Vascular Protection
A Novel Nonangiogenic Cardiovascular Role for Vascular Endothelial Growth Factor

Ian Zachary, Anthony Mathur, Seppo Yla-Herttuala, John Martin

Abstract—There is widespread interest in the use of the angiogenic cytokine, vascular endothelial growth factor (VEGF), for the treatment of cardiovascular disease. The main paradigm for VEGF cardiovascular therapy is the stimulation of “therapeutic angiogenesis” in ischemic myocardial and peripheral vascular limb disease. In this review, approaches to VEGF therapy based on the therapeutic angiogenesis model are critically assessed, and the alternative mechanism of vascular protection is advanced. Vascular protection is defined as the VEGF-induced enhancement of endothelial functions that mediate the inhibition of vascular smooth muscle cell proliferation, enhanced endothelial cell survival, suppression of thrombosis, and anti-inflammatory effects. VEGF-induced synthesis of NO and prostacyclin are both likely to be key mediators of VEGF-dependent vascular protection. Investigation into vascular protection should help us to gain insight into the underlying mechanisms of the cardiovascular actions of VEGF and should prove valuable in the development of novel therapeutic approaches based on local VEGF gene delivery. (Arterioscler Thromb Vasc Biol. 2000;20:1512-1520.)

Key Words: angiogenesis ■ atherosclerotic ■ prostacyclin ■ nitric oxide ■ endothelium

In embryogenesis, vascular endothelial growth factor (VEGF) is essential for vasculogenesis (the process of endothelial cell differentiation and the development of the primitive vascular system) and for angiogenesis (the sprouting of new capillaries from preexisting vessels). A large body of evidence now shows that VEGF plays a central role in postnatal angiogenesis in human pathophysiology, including cancer, rheumatoid arthritis, ocular neovascularizing disorders, and cardiovascular disease. In most diseases involving neovascularization, having too much VEGF is likely to contribute to disease progression, but in ischemic heart and peripheral vascular disease, the problem is one of vascular insufficiency, and an exciting recent development has been the use of VEGF as a proangiogenic cytokine able to stimulate collateral blood vessel formation in the ischemic heart and limb, an approach called “therapeutic angiogenesis.” Understanding the mechanisms through which VEGF exerts its effects on the cardiovascular system is clearly an essential prerequisite for realizing the therapeutic potential of this molecule. Recent findings indicate that VEGF is a multifunctional cytokine that regulates diverse biological functions in endothelial cells in vitro and in the adult vasculature in vivo. In view of the present interest in VEGF and the increasing awareness of the complexity of VEGF biology, it seems timely to challenge the view that “therapeutic angiogenesis” is the only mechanism through which VEGF may act therapeutically in cardiovascular disease. In the present review, a different framework for interpreting the cardiovascular actions of VEGF is considered. Based on studies of extravascular VEGF gene transfer in vivo and biological actions of VEGF in cultured endothelial cells, we have developed the concept that vascular protection is an important mode of action for VEGF in the adult vasculature. Whereas “therapeutic angiogenesis” is the formation of new blood vessels and involves the stimulation of endothelial cell proliferation, vascular protection is a distinct mechanism through which VEGF can enhance antiproliferative, anti-thrombotic, and other protective functions of essentially intact endothelia independently of significant promitogenic or angiogenic effects. Furthermore, VEGF-mediated arterial protection may prove to be useful in the treatment of occlusive cardiovascular disease.

Effects of VEGF in the Cardiovascular System

There is now a large body of experimental evidence (summarized in Table 1) that either VEGF protein or VEGF gene transfer accelerates reendothelialization and reduces intimal thickening and thrombus formation after balloon endothelial denudation and stent implantation. VEGF and another angiogenic cytokine, basic fibroblast growth factor (bFGF), have also been widely reported to increase blood flow and
promote angiogenesis in the myocardium and in peripheral vessels in several animal models of vascular insufficiency (Table 1).

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<tr>
<th>Model</th>
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<td>Rat carotid</td>
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The results of most studies provide support for the conclusion that VEGF protein and cDNA are able to promote reendothelialization and reduce intimal hyperplasia after balloon catheter–induced endothelial injury and stent implantation and to improve blood supply to areas of peripheral limb and myocardial ischemia. VEGF is the 165–amino acid isoform unless otherwise stated. MRI indicates magnetic resonance imaging.

Next, trials of VEGF in human cardiovascular disease have provided support for VEGF-induced therapeutic angiogenesis in patients with ischemic limb<sup>25,26</sup> and cardiac disease (Table 2)<sup>,27,28</sup>

Because hypoxia is a stimulus for VEGF production, it is probable that endogenous collateral vessel formation in the ischemic heart could occur through sprouting angiogenesis from preexisting vessels mediated by locally produced VEGF. An important new insight into the mechanism through which VEGF can stimulate neovascularization in adults has come from the discovery of circulating endothelial progenitor cells (also called angioblasts) and the illumination of the role played by these cells in VEGF-driven postnatal vasculogenesis and angiogenesis<sup>,29–31</sup> These findings not only add an important new facet, as well as further complexity, to the mechanisms of postnatal blood vessel formation, but importantly, they also broaden the scope of therapeutic angiogenesis to embrace strategies based on cell delivery as well as cytokine therapy. The use of endothelial progenitor cells further enlarges the range of therapeutic options because they can be genetically engineered to express proangiogenic cytokines or other therapeutically useful molecules.

Clearly, these studies offer enormous potential for the therapeutic use of VEGF. Nevertheless, there are several outstanding problems that proponents of VEGF therapy need to consider and that are likely to modify our understanding of the role of VEGF in cardiovascular disease and practical approaches in using VEGF as a therapeutic cytokine. These problems can be summarized under the following headings: (1) risks associated with unwanted angiogenesis, (2) uncertainty concerning the sufficiency of VEGF for arteriogenesis and viable collateral formation, and (3) the preliminary nature of the studies performed so far in humans.

### Risks of Unwanted Neovascularization

A potentially important, though still unquantifiable, concern is that systemic leakage from bolus intracoronary administration of VEGF protein or cDNA will generate unwanted angiogenesis in other tissues with the attendant risk that this could promote unwanted and potentially disease-promoting angiogenesis in other tissues. The risk of angiogenesis in tissues distant from the site of action may be small, but paradoxically, the greatest problem could be that plaque angiogenesis will itself promote plaque growth and instability.<sup>32</sup> Several studies show a close relationship between plaque formation and the development of an adventitial vasa vasorum and plaque neovascularization.<sup>33–35</sup> It remains un-

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**TABLE 1. Studies of VEGF Delivery in Animal Models of Vascular Insufficiency and Neointima Formation**

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known whether vascularization in the diseased vessel is a prerequisite or a contributory factor in plaque growth. However, the role of angiogenesis in plaque formation has recently been highlighted by the finding that antiangiogenic molecules inhibit plaque vascularization and reduce plaque size in the apoE-deficient mouse model of atherosclerosis. These findings provide the first direct evidence that neovascularization, if not sufficient for plaque formation, may contribute to lesion growth and perhaps be essential for it. It is not yet known whether intracoronary administration of VEGF can stimulate neovascularization in preexisting atherosclerotic lesions, but if the findings of Moulton et al extend to human atherosclerosis, then the balance between stimulation of collateral formation and intraplaque new vessel formation may be an important consideration in evaluating the benefits of therapeutic angiogenesis.

Is VEGF Sufficient for Collateral Artery Formation?

A second area of uncertainty is whether VEGF and its receptors are by themselves sufficient to induce the formation of a viable collateral blood vessel network in the ischemic heart or in peripheral ischemic limb disease. Studies of cytokine treatments in animals have not always confirmed the ability of VEGF (or other direct angiogenic cytokines) to promote therapeutic angiogenesis. In a direct comparison of effects of bFGF and VEGF in a canine ischemic hindlimb model, Lazarou et al found that whereas bFGF increased collateral blood flow, VEGF had no significant effect. Another report found that intracoronary administration of bFGF reduced infarct size in a canine model of myocardial ischemia without increasing vascular density. Such reports must be set against a larger body of evidence supporting a role for VEGF and FGFs in improving the collateral blood supply in various animal models of ischemic disease (see Table 1). It is possible, however, that combined therapies involving VEGF and FGFs (eg, bFGF and FGF-5) may be a more effective strategy. Support for this comes from studies showing synergism between VEGF and FGF in promoting angiogenesis.

A more fundamental reason why VEGF may be insufficient for collateral formation is that angiogenesis (the sprouting of capillaries) is a process different from the proliferation of preexisting arteriolar networks to produce collateral arteries, a process called arteriogenesis. Thus, it can be argued that whereas new microvessels induced by exogenous VEGF provide a limited and short-term palliative to ischemic heart tissue, only the formation of true collaterals constitutes an effective therapeutic strategy. An important insight into the mechanism of arteriogenesis is the finding that monocye activation plays a major role in angiogenesis and collateral artery formation. However, because VEGF promotes monocyte chemotaxis, it is plausible that VEGF could still be a key orchestrator of arteriogenesis by stimulating monocyte recruitment. At present, it seems that judgment as to whether VEGF is sufficient to trigger an arteriogenic (as distinct from an angiogenic) response is suspended. Even if VEGF can initiate arteriogenesis, it is nevertheless becoming increasingly apparent that other cooperating factors and receptor-mediated mechanisms are required for different stages in the development of mature vascular networks. Tie receptors and their ligands, the angiopoietins, and other factors, such as platelet-derived growth factor, are crucial for sprouting angiogenesis, for the recruitment of vascular smooth muscle cells (SMCs), and for the pruning and stabilization of blood vessels in the later stages of angiogenesis. Such remodeling may be essential for the formation of mature viable collateral vessels.

Results of Studies of Therapeutic Angiogenesis in Humans

The value of VEGF-mediated therapeutic angiogenesis for the treatment of human cardiovascular disease is still unclear. The Isner group (Losordo et al) reported decreased angina and increased cardiac perfusion in 5 patients given direct myocardial injection of naked VEGF cDNA. However, this and similar studies whose results have so far been published have been designed to establish the feasibility and safety of using VEGF for human cardiovascular therapy and have
by VEGF-induced Ca\(^{2+}\) mobilization,\(^{60,61}\) in common with other activators of eNOS. Another mechanism for VEGF-dependent NO synthase activation may be through activation of the heat shock protein Hsp 90 or an Hsp 90–associated protein.\(^ {62}\) Activation of Hsp 90 seems to increase its affinity for and association with eNOS to stimulate eNOS activity.

What are the likely biological consequences of VEGF-induced NO and PGI\(_2\) production? An important function of these 2 intercellular mediators is vasodilatation, but NO and PGI\(_2\) have several other effects that may perform vascular protective roles, including the inhibition of SMC proliferation, antiplatelet actions, and, in the case of NO, inhibition of leukocyte adhesion.

Antimitogenic effects of NO and PGI\(_2\) on SMCs have been demonstrated in vitro and in vivo and act via the production of the intracellular messengers cGMP and cAMP, respectively.\(^ {53–68}\) Clinical application of PGI\(_2\) has been frustrated by the failure of short-term PGI\(_2\) administration to inhibit restenosis after balloon injury and by the intolerable side effects of high PGI\(_2\) doses.\(^ {69,70}\) Recently, however, gene transfer of PGI\(_2\) synthase was shown to accelerate reendothelialization and to reduce neointimal formation after balloon injury.\(^ {71}\) eNOS gene transfer also reduces neointimal hyperplasia in balloon injury models of restenosis.\(^ {54–72}\) Inhibition of neointimal SMC hyperplasia after VEGF delivery in the rabbit collared carotid artery or balloon denudation and stent implantation may be mediated in part through the antimitogenic effects of these 2 intercellular mediators.

Another important vascular protective effect of NO and PGI\(_2\) that is predictable from in vitro studies is the inhibition of platelet aggregation and, hence, an antithrombotic effect.\(^ {73,74}\) There is no direct evidence so far that VEGF is antithrombotic, but some findings are very suggestive. VEGF increases the expression and activation of the serine proteases, urokinase and tissue-type plasminogen activator, which cleave plasminogen to generate the key thrombolytic enzyme, plasmin.\(^ {75}\) In vivo studies of vascular effects of VEGF have provided no evidence that VEGF increases the risk of thrombus formation, and 4 studies have demonstrated that VEGF delivery markedly reduces mural thrombus formation after balloon injury–induced intimal thickening.\(^ {9–12}\) Paradoxically, VEGF induces the secretion of von Willebrand factor (vWF)\(^ {76,78}\) and the expression of tissue factor\(^ {15}\) in human umbilical vein endothelial cells, effects that, in contrast to NO and PGI\(_2\), could play a role in thrombogenesis. vWF plays a crucial role in the adhesion of platelets to subendothelial collagen,\(^ {77}\) and tissue factor expression and activation are essential for the extrinsic pathway of coagulation and clot formation.\(^ {78}\) However, VEGF appears only to increase the surface expression of active tissue factor on endothelial cells in cooperation with tumor necrosis factor-\(\alpha.\)\(^ {79}\) Other findings may point toward a role for vWF and tissue factor in angiogenic functions of VEGF. Mice deficient in tissue factor have an impaired pattern of extraembryonic angiogenesis during embryogenesis,\(^ {80,81}\) and vWF increases endothelial cell adhesion, suggestive of a role in the maintenance of endothelial integrity.\(^ {82}\) Interestingly, VEGF is released by platelets, its synthesis is increased by thrombopoietin in megakaryocytic cell lines, and increased levels of VEGF are found at the site of hemostatic plugs in humans.\(^ {83–85}\) It remains enigmatic whether VEGF plays a
Mechanisms of VEGF-mediated vascular protection. VEGF production in arteries may be increased by gene transfer, or endogenous production may be upregulated in SMCs by hypoxia, growth factors (bFGF and platelet-derived growth factor-BB), or cytokines. Intimal thickening could reduce oxygen tension and lead to increased expression of regulatory factors in medial SMCs in vivo, leading to increased VEGF production. VEGF is most likely to act through receptors (KDR/Fk-1 and possibly Flt-1) in the endothelium to increase production of NO and PGI2 and augment intracellular endothelial cell survival signaling. NO and PGI2 are predicted to have 3 major biological consequences: vasodilatation, inhibition of SMC proliferation, and decreased platelet aggregation and thrombosis. NO is also predicted to act in an anti-inflammatory manner by inhibiting leukocyte adhesion. The combined effect of these biological actions is vascular protection.

A further key component in a vascular protective function of VEGF-induced NO production is likely to be the ability of NO to inhibit leukocyte recruitment to blood vessels. It is now well established that endogenous NO synthesis inhibits leukocyte rolling and adhesion as well as the upregulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1. Given the important role played by adhesion molecule expression and leukocyte adhesion in the early stages of atherosclerosis, VEGF-induced NO synthesis might be predicted to have antiatherogenic properties.

PGI2 and, particularly, NO are short-lived intercellular mediators, and if they play a role in the long-term protective effects of VEGF, it is likely that mechanisms might exist for increasing the effective longevity of the signal. In the case of NO, an insight into how production of NO might be prolonged has come from the finding that VEGF can increase the expression of eNOS.

Another important mechanism through which VEGF may augment endothelial function is by increasing endothelial cell survival (Figure). VEGF was originally shown to act as a survival factor for retinal endothelial cells. More recently, VEGF has been reported to inhibit human umbilical vein endothelial cell apoptosis by activating the antiapoptotic Akt/PKB pathway via a phosphatidylinositol 3'-kinase-dependent pathway. VEGF also increases tyrosine phosphorylation and the focal adhesion association of focal adhesion kinase (FAK) and the FAK-associated protein paxillin. Because FAK appears to be critical for maintaining survival signals in adherent cells and because in endothelial cells, FAK tyrosine dephosphorylation (M. Lobo, I. Zachary, unpublished data, 1999) and caspase-mediated proteolytic cleavage are early responses to apoptogenic stimuli, it is possible that VEGF-dependent survival signaling may also be relayed through increased FAK tyrosine phosphorylation.

The receptor mediating VEGF-induced NO and PGI2 production in human umbilical vein endothelial cells is likely to be KDR (VEGF receptor-2) because this is the major receptor for VEGF in these cells, and PlGF, a specific ligand for the high-affinity Flt-1 receptor (VEGF receptor-1), had no effect on these biological functions. Whether KDR is the receptor that mediates the arterioprotective functions of VEGF in vivo or whether there is a role for Flt-1 and the putatively identified KDR coreceptor, neuropilin-1, is currently being investigated. Other VEGF-related cytokines, (VEGF-B, -C, and -D and PlGF), could also play a role in cardiovascular protective functions either therapeutically or physiologically depending on the expression profiles for different VEGF receptors in cardiovascular tissues. VEGF-C has been shown to be angiogenic in the rabbit ischemic hindlimb model, and our recent unpublished data show that VEGF-C gene transfer can accelerate neovascularization and inhibit intimal hyperplasia in the balloon-injured rabbit aorta (M.O. Hiltunen, K. Alitalo, S. Yla-Herttuala, et al, unpublished data, 1999). It is not yet clear whether vascular endothelial effects of VEGF-C are mediated via KDR or Flt-4 (VEGF receptor-3).

An intriguing speculation that arises from these findings is whether VEGF functions as an endogenous vascular protective factor. The ability of VEGF to induce NO and PGI2 production, increase endothelial integrity and survival, and inhibit intimal SMC proliferation makes it a particularly attractive candidate for such a role. SMCs produce VEGF in response to hypoxia, growth factors, and cytokines (see Figure). Intimal thickening and plaque formation are associated with increased production of growth factors and cytokines and may cause reduced oxygen tension in medial SMCs by increasing the diffusion distance of oxygen from the lumen. Therefore, the atherosclerotic milieu may promote endogenous VEGF synthesis, and in agreement with this hypothesis, VEGF expression has been demonstrated in atherosclerotic lesions. Reduced expression or impaired function of VEGF would, in turn, be predicted to attenuate endothelial antiproliferative and antithrombotic functions and, hence, encourage SMC proliferation and promote atherogenesis.

The notion of vascular protection emphasizes the consequences of VEGF biological functions for the cardiovascular system that are not readily predictable from the perspective of therapeutic angiogenesis. However, the discussion of the ramifications of VEGF-mediated biological actions for thrombosis highlighted the difficulty of integrating these diverse actions into the vascular protection model. It is also likely that the context, in terms of pathophysiology, tissue type, and the cytokine milieu, will be crucial for determining the overall outcome of VEGF treatment. In turn, this suggests that VEGF may even have deleterious as well as beneficial consequences for the cardiovascular system depending on the site of action, the specific type of disease or therapeutic intervention (eg, bypass graft or angioplasty) being targeted, and the presence of other cooperating cytokines. Thus, VEGF delivered locally to the site of anastomosis in a bypass graft
may reduce the risk of stenosis, whereas VEGF within an existing atherosclerotic plaque could have the contradictory effects of enhancing endothelium-dependent protective functions on one hand and inducing neovascularization on the other. These suppositions indicate that the careful selection of the pathophysiological context in which VEGF is delivered to patients and the need for targeted delivery are likely to be crucial for ensuring successful VEGF therapy.

Feasibility of Local Human VEGF Gene Transfer

Extravascular (adventitial) and luminal gene transfer have both been used to achieve gene transfer and expression in animal and human arteries.\textsuperscript{110} Endoluminal gene delivery is likely to have its major application in ameliorating restenosis after angioplasty and stenting. Extravascular gene (or drug) transfer has so far been less widely used but is of potential value for targeting therapeutic genes and compounds in a variety of vascular surgical operations, including bypass procedures, tissue transplantation, endarterectomies, and access for renal dialysis.

Preliminary results have described the beneficial effects of nontargeted VEGF gene transfer in human peripheral vascular disease and ischemic myocardium.\textsuperscript{26,28} The feasibility of local VEGF gene therapy in humans was studied by using an infusion-perfusion catheter to transfer VEGF plasmid to human coronary arteries immediately after angioplasty in 15 patients with angina pectoris that was due to a single lesion in 1 coronary artery.\textsuperscript{36,47} The results showed that 1000 \( \mu \)g of VEGF plasmid cDNA was well tolerated. Systemic leakage of the VEGF transgene was minimal, as judged by polymerase chain reaction, but in a patient with critical leg ischemia subjected to the same gene transfer procedure, VEGF transgene expression could be detected in peripheral tibial artery segments up to 180 days after angioplasty.\textsuperscript{47} Arterial pieces distal and proximal to the site of angioplasty did not express the transgene, indicating minimal lateral diffusion of the VEGF plasmid. Mouse VEGF was used in that study (Laitinen et al\textsuperscript{47}) to allow detection of any increase in VEGF protein specifically arising from gene transfer. Mouse VEGF could not be detected in the systemic circulation by specific ELISA, indicating minimal systemic leakage of transduced protein. Laitinen et al show that local VEGF transfer is feasible, safe, and well tolerated. The failure to detect VEGF protein systemically could indicate either that expression is truly local or that expression is very low. However, long-term low-level expression may be sufficient to achieve beneficial effects locally without raising systemic VEGF protein levels sufficiently to promote angiogenesis at distant sites.

Conclusions and Perspectives

There is a clearly established role for NO and PGI\textsubscript{2} in mediating the biological actions of VEGF in vitro and in vivo. In the present review, it has been argued that as well as being angiogenic, VEGF also acts as a vascular protective factor via increased NO and PGI\textsubscript{2} production and the maintenance of antiapoptotic signaling pathways to enhance endothelial integrity, inhibit SMC proliferation, and enhance the antithrombogenic and anti-inflammatory properties of the endothelium. An advantage of VEGF therapy over approaches using either PGI\textsubscript{2} or eNOS gene delivery is that VEGF is predicted to combine the therapeutic effects of both factors. Whereas vascular protection may provide an attractive alternative mechanistic framework for understanding the impact of VEGF on the cardiovascular system, it should be stressed that the vascular protective and therapeutic angiogenic models of VEGF action are not mutually exclusive. As examples, NO has been implicated in mediating the effects of VEGF on vasorelaxation, and PGI\textsubscript{2} and NO were shown to mediate permeability-increasing effects of VEGF in vivo.\textsuperscript{50} NO has also been implicated in the scalar movement (podokinesis) of endothelial cells and in playing a permissive role in VEGF-induced endothelial cell migration\textsuperscript{111,112} and angiogenesis.\textsuperscript{113}

From a therapeutic standpoint, the vascular protection paradigm may have the greatest relevance for pathophysiological contexts in which stenosis occurs in previously normal vessels characterized by relatively undamaged or undiseased endothelia. Theoretically, clinical situations that could be suitable for local extravascular VEGF gene delivery are bypass grafting, tissue transplantation, and access for renal dialysis arteriovenous access loops. In all these situations, a major cause of nonacute failure is stenosis of a previously unoccluded vessel at or near the anastomosis. An additional important feature of these clinical procedures is that they allow perivascular surgical access and are therefore potentially useful for local extravascular VEGF gene therapy.

The potential for using VEGF therapy in cardiovascular diseases is an exciting one, but effectively harnessing this potential clearly poses challenges for scientists and clinicians alike. In meeting these challenges, an improved understanding of how this multifunctional cytokine works, one that fully encompasses the complexity of VEGF biology, is essential. The concept of VEGF-directed vascular protection may add an important new dimension to this understanding.

Acknowledgments

John Martin is a British Heart Foundation (BHF) Professor of Cardiovascular Medicine. Ian Zachary is a BHF Senior Lecturer. Anthony Mathur is a Medical Research Council Clinical Training Fellow.

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