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Neovascularization in Human Atherosclerosis

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In the absence of disease, the vasa vasorum nurture the outer component of the vessel wall, and the intima is fed by oxygen diffusion from the lumen. As disease progresses, the intima thickens, and oxygen diffusion is impaired. As a result, vasa become the major source for nutrients to the vessel wall.1

The vasa vasorum structure consists of a network of small arteries and veins, as shown in Figure 1. In the coronary arteries, vasa originate from bifurcation segments of epicardial vessels; in the ascending aorta, vasa originate from coronary and brachiocephalic arteries; and in the descending thoracic aorta, vasa originate from intercostal, lumbar, and mesenteric arteries.1

Regulation of Blood Flow

Sympathetic fibers help vasa to regulate blood flow, as shown in Figure 2. Vasa react to adenosine and endothelin-1.2,3 However, vasa appear to be relatively insensitive to thromboxane A2, norepinephrine, and angiotensin II, providing neuronal protection against ischemia during sustained sympathetic activity.3 The vasa vasorum are also sensitive to acetylcholine, histamine, isoprenaline, adenosine triphosphate, adenosine diphosphate, adenosine, and sodium nitroprusside.4 Additionally, precontracted vasa exhibit endothelium-dependent vasodilatation to bradykinin and substance P, which are mediated by endothelium-dependent hyperpolarization and nitric oxide, respectively.4 As a result, vasa response to some agonists seems different from that of other vessels of similar caliber. The molecular mechanisms underlying this selectivity are not completely elucidated but may involve hypoxia, primarily mediated by the hypoxia-inducible factor-1α (HIF-1α). The HIF transcription factor is composed of 2 subunits: an ubiquitous HIF-1β subunit and a hypoxic responsive subunit HIF-1α.5 Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylases at 2 distinct proline residues. This modification is oxygen dependent and requires the cofactors 2-oxoglutarate, vitamin C, and iron. The hydroxylation in turn targets HIF toward the ubiquitin-proteasomal pathways for degradation. However, under hypoxic conditions, the inactivation of the prolyl hydroxylases allows HIF-1α protein stabilization and dimerization with HIF-1β subunit, upregulating HIF-dependent pathways and multiple target genes, including nitric oxide synthase and the angiogenic vascular endothelial growth factor-A.6 The hypoxic stabilization of HIF clearly represents a mechanism of plaque angiogenesis. Additionally, reactive oxygen species (ROS) generated within atherosclerotic plaques may independently regulate HIF expression through prolyl hydroxylase–dependent and –independent mechanisms.7

Removing Vasa Vasorum: Experimental Results

The effects of removing vasa were analyzed in several experimental animal models.8–10 Medial necrosis and macrophage and smooth muscle cell infiltration occurred within a period of 7 days even with a morphologically intact endothelium, suggesting a role for adventitial vasa vasorum in the initial phases of the disease.10

Neovascularization and the Atherosclerotic Process

Neovessels and Plaque Progression

Neovascularization is the process of generating new blood vessels mediated primarily by progenitor and/or endothelial cells leading to tube formation, resulting in a stabilized neovascular channel.11 Angiogenesis, the predominant form of neovascularization in atherosclerosis, is mediated by endothelial cells sprouting from postcapillary venules, leading primarily to new capillaries.12 As discussed above, the molecular mechanisms responsible for neovessel formation are related predominantly to hypoxia.12 More recently, hypoxia-independent pathways have also been described, mediated primarily by inflammation and activation of the Toll-like receptor.13

Neovascularization occurs when the tunica intima thickens up to 500 μm, although recent observations suggest that it may occur earlier.14 Groszek and Grundy suggested that angiogenesis facilitates atherosclerosis through lipoprotein “leak” into the intima from permeable microvessels.15 The same authors speculated that atherogenesis occurs when there is a relative paucity of vasa vasorum because this would lead to a decreased efflux of lipoproteins from the artery wall.15 However, neovascularization is extremely rare in the absence of atherosclerosis, suggesting that intimal disease occurs first and angiogenesis follows.

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Coronary neovascularization may also accompany the early process of vessel wall remodeling, as observed in hypercholesterolemic swine. In this animal model, neovascularization is present as early as 4 to 6 weeks after beginning a hypercholesterolemic diet, preceding endothelial dysfunction, which became evident only after 6 to 12 weeks. As a result, neovessels may play an important role in early atherogenesis.

Jeziorska and Woolley evaluated neovessels in early human lesions. Fatty streaks and preatheromas showed either sparse or extensive neovascularization, usually at the site of inflammatory cells, as shown in Figure 3. Of note, apolipoproteins A-I and B were observed around neovessels, suggesting local lipid depositions derived from the microvasculature. It is noteworthy that extravasation of red blood cells (RBCs) or intraplaque hemorrhage was not observed. As a result, it is clear that neovascularization in early atherosclerosis is associated with inflammation and lipid deposition, leading to plaque progression into more advanced lesions.

Plaque neovessels in advanced lesions were evaluated by Barger et al using cinematography, as shown in Figure 4. Neovascularization was distributed from the epicardial fat to the plaque throughout vessel wall. A decade later, Zhang et al identified adventitial vasa vasorum as the only source for microvessels in human coronary arteries. Total microvessel content correlated with intimal thickness and luminal stenosis. Most importantly, extravasation of albumin within the plaque showed an impressive rank correlation of 0.99 with microvessels. However, this study only identified neovessels originating from adventitial vasa and omitted the lumen as a potential source. To further elucidate this issue, Kumamoto et al identified the vessel lumen as a source for microvessels. Nevertheless, neovessels from adventitial vasa were 28 times more numerous (96.5%) than those from the lumen (3.5%). Neovessels from vasa origin characterized severely stenotic lesions and correlated with the extent of inflammatory cell infiltration and lipid core size. On the contrary, neovessels from lumen origin were found in plaques with 40% and 50% stenosis and were associated more often with intraplaque hemorrhage or hemosiderin deposits.

Neovascularization and Leukocyte Recruitment
Neovessels may also serve as a pathway for leukocyte recruitment to high-risk areas of the plaque, including the cap and shoulders. The pivotal work of O’Brien et al documented the mechanisms underlying neovessel recruitment of plaque leukocytes in human atherosclerosis. The expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin were 2- to 3-fold higher on neovessels than on arterial luminal endothelium, confirming the predominant role for neovessels as a pathway for leukocyte infiltration in human coronary plaques. More recently, our group documented histological evidence for atherosclerotic neovascularization as a pathway for macrovessels.
phage infiltration in advanced, lipid-rich plaques, as shown in Figure 5. To further evaluate the links between neovascularization and leukocyte infiltration, our group quantified neovessels, macrophages, and T lymphocytes in 269 aortic plaques evaluated by double immunohistochemistry. Neovessel content was significantly increased in plaques with moderate and severe inflammation. Moreover, ruptured plaques exhibited the highest degree of neovascularization. In addition, fibro-

calcific plaques, which are characterized by the lowest content of intimal fat, exhibited the lowest degree of neovascularization. Further analysis of plaque angiogenesis in diabetes documented a complex morphology including sprouting, RBC extravasation, and perivascular inflammation. Finally, perihemorrhagic inflammation and macrophage erythrophagocytosis were also increased in diabetic lesions, suggesting a role for microvessels in diabetic atherosclerosis.

On the basis of the aforementioned observations, it is reasonable to develop the following hypothesis: Adventitial-derived vasa vasorum neovascularization develops under the trigger of oxidized low-density lipoprotein deposits in the intima, mediated by hypoxia and Toll-like receptors. Such neovessels may contribute to the removal of intimal fat when the concentration of low-density lipoprotein is lower in the neovessel circulation than in the intima (low-density lipoprotein concentration gradient). However, extravasation of RBCs from leaky neovessels attracts macrophages to the field, both at the intima-media junction and the shoulders of the plaque. Macrophage erythrophagocytosis leads to cell activation at these crucial sites of the plaque. Then macrophage-derived matrix metalloproteinase secretion leads to rupture of the internal elastic lamina and fibrous cap collagenolysis, precipitating plaque rupture and thrombosis.
leading to iron deposition, macrophage activation, ceroid production, and foam cell formation, which promotes lipid accumulation within atherosclerotic plaques.39

To further explore the role of RBC deposition in human atherosclerosis, Arbustini et al40 identified an erythrocyte membrane protein, glycophorin A, in pulmonary plaques from patients with thromboembolic disease. These findings were reproduced in coronary plaques from patients with sudden cardiac death, suggesting that intraplaque hemorrhage and RBC lysis contributed to lipid deposition in high-risk atherosclerosis.41

**Free Hemoglobin, Haptoglobin, and Macrophage Activation**

The second mechanism of accelerated atherosclerosis after RBC extravasation involves macrophage activation from free Hb. After RBC membrane lysis, extracorpuscular Hb can induce oxidative tissue damage by virtue of its heme iron,42 with subsequent production of ROS. Extracorpuscular Hb can also activate the proinflammatory transcription factor NF-κB,36 leading to inflammation and angiogenesis, as discussed above.37

The primary defense mechanism against Hb-induced oxidative damage is provided by the protein haptoglobin (Hp), which rapidly and irreversibly binds to extracorpuscular Hb, forming a Hp-Hb complex. The antioxidant protection provided by Hp appears to be due to its ability to prevent the loss of heme iron from Hb.43 Haptoglobin also serves to promote the clearance of free Hb. In the vascular compartment, the Hp-Hb complex is cleared by 2 pathways: the liver (90%) or the monocyte (10%).44,45 However, in extravascular sites like atherosclerotic plaques, the only route for clearance of the Hp-Hb complex is via the macrophage, which is mediated by the membrane receptor CD163.45 In addition to its scavenging role, cross-linking of the CD163 receptor induced by the Hp-Hb complex plays an immunomodulatory role by controlling the expression of proinflammatory46 or antiinflammatory cytokines47 that may be deleterious48 or protective49 in atherogenesis. Most importantly, the ultimate effect of intraplaque hemorrhage on macrophage activation, as mediated by the interaction of the Hb-Hp complex with the CD-163 receptor, may be determined by the Hp genotype.50 Two classes of alleles (Hp-1 and Hp-2) have been identified at the Hp locus on chromosome 16q22.44,51 The protein products of the 2 Hp alleles are structurally and functionally distinct. The Hp-1 allele product forms only linear dimmers, whereas the Hp-2 allelic protein product forms large cyclic polymers.44 The larger size of the Hp-2 protein may impair its ability to permeate into the extravascular compartment at sites of hemorrhage.51 Functionally, the Hp-1 allele protein product is superior to the Hp-2 allele protein in preventing Hb-induced oxidative stress45,52 and the formation of ROS. These differences in antioxidant protection provided by Hp allelic protein products are magnified because of differences in the rate at which Hp-1–Hb or Hp-2–Hb complexes are scavenged by the CD163 receptor. We have shown that Hp-1–Hb complexes are scavenged much more rapidly than Hp-2–Hb via this CD163 mechanism,53 the chief pathway for disposing of Hp-Hb in the extravascular space. Furthermore, there are
differences between Hp-1–Hb and Hp-2–Hb complexes in how they modulate macrophage activation via their interaction with CD163. The Hp-1–Hb complex induces a novel antiinflammatory and cytoprotective effector pathway by the induction of the antiatherogenic cytokine interleukin-10.47 On the other hand, the Hp-2–Hb complex markedly increases macrophage oxidative stress, Ca mobilization, and IP3 generation compared with Hp-1–Hb complexes.53,54 As a result, Hp genotype may modulate macrophage activation toward plaque stabilization and quiescence (Hp-1) or toward plaque progression and instability (Hp-2), as shown in Figure 6.

The cardiovascular effects of the Hp polymorphism are especially relevant in patients with diabetes mellitus (DM).53–56 Multiple independent epidemiological studies examining incident cardiovascular disease have demonstrated that subjects with DM with the Hp-2 genotype, a redox-active Hb–Hp-2 complex is generated that produces ROS and induces macrophages to secrete proinflammatory cytokines by both CD163-dependent and -independent pathways, as shown. With permission from Moreno P.R. and Levy A.P., 2006.

Neovessels and Plaque Regression
As described above, vasa vasorum may provide a pathway for reverse lipid transport, allowing for active efflux of plaque lipoproteins from the intima through the adventitia,15 followed by neovessel regression after lipid removal. In fact, when compared with lipid-rich plaques, fibrocalcific lesions, also known as regression-type lesions, had the lowest microvessel content.59 As stated before, fibrocalcific plaques from DM are no longer vascularized, suggesting that microvessel involution may be a marker for plaque stabilization.60 Of clinical relevance, Corti et al61 elegantly documented the human adventitial pathway for plaque regression in vivo. Hence, as cholesterol exits the plaque, neovascularization may also experience regression, and the flow across vasa vasorum will be reduced to normal values. This observation, previously validated in experimental animal models,62 may also apply for human disease.

The pivotal experiments of plaque regression with the use of high-density lipoprotein were characterized by a significant reduction in plaque macrophage content.63,64 Recently, a strong correlation between macrophages and neovessels was documented in extremely effective models of plaque regression with the use of the angiogenic inhibitor endostatin.65,66 Quite the opposite to the process of plaque rupture, in which the battle between stability and instability is won by RBC extravasation and macrophage infiltration, neovessels in disease regression may play a protective role allowing for passivation of the plaque, leading to restoration of healthy tissue. Of note, circulating progenitor cells may also contribute to this pathway,67 but further studies are needed to confirm this hypothesis.

Neovascularization and Imaging
Although scarce and early in its development, magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound provide preliminary data imaging atherosclerotic neovascularization. MRI-derived fractional blood volume successfully correlated with microvessel density in human carotid plaques ($r=0.8$).68 In addition, superparamagnetic contrast agents such as iron oxide particles (ultrasmall superparamagnetic iron oxide) successfully determined blood volume and tumor vasculature on the basis of the phagocytic activity of macrophages.69 Nevertheless, the evaluation of plaque neovessels with the use of MRI–ultrasmall superparamagnetic iron oxide has not been published yet.

CT has only generated ex vivo microscopic images, with exquisite delineation of coronary adventitial neovascularization, as shown in Figure 1. No data have been reported in vivo. Finally, contrast-enhanced ultrasound (microbubbles) provides high-quality images of human plaque microvessels in carotid lesions70 and may also facilitate imaging neovessels in coronary lesions.71 Despite these preliminary results, multiple limitations of each of these techniques oriented the field toward molecular imaging.72 As a general principle, molecular imaging of angiogenesis targets the endothelial cell. One study evaluated atherosclerotic neovessels with the use of $\alpha$, $\beta$, targeted paramagnetic nanoparticles in the context of experimental atherosclerosis.73 Increased adventitial angiogenesis was detected as a 47±5% enhancement in MRI signal averaged throughout the abdominal aortic wall with the use of
routine MRI (1.5 T), 2 hours after injection, as shown in Figure 7.75

Recently, the extra-domain B of fibronectin, which is typically inserted in the fibronectin molecule in atherosclerotic lesions.74 With the use of radiographic and fluorescent imaging, extra-domain B areas correlated with lipid areas of atheroma, predominantly around vasa vasorum.75

Finally, vascular cell adhesion molecule-1, a critical component of the leukocyte-endothelial adhesion cascade, was successfully targeted with phage display-derived peptide sequences and multimodal nanoparticles for MRI and fluorescence molecular imaging in apolipoprotein E knockout mice, adding an additional method to interrogate angiogenesis in atherosclerosis with the use of molecular imaging techniques.76

These methods are, however, still under development to specifically target angiogenesis in the setting of atherosclerosis and neovascularization. More studies are needed for clinical application in humans.

Conclusion
Vasa vasorum–derived microvessels nurture the atherosclerotic plaque, with an organized system regulated by sympathetic and hormonal stimuli. They also provide a permanent communication between the systemic circulation and the atheroma, increasing leukocyte, albumin, and RBC extravasation, leading to ROS generation and tissue damage mediated by the potent oxidative effects of free Hb. Hp neutralizes the free Hb, and its protective effects may be genetically mediated, encoded at the Hp locus, as shown in basic, experimental, and human epidemiological studies. Furthermore, microvessels may play a role in plaque regression, as suggested by a dramatic reduction of intima-medial blood flow after regression in atherosclerotic monkeys. These results are in agreement with human data showing reduced microvessels in fibrocalcific plaques compared with lipid-rich and ruptured plaques. The second fact relating neovessels to plaque regression is the impressive 85% and 70% reduction of atherosclerosis in apolipoprotein E knockout mice treated with the angiogenic inhibitors endostatin and TNP-470, respectively. Inhibition of plaque angiogenesis induces a subsequent reduction of plaque macrophages and may have beneficial effects in the treatment of advanced atherosclerosis. Finally, plaque neovessels may be suitable for in vivo evaluation with the use of molecular imaging. As a result, plaque neovessels may serve for risk stratification and therapy in the future. Nevertheless, more studies are urgently needed to expand the multiple pathways this field can offer in the fight against cardiovascular disease.

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Disclosures
Dr Levy is a consultant to and has received stock options in the company Haptoguard, Inc, which owns a patent claiming to predict the susceptibility of a diabetic individual to develop cardiovascular disease on the basis of the haptoglobin genotype. Dr Levy has also received funding from the Israel Science Foundation. The other authors report no conflicts.

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