EDITORIAL COMMENT

Vascular Endothelial Growth Factor-Induced Angiogenesis: Crouching Tiger or Hidden Dragon?*

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Over 50 years ago Geiringer (1) noted that the vasa vasorum must extend into the media if vessel wall thickness is >0.5 mm, implying that thickening of the arterial wall required new blood vessel growth. Many years later Williams et al. (2) showed that regression of atherosclerosis, achieved by reducing the dietary cholesterol in a primate model, was accompanied by a proportional regression of the vasa vasorum. More recently, Kwon et al. (3) performed the opposite analysis, using three-dimensional computed tomography to calculate the area of the vasa vasorum, and they demonstrated a significant increase in the area of the vasa in association with the development of atherosclerosis in a hypercholesterolemic pig model. Moulton et al. (4) then took advantage of endothelial-specific inhibitors to attenuate angiogenesis in a hypercholesterolemic mouse model of atherosclerosis, revealing that plaque progression was inhibited by administration of agents designed to block new vessel growth. The reductions in plaque area occurred despite equivalent cholesterol levels in all animals and were accompanied by a decrease in the percentage of aortic plaques containing vessels. Together, these studies have provided strong evidence that neovascularization is *necessary* for the growth of atherosclerotic plaque. What these studies did not examine, however, is whether neovascularization, in and of itself, is a *sufficient* condition for plaque expansion.

The study by Celletti et al. (5) in this issue of the *Journal* addresses this very question. The investigators report that a single intramuscular injection of recombinant human vascular endothelial growth factor (VEGF) protein, in a classic hypercholesterolemic rabbit model, results in an increase in aortic plaque size and "endothelial area" within one week, with a persistent difference between control and VEGF-treated animals at three weeks. An increase in macrophage infiltration of the vessel wall is also reported.

The investigators took a novel approach in evaluating plaque vascularity, counting pixels instead of vessels. Therefore, the current findings are difficult to compare with previous investigations, especially given the absence of examples of the immunohistology in this report. It would have been interesting, for example, to see where the "pixels" were located—luminal surface, plaque, adventitia—and to visualize the relationship of numbers of pixels and actual vessels. In addition, the investigators decided to normalize plaque area to luminal area, which can vary markedly from animal to animal, depending on fixation technique (see their Figs. 1 and 2), rather than to medial area, which is not as prone to this variability. Nevertheless, all the data, including maximum plaque thickness and circumferential plaque extension, point to a significant increase in early plaque growth induced by VEGF.

These findings provide the counterpart to the findings of Moulton et al. (4). Here, instead of *blocking* angiogenesis and inhibiting atheroma formation, the researchers have shown that, in a milieu compatible with the development of atherosclerosis, administration of an angiogenic cytokine can result in accelerated nascent plaque growth. Given the growing interest in the use of endothelial growth factors for therapeutic angiogenesis, these findings could amplify concerns regarding the potential for these therapies to worsen the disease they are designed to treat.

Before reaching this conclusion, however, it is worth putting these important findings into context. The data clearly show that treatment with VEGF during the very earliest stage of atherosclerosis can accelerate fatty streak formation. It is equally important to note the hypothesis *not* tested—whether the same cytokine can worsen *already severe* atherosclerosis of the type that would lead to consideration for therapeutic angiogenesis. The potential significance of this discrepancy is underscored by the finding in the study by Moulton et al. (4) that inhibition of plaque progression was attenuated when antiangiogenic therapy was delayed by 32 weeks.

Indeed, the crucial factor to consider in terms of putting the findings of Celletti et al. (5) into perspective is how they bear upon a possible effect of VEGF on already established atherosclerosis.

Atherosclerosis has been inferred to result from a constellation of events, including inflammatory cell invasion of the arterial wall, lipid accumulation and smooth muscle cell proliferation. Central to these events is the failure of the endothelium to exert its normal homeostatic control over these processes. The concept that "injury" to the endothelium or a loss of endothelial integrity could play a seminal role in atherogenesis has, in fact, driven many of the investigations into the pathogenesis and treatment of atherosclerosis. This concept has been fueled by the finding that human atherosclerotic arteries have measurable deficiencies in endothelium-dependent functions, such as nitric

oxide (NO) production (6) and that atheroma formation is accelerated when the endothelial function is impaired chemically (7) or mechanically (8). Data regarding the relationship between endothelial integrity and neointimal thickening in human arteries, although limited, are consistent with the results of animal experiments. Davies et al. (9) harvested coronary arteries from explanted hearts of six transplant recipients and found that the severity and extent of endothelial cell defects varied directly with the severity and extent of intimal disease. Schwarcz et al. (10) found foci of—but not complete—re-endothelialization in only 6 (38%) of 16 carotid specimens of patients with recurrent carotid artery narrowing following an initially successful endarterectomy.

These studies support the notion that certain functions of the endothelium—including barrier regulation of permeability, thrombogenicity and leukocyte adherence, as well as production of growth-inhibitory molecules—are critical in the prevention of neointimal thickening (11). This concept has stimulated efforts to preserve intact the endothelium of native veins used for bypass surgery (12,13); to accelerate re-endothelialization following balloon-induced arterial injury (14,15); and to facilitate endothelialization of prosthetic conduits (16–19) or endovascular stents (20,21).

The capability of certain cytokines to serve as mitogens for endothelial cells (ECs) in vitro suggests that such growth stimulatory molecules might be exploited to accelerate endothelial recovery consequent to natural plaque rupture or mechanical balloon injury. Application of EC mitogens to a freshly injured arterial segment has been shown previously to exert favorable reparatory effects. Lindner et al. (22), for example, established clear evidence for the mitogenic effect of basic fibroblast growth factor (bFGF) on EC replication in the rat carotid artery model of balloon injury; total EC regrowth was achieved by administration of bFGF in doses of 12 μg twice weekly for up to eight weeks. In the case of acidic FGF—like bFGF, a known mitogen for vascular smooth muscle cells (SMCs) as well as ECs—low doses (<1% of the dose of bFGF employed in the aforementioned studies) administered to this same animal model were shown to have an inhibitory effect on neointimal thickening (23).

Vascular endothelial growth factor (24), known also as vascular permeability factor (25) and vasculotropin (26), has been previously shown to be an EC-specific mitogen and to promote EC migration in vitro (25,27,28) and as an angiogenic growth factor in vivo (24,29–31). Potentially contributory as well, however, and beyond its mitogenic effects, is the potential for VEGF to modulate qualitative aspects of EC function. The finding by Peters et al. (32) of the *fms*-like tyrosine kinase receptor in the endothelium of mature (quiescent) endothelium of adult organs was interpreted as evidence for the concept that VEGF may be important for the maintenance and repair of the endothelium. Observations that VEGF directly augments EC release of NO (33) and induces endothelium-dependent hypotension in healthy (as well as hypercholesterolemic) adult rabbits and swine (34) are consistent with the notion that VEGF constitutes a principal determinant of endothelial maintenance and repair. Indeed, this functional aspect of the effect of VEGF on EC biology has been shown to be critical for promotion of angiogenesis in vivo (35), and it is clearly critical for restored endothelium to inhibit SMC proliferation and/or neointimal thickening (36).

Based on such mitogenic, migratory, and functional modulation of ECs in response to VEGF, we performed a series of preclinical studies to investigate the direct application of VEGF as naked DNA or recombinant protein to arteries that were aggressively injured by balloon endothelial denudation, with (37,38) or without (39,40) deployment of an endovascular stent. In each of these animal experiments, local administration of VEGF markedly accelerated endothelial recovery of a freshly injured arterial segment.

In all four studies, accelerated endothelial recovery led to marked reduction in intimal thickening and/or mural thrombus formation. These findings implied that the anatomically restored endothelium was functionally competent, consistent with the principle that the endothelium, as described by Clowes (41), represents "the controlling element governing the function of biomaterials in the vasculature."

Indeed, what was perhaps the most striking aspect of these animal studies was the impact on direct functional assessment of endothelium-dependent function. Previous investigations of re-endothelialization have demonstrated that restoration of anatomic integrity and recovery of physiologic function do not proceed simultaneously (42). Using quantitative angiography, we determined the vasomotor response of the VEGF-transfected, balloon-injured arterial segments to endothelium-dependent agonists. Consistent with the previous experience of Weidinger et al. (43), control rabbits transfected with plasmid DNA encoding for beta-galactosidase (LacZ) demonstrated persistent impairment in vasomotor response to endothelium-dependent agents at four weeks' postinjury. In contrast, arteries transfected with phVEGF$_{165}$ disclosed recovery of near-normal endothelium-dependent vasoreactivity within one week. Thus, these findings are consistent with the notion that VEGF functions as an endogenous regulator of endothelial integrity—both physiologic and anatomic—in the artery wall (36).

Another feature of the VEGF gene—the presence of a secretion signal at its amino terminus (44) permitting VEGF to be naturally secreted by intact cells—is particularly critical to its investigation in strategies of naked DNA gene transfer (45). In experiments that have relied exclusively on the use of nonsecreted gene products, examination by histochemical staining, in situ hybridization and/or polymerase chain reaction has suggested that the transfection efficiency of direct gene transfer to vascular SMCs within the arterial wall was considerably <1% and, therefore, might preclude a meaningful biological response.

In contrast, genes encoding for a secreted protein such as VEGF may overcome the handicap of inefficient transfection by a paracrine effect, secreting adequate protein to achieve local levels that may be physiologically meaningful. The relation between a secreted gene product and transfection efficiency after in vivo arterial gene transfer was graphically documented by in vitro (46) and in vivo (47) experiments performed in our laboratory to serially monitor expression of a gene encoding for a secreted protein. In vivo analyses were performed using the central artery of the rabbit ear. Liposome-mediated transfection of plasmid DNA containing the gene for human growth hormone (hGH) was successfully performed in 18 of 23 arteries. Serum hGH levels measured five days after transfection ranged from 0.1 to 3.8 ng/ml (mean, 0.97 ng/ml); in contrast, serum drawn from the control arteries demonstrated no evidence of hGH production. Serial measurement of hGH from transfected arteries demonstrated maximum hGH secretion five days after transfection and no detectable hormone after 20 days. Despite these levels of secreted gene product documented in vivo, immunohistochemical staining of sections taken from the rabbit ear artery at necropsy disclosed evidence of successful transfection in <0.1% of cells in the transfected segment. Thus, low-efficiency transfection with a gene encoding for a *secreted* protein might achieve therapeutic effects not realized by transfection with genes encoding for proteins that remain intracellular.

These favorable preclinical studies led to the design of phase I clinical trials to test the hypotheses that VEGF gene transfer could: 1) augment angiogenesis in patients with symptomatic ischemia and no options for conventional revascularization, or 2) accelerate endothelial recovery after balloon angioplasty and thereby inhibit restenosis. In the context of these trials over 40 patients have now received VEGF by direct intra-arterial gene transfer of naked DNA delivered to a freshly injured arterial surface. The VEGF plasmid was administered in 12 patients in normal or moderately diseased arterial segments using a hydrogel-coated angioplasty to which the DNA, in saline solution, was applied in an attempt to augment collateral blood vessel growth (48). Follow-up angiography and intravascular ultrasound have disclosed no evidence of progression of atherosclerosis in these vessels (J. Isner, unpublished data, 2000). In 30 additional patients, the same delivery technique was employed following percutaneous revascularization of severely atherosclerotic femoral arteries in a phase I study to examine the potential of this approach to prevent restenosis. Follow-up examination up to 18 months' post-gene transfer (49) disclosed no evidence of new atherosclerotic lesion development, and the incidence of restenosis was at the very least no higher—and perhaps lower—than that observed among contemporary controls (50).

Thus, the animal and human studies performed in our laboratory (38–40,49,51–54) and by other investigators (55–57) (Table 1) in fact fail to support the notion that

Table 1. Studies Refuting the Notion That VEGF Accelerates Atherosclerosis

First Investigator	Year	Species	Target Vessel	Trauma	VEGF Gene	VEGF Protein	Mode of Delivery	Neointimal Thickening Increased	Neointimal Thickening Decreased	Neointimal Thickening Unchanged
1 Asahara (39)	1995	Rat	Carotid artery	Balloon		X	Dwell		X	
2 Asahara (40)	1996	Rabbit	Femoral artery	Balloon	X		Balloon catheter		X	
3 Van Belle (51)	1996	Rabbit	Iliac artery	Balloon/stent		X	Balloon catheter		X	
4 Van Belle (38)	1997	Rabbit	Iliac artery	Balloon/stent	X		Balloon catheter		X	
5 Van Belle (52)	1997	Rabbit	Iliac artery	Balloon/stent		X	Balloon catheter		X	
6 Luo (53)	1998	Rabbit	Vein graft	Surgery	X		Topical		X	
7 Isner (54)	1998	Human	SFA/Profunda	Balloon	X		Balloon catheter			X
8 Vale (49)	1998	Human	SFA	PTA	X		Balloon catheter		X*	
9 Laitinen (55)	2000	Human	Coronary	PTCA	X		Balloon catheter			X
10 Henry (56)	1999	Human	Coronary	None	X		IC/IV			X
11 Hiltunen (57)	2000	Rabbit	Aorta	Balloon/cholesterol	X		Balloon catheter		X	

*Versus historical controls.
IC = intracoronary; IV = intravenous; PTA = percutaneous transluminal angioplasty; PTCA = percutaneous transluminal coronary angioplasty; SFA = superficial femoral artery; VEGF = vascular endothelial growth factor.

accelerated atherosclerosis is a likely consequence of administering angiogenic cytokines. The outcome was quite the opposite in that administration of VEGF led to statistically significant *reductions* in intimal thickening.

Nevertheless, the findings of Celletti et al. (5) will doubtless serve as the springboard for additional studies to examine the potential impact of angiogenic therapies, perhaps employing animal models of established atherosclerosis. In addition, these findings will also likely heighten the already intense scrutiny of patients enrolled in clinical trials of therapeutic angiogenesis (58–62). In this sense, the success of this study is irrefutable.

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