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Anti-angiogenic Activity of rPAI-1₂₃ and Vasa Vasorum Regression

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Abstract

The vasa vasorum are a unique network of vessels that become angiogenic in response to changes in the vessel wall. Structural studies, using various imaging modalities, show that the vasa vasorum form a plexus of microvessels during the atherosclerotic disease process. The events that stimulate vasa vasorum neovascularization remain unclear. Anti-angiogenic molecules have been shown to inhibit/regress the neovascularization; they provide significant insight into vasa vasorum function, structure and specific requirements for growth and stability. This review discusses evidence for and against potential stimulators of vasa vasorum neovascularization. Anti-angiogenic rPAI-1₂₃, a truncated isoform of plasminogen activator inhibitor-1 (PAI-1) stimulates a novel pathway for regulating plasmin activity. This mechanism contributes significantly to vasa vasorum regression/collapse and will be discussed as a model of regression.

Vasa Vasorum Origination

The vasa vasorum are a network of microvessels that originate primarily in the adventitial layer of large arteries. They branch from arteries at various anatomical locations. Vasa vasorum in the ascending aorta originate from the brachiocephalic and coronary arteries. They branch from intercostals in the descending thoracic artery and from the lumbar and mesenteric arteries in the abdominal aorta. The vasa vasorum in coronary arteries originate from bifurcation segments of epicardial vessels (Clarke, 1965). The adventitial vasa vasorum have heterogeneous structure and density among different vascular beds (Galili et al., 2004).

Vasa Vasorum Structure

In 1984, Barger, *et al.* demonstrated that the vasa vasorum are rarely detected in human coronary arteries in the absence of atherosclerotic plaque (Barger et al., 1984). However, in diseased vessels, they form a dense plexus of microvessels. Others, who examined the vasa vasorum structure in atherosclerotic rabbit carotid arteries describe them as a longitudinal vessel that branches to form a circumferential plexus around the vessel wall (Barker et al., 1992).

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More recent advances in imaging technology have enabled acquisition of high resolution images of the vasa vasorum (Zagorchev and Mulligan-Kehoe, 2011), for review. Micro computed tomography (mCT) images of the vasa vasorum in diseased vs. non-diseased porcine coronary arteries show distinct anatomical differences (Kwon et al., 1998a), which are comparable to what Barger found in humans (Barger et al., 1984). The mCT images revealed two types of vasa vasorum which the authors describe as first and second order. The first order originates from the coronary artery and runs longitudinally along the adventitia while the 2nd order branches from the first order, forming a plexus circumferentially around the vessel wall. The pigs with normal hearts have significantly greater first order vasa vasorum density compared to the second order (ratio 3:2). However, the 2nd order vessel density is 2-fold greater than the 1st order in hypercholesterolemic pigs. The increased vessel density correlates with an increased arterial wall area, but precedes plaque development (Kwon et al., 1998a; Kwon et al., 1998b). The 2nd order vasa vasorum can also be detected by mCT in mouse models of atherosclerosis (Supplemental Figure 1). These studies are discussed in detail elsewhere (Mulligan-Kehoe, 2010).

Our laboratory uses confocal microscopy at 0.7 μ m resolution to detect a vasa vasorum plexus in hypercholesterolemic LDLR^{-/-}/ApoB^{100/100} mice perfused with FITC-labeled lectin (Figures 1B and E), which collapses in response to rPAI-1₂₃ (Figures 1C and F). The vessels in the plexus have a diameter in the 5 μ m range and are located between larger branches of a vascular tree. The plexus is not present in chow-fed mice (Mollmark et al., 2012).

Micro CT analysis of vasa vasorum branching patterns in non-diseased pigs shows a dichotomous tree structure with a hierarchical structure in which each branch pair is at a higher generation than the parent branch (Gossl et al., 2003). However the branching lacks uniformity in that some continue to branch while others discontinue the process. Although we can detect the vasa vasorum in the adventitia of chow-fed LDLR^{-/-}/ApoB^{100/100} mice, they do not exhibit a branching pattern (Figures 1A and D).

The conclusion that can be drawn from the cumulative studies using various animal models and imaging modalities is that the angiogenic vasa vasorum form a plexus in response to the disease process. The plexus is not present in healthy animals.

Vasa Vasorum Function

Structural studies clearly show that the vasa vasorum are poised to rapidly respond to changes in the vessel wall. A primary response is to provide nourishment and oxygen to the outer third of the avascular media when the aorta exceeds a certain thickness, which in mammals is 0.5 mm or 29 lamellar units (Wolinsky and Glagov, 1967). Several studies provide evidence to support this concept. For example, intercostal arteries in the descending aorta of dogs were ligated to block blood flow to the vasa vasorum. Necrotic tissue was detected in the middle media ten days post ligation, while the inner third and outer third layers were not necrotic (Wilens et al., 1965).

Other studies used probes to examine diffusion of oxygen from the luminal or abluminal side of the canine femoral artery wall. They found that oxygen levels are highest in outer layers of the vessel wall and they decrease as the probe approaches the lumen. The data suggest that circumferential adventitial vessels are the primary source of oxygen in the femoral artery (Moss et al., 1968).

These studies substantiate the idea that vasa vasorum expand to meet the nutritional needs of the vessel wall in mammals. The cue for initiation of vasa vasorum neovascularization is seemingly linked to an increase in vessel wall thickness. However, the adventitial vasa

vasorum in mouse models of atherosclerosis becomes highly vascularized (Drinane et al., 2009; Mollmark et al., 2011; Mollmark et al., 2012; Moulton et al., 1999; Moulton et al., 2003) despite the fact that their artery walls do not exceed the 0.5 mm limit for diffusion. Moreover, there is evidence that the coronary artery undergoes neovascularization prior to thickening of the vessel wall (Kwon et al., 1998b). Other groups demonstrated that significant vasa vasorum neovascularization in the porcine coronary artery precedes development of early atherosclerotic endothelial dysfunction and plaque development (Herrmann et al., 2001; Kwon et al., 1998b).

From these studies it remains unclear if the angiogenic vasa vasorum contribute to atherosclerotic disease progression or are a response to the disease response. Despite this uncertainty, we know there is a significant correlation between vasa vasorum density and plaque progression in humans (Kolodgie et al., 2004; Virmani et al., 2000). Human studies demonstrate that vasa vasorum density is significantly increased in plaques categorized as vulnerable and those that rupture when compared to stable plaques having significant luminal narrowing. Furthermore they show that increased microvessel density is associated with intraplaque hemorrhage, a characteristic of unstable lesions (Kolodgie et al., 2004; Tenaglia et al., 1998; Virmani et al., 2002; Virmani et al., 2005).

The work of Khurana *et al.* addressed the specific role of angiogenesis in relationship to intimal thickening (Khurana et al., 2004). They used two distinctly different animal models to induce neointimal formation, each by a different mechanical stimulation. In both sets of experiments, pro-angiogenic molecules, anti-angiogenic molecules or a combination of both were applied to the adventitial space to examine smooth muscle cell accumulation. The data show that stimulation of angiogenesis in adventitial vessels is not sufficient to induce neointima thickening, but once the event is initiated by way of injury or mechanical stimulus, the pro-angiogenic molecules promote growth of the intimal lesion. Angiogenesis inhibitors block the effect of neointima thickening induced by pro-angiogenic factors.

Factors That Stimulate Vasa Vasorum Neovascularization

Hypoxia has been considered to be a link between vasa vasorum angiogenesis and plaque growth. One hypothesis is that hypoxia induces transcription of vascular endothelial growth factor (VEGF), a predominant angiogenic growth factor. Studies have shown that extensive neovascularization of the vasa vasorum corresponds with up regulation of VEGF and its transcriptional regulator, hypoxia inducible factor-1 α (HIF-1 α) (Bayer et al., 2002). Others have shown that VEGF is expressed in 97% (32/33) of atherosclerotic and restenotic human coronary arteries that were examined. However, it co-localizes with a marker of lymphocyte activation and has no correlation with vasa vasorum neovascularization (Couffignal et al., 1997). Other studies show that VEGF in atherosclerosis has a more prominent pro-inflammatory than pro-angiogenic role (Celletti et al., 2001; Zhao et al., 2004).

Recent studies demonstrate that hypoxia in advanced human carotid plaques correlate with the presence of macrophages and angiogenesis (Sluimer et al., 2008). They further demonstrate comparable findings in hypercholesterolemic LDLR^{-/-} mice. In both cases, hypoxia is predominantly determined by inflammatory cell content and not vessel wall thickness. The authors conclude that hypoxia is stimulated by the high metabolic demand of inflammatory cells.

Our laboratory has shown in hypercholesterolemic LDLR^{-/-}ApoB^{100/100} mice that VEGF mRNA is not elevated and its protein expression is mostly undetectable in the adventitia and plaque (Drinane et al., 2009). On the other hand, FGF-2 mRNA is elevated 8-fold in hypercholesterolemic mice compared to the chow-fed control and mice treated with the angiogenesis inhibitor, rPAI-1₂₃ (Drinane et al., 2009). These data suggest that FGF-2 has a

significant role in stimulating angiogenesis in the vasa vasorum and contributes to plaque growth while VEGF appears to have no influence, at least within the examined time frame. The data further suggest that FGF-2 plays a role in defining the vasa vasorum plexus architecture (Supplemental Figure 2)(Mollmark et al., 2012).

Others have shown that FGF-2 delivered to the adventitia stimulates significant angiogenesis in rats (Cuevas et al., 1991; Edelman et al., 1992). On the other hand, when it is delivered to the media, FGF-2 stimulates smooth muscle cell proliferation. There is evidence that endothelial cells respond to FGF-2 stimulation under hypoxic conditions. This response is due to hypoxia stimulated synthesis of heparan sulfate chains that increase the number of heparan sulfate binding sites for FGF-2 (Li et al., 2002). This in turn enables FGF-2 signaling through its receptor.

These data suggest that hypoxia may be the basis for activation of both FGF-2 and VEGF-A. However, FGF-2 appears to stimulate the angiogenesis while VEGF influences the inflammatory process.

Vasa Vasorum and Inflammation

The seminal work of Moulton, *et al* (Moulton et al., 2003) demonstrated that angiostatin, a plasminogen cleavage product and angiogenesis inhibitor (O'Reilly et al., 1994), blocks expansion of adventitial vasa vasorum, which correlates with reduced macrophage accumulation in plaque. Although these results were correlative, they introduced the concept that the vasa vasorum may serve as a conduit for infiltration of inflammatory cells into the arterial wall and plaque. The work of others supports this “outside in” theory to partially explain inflammatory cell infiltration into the vessel wall (Galkina et al., 2006; Maiellaro and Taylor, 2007).

More recent studies by Eriksson (Eriksson, 2011) used multiple imaging modalities to show in ApoE^{-/-} mice that the adventitial microvasculature recruits leukocytes. Interactions of leukocytes with the endothelium occur primarily in lesion venules rather than the arterial lumen. Fluorescently labeled myelomonocytic cells provided further evidence that extravasation is from venules into plaque and not from arterioles or capillaries. This study supports the “outside-in” theory.

Vasa Vasorum Regression

Experiments in monkeys were among the first to analyze the impact of plaque regression on vasa vasorum blood flow. Monkeys were fed an atherogenic diet for 18 months, half of the group was sacrificed at the end of the diet while the other half was removed from the atherogenic diet and fed normal chow for an additional 18 months. Blood flow through the vasa vasorum was measured by injecting isotope-labeled microspheres into the left atrium. Regression of the plaque resulted in loss of vasa vasorum accompanied by a significant reduction in blood flow through the vasa vasorum into the intima-media of the coronary arteries (Williams et al., 1988).

The discovery of angiogenesis inhibitors and development of genetically modified mice have enabled scientists to test the effects of an inhibitor on vasa vasorum neovascularization and corresponding plaque growth as well as define mechanistic pathways. Interestingly, many of the inhibitors are cleavage products of an extracellular protein (Folkman, 2004) several of which have been shown to inhibit vasa vasorum neovascularization (Drinane et al., 2009; Moulton et al., 1999; Moulton et al., 2004; Moulton et al., 2003).

The anti-angiogenic activity of endostatin, a 20-kDa C-terminal fragment of type XVIII collagen, was shown to significantly inhibit plaque growth and vasa vasorum density in atherogenic ApoE^{-/-} mice (O'Reilly et al., 1997). Subsequent studies identified an endostatin interaction with the proteoglycan biglycan that interferes with LDL retention. Biglycan/LDL/subendothelial matrix interactions promote LDL retention; blockage of the interaction prevents macrophage uptake of LDL (Zeng et al., 2005), a primary cause of atherosclerosis.

Studies that examined the anti-angiogenic effects of angiostatin in atherogenic ApoE^{-/-} mice found that the vasa vasorum were expanded in some but not all plaques (Moulton et al., 2003). They found a significant linear correlation between infiltrated mononuclear cells and vasa vasorum density, but not with plaque size.

Others examined the effects of thalidomide (Gossl et al., 2009), an anti-angiogenic (D'Amato et al., 1994) and anti-inflammatory drug (Aydogan et al., 2008), on neovascularization in hypercholesterolemic pigs at early stages of atherogenesis. They found that chronic thalidomide administration prevented vasa vasorum expansion presumably through reduced VEGF expression, but did not alter early inflammation associated with vasa vasorum ECs. The reduced vasa vasorum density was associated with reduced intima-media thickness (Gossl et al., 2009).

Anti-angiogenic rPAI-1₂₃, a truncated isoform of plasminogen activator inhibitor-1 (PAI-1), causes regression of angiogenic vasa vasorum and atherosclerotic plaque (Drinane et al., 2009; Mollmark et al., 2011). It stimulates endothelial cell specific apoptosis and is an antagonist of its parent protein's anti-proteolytic function (Mollmark et al., 2011).

Vascular homeostasis requires both pro- and anti-angiogenic factors. The activity of these factors is tightly regulated in quiescent tissues. Pathological conditions alter the regulated balance, which may result in abnormal growth of the vasculature or defective repair processes. Since pro- and anti-angiogenic factors are primarily secreted molecules, the extracellular matrix (ECM)/basement membrane (BM) plays a critical role in regulating angiogenesis. It provides an appropriate scaffold to support endothelial cell adhesion, vessel formation, stability, maturation and increased density. ECM/BM compositional changes are required to provide an environment suitable for activation of angiogenic factors and neovascularization (Rhodes and Simons, 2007), for review.

Degradation of the ECM/BM leads to vessel collapse/regression (Davis and Saunders, 2006; Davis and Senger, 2005). Proteases that degrade the ECM/BM play a key role in matrix remodeling during normal wound healing and in vascular diseases. Plasmin, a primary ECM/BM protease, contributes to matrix remodeling through its own proteolytic activity and by activating numerous matrix metalloproteinases (MMPs). Of those, MMP-1, -3, -9, -10 and -13 promote capillary network regression (Davis et al., 2001; Saunders et al., 2005). Plasmin also contributes to ECM remodeling by degrading fibrin, an ECM protein that forms a supportive scaffold for angiogenic vessels (Dvorak et al., 1983). Fibrin, the major constituent of provisional matrix, enables endothelial cells to adhere, spread and proliferate (Cheresh et al., 1989). Plasmin also degrades nidogen (Mayer et al., 1993) and perlecan (Whitelock et al., 1996), two of the four key components of the ECM/BM that are important in blood vessel formation (Rhodes and Simons, 2007), for review.

PAI-1 is the primary inhibitor of plasmin production. It binds plasminogen activators tPA and uPA to prevent them from converting plasminogen to proteolytic plasmin. We have studied extensively the mechanisms by which rPAI-1₂₃ activity blocks the function of PAI-1 and how it modifies vasa vasorum neovascularization in LDLR^{-/-}-ApoB^{100/100} mice. In this

model, PAI-1 protein expression is elevated 2-fold in hypercholesterolemic mice. Mice treated with rPAI-1₂₃ have a 2-fold reduction in PAI-1 activity that is at levels measured in chow-fed, non-hypercholesterolemic mice. The reduction in PAI-1 activity is accompanied by a 1.6-fold elevation in plasmin activity when compared to the saline treated counterpart. Despite the reduction in PAI-1 activity, its protein expression level remains elevated (Mollmark et al., 2011). These data suggested that rPAI-1₂₃ could counter the elevated PAI-1 expression by preventing its inhibition of tPA conversion of plasminogen to plasmin.

Further mechanistic studies uncovered a novel mechanism(s) by which rPAI-1₂₃ blocks the anti-proteolytic function of PAI-1 (Mollmark et al., 2011). Biochemical interactions of rPAI-1₂₃ and PAI-1 with plasminogen (Plg) and tissue plasminogen activator (tPA) show that rPAI-1₂₃ and PAI-1 act synergistically to enhance plasmin activity (Figures 2A and 2B). This is achieved through a rPAI-1₂₃/Plg complex that stimulates binding of PAI-1 to Plg (Mollmark et al., 2011). The complex seemingly sequesters PAI-1 such that it is no longer available to bind and inhibit tPA (Supplemental Figure 3).

The elevated plasmin activity stimulated by rPAI-1₂₃ has a dramatic effect on vasa vasorum stability; it degrades perlecan, nidogen and fibrin(ogen), all key components of the BM/ECM. The result is endothelial cell death and collapse of the vasa vasorum. These three key components of the ECM/BM have an enormous impact on vessel stability; altogether they contribute to the supportive scaffold for angiogenic vessels (Mollmark et al., 2011) and angiogenic growth factors (Mollmark et al., 2012).

These data are counter intuitive to the long-held belief that plasmin is angiogenic, because it is the primary protease that degrades fibrin, the provisional matrix on which angiogenic endothelial cells migrate. However, more recent studies show that angiogenic endothelial cells invade fibrin matrices in a plasmin-independent manner (Hiraoka et al., 1998).

Confocal images of the vasa vasorum in hypercholesterolemic LDLR^{-/-}ApoB^{100/100} mice suggest that the angiogenic plexus branches form a tree-like structure (Figure 3B) and the plexus vessels are the ones which undergo apoptosis and collapse in response to rPAI-1₂₃ treatment (Figure 3C). The plexus vessels in saline treated LDLR^{-/-}ApoB^{100/100} / PAI-1^{-/-} mice (produce excess plasmin) undergo pruning/degradation (Figure 3D) and completely collapse in response to rPAI-1₂₃ (Figure 3E). These data demonstrate that the plexus vessels are susceptible to plasmin remodeling/pruning or collapse.

Their development may be dependent upon distinct spatial distribution of BM/ECM components that provide the supportive scaffold necessary for angiogenic vessel stability. The data also suggest that the plexus is transient depending upon the physiological status of the vessel wall, while the larger branches are stable and poised to support angiogenesis.

Summary

Development of an angiogenic plexus is a unique characteristic of the vasa vasorum; one that is not found in other post-natal vessels. This is seemingly due to its importance in responding rapidly to the nutritional needs of the vessel wall. Once the nutritional demands have been met, the plexus collapses. Pathological conditions, particularly atherosclerosis, maintain the angiogenic process. However, the events and specific factors that stimulate angiogenesis need further clarification. Our studies indicate that factors, which respond to normal wound healing, play a significant role in expansion and collapse of the vasa vasorum plexus.

All of the described anti-angiogenic molecules inhibit the vasa vasorum and reduce plaque size. Each molecule achieves that effect through a different mechanism. Various

combinations of the treatments may achieve a more effective therapeutic. The rPAI-1₂₃ stimulated increase in plasmin activity and reduced PAI-1 activity holds the potential for monitoring the progress of rPAI-1₂₃ treatment through well established assays. This could provide a means for individualized treatment regimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Vasa vasorum form a plexus in hypercholesterolemic mice

LDLR^{-/-}/ApoB^{100/100} (DKO), were fed normal chow diet (CH) or high fat, high cholesterol Paigen's diet without cholate (PD). The PD-fed mice develop plaque and angiogenic adventitial vasa vasorum. The PD mice were treated with saline or anti-angiogenic rPAI-1₂₃ for the last 6 weeks of PD. Mice were perfused with FITC-labeled lectin (white).

Descending aorta whole mounts were imaged by confocal microscopy at 20× magnification (A) CH; (B) PD, saline; (C) PD, rPAI-1₂₃ and at 63× magnification (D) CH; (E) PD, saline; (F) PD, rPAI-1₂₃. Arrows indicate position of plexus. N=8 per group. Scale bar = 20μm.

Courtesy of J Mollmark, A Park et al., *Arteriogenesis, Thrombosis and Vascular Biology*, 9/2012, in press.

Figure 2. rPAI-1₂₃ and PAI-1 act synergistically to modulate plasmin activity

(A) Biochemical reactions containing plasminogen, tPA, rPAI-1₂₃ and PAI-1 were performed. The concentrations of rPAI-1₂₃ and PAI-1 were varied. The reactants were combined simultaneously in a reaction mixture and incubated at 37° C. for 1h. Plasmin activity was measured in a Chromozym PL assay. Data shown as mean ± standard deviation and probability values were determined by ANOVA. *p 0.05, **p 0.001 vs. 90 nM rPAI-1₂₃.

S = reactants added simultaneously; L = plasminogen incubated for 1h at 37° C before adding tPA.

Note that PAI-1 anti-proteolytic activity is blocked by rPAI-1₂₃ in a dose dependent manner.

(B) Schematic illustrating the effects of rPAI-1₂₃ and PAI-1 interactions with plasminogen.
Courtesy of J Mollmark, S Ravi et al., *Circ. Res.*, 2011.

Figure 3. Plasmin remodels the angiogenic vasa vasorum

Atherosclerosis mouse model LDLR^{-/-}ApoB^{100/100} (DKO) and PAI-1 deficient LDLR^{-/-}ApoB^{100/100}/PAI-1^{-/-} (DKO/PAI-1^{-/-}, plasmin in excess) mice were CH-fed for 32 weeks or CH for 12 weeks followed by PD from 12–32 weeks of age and treated with saline or rPAI-1₂₃ for the last 6 weeks of PD. Mice were perfused with FITC-labeled lectin to detect plasmin effects on adventitial vessel structure. Descending aorta whole mounts were imaged by confocal microscopy at 20× magnification. (A) DKO, CH (B) DKO, PD, saline; (C) DKO, PD, rPAI-1₂₃; (D) DKO/PAI-1^{-/-}, PD, saline; (E) DKO/PAI-1^{-/-}, PD, rPAI-1₂₃. Arrows indicate position of plexus. Scale bar = 20 μm. Courtesy of J Mollmark, A Park et al., *Arteriogenesis, Thrombosis and Vascular Biology*, 2012.