, using the SPSS 12.0.1 statistical software package.

Results
rPAI-123 Inhibits Adventitial Vessel Growth
rPAI-123 Inhibits Adventitial Vessel Growth
Whole mount DAs from each test group were probed for endothelial marker CD31 to determine whether rPAI-123 treatment had a corresponding effect on adventitial vasa vasorum. Confocal Z-stack microscopic images of adventitial vessels showed substantial structural and density differences between rPAI-123 (Figure 2D) and saline-treated mice fed PD for 20 weeks (Figure 2A), whereas similarities were observed between rPAI-123 and the 14-week PD control (supplemental Figure I). Quantification of unskeletonized (Figure 2B and 2E) and skeletonized (Figure 2C and 2F) reconstructed vessel images demonstrated a 37% (P=0.01) reduction in total vessel area (Figure 2G) and a 43% (P=0.004) reduction in vessel length (Figure 2H) in rPAI-123–treated mice maintained on PD compared to the saline counterpart.

MicroCT images of vasa vasorum were acquired to confirm the confocal microscopy results. Microfil, a microvascular contrast agent, was infused into the ascending aorta of mice fed PD for 14 weeks or 20 weeks with rPAI-123 or saline treatment for the final 6 weeks of diet. DAs were removed for ex vivo microCT scanning and 3D reconstruction of the scanned images. Reconstructed images of rPAI-123–treated mice showed an absence of second order vasa vasorum (Figure 3C) that were abundantly dense in the saline counterpart.

Plaque cholesterol in rPAI-123–treated mice was significantly reduced by 49% when compared to control mice fed PD for 20 weeks (rPAI-123, 8.8±0.4 μg/mL; versus saline, 17.2±2.9 μg/mL; P<0.01) but remained 39% higher than Chow fed mice (5.4±0.7 μg/mL; P<0.001) (Figure 1E). These experiments demonstrate that rPAI-123 treatment has a dramatic effect on reducing plaque area and plaque cholesterol levels.
terpart (Figure 3B, white arrows) and beginning to develop in the 14-week-diet group (Figure 3A). The microCT images were rotated to visualize plaque in the luminal side of the DA. Plaque was not detected in the lumen of rPAI-123–treated mice (Figure 3F), but the saline group had extensive plaque (Figure 3E) and the 14 week atherogenic control group had detectable plaque (Figure 3D).

**rPAI-123 Inhibits Vessel Growth Into Plaque Area**

Confocal microscopic Z-stack images of DA cross-sections were examined for vessels within the plaque and vessel wall of atherogenic mice treated with rPAI-123 or saline for 6 weeks. Vascular structures were probed for lectin and smooth muscle actin. Mice treated with saline had distinct vessels in the plaque (Figure 4C) and DA wall (Figure 4B), while vessels in the plaque and DA wall of atherogenic mice treated with rPAI-123 were absent (Figure 5B and 5C). Moreover, these vessels abutted highly vascularized adventitia (Figure 4A), which was absent in rPAI-123–treated mice (Figure 5A).

Blood vessels in plaque, adventitia, and vessel wall were visualized by aligning confocal Z-stacks images in a 3D volumetric image and increasing resolution by trilinear interpolation. Reconstructed Z-stacks were manually segmented to represent the detected colocalized probes in consecutive axial slices. Contours of blood vessels going all the way through the interpolated volumes were modeled and stacked in 3D to provide volumetric surface representation. The reconstructed images show the presence of fully formed vessels in the DA wall, plaque and adventitia of saline-treated mice (Figure 4D through 4F) and their absence in the rPAI-123–treated mice (Figure 5D through 5F). The collective data from this series of experiments clearly demonstrate that rPAI-123 has a profound inhibitory effect on second order vasa vasorum. The absence of these vessels corresponds to a lack of vessels invading the...
plaque and to dramatically reduced plaque area (supplemental Figure II, C and D), which is the opposite of what was found in control mice that were not treated with the angiogenesis inhibitor (supplemental Figure II, A and B).

Adventitial and Intraplaque FGF-2 Expression Levels

The adventitia was probed for the angiogenic growth factors FGF-228 and VEGF-A16511 to determine whether they were influential in expansion of the vessels observed in Figures 2A, 3B, and 4A. VEGF-A165 was not detected, but FGF-2 was expressed abundantly along vascular structures in the adventitia of saline-treated atherogenic mice (Figure 6A) and to a significantly lesser degree in rPAI-123–treated atherogenic mice (Figure 6B). Vessels in the saline-treated group have a lumen and follow the well-defined pattern of FGF-2–bound vascular structures. Lectin-probed vessels in rPAI-123–treated mice do not have a lumen. Although they follow the
FGF-2-bound vascular structures, the pattern of the structures is disordered and in many instances disrupted. Similarly, FGF-2 was detected in plaque from saline-treated mice (Figure 6C) and was associated with vessels, whereas FGF-2 and lectin were barely detectable in rPAI-123-treated mice (rPAI-123, 0.25±0.25 vessels per field at ×65 magnification; versus saline, 7±0.9 vessels per field at ×65 magnification).

Quantitative PCR showed no significant reduction in FGF-2 mRNA copy number in rPAI-123–treated mice compared to age-matched chow-fed mice. However, it was reduced 8-fold compared to the saline counterpart (P=0.003). Quantitative analysis of VEGF-A165 mRNA levels showed no significant differences among the 3 groups. These data suggest that FGF-2 has a significant role in stimulating angiogenesis in the vasa vasorum and contributes to plaque growth. Furthermore, it shows that rPAI-123 blocks FGF-2-stimulated angiogenesis by regulating its transcription.

Innominate Artery Morphological Changes in Response to rPAI-123

The relationship between plaque growth and vessel remodeling was addressed by using morphological analyses of innominate artery cross-sections. Differences in vessel circumference, plaque and luminal area, and plaque composition at a site more proximal to the origin of diseased vessels were examined. Measurements of innominate artery circumferences in H&E-stained sections did not show a difference between rPAI-123–treated (Figure 7C) and saline-treated (Figure 7B) mice fed PD for 20 weeks (rPAI-123, 2.62±0.11 mm; saline, 2.46±0.18 mm; P=NS) (Figure 7D). However, the average circumference length of both groups was significantly greater than the 14-week PD group (1.85±0.06 mm; P<0.001) (Figures 7A and 6D). A comparison with age-matched chow-fed mice determined that the average circumference of 14-week PD mice was 18% larger than its chow-diet control (1.52±0.02 mm; P<0.001) (Figure 7D). Similarly, the 20-week PD groups had an average innominate artery circumference that was 20% larger than age-matched chow controls (1.98±0.18; P=0.01). The combined data indicate that larger vessel circumferences in 20-week atherogenic diet groups are attributable to outward remodeling and age-related vessel size. The data also demonstrate that vessel wall outward remodeling began to occur by 14 weeks of atherogenic diet.

Measurements of innominate artery luminal area in rPAI-123–treated mice were 2.14-fold greater than saline-treated mice (rPAI-123, 0.3±0.04 mm²; versus saline, 0.14±0.04 mm²; P<0.001) and 3.2-fold more than the 14-week PD group (0.09±0.01; P<0.001). Conversely, plaque area in rPAI-123–treated mice was reduced 64% when compared to saline treatment (rPAI-123, 0.09±0.01 mm²; versus saline, 0.25±0.02 mm²; P=0.01) and 25% less than 14-week PD mice (0.12±0.02) (Figure 7E). We conclude that both groups fed PD for 20 weeks experienced the same degree of outward remodeling in the innominate artery. However, the effects of...
rPAI-123 over a 6-week treatment period significantly reduced plaque area, which in combination with the enlarged circumference dramatically increased luminal area.

A comparison of average cell number in innominate artery plaques relative to plaque area determined that rPAI-123–treated mice had 1.9-fold more cells/mm² than saline-treated mice and 1.7-fold more than 14-week PD controls (rPAI-123, 11.16±1.6; saline, 5.6±0.87 [rPAI-123 vs saline, P=0.01]; 14-week PD, 6.4±1.1 [rPAI-123 vs 14-week PD, P=0.05]). Measurements of collagen area as a percentage of total plaque area show that rPAI-123–treated plaques had 1.7-fold more collagen/mm² than either the saline or 14-week PD control (rPAI-123, 72%; saline, 42%; 14-week PD, 43%; P=0.01).

Similarly, cell number/mm² of H&E-stained DA plaques was 1.58-fold greater in rPAI-123–treated mice (rPAI-123, 11.5±0.6; versus saline, 8.5±1.0; P=0.003), whereas collagen degradation was significantly reduced in these mice compared to the saline counterpart (59%; P=0.002). Analysis of plaque composition showed the presence of macrophages and MHCI cells in rPAI-123 and saline-treated mice (Figure 8A, 8B, 8D, and 8E). MHCI cells were relatively few in number (rPAI-123, 19±5 vs. saline, 26±5, P=NS) (Figure 8A and 8D), whereas macrophages were abundant in both treatment groups (Figure 8B and 8E). The significant difference between the 2 groups was the excess of apoptotic cells in macrophage-rich and/or collagen degraded areas in plaques of saline-treated mice (Figure 8C), which are absent in plaques responding to rPAI-123 treatment (Figure 8F) (rPAI-123, 3±1; versus saline, 18±1.5 TUNEL-positive degraded areas >2 mm² at ×100 magnification; P<0.001). Increased

Figure 7. Morphology measurements of the innominate artery. Innominate arteries, from mice fed PD for 14 or 20 weeks with variable treatment from weeks 14 to 20 were stained with H&E. Diet and treatment groups are: PD for 14 weeks (n=8) (A); PD for 20 weeks plus saline from weeks 14 to 20 (n=11) (B); PD for 20 weeks plus rPAI-123 from weeks 14 to 20 (n=13) (C). Regions of Interest software was used to obtain measurements of vessel circumference (D) and plaque area and lumen area (E). White bars indicate normal chow diet; gray bars, PD for 14 weeks; black bars, PD for 20 weeks plus saline from weeks 14 to 20; striped bars, PD for 20 weeks plus rPAI-123 from weeks 14 to 20. Data are shown as means±SEM, and probability values were determined by ANOVA. *P<0.05 vs control, **P<0.001 vs control.

Figure 8. Characterization of DA plaque composition. Sections of DA plaque from mice fed PD for 20 weeks and treated with saline (A through C) or rPAI-123 (D through F) were probed for: I-A/I-E (A and D); MOMA-2 (B and E); TUNEL (C and F). Magnification, ×100 (insets, ×40). N=5 mice per group.
apoptosis appears to explain the presence of large acellular areas in saline-treated plaques, a characteristic indicative of advanced plaque progression.29

Discussion

In this study, we found that inhibition of adventitial angiogenesis by a novel antiangiogenic protein, rPAI-123, dramatically reduced the number and size of second order vasa vasorum in atherogenic female LDLR−/− ApoB-48−deficient mice. Inhibition of angiogenesis led to a highly statistically significant reduction in plaque growth and plaque cholesterol levels. These results strongly suggest that adventitial angiogenesis and vasa vasorum play a key role in plaque growth and atherosclerotic arterial remodeling and that targeting this process can induce regression of atherosclerotic lesions.

We demonstrate that increased vasa vasorum density corresponds with adjacent vessels in significantly larger plaques accompanied by vessels in the DA wall. This evidence is supported by the antiangiogenic activity of rPAI-123 that significantly inhibits vasa vasorum expansion to the second order to result in an absence of vessels invading the plaque and reduced plaque area.

Plaque growth is a complex and poorly understood process. A previous study9 suggested that inhibition of plaque vasculature may reduce plaque progression, but the relationship between plaque vasculature and arterial wall vasculature remains undefined. A number of studies suggest that increased second order vasa vasorum is associated with increased atherosclerotic lesion size in humans and hypercholesterolemic animal models.8,9,30,31 The primary function of these vessels is thought to be transport of nutrients to the vessel wall. Increased microvessel density in the adventitia and plaque in humans is associated with plaque instability, hemorrhage, and rupture, all life-threatening events that can accompany atherosclerosis.32,33

Confocal Z-stack and microCT images were reconstructed to show that rPAI-123 inhibits adventitial vessel density in LDLR−/− ApoB-48−deficient atherogenic mice such that the vascular architecture no longer resembles the saline control. Vessels observed in reconstructed confocal images of rPAI-123–treated mice are short and discontinuous and in many cases do not appear to have a lumen.

MicroCT images of 14- and 20-week saline-treated atherosclerotic mice clearly show that second order vasa vasorum are associated with neighboring luminal plaque, whereas the absence of second order vasa vasorum in rPAI-123–treated mice is accompanied by undetectable plaque. These data demonstrate a distinct association between rPAI-123-induced reduction of vasa vasorum density and plaque growth. The association is validated by reconstructed 3D confocal images, which show the presence of fully formed vessels in the DA wall, plaque, and adventitia of saline-treated mice and their absence in rPAI-123–treated mice. These collective data provide evidence to support the concept that angiogenic vasa vasorum supply the plaque nutritional requirements for enhanced growth.

The high levels of FGF-2 expression detected in adventitial vascular structures of saline-treated mice appear to stimulate tubulogenesis and provide patterning guidance to vessels with a lumen. The significantly diminished FGF-2 levels in rPAI-123–treated mice are accompanied by short discontinuous vessels that lack a lumen. The association of FGF-2 with intraplaque vessels in saline-treated mice and its absence in rPAI-123–treated mice suggests that FGF-2 expression contributes to plaque growth. These conclusions are further validated by the highly significant loss of FGF-2 gene expression in response to rPAI-123 treatment, which inhibited plaque growth and progression. These data are consistent with our in vitro studies, which demonstrate rPAI-123 inhibition of FGF-2 angiogenic functions.26

We examined plaque growth in relationship to outward remodeling in the innominate artery, a smaller artery located in an area where blood flow would be more impaired by plaque growth. Treatment with rPAI-123 or saline was initiated after mice were fed PD for 14 weeks; therefore, both treatment groups would have plaque content similar to 14-week control mice at the onset of treatment. Plaque area in the DA and innominate arteries of rPAI-123–treated mice was reduced 73% and 64%, respectively, when compared to 20-week saline control mice and comparable to or less than the 14 week saline control. The results suggest that rPAI-123 inhibits plaque progression and promotes plaque regression.

Further analysis of the innominate artery indicates that the circumferences are expanded in all mice fed PD. However, the outward remodeling is more extensive in rPAI-123 and saline treatment groups that were fed PD for 20 weeks when compared to mice fed PD for 14 weeks. Expansion of the innominate circumference in rPAI-123–treated mice could partially explain their larger lumen area when compared to 14-week PD controls. However, the differences in lumen area between rPAI-123 and saline-treated mice fed PD for 20 weeks are clearly attributable to reduced plaque in response to rPAI-123. The collective data indicate that rPAI-123 inhibitory effects do not immediately prevent progression of the disease process but reduce plaque size over the course of treatment. This is supported by the presence of macrophages and MHC II cells in both treatment groups that presumably enter the plaque via the vasa vasorum before the onset of treatment. These data, combined with reduced plaque cholesterol levels, further suggest that rPAI-123 promotes plaque regression.

The vasa vasorum has been implicated as the origin of plaque vasculature and the promoter of plaque growth; however, there has not been any conclusive evidence that angiogenesis in the vasa vasorum promotes plaque development. Others have shown that cleavage products of extracellular matrix proteins with antiangiogenic activity can also reduce vasa vasorum and plaque growth, but they have not demonstrated the existence of plaque vessels.9,10 Moulton et al demonstrated that angiostatin, a plasminogen cleavage product, reduces plaque progression.9 Seventy-five days of angiostatin treatment at a concentration of 20 mg/kg per day reduced plaque area in the DA by 36%.9 Similarly, ApoE77– mice treated with endostatin, a cleavage product of collagen XVIII, at a dose of 20 mg/kg per day for 16 weeks achieved 57% reduction in DA plaque area.10 The rPAI-123 protein, a cleavage product of PAI-1, reduced plaque area by 62% in
LDLR<sup>−/−</sup>-ApoB-48-deficient mice within 6 weeks of treatment at a dose of 5.4 μg/kg per day.

This study supports our in vitro and ex vivo studies, which show that rPAI-1<sub>23</sub> is a potent inhibitor of arterial endothelial cell tubulogenesis, migration, and proliferation.<sup>34–36</sup> Taken together, the data suggest that rPAI-1<sub>23</sub> could have significant therapeutic potential in the treatment of atherosclerosis.

**Sources of Funding**

This study was supported by NIH grants HL69948 (to M.J.M.-K.) and HL53793 (to M.S.).

**Disclosures**

None.

**References**


The Antiangiogenic Activity of rPAI-1 Inhibits Vasa Vasorum and Growth of Atherosclerotic Plaque

Mary Drinane, Jessica Mollmark, Lyubomir Zagorchev, Karen Moodie, Baiming Sun, Amy Hall, Samantha Shipman, Peter Morganelli, Michael Simons and Mary Jo Mulligan-Kehoe

_Circ Res._ 2009;104:337-345; originally published online January 2, 2009; doi: 10.1161/CIRCRESAHA.108.184622

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/3/337

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/01/02/CIRCRESAHA.108.184622.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Online Figure 1. Detection of adventitial vessels in mice fed Paigen’s diet until the onset of treatment. Twelve week old female LDLR<sup>−/−</sup>ApoB-48 deficient mice were fed Paigen’s diet without cholate for 14 weeks. Adventitial vessels in the descending aorta were probed for CD31 and detected in reconstructed confocal Z-stack images.
Online Figure II. Morphological staining of descending aorta plaque. Histological cross sections of plaque in descending aortas of atherogenic mice treated with saline (A and B) or rPAI-1$_{23}$ (C and D) were stained with hematoxylin and eosin (A and C) or picro-sirius red (B and D).
Online figure III. Negative controls for tissue probes. 
Cross sections of descending aorta plaque from mice fed Paigen’s diet without cholate for 20 weeks and received daily intraperitoneal injections of either saline or rPAI-1_{23} served as negative controls for: (A) MHCII marker I-A/I-E; (B) MOMA-2 or (C) TUNEL. The sections were subjected to the same procedure as Figure 8 except the primary antibody was not incubated with the sections.
SUPPLEMENTAL METHODS

Online Method 1
B6; 129S-Apob<sup>tm2Sgy</sup> Ldlr<sup>tm1Her</sup>/J mice
This strain was selected because it is reported to more closely mimic human atherosclerosis than other models. This strain is deficient in low density lipoprotein receptor (LDLR<sup>−/−</sup>) and has a point mutation in the apolipoprotein B-48 codon so that only ApoB-100 is expressed.

Online Method 2
Paigen’s diet composition
Paigen’s atherogenic diet<sup>1</sup> without cholate = 20% protein, 45% carbohydrate, 35% fat, no cholate

Online Method 3
Determination of rPAI-1<sub>23</sub> in vivo dose.
The rPAI-1<sub>23</sub> dose was based on in vivo Matrigel plug assays in C57B6 mice that tested the ability of 5.4 mg/kg/day and 20 mg/kg/day of rPAI-1<sub>23</sub> to inhibit FGF-2 and VEGF stimulated vessel migration. The dose that was one log below the lowest inhibitory dose (5.4 µg/kg/day) was tested in breast and pancreatic tumor models.

Online Method 4
Preparation of tissue.
At the end of each treatment period the animals were fasted overnight, then given an injection of 0.1 ml ketamine per 30 grams of weight. The chest was opened and blood was drawn by heart puncture. The mice were euthanized, perfused with phosphate buffered saline (PBS) followed by 3.5% paraformaldehyde under 110-120 mm/Hg pressure. The innominate artery, carotid arteries, aortic arch and descending aorta (DA) to the iliac bifurcation were surgically removed. Innominate arteries were removed at the interface with the aortic arch and placed in O.C.T. tissue embedding compound (Sakura Finetek USA, Inc., Torrance, CA). Five micron serial sections began at the base of the artery to obtain consistent measurements among all mice. Serial sectioning continued through 12 sections followed by removal of a 10 micron section and then 0.5 micron serial sectioning for an additional 12 sections. Descending aorta sections were 15 micron.

Online Method 5
Plaque cholesterol measurement. Mice were sacrificed, descending aortas were removed and weighed. Cholesterol was extracted in 2 parts chloroform: 1 part methanol for 16h at room temperature. Each sample was aliquoted into glass vials and evaporated to dryness. Cholesterol was rehydrated in PBS, equal volume aliquots were placed in cholesterol reagent (Raichem, San Diego, CA) and incubated at 37° C for 10 minutes before reading absorbance at 500 nm. Cholesterol concentration was calculated from a purified cholesterol standard curve. Six aortas per test group were measured in triplicate.
Confocal microscopy and vessel reconstruction.

Descending aortas were removed with a wide margin and made permeable in an overnight incubation at 4°C in PBS containing 1% BSA and 0.1% Triton X-100. The aortas were then incubated overnight at 4°C with 5 µg/ml anti-mouse CD31 (BD Biosciences, Franklin Lakes, NJ). A fluorescein-conjugated goat anti-rat secondary antibody (Invitrogen-Biosciences, Carlsbad, CA) amplified the binding reaction and enabled detection at 488 nm. CD-31 probed adventitial vessels were imaged on a Zeiss LSM-510 META point scanning confocal microscope and Z-stacks were collected (40X objective, 0.7 scan zoom, 0.7 scan zoom, 135 µm pinhole, and 325.8 x 325.8 x 9.0 µm stack size). The Z-stack images were reconstructed using Volocity V3.7 software (Improvision, Coventry, UK).

Confocal microscopy and vessel reconstruction (lectin perfusion)

Fifteen micron sections of the descending aorta were analyzed for vessels in the adventitia, DA wall and plaque. Sections were incubated with Cy5-conjugated smooth muscle actin (SMA) monoclonal antibody (Sigma, St. Louis, MO). The unbound antibody was removed and 5 µg/ml of fluorescein-labeled Lycopersicon esculentum lectin (Tomato) (Vector Labs, Burlingame, CA) was added for 5 minutes at room temperature. Confocal Z-stack images of SMA and lectin probed adventitia, DA wall and plaque were collected with a 63x objective, 1.0 scan zoom, 218 µm pinhole, 146.2 x 146.2 x 7.0 µm stack size.

Quantitative realtime PCR of growth factor RNA.

RNA was isolated and purified from the DA of atherogenic mice treated with rPAI-123 or saline for 6 weeks and age-matched chow fed mice using Tri Reagent (Sigma) as described in the manufacturer’s instructions (5 mice per group). RNA was reverse transcribed into cDNA using a First Strand Synthesis kit (Roche, Indianapolis, IN). Quantification of VEGF-A165, FGF-2 and β-actin gene expression was performed by the Realtime PCR technique using the GeneAmp 5700 system with SYBR Green reagents (PE Applied Biosystems, Foster City, CA). Primer design and optimal primer pair selection were done with OligoPerfect™ Designer software (Invitrogen, Carlsbad, CA). PCR reactions were run under PE Applied Biosystems universal thermal cycling conditions. All samples were tested in triplicate in two separate experiments. Relative mRNA copy number was calculated by the 2^(-ΔΔCt) method.
through the interpolated volumes, as detected by the co-localized probes, were considered. The obtained contours were modeled with cubic B-Spline curves and stacked in 3-D to provide volumetric surface representation as a closed triangulated mesh.

**Online Method 10**  
**Infusion of microfil into vessels.**  
Mice were anesthetized with Ketamine (80 mg/kg) / Xylazine (10mg/kg) (Webster Veterinary Supply, Sterling, MA) and at the same time heparinized, to ensure effective removal of blood volume during perfusion. A 21G butterfly needle was inserted into the ascending aorta through the left ventricle and the inferior vena cava was cut to allow venous drainage. The animals were perfused with PBS and adenosine (1mg/ml). Next, a mixture containing silicone rubber compound Microfil Blue (1 ml), Microfil Clear (3 ml), diluent (8 ml) and curing agent (0.6 ml)(Flow Tech, Inc., Carver, Massachusetts) was infused through the aortic cannula. Descending aortas with a wide adventitial margin were removed after polymerization was complete. N= 3 per group.

**Online Method 11**  
**MicroCT imaging of the vasa vasorum.**  
Descending aortas containing microfil were scanned with a GE eXplore Locus SP microCT scanner. After optimizing the scanning protocol for soft tissue imaging, the animals were scanned at a voltage and current of the x-ray tube of 52 kV and 118 mA, respectively. The x-ray exposure time for a single projection was set to 1700 ms and a total of 720 projections per scan were acquired at the maximum resolution of the x-ray detector, 6.5 microns. Following the scan, three-dimensional volumetric images were reconstructed from the acquired two dimensional projections without averaging, yielding a final voxel size of 6.5 microns and data volumes of approximately 1.5Gbs.

**References**