Angiogenesis

Guest commentary

The pharmacology of angiogenesis in cardiovascular disease - H. A. J. Struijker Boudier

Lead Article


Expert Answers to Three Key Questions

What are the candidate pathologies for therapeutic angiogenesis? - P. Carmeliet
Angiogenesis and cardiovascular disease: what are the risks? - I. Baumgartner
Angiogenesis and cardiovascular disease: how long will angiogenesis last and how can we stop it? - S. Nikol

Fascinoma Cardiologica

Plants and the heart: At the heart of the fuel supply: the cereal grasses - A. Banerjee

Summaries of Ten Seminal Papers - M. S. Marber and P. D. Lambiase

Therapeutic angiogenesis. A single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb models – S. Takeshita and others

Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart – F. J. Giordano and others

Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb – J. M. Isner and others

Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor stimulates collateral development in vivo – S. Takeshita and others

Isolation of putative progenitor endothelial cells for angiogenesis – T. Asahara and others

Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia – I. Baumgartner and others

Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization – C. Kalka and others

Vascular endothelial growth factor (165) gene transfer augments circulating endothelial progenitor cells in human subjects – C. Kalka and others

Left ventricular electromechanical mapping to assess efficacy of phVEGF165 gene transfer for therapeutic angiogenesis in chronic myocardial ischemia – P. R. Vale and others

Favorable effect of VEGF gene transfer on ischemic peripheral neuropathy – P. Schratzberger and others

Bibliography of One Hundred Key Papers
Obituary

As this issue of
*Dialogues in Cardiovascular Medicine*
was going to press,
the news came of the death of
Doctor Jeffrey M. Isner,
on October 31, 2001.

The Editors and Publisher
wish to express their deep regret
concerning this untimely death
and their admiration for
Dr Isner’s outstanding research.

This issue of *Dialogues*, for which
Dr Isner and his colleagues
contributed the Lead Article,
is dedicated to his memory.
This issue is devoted to the role of angiogenesis in cardiovascular disease. The lead article and the three respondent articles give excellent reviews on various aspects of modern angiogenesis research. Although this field of research initially emerged from investigations into tumor metastases, it has its roots in cardiovascular embryology. Already in 1893, Thoma described the principles of vascular development in an elegant monograph. He defined three rules for vascular development: (i) the number of vessels that grow in a tissue is determined by genetic constitution and the metabolic needs of the tissue; (ii) the diameter of a vessel depends on the flow of blood through its lumen; and (iii) the thickness of the vessel wall is determined by transmural tension. These basic rules remain unchallenged even in this early part of the 21st century. The past two decades have witnessed a tremendous increase in knowledge of the molecular mechanisms underlying these basic features of vascular growth.

In the lead article, Isner and coworkers describe how a cascade of growth factors is required to orchestrate a vascular network, both in conditions of normal, embryological development and in situations of tissue ischemia.

The vascular endothelial growth factor (VEGF) is regarded as playing first violin in this orchestra, but other major players have entered the picture, such as various fibroblast growth factors, angiopoietins, ephrins, and angiogenesis. These various factors target different mechanisms involved in vascular growth. As pointed out by Carmeliet in his contribution, an orchestrated response involving different factors is needed to obtain a fully functional vascular tree.

Both Isner and coworkers and Carmeliet discuss the impaired angiogenic response in various pathological conditions, such as aging, hypercholesterolemia, and diabetes. In our own research in the past decade we have shown that hypertensive disease constitutes another pathological consequence of impaired angiogenesis. Decreased microvascular density (“rarefaction”) is a major contributor to the increased vascular resistance in hypertension. It is also a major source of end-organ damage in hypertensive disease, such as decreased coronary flow reserve and microvascular brain damage (lacunar infarcts).

An intriguing aspect discussed by both Isner and coworkers and Carmeliet is the large interindividual variability in switching to an angiogenic response during pathological conditions. The reasons for this variability are virtually unexplored and may involve an important genetic component. With the current powerful technologies in the area of functional genomics, we can expect rapidly increasing insights into the genetic basis of the interindividual variability of angiogenic response.

An important focus in all of the aforementioned contributions in this issue of Dialogues is therapeutic angiogenesis as a treatment for ischemic diseases. Isner’s group has pioneered this field and provided many important contributions. Their paper gives an update of their recent research with a particular focus on the application of bone marrow–derived endothelial progenitor cells. This form of cell therapy may circumvent some of the formidable difficulties encountered in the use of gene or protein applications of angiogenic growth factors. In her contribution, Baumgartner raises important questions about potential risks of the use of molecules like VEGF. Potential problems include angioma-genesis, plaque growth, tumor angiogenesis, and ocular neovascularization. Initial analyses in the first series of patients treated indicate that adverse effects are rare. However, the number of treated patients is still small when compared with more classic forms of drug treatment.

Nikol, in her contribution, discusses various aspects related to the optimal delivery of vascular growth factors. A number of vector systems and sophisticated local delivery strategies are now used in combination with protein or gene therapy to optimize treatment. Localized delivery of genes or proteins to the ischemic heart is still difficult to achieve. Recent research by Laham et al and Hermans (in our own group, unpublished observations), indicates...
that chronic low-dose infusion of angiogenic proteins into the pericardial fluid may be a particularly effective route of delivery. Clinical follow-up work will be needed to establish the therapeutic value of this approach.

Another issue that deserves attention is the use of more classic, non–gene-derived and nonprotein drugs to influence impaired angiogenesis in cardiovascular disease. More research on the pathways utilized by the peptidergic angiogenic growth factors will undoubtedly lead to new small-molecular-weight molecules to target sections of these pathways. Many such drugs already exist in the area of antiangiogenic therapies for tumor metastasis. Tyrosine kinase inhibitors, for instance, are an important class of antiangiogenic molecules. Similarly, certain steroids and heparin antagonists also act as potent antiangiogenic drugs. By analogy, proangiogenic small-molecular-weight molecules could be developed. Such molecules would probably be greatly advantageous from a biopharmaceutical point of view.

A final approach is to reinvestigate some of the existing drug classes in cardiovascular pharmacotherapy in search of proangiogenic activity. Recent research indicates that certain classes of vasodilators have a proangiogenic effect. Drugs interfering with the renin-angiotensin system are a particularly interesting class of drugs in view of the many vascular actions of angiotensin II. The angiogenic actions of angiotensin-converting enzyme (ACE) inhibitors and angiotensin-II–receptor blockers are still a subject of controversy, some reporting a proangiogenic effect and others an antiangiogenic effect. These different effects are probably due to the segment of the microcirculation investigated (capillaries or arterioles) and the relative involvement of different angiotensin-II–receptor subtypes. A more consistent proangiogenic effect has been reported with a very-low-dose ACE-inhibitor (perindopril)/diuretic (indapamide) combination.

At least part of the effect of vasodilator drugs on the angiogenic process is not due to direct effects of these drugs. Instead, altered hemodynamic forces within the microcirculation trigger proangiogenic or antiangiogenic responses. Such an important role of hemodynamic forces was already suggested by Thoma in his original work.

REFERENCES

1. Thoma R.

2. Le Noble JLML, Tangelder GJ, Slaaf DW, Van Essen H, Reneman RS, Struijker Boudier HA.
   A functional morphometric study of the cremaster muscle microcirculation in young SHR.
   J Hypertens. 1990;8:741-748.

3. Le Noble FA, Stassen FR, Hacking WJ, Struijker Boudier HA.
   Angiogenesis and hypertension.

   Local perivascular delivery of fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase-I randomized double-blind, placebo-controlled trial.

5. Fan TP, Jaggar R, Bicknell R.
   Controlling the vasculature: angiogenesis, anti-angiogenesis and vascular targeting of gene therapy.

   Microcirculation in hypertension: a new target for treatment?
Angiogenesis and cardiovascular disease

Jeffrey M. Isner†, MD; Peter Vale, MD; James Symes, MD; Douglas W. Losordo, MD; Takayuki Asahara, MD, PhD

Tufts University School of Medicine - St Elizabeth’s Medical Center - Boston - Mass - USA

Keywords: angiogenesis; neovascularization; gene therapy; gene transfer; vascular endothelial growth factor; cytokine; endothelial cell; myocardial ischemia; diabetic retinopathy; ischemic peripheral neuropathy; limb ischemia

† Jeffrey M. Isner died October 31, 2001

Address for correspondence: Peter Vale, MD, St Elizabeth’s Medical Center, 736 Cambridge St, Boston, MA 02135, USA

A comprehensive review is offered of recent fundamental and clinical research, much of it by the authors, into the mechanisms and applications of neovascularization, a term encompassing both angiogenesis, where mature endothelial cells (ECs) leave the basement membrane and proliferate as sprouts from parental vessels, and vasculogenesis, where bone marrow-derived endothelial progenitor cells (EPCs) circulate to ischemic sites and differentiate into mature ECs. EPCs act as a substrate for growth factors, notably vascular endothelial growth factor (VEGF), released endogenously in response to tissue ischemia or administered exogenously for therapeutic neovascularization in subjects (elderly, diabetics) unable to upregulate their cytokine expression. Phase 1 trials in critical limb ischemia with intramuscular injection of naked plasmid DNA encoding the 165-amino-acid isoform of human VEGF show increased gene product expression, magnetic resonance angiography evidence of improved blood flow, and concomitantly reduced rest pain. Results are similar in class III-IV angina where electromechanical mapping evidence of hibernating myocardium salvage is associated with decreased anginal episodes. VEGF also reverses peripheral neuropathy via its ability to preserve the vasa nervorum. Optimal therapeutic strategy comprises stimulation of the EPC substrate combined with VEGF administration. No potential adverse effects of neovascularization—increased malignancy, proliferative retinopathy—have yet been reported.

ANGIOGENESIS AS AN ENDOGENOUS RESPONSE TO ISCHEMIA

The sequence of biological events that permits an organism to maintain tissue viability in the face of acute or chronic ischemia constitutes a fundamental survival response. Among mammalian species, this response may be best exemplified by the Israeli mole rat,1 a creature unique to the Middle East, living only in Egypt, Israel, and Syria. What is fascinating about this animal is that its entire life span is spent underground in subterranean burrows at decidedly low oxygen tensions; accordingly, the tissues of this animal have been shown to be highly vascularized, and the vascular density is associated with upregulated endogenous expression of vascular endothelial growth factor (VEGF).

Among supraterranean species confronted with tissue ischemia localized to cardiac or skeletal muscle, at least two categories of options are available. The first is to reduce demand for tissue oxygenation. This may be accomplished by the Israeli mole rat,1 a creature unique to the Middle East, living only in Egypt, Israel, and Syria. What is fascinating about this animal is that its entire life span is spent underground in subterranean burrows at decidedly low oxygen tensions, accordingly, the tissues of this animal have been shown to be highly vascularized, and the vascular density is associated with upregulated endogenous expression of vascular endothelial growth factor (VEGF).
NATURAL RESPONSE INVOLVES CYTOKINE AND CELLULAR ELEMENTS

Nature’s response to the development of profound muscle ischemia includes upregulation of angiogenic growth factors and mobilization of circulating cellular elements that together enable development of an accessory vasculature. The involved paradigm, not surprisingly, recapitulates many aspects of embryonic circulatory development.

Certain experimental findings suggest that, as is the case for the Israeli mole rat, VEGF is the key, if not the principal, regulatory cytokine orchestrating the response to postnatal ischemia. In a murine model of hindlimb ischemia, for example, we observed that excision of the iliac and femoral arteries was followed by reduced blood flow and evidence of tissue necrosis documented by histochemical staining. Within 2 to 4 days, tissue immunostaining and Western blots of skeletal muscle harvested from the ischemic limb documented upregulation of VEGF protein. This lasted for 28 to 35 days. Similar findings have been reported in response to transient myocardial ischemia.

Evidence that VEGF constitutes the principal regulatory mediator of endogenous neovascularization of ischemic tissues was established in the murine hindlimb ischemia model by two interventions. The first involved administration of recombinant platelet factor–4 (PF-4), which inhibits angiogenesis by disrupting VEGF receptor–mediated signal transduction and/or disrupting the binding of VEGF to cell surface heparan sulfates. To more specifically isolate the role of VEGF in modulating angiogenesis in this mouse model, we administered a VEGF-neutralizing antibody. Similar to findings observed in mice receiving PF-4, recovery of blood flow, capillary density, and proliferative activity measured by incorporation of bromodeoxyuridine (BrdU) were all significantly depressed in the ischemic limb of mice treated with neutralizing VEGF antibody compared with control mice. Similar attenuation of spontaneous angiogenesis in freshly cut aortic rings cultured in a serum-free collagen gel and treated with a neutralizing VEGF antibody was reported by Nicosia et al. While such a time-course analysis of tissue expression is not feasible in human subjects, at least two groups have now studied patients following acute myocardial infarction and documented a similar rise and fall in VEGF expression.

More recently, D’Arcangelo et al have suggested that tissue acidoisis may constitute an independent stimulus to upregulated VEGF expression. While the mechanism for upregulated VEGF expression in response to acidoisis remains enigmatic, it is clear that hypoxia-induced VEGF expression is mediated by the binding of the transcription factor hypoxia-inducible factor–1 (HIF-1) to a hypoxia response element in the VEGF promoter. Analyses of endomyocardial biopsy specimens retrieved from patients undergoing coronary artery bypass surgery documented an increase in tissue expression of HIF-1 protein associated with acute ischemia or early infarction.
Increase in mRNA stability constitutes a second important control point for the hypoxic induction of VEGF in different cell lines. Stabilization of VEGF mRNA by hypoxia is thought to be mediated by the binding of sequence-specific RNA-binding proteins to sequences in both the 3’- and 5’-untranslated regions (UTRs) of VEGF mRNA. This mechanism thus acts to extend under the stress of hypoxia the intrinsically short half-life of VEGF mRNA (approximately 30 min).

A third feature of VEGF that is critical for facilitating an efficient and sensitive response to hypoxia is the presence of an internal ribosome entry site (IRES) that permits cap-independent translation by ribosomal scanning of its mRNA.9 This may be particularly important in the case of VEGF due to the fact that the 5’UTR of its mRNA has several features that are incompatible with efficient ribosomal scanning, including its length, high G+C content that permits secondary structure formation, and a short open reading frame bounded by in-frame initiation and termination codons. Importantly, Stein et al9 have shown that internal ribosome entry, at least in the case of VEGF, is not adversely impacted by the development of hypoxia.

In vivo and in vitro studies have documented that skeletal myocytes4 and cardiomyocytes10 constitute important sites of VEGF synthesis, as do vascular endothelial cells under conditions of hypoxia.4,11 Infiltrating T cells12 and monocytes,13 however, comprise additional circulating cellular sources by which VEGF is imported, Trojan horse–like, into the necrotic/ischemic milieu to acutely upregulate local VEGF expression. This property of infiltrating T cells was first described in the development of tumor neovasculature by Freeman et al.14 The critical nature of this contribution has perhaps been best demonstrated in T cell–deficient nude mice that undergo necrotic autoamputation in response to hindlimb ischemia due to retarded angiogenesis.12,15

There is also good evidence to suggest that coordinated upregulation of VEGF receptor expression is important for not only enabling, but indeed for localizing neovascularization. VEGF receptors are typically expressed at exquisitely low levels under quiescent circumstances. With the onset of hypoxia, however, expression of VEGF receptor-2 (KDR), has been shown to increase up to 13-fold in skeletal16,17 or cardiac18 muscle. The consequence differential in upregulated VEGF expression by ischemic versus normal tissues may play a critical role in limiting neovascularization to those sites where augmented perfusion is required.

Previous reports by Murohara et al19 and Ziche et al20 and more recent data by Fujio et al21 suggest that cytokine-induced angiogenesis is mediated in large part by Akt-mediated upregulation of nitric oxide (NO) expression. Although reconstitution of NO expression by regenerated endothelium has been shown to act via a negative feedback mechanism to downregulate VEGF expression in the arterial wall following endothelial denudation,22 whether NO or other mechanisms act similarly to limit VEGF expression following reconstitution of limb perfusion remains to be clarified.

Endogenous revascularization often has a distinctive appearance when visualized radiographically using iodinated contrast agents. Such angiograms typically disclose a “corkscrew” appearance, once alleged to be specific for so-called Buerger’s disease, but now recognized to be a characteristic feature of collateral vessels in general. Why such collaterals are “crooked” remains uncertain. It is intriguing, however, to consider two pathogenetic bases. The first is the possibility that such vessels represent the fusion of multiple neovascular segments that are joined together under the influence of certain angiogenic growth factors such as VEGF. The second is that such vessels represent the consequence of a not-so-random walk in which the developing vessels transiently deviate before reestablishing a correct course in the direction of a putative ischemic stimulus.

While the basis for the appearance of such angiographically visible collateral vessels remains to be elucidated, it is quite clear from studies performed in animal models that the caliber of most vessels comprising the neovascularature that develops in response to ischemia are in fact beyond the resolution (180-200 µm) of what can be recognized with conventional angiographic imaging. DNA labeling studies in pig and dog models of myocardial ischemia established that improvement in collateral-dependent flow typically results from proliferation of vessels of <200 µm in diameter. This observation was confirmed by more recent in vivo imaging studies performed by Takeshita et al23 who used synchrotron radiation microangiography to determine that neovascularization following VEGF gene transfer (GTx) predominantly involved vessels <180 µm in diameter. Contrast angiography cannot provide images of arteries measuring <200 µm in diameter, the spatial resolution of images obtained by magnetic resonance angiography is even less. Thus, current imaging techniques are suboptimal for evaluation of neovascularization in response to myocardial or lower-extremity ischemia.
The maturity and durability of the vessels that form in response to de novo or therapeutic neovascularization is currently the subject of intensive inquiry. Most investigators currently view the full maturation of developing neovasculature as a process that borrows from embryonic paradigms, including evidence that a cascade of angiogenic growth factors (Table I) are required to elaborate a normal vascular network. Gene targeting experiments, for example, have indicated that the Tie-2 ligand angiopoietin-1 (Ang-1) plays a critical role in neovascular maturation. Evidence that tissue expression of Ang-1 is upregulated in response to tissue ischemia, however, has not been published, consistent with the fact that in vitro studies have failed to show that Ang-1 (in contrast to its relative, angiopoietin-2 [Ang-2]) is upregulated in response to hypoxia, or for that matter VEGF. Indeed, postnatal upregulation of Ang-1 expression remains largely undefined.

---

**Table I.** Angiogenic cytokines and genes used in clinical angiogenesis trials.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Protein</th>
<th>Gene ph</th>
<th>Gene ad</th>
<th>EC specific</th>
<th>Pleiotropic</th>
<th>Secretory sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF165</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF121</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>VEGF-2 (VEGF-C)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>FGF-1 (aFGF)</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-1 modified</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-2 (bFGF)</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-4</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend: a, acidic; ad, adenoviral vector; b, basic; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; ph, plasmid human; VEGF, vascular endothelial growth factor.

---

**Figure 1.** Neovascularization encompasses both angiogenesis and vasculogenesis. Angiogenesis represents the classic paradigm for new vessel growth, as mature, differentiated endothelial cells (ECs) break free from their basement membrane and migrate as well as proliferate to form sprouts from parental vessels. Vasculogenesis involves participation of bone marrow (BM)-derived endothelial progenitor cells (EPCs), which circulate to sites of neovascularization where they differentiate in situ into mature ECs. Growth factors, cytokines, or hormones released endogenously in response to tissue ischemia, or administered exogenously for therapeutic neovascularization, promote EPC proliferation, differentiation, and mobilization from BM, via the peripheral circulation, to neovascular foci.
There is a similar lack of evidence to implicate upregulated tissue expression of fibroblast growth factor (FGF) in response to ischemia, although there are limited data to suggest that activity of available FGF ligand may be augmented by receptor upregulation. VEGF has been shown to upregulate endothelial cell (EC) expression of both platelet-derived growth factor isoforms A and B (PDGF-A and PDGF-B). Together with gene targeting data that have demonstrated an absence of pericytes in PDGF-B−/− mice, these findings suggest that VEGF-induced EC secretion of PDGF may function to recruit smooth muscle cells, including pericytes, to facilitate maturation of the neovasculature.

The durability of neovascularity that develops in response to ischemia does not appear, however, to depend upon persistently upregulated ligand expression. Clinical experience with patients in whom collateral vessels form in response to occlusion of coronary or lower-extremity medium-sized arteries has repeatedly demonstrated that such collateral vessels persist indefinitely. This is in marked contrast to neovasculature that develops in association with wound healing, including wounding induced by myocardial laser revascularization. The latter is ultimately associated with vascular regression, once the wound has healed. In contrast, vessels that have developed for the purpose of providing nutrient blood flow persist as long as the need for such accessory flow exists, this postnatal observation is consistent with the embryonic paradigm that blood flow is the principal determinant that acts on the plethora of blood vessels that form during embryogenesis and undergo apoptosis or survive to term.

An additional cellular component of the response to ischemia involves bone marrow (BM)-derived circulating EC precursors, termed endothelial progenitor cells (EPCs) (Figure 1). Experimental hindlimb ischemia in mice, for example, increases the frequency of the EPC-enriched population in the circulation by >400%. EPC differentiation, assessed by the number of cultured EPCs among mononuclear blood cells under EC-specific conditions, is similarly increased. To investigate the impact of enhanced EPC mobilization induced by ischemia on neovascularization, the mouse cornea micropocket assay was applied to animals in which hindlimb ischemia had been surgically created 3 days earlier (Figure 2). Slit-lamp biomicroscopic photographs and fluorescent photomicrographs documented that neovascularization of avascular mouse cornea was enhanced in animals with hindlimb ischemia compared with nonischemic sham-operated controls. Furthermore, in FVB/N mice transplanted with bone marrow of diabetic donors, there was evidence of enhanced EPC mobilization and neovascularization in response to ischemia in recipient animals.

**Figure 2.** Bone marrow (BM) transplantation (T) model employed to study the contribution of postnatal vasculogenesis to neovascularization of ischemic tissues. Transgenic mouse constitutively expressing lacZ gene transcriptionally regulated by an endothelial cell (EC)-specific promoter, flk-1 or tie-2, is used as BM donor. BM is harvested and transplanted to mouse of same genetic background, in which BM has been lethally irradiated. After a period of 4 weeks to allow for BM reconstitution of transplanted BM, recipient mouse undergoes one or more interventions, all of which are intended to serve as stimulus for neovascularization. At arbitrarily selected time points following these interventions, animals are sacrificed and the respective tissues stained with X-gal to histologically identify cells in which expression of β-galactosidase produces blue cells. Use of EC-specific promoter permits identification of blue cells, which have incorporated into foci of neovascularization as endothelial lineage cells.
planted with BM from transgenic mice constitutively expressing β-galactosidase encoded by lacZ under the transcriptional regulation of an EC-specific promoter, Tie-2, corneas excised 6 days after micropocket implantation and examined by light microscopy demonstrated a statistically significant increase in corneal expression of β-galactosidase among mice with hindlimb ischemia versus a sham-operated group. EPCs may thus constitute a reparative response to ischemic injury, controlled by the BM via circulating cytokines and soluble receptors and/or adhesive molecules; the identity of such putative mediators remains to be defined.

**PATHOLOGIC ATTENUATION OF ENDOGENOUS NEOVASCULARIZATION**

Animal studies performed in a variety of species suggest that endogenous neovascularization in response to ischemia may be impaired in association with certain “clinical” phenotypes. In old (2 years) mice and old (5 years) rabbits, angiogenesis in response to hindlimb ischemia was markedly impaired. Reductions in perfusion pressure, angiographically visible collaterals, hindlimb blood flow, and capillary density were associated with reduced levels of VEGF expression. Subsequently, certain elements of this impaired response have been clarified. Activation of HIF-1 by hypoxia is primarily determined by the stabilization of HIF-1α protein. In vitro studies from our laboratory and others of HIF-1α protein expression under hypoxic conditions suggest that the stabilization of HIF-1α is impaired with aging. Hypoxia-induced VEGF expression was significantly lower in old versus young rabbit smooth muscle cells. Transient transfection experiments with full-length and deletion constructs of the luciferase reporter gene transcriptionally regulated by the VEGF promoter indicated that this differential was attributable to the HIF-1 binding site. Indeed, HIF-1 protein and DNA binding activity were significantly reduced in old versus young cells. The exact mechanisms involved in this age-dependent reduction in HIF-1 protein expression are unknown. HIF-1 protein has been shown to be rapidly degraded by the ubiquitin-proteasome system under normoxic conditions, and stabilized by hypoxia through redox-induced changes. It is also possible that aging leads to a reduction in the ability of HIF-1 to bind to the hypoxia response element (HRE) within the VEGF promoter, or that the ability of HIF-1 to form active heterodimers is reduced. Such posttranslational loss of function has been previously described with aging for other proteins and transcription factors. Whether these mechanisms are affected by aging remains to be determined.

However, consistent with the notion that endogenous neovascularization represents a combination of cytokine and cellular responses, age-related “defects” are not confined to ligand upregulation. Recently completed analyses of patients with critical limb ischemia have documented that mobilization of BM-derived endothelial progenitor cells is significantly attenuated in old versus young individuals following VEGF GTx. Impairment of endogenous neovascularization has also been demonstrated in murine (apolipoprotein E [ApoE]-/-)12 and rabbit (Watanabe)31 models of hypercholesterolemia. Similar to findings described in animal models of advanced age, VEGF expression was markedly reduced in tissue sections retrieved from the ischemic limbs. Inferential evidence that the defect in VEGF expression associated with hypercholesterolemia is—like in old age—ultimately attributable to defects in HIF-1 expression and/or binding, was demonstrated in the Watanabe rabbit. In this hypercholesterolemic animal model, impaired angiogenesis was successfully rescued by GTx of a HIF-1α/VP16 naked DNA hybrid. While we have recently found that hypercholesterolemia augments the population of circulating EPCs (C. Kalka, unpublished data), homing and integration of EPCs into foci of neovascularization appears to be markedly impaired.

Diabetes constitutes a third phenotype that is associated with impaired angiogenesis. First demonstrated in a murine (nonobese diabetic [NOD]) model of diabetes, this finding has been recently confirmed in studies of human coronary collateral development. Reconstitution of hindlimb perfusion in the NOD mouse by VEGF GTx again implicates VEGF as the critical angiogenic growth factor responsible for endogenous neovascularization in the setting of diabetes.

**THE HETEROGENEITY OF ENDOGENOUS NEOVASCULARIZATION**

Even in the absence of the specific pathologic phenotypes cited above, there are now good data, both in animals and patients, to indicate that natural heterogeneity is a characteristic and important feature of endogenous neovascularization. In mice, for example, Rohan et al have documented a 10-fold range of response in growth factor–stimulated angiogenesis.
from Schultz et al36 who evaluated the response of patients with coronary artery disease have suggested that clinical prognosis may be determined by the extent of endogenous coronary collaterals supplying blood flow to the myocardial bed subtended by an occluded artery. Past investigations of patients with coronary artery disease who, in spite of extensive lower-extremity arterial occlusions, remain nearly asymptomatic as a result of a naturally robust collateral network. The possibility that this longstanding clinical notion of endogenous coronary collaterals supplying blood flow to the myocardial bed subtended by an occluded artery.

The possibility that this longstanding clinical notion has a genuine molecular basis is supported by work from Schultz et al36 who evaluated the response of VEGF mRNA to hypoxia in monocytes harvested from patients undergoing coronary angiography, and found that hypoxic induction of VEGF was significantly reduced in patients with poor versus rich collateral development. The significant difference in the induction of VEGF was maintained even after adjustment of data for variables such as age, diabetes, and hypercholesterolemia. Mice in which two specific isoforms (164 and 188) of the VEGF-1 gene have been deleted go on to develop an ischemic cardiomyopathy that appears to be the result of incomplete vascularization associated with defective VEGF expression.37

To what extent individual variations in the potential for endogenous neovascularization may reflect upstream dysregulation of HIF-1—mediated VEGF expression allowed to above, versus defective expression of tissue metalloproteinases, tissue plasminogen activators, other components of the cascade responsible for neovascularization, or even variations in intracellular signaling,38 remains to be defined. Such whole organism heterogeneity is further compounded by what appears to be tissue-specific variation as well.39 For example, the consequence of retinal hypoxia in diabetes is VEGF upregulation followed by pathologic neovascularization; these same patients may present with limb ischemia due to the above-described paucity of limb collaterals possibly related to locally reduced expression of VEGF. Such tissue-specific variations may not be limited to volutional differences, but may involve tissue-specific morphologic features in neovascularity as well.

**THERAPEUTIC ANGIOGENESIS FOR CRITICAL LIMB ISCHEMIA**

To date, reports of efforts to employ angiogenic growth factors to promote neovascularization in patients with critical limb ischemia have been limited to GTx, as opposed to the use of recombinant protein. The natural history of critical limb ischemia has been well documented to have an inexorable downhill course. Preclinical studies established that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral ischemia.40,41 We subsequently demonstrated angiographic and histologic evidence of angiogenesis after intra-arterial GTx of naked plasmid DNA encoding the 165-amino-acid isoform of human VEGF (phVEGF165) in a patient with critical limb ischemia.42

Following the demonstration that intramuscular injection could be equally effective43 and technically simpler as well as safer (particularly in patients with compromised and/or calcified lower-extremity vasculature), we initiated a phase 1 clinical trial comprising 9 patients with 10 critically ischemic limbs.44 Seven of the 10 limbs had developed frank gangrene. While inclusion criteria required a minimum of 4 weeks of conservative measures without evidence of improvement, in reality signs and/or symptoms of critical limb ischemia had been present in all cases for 2 to 12 months prior to gene therapy. Among this series of 9 patients (10 limbs), 6 developed critical limb ischemia despite having undergone as many as 2 surgical reconstructive procedures. Seven patients had been specifically advised to undergo limb amputation. All were using analgesic, typically ≥1 narcotic, medications. Spontaneous resolution of rest pain and/or healing of an ischemic ulcer in patients such as these with critical limb ischemia has not to our knowledge been previously reported.45 Furthermore, because VEGF had not been previously administered as recombinant protein, no data were available from any source to indicate either the safety or bioactivity of any dose of phVEGF165. Accordingly, the design of this phase 1 trial, unanimously approved by the Recombinant DNA Advisory Committee (RAC) and the US Food and Drug Administration (FDA), was conducted as a nonrandomized, consecutive treatment series, similar to phase 1 oncology protocols employed to study new chemotherapeutic agents administered to human subjects. A total dose of 4000 µg phVEGF165 was injected directly into the muscles of the ischemic limb. Analysis of gene expression at the protein level using an enzyme-
linked immunosorbent assay (ELISA) assay for VEGF documented a transient peak of the gene product in the systemic circulation 1 to 3 weeks after GTx in 7 cases (Figure 3). Further evidence of gene expression was observed in 6 patients, who developed temporally related peripheral edema (Figure 4), including 2 with bilateral edema. Parenthetically, the latter finding constitutes what to our knowledge is the first demonstration that VEGF may augment vascular permeability in human subjects.

In most patients, treatment was sufficient to achieve clinically significant modulation of the recipient phenotype. Noninvasive studies documented hemodynamic evidence of improved limb perfusion that satisfies outcome criteria proposed to assess the results of surgical reconstruction or percutaneous revascularization. Absolute ankle and/or toe pressure increased in 9 limbs after gene therapy (P=0.008). The ankle-brachial blood pressure index (ABI) and/or the toe-brachial blood pressure index (TBI) increased from 0.33±0.05 at baseline to 0.48±0.03 at 12 weeks (P=0.017). To put this in perspective, an increase of >0.1 in the ABI is considered indicative of a successful surgical or percutaneous intervention. To our knowledge, such improvement has not been previously achieved spontaneously or with medical therapy in patients with critical limb ischemia.

Similarly, angiographic demonstration of newly visible collateral vessels — accompanied here by noninvasive (magnetic resonance angiography [MRA]) evidence of improved blood flow — has to our knowledge not been previously reported in response to any therapeutic intervention. Indeed, previous reports have indicated that current methods used to perform diagnostic contrast angiography cannot provide images of arteries measuring <200 μm in diameter; the spatial resolution of images obtained by magnetic resonance angiography is even less. Using synchrotron radiation microangiography to assess collateral artery development following VEGF GTx in a rat model of hindlimb
ischemia, Takeshita et al.\(^2^3\) showed that neovascularization included a substantial contribution of vessels <180 µm in diameter. Thus, conventional angiographic techniques employed in the current study may have failed to depict the full extent of angiogenesis achieved after phVEGF\(_{165}\) transfection, particularly given that most newly visible collaterals were diminutive (<180 µm).

That angiogenesis was in fact therapeutic in the present investigation was shown by concomitant reduction in rest pain and/or a favorable impact on limb integrity. Among the 3 patients who presented with rest pain alone, rest pain resolved in all. Ischemic ulcers present in 7 limbs healed or improved markedly in 4 patients; this included 3 patients recommended for below-knee amputation in whom successful limb salvage was achieved. Given the poor prognosis for patients with chronic critical limb ischemia, in whom the possibility of spontaneous improvement is remote, the outcome in this initial cohort is thus encouraging. Subsequent experience with larger cohorts of patients, including patients receiving the VEGF-2 gene as well as the gene encoding a HIF-1α/VP16 hybrid, have demonstrated that younger patients with critical limb ischemia due to Buerger’s disease (thromboangiitis obliterans) appear to respond most consistently (25%) to strategies of therapeutic angiogenesis for critical limb ischemia\(^2^7\) consistent with the finding of an age-dependent angiogenesis in animals.\(^2^8,4^8\)

The failure of previous gene therapy trials to yield evidence of clinical success has been attributed to gene delivery, specifically the inability to deliver genes efficiently and obtain sustained expression. Those cases in which phVEGF\(_{165}\) gene therapy led to successful clinical outcomes in this clinical trial suggest that the success of gene therapy is not solely a function of transfection efficiency, nor is it necessarily dependent upon protracted gene expression. Several aspects of the gene, protein, and target tissue may have contributed to successful modulation of the host phenotype, despite the relatively low transfection efficiency typically associated with naked DNA. First, VEGF, as noted above, is actively secreted by intact cells; previous studies in our laboratory\(^4^9\) have documented that genes that encode for secreted proteins—as opposed to proteins that remain intracellular—may yield meaningful biological outcomes due to paracrine effects of the secreted gene product. Second, heparin-avidity of the VEGF\(_{165}\) isoform promotes binding to cell surface and matrix heparan sulfates that may create a biological reservoir of the secreted protein, enhancing the temporal opportunity for bioactivity. Third, while ECs were previously viewed solely as the target for VEGF, it is now clear that ECs subjected to hypoxia can synthesize VEGF as well.\(^1^1\) This autocrine feature of VEGF creates the opportunity for amplifying the effects of even a small amount of exogenous VEGF, as EC proliferation in the ischemic territory creates additional potential cellular sources of VEGF synthesis and secretion. Third, VEGF inhibits apoptosis,\(^5^0\) in part by up-regulating EC expression of fibronectin and α\(_1\)β\(_3\), thus preserving the survival signal generated by attachment of ECs to their extracellular matrix. Such reduction in EC apoptosis would be expected to complement the mitogenic effect of VEGF, resulting in a further net in-

---

**Figure 4.** Representative examples of lower-extremity edema (**) according to clinical grade in 4 patients after intramuscular plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor (phVEGF\(_{165}\)) gene transfer.
crease in EC viability. Fourth, with regard to the target of gene therapy, it has been noted that VEGF-induced angiogenesis is not indiscriminate or widespread, but is instead restricted to the sites of ischemia. This appears to result from paracrine upregulation of the principal high-affinity VEGF receptor (KDR) in response to factors released by hypoxic skeletal myocytes. Receptor upregulation on ECs within the region of lower-limb or myocardial ischemia thus enables these cells to act as magnets for any VEGF secreted into the ischemic milieu. Finally, the fact that the host tissues are by definition hypoxic may directly aid intramuscular transfer of naked DNA, due to the fact that transfection efficiency is augmented when the injected skeletal muscle is ischemic.

Previous work from our laboratory established that phVEGF 165 transgene expression is limited to less than 30 days in animal models of limb ischemia. Although Southern blot and polymerase chain reaction (PCR) analyses indicated that small amounts of plasmid DNA were preserved in tissue specimens derived from two amputees in this clinical trial, we have no evidence to suggest that transgene expression is more protracted in human subjects than in our animal models. Fortuitously, however, it appears that both in animals and humans, collateral vessel development sufficient to restore limb perfusion to satisfactory resting levels occurs within this time interval. Cessation of gene expression beyond this time point can be considered to constitute an inherent safety feature of phVEGF 165 GTx, which protects the organism from indefinite constitutive expression of an angiogenic growth factor.

Several caveats regarding this preliminary clinical experience must be acknowledged. First, it is theoretically possible that VEGF expression resulting from GTx could promote the development of a tumor that is currently too small to be recognized. Previous laboratory studies, however, have established that VEGF expression, although sufficient to promote a growth process, did not lead to malignant proliferation or to metastasis, a finding in agreement with the notion that stimulation of angiogenesis is necessary, but not sufficient for malignant growth. It is also theoretically possible that VEGF may aggravate deteriorating eyesight due to diabetic retinopathy. To date, however, no change in visual acuity has been observed in any patient treated with phVEGF 165 GTx. Nevertheless, these findings are preliminary and do not establish the long-term safety of VEGF, administered either as a gene or gene product. Second, while it is conceivable that continuous, predominantly local production of VEGF resulting from the transgene may be preferable, both from the standpoints of safety and efficacy, to a single larger dose of recombinant protein, this notion remains to be proven. Preliminary clinical trials of recombinant VEGF protein therapy have confirmed that mild hypotension seen in preclinical studies may be seen in humans as well (unpublished data). Presumably, the route and/or dose of recombinant protein delivery can be adjusted to address this issue. Clearly, further clinical studies of both recombinant protein as well as alternative dosing regimens of gene therapy will be required to define the relative merits of each approach. Third, we cannot exclude the possibility that these encouraging preliminary results might have been made more substantial and/or uniform by the use of alternative vector systems and/or dosing strategies.

**THERAPEUTIC ANGIOGENESIS FOR MYOCARDIAL ISCHEMIA**

Preliminary clinical trials established that the results obtained in human subjects with critical limb ischemia may extend to patients with myocardial ischemia. In particular, investigations of therapeutic neovascularization in patients experiencing functional class III-IV angina refractory to medical therapy and not amenable to conventional revascularization have reported significant symptomatic benefit.

Initial studies performed in our laboratory documented that symptomatic improvement in patients with myocardial ischemia was associated with improvement in the outcome of single photon emission computed tomography (SPECT)-sestamibi myocardial perfusion imaging, not only was there a reduction in the perfusion deficits associated with pharmacological stress, but large rest defects often resolved as well. These findings constituted objective evidence of improved myocardial perfusion following therapeutic neovascularization, including the possibility that foci of hibernating myocardium might be successfully rescued.

To determine if the implications of SPECT imaging could be confirmed by an independent diagnostic technique, we employed a novel strategy of catheter-based electromechnical assessment of myocardial perfusion (NOGATM system, Biosense-Webster, JCl, Warren, NJ, USA). This system utilizes electromagnetic field sensors to combine and integrate real-time information from percutaneous, intracardiac electrograms acquired at multiple endocardial locations. The result-
ing interrogations can be used to distinguish between infarcted and normal myocardium and thus permit online assessment of myocardial function and viability.

Accordingly, NOGA™ electromechanical mapping (EMM) was prospectively performed in 13 consecutive patients before and 60 days following GTx of phVEGF165, administered intraoperatively by direct myocardial injection in patients with chronic myocardial ischemia. Electromechanical maps of the left ventricle (LV) recorded during sinus rhythm were successfully generated in all patients before and 60 days after GTx. During the mapping procedure, there were no significant changes in mean heart rate or blood pressure. EMM was associated with transient ventricular ectopic activity, but neither sustained ventricular arrhythmias nor other arrhythmias were observed. In all patients, NOGA™ maps were reliably reproduced following GTx in terms of number of points, end-diastolic volume, end-systolic volume, and average loop stability (data not shown). The LV ejection fraction (LVEF), calculated on the basis of algorithms incorporated in the NOGA™ system, increased from 31.3±2.7% pre-GTx to 36.9±2.3% post-GTx (P=0.023).

Foci of ischemic myocardium, identified by preserved viability associated with impaired linear local shortening (LLS), ie, electromechanical uncoupling, were demonstrated in all patients prior to GTx. Foci of ischemia involved the anterior (n=1), anteroseptal (n=1), lateral (n=1), inferolateral (n=2), posterior (n=3), posterolateral (n=2), septal (n=2), and inferoseptal (n=1) walls. Mean unipolar and bipolar voltage recordings ≥5 mV and ≥2 mV, respectively, defining myocardial viability in the ischemic zone, did not change significantly following GTx. Mean LLS in areas of myocardial ischemia, however, improved significantly from 9.94±1.53 cm² pre-phVEGF165 GTx to 15.26±0.98 cm² post-phVEGF165 GTx (P=0.004). The area of ischemic myocardium was consequently reduced from 6.45±1.37 cm² pre-phVEGF165 GTx to 0.95±0.41 cm² post-phVEGF165 GTx (P=0.001). Clinically, these 13 patients reported significant reduction in anginal episodes/wk (48±4.9 vs 2.0±0.8, P<0.0001), and weekly consumption of nitroglycerin (NTG) tablets (55.0±7.1 vs 1.9±0.8, P<0.0001). Standard Bruce protocol exercise tolerance testing was performed in all patients at days 90 and 180 following GTx. The mean duration of exercise increased from 272 s to 453 s (P=0.001) up to 180 days following GTx. LVEF remained the same (n=5) or increased (n=8, mean increase 5%) up to day 180 post-GTx (mean EF pre-GTx=53.5%±3.7% vs post-GTx=58.1%±3.8%, P=0.004). The results of EMM corresponded to improved perfusion scores calculated from SPECT-sestamibi myocardial perfusion scans recorded at rest (7.4±2.1 pre-GTx vs 4.5±1.4 post-GTx, P=0.009), as well as with pharmacological stress (12.8±2.7 vs 8.5±1.7, P=0.047) (Figures 5-8, see next pages). A positive correlation existed between the change in rest perfusion score for ischemic myocardium and the reduction in ischemic area as measured by NOGA™ mapping (P=0.042, r=0.567). The collated electrical and mechanical results of percutaneous EMM provide both an assessment of myocardial viability (ie, the presence of normal versus reduced voltage) and wall motion (presence of normal versus reduced fractional shortening). Validation of intracardiac signal recording and location accuracy has been previously established, both in vitro and in vivo. Clinical investigations have demonstrated that the mapping capabilities of the NOGA™ system may be used to distinguish between infarcted and normal myocardium. Gepstein et al59 found significantly lower LLS (<4%) and bipolar voltages (<2 mV) in infarcted versus noninfarcted myocardium. Furthermore, comparison with pathologic specimens confirmed that the location and extent of infarction could be accurately defined by EMM.

These earlier findings were confirmed by Kornowski et al60 who showed that patients with prior myocardial infarction had reduced unipolar (7.2±2.7 mV) and bipolar (1.4±0.7 mV) voltage recordings compared with patients without prior infarction (19.7±4.4 mV and 5.8±1.0 mV for unipolar and bipolar, respectively), and that these patients had reduced local endocardial shortening compared with patients without prior infarction. Moreover, Kornowski et al demonstrated that mean voltage and LLS values are highest when measured in myocardial segments with normal perfusion, and lowest when measured from segments with fixed perfusion defects; intermediate LLS (4%-12%) and voltage (≥5 mV) recordings were documented for myocardial segments with reversible perfusion defects.61 Resolution of rest defects observed in the SPECT scans post-GTx is particularly intriguing. In this population of severely disabled, so-called “no-option” patients, the rest defects were presumed to represent sites of myocardial scar associated with the clinical history of myocardial infarction in 13/13 patients. Partial or complete resolution of these rest defects post-GTx is consistent with the notion that these preexisting defects constitute foci of hibernating myocardium, and may have been successfully resuscitated as a result of therapeutic neovascularization.
The corresponding NOGA™ maps likewise showed reduced evidence of ischemia post-GTx. EMM provides separate assessments of viability (endocardial voltage recording) and function (LLS). Thus, those areas of the NOGA™ map that showed viable myocardium with impaired function pre-GTx, versus viable myocardium with improved function post-GTx, support the notion that the defects that resolved on the SPECT scans constitute sites of hibernating myocardium that have been resuscitated as a result of myocardial neovascularization. These findings further suggest that LV EMM represents an independent diagnostic tool that may be useful for defining the myocardial consequences of improved perfusion.

Figure 5. NOGA™ left ventricular (LV) electromechanical mapping (EMM) performed in a 48-year-old male. NOGA™ images in the right anterior oblique (RAO) projection prior to gene transfer (GTx) show the unipolar voltage (UpV) map (upper left panel) with normal voltages suggestive of viable myocardium (purple/pink/blue/green) and the linear local shortening (LLS) map (upper right panel) with a large zone of abnormal wall motion (red, arrow) representing electromechanical uncoupling that suggests ischemic or hibernating myocardium predominantly involving the septum. UpV and LLS maps in the RAO projection 60 days following GTx (lower left and right panels, respectively) demonstrate almost complete resolution of the ischemic zone (ischemic area 9.57 cm² pre-GTx vs 0.39 cm² post-GTx). Changes in LLS correspond with changes observed on single photon emission computed tomography (SPECT) scan (Figure 6). Red line represents long axis through the apex.

Figure 6. Persantine single photon emission computed tomography (SPECT)-sestamibi myocardial perfusion scanning. Selected short and horizontal axis stress and rest images taken prior to and following plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor gene transfer (pVEGF165 GTx) in the same patient as shown in Figure 5. White/yellow color depicts maximal uptake of radionuclide and red depicts impaired uptake. Pre-GTx scans (upper panel) show a fixed anteroapical defect (arrowhead) and a partially reversible inferoseptal defect (arrow). Post-GTx scans (lower panel) show complete normalization of resting perfusion with a small persistent reversible anteroapical defect following pharmacological stress.
PERCUTANEOUS GENE TRANSFER FOR THERAPEUTIC ANGIOGENESIS IN PATIENTS WITH MYOCARDIAL ISCHEMIA

The above clinical findings,62 as well as preliminary studies performed in swine with myocardial ischemia,63 suggest that mapping the extent of ischemia may also be used online to direct percutaneous myocardial GTx. Such an adjunct may be particularly advantageous for optimizing low-efficiency strategies such as naked DNA GTx, in which EMM may direct injection of naked DNA to ischemic muscle, shown previously to yield higher levels of gene expression.43 We thus designed a pilot study to assess the feasibility, safety, and potential efficacy of catheter-based, percutaneous myocardial GTx.

Figure 7. NOGATM left ventricular (LV) electromechanical mapping (EMM). Unipolar voltage and linear local shortening (LLS) NOGATM images in the left anterior oblique (LAO) projection (upper left and right panels, respectively) of a 53-year-old male prior to plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor gene transfer (phVEGF165 GTx) showing an area of electromechanical uncoupling suggestive of ischemic or hibernating myocardium that involves the inferolateral region (arrow). Sixty days following GTx, Unipolar voltage (UpV) and LLS images (lower left and right panels, respectively) show complete resolution of ischemia (1.39 cm² pre-GTx vs 0.00 cm² post-GTx) that corresponds to changes observed on single photon emission computed tomography (SPECT) scan (see Figure 8).

Figure 8. Persantine single photon emission computed tomography (SPECT)- sestamibi myocardial perfusion scanning. Selected short and horizontal axis stress and rest images (same color scale as Figure 6) taken prior to and following plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor gene transfer (phVEGF165 GTx) in the same patient as shown in Figure 7. Pre-GTx scans (upper panel) show a reversible inferolateral defect (arrows). Post-GTx scans (lower panel) show complete normalization of resting perfusion.
of naked DNA encoding VEGF-2 administered via a novel needle-injection catheter, and compared this in a single-blind fashion with a mock procedure. A steerable, deflectable 8F catheter incorporating a 27-gauge was advanced percutaneously to the LV myocardium of 6 patients with chronic myocardial ischemia. Patients were randomized (1:1) to receive phVEGF-2 (total dose 200 µg) administered as 6 injections into ischemic myocardium (total 6.0 mL) or placebo (mock procedure), guided by NOGATM LV EMM. Patients initially randomized to placebo became eligible for phVEGF2 GTX if there was no clinical improvement by 90 days following their initial procedure. Catheter injections (total=36) caused no changes in heart rate or blood pressure. No sustained ventricular arrhythmias, ECG evidence of infarction, or ventricular perforations were observed. VEGF-2-transfected patients reported reduced angina (pre-GTx=36.2±2.3 vs post-GTx=3.5±1.2 episodes/wk) and reduced nitrate tablet consumption (33.8±2.3 vs 4.1±1.5 tablets/wk) up to 360 days post-GTx. EMM documented a reduction in the myocardial ischemia (mean area of ischemia pre-GTx=10.2±3.5 cm² vs post-GTx=2.8±1.6 cm², P<0.04). Finally, evidence of improved myocardial perfusion was documented by SPECT-sestamibi scan up to 90 days post-GTx compared with images obtained post-mock procedure.

This randomized trial of catheter-based VEGF-2 myocardial GTX thus provides preliminary evidence for the safety, feasibility, and potential efficacy of percutaneous myocardial GTX. The findings permitted initiation of a prospective, double-blind, placebo-controlled trial that recruited 19 patients (total=114 catheter injections) before it was interrupted by the US FDA, as of this date, nearly all patients have been followed for 1 year or more with no mortality and no morbidity related to the interventions. These findings may ultimately permit catheter-based myocardial GTX, obviating the need for surgery.

FAVORABLE IMPACT OF VEGF GENE TRANSFER ON ISCHEMIC PERIPHERAL NEUROPATHY

An unanticipated finding emerged with the very first patient to receive intramuscular (IM) GTX of phVEGF165. This patient, a 33-year-old New York school teacher, presented with a 9×2 3-cm ulcer in her left leg. The ulcer had failed to resolve with 6 months of conservative therapy and she was recommended to undergo below-knee amputation. At the time of her presentation she was using methadone, oxycodone, and a fentanyl patch for severe rest pain. Within 12 weeks post–gene therapy, the size of the ulcer had decreased sufficiently to permit a split-thickness skin graft, which promptly healed. She was weaned from all analgesics over the next 6 weeks. At 3-year follow-up, she is free of rest pain and the graft remains entirely intact. While this clinical response to the first application of intramuscular phVEGF165 GTX was in itself remarkable, what was particularly intriguing was that the patient reported to us that inability to perceive touch, which had extended from the toes to the mid-calf, had resolved as well. A similar finding was reported by 2 other of the first 8 patients in this protocol. In the case of the third patient, a 64-year-old man, in whom such hypesthesia extended to the knee level, we began to systematically track the residual extent of the sensory deficit as it resolved over a 16-week period; this man remains asymptomatic with no residual sensory deficit at 18-month follow-up.

On the basis of these anecdotal observations, we initiated a prospective evaluation of patients undergoing intramuscular phVEGF165 gene therapy for critical limb ischemia. Patients were evaluated by two neurologists, one performing the clinical assessment, and one performing electrophysiologic testing. Both were blinded to each other’s results, and both were blinded to the results of the patients’ vascular exams. Furthermore, at the time of the follow-up exam, both were blinded to the results of previous exams. A total of 24 limbs have thus far been analyzed before and 3 months after GTX; 19 of these have been followed out to 6 months. Similar to what was observed in our index patient, improvement in neurogenic symptoms and reduction in neurologic disability was evident in these patients as early as 3 months, and to a greater extent at 6 months. Motor nerve conduction studies and quantitative sensory testing disclosed objective evidence of improved peripheral nerve function, in comparison with untreated legs and baseline studies; specifically, improvement was noted in peroneal nerve amplitude and vibratory threshold at 6 months following phVEGF165 GTX. These findings suggested that therapeutic angiogenesis may have a favorable impact on established ischemic peripheral neuropathy.

To further investigate the impact of administered EC mitogens on ischemic peripheral neuropathy (IPN), we established appropriate animal models to investigate whether IPN could be prevented and/or reversed by GTX of an EC mitogen designed to promote therapeutic angiogenesis.66
When intramuscular GTx of naked DNA encoding VEGF was performed simultaneously with induction of hindlimb ischemia in rabbits, severe depression of motor and sensory nerve parameters was aborted and nerve function recovered promptly. When GTx was administered 10 days after induction of ischemia, nerve function was restored earlier and/or recovered faster than in untreated rabbits. Neurophysiologic results were paralleled by improvements in perfusion parameters. Additionally, in vitro experiments indicating functional VEGF receptor expression by Schwann cells suggested the contribution of a direct effect of VEGF on neural integrity as well. These findings thus constitute a novel paradigm for the treatment of IPN.

The development of IPN concurrently with reduced hindlimb blood flow is consistent with previous reports indicating that compromised blood flow causes pathologic alteration of peripheral nerves, including loss of myelin and axonal degeneration; these findings are typically associated with altered nerve electrophysiology and attenuated sensory and motor function. The demonstration that hindlimb ischemia leads to a severe peripheral neuropathy thus provided the opportunity to determine in a preliminary fashion if such neurologic findings could be attenuated by strategies of therapeutic angiogenesis employed previously in this animal model. More recently, experiments performed in two animal models of diabetic neuropathy—unassociated with macrovascular ischemia—have established that diabetic peripheral neuropathy results from a loss of vasa nervorum, and that therapeutic angiogenesis may successfully preserve the vasa nervorum and thus attenuate the associated neuropathy.

These findings thus suggest a protective effect of therapeutic angiogenesis on the development of IPN, and raise intriguing questions regarding the basis for the putative impact of plVEGF165 GTx on symptoms and signs of IPN. We have inferred that our preliminary findings are at least in part attributable to enhanced perfusion via vasa nervorum, the nutrient arteries derived from a main artery or muscle artery that form an anastomotic complex within the nerve. In this regard, it is important to note that in vivo and postmortem studies have suggested that neovascularization that develops in response to angiogenic cytokines principally involves vessels <180 µm in diameter, a dimension that would include the vasa nervorum.

Alternatively, the preliminary results of our in vitro experiments indicate the possibility of a direct effect of VEGF on neural elements. As indicated above, we found that VEGF promotes both survival of Schwann cells in vitro and migration of Schwann cells in a modified Boyden chamber assay. Moreover, reverse transcriptase polymerase chain reaction (RT-PCR) and Western blots performed on cultured Schwann cells disclosed expression of VEGF receptors Flt-1 and Flk-1. While further work is required to clarify the extent to which a direct effect of VEGF is responsible for in vivo observations described in this paper, the in vitro observations at the very least strengthen the theoretical basis for direct interaction between VEGF and neuronal elements in ischemic peripheral neuropathy.

Our preliminary clinical findings in patients and experimental findings in the rabbit ischemic hindlimb also raise questions regarding a potential role for endogenous VEGF expression in modulating peripheral nerve integrity, indirectly via effects on vascularity and/or directly via effects on neural elements. In this regard, it is intriguing that Mellick and Cavanagh noted over 30 years ago:

... a direct relationship between the known growth rates of regenerating axons and the pattern of increased blood vessel permeability... [following nerve injury]. During the first 24 hours after nerve injury the site of leakage is in its immediate vicinity... By the fourth day, however, the blood vessels in the more distal segments begin to show increased permeability. The extravascular albumin content of these increased from the fourth day to reach a maximum on the 14th day and then decreased until the 32nd day. By the 32nd day, these segments still show greatly increased permeability. The delay in onset of the increased permeability of more than 24 hours in the segments more than 12 mm from the injury suggests that the leakage is not a direct and immediate consequence of the injury. [italics added]

They suggested that these findings were consistent with the demonstration “...as early as 1900, [of] a histamine-like substance ... in peripheral nerves...” VEGF, originally known as vascular permeability factor (VPF) is 50 times more potent than histamine in promoting permeability. Current studies in our laboratory indicate that VEGF is indeed expressed by cultured Schwann cells (P. Schratzberger, unpublished data). The extent to which VEGF and its receptors may constitute an endogenous regulatory system for maintaining the integrity of the vasa nervorum—and directly or indirectly the peripheral nerves themselves—requires further study.
**IMPACT OF CLINICAL PHENOTYPE ON NEOVASCULARIZATION**

Preliminary clinical findings in patients with critical limb ischemia indicated that the response to phVEGF GTx was most robust and expeditious in young patients with premature atherosclerosis involving the lower extremities, so-called Buerger’s disease. This clinical observation was supported by experiments performed in live animal models, specifically young (4-5 y) versus old (6-8 mo) rabbits and young (8 wk) versus old (2 y) mice. In both cases, native neovascularization of the ischemic hindlimb was markedly retarded in old versus young animals. Retardation of neovascularization in old animals appeared in part to result from reduced expression of VEGF in tissue sections harvested from the ischemic limb. Recent studies in our laboratory have established that dysregulated VEGF expression ultimately results from deficient expression and/or binding of HIF-1 transcription factor to the VEGF promoter.

Similarly retarded neovascularization and reduced VEGF expression was observed in diabetic (NOD) and hypercholesterolemic (ApoE-/-) mice. Cell-specific immunostaining localized VEGF protein expression to skeletal myocytes and infiltrating T cells in the ischemic limbs of C57 mice; in contrast, VEGF-expressing T cell infiltrates were found to be severely reduced in ischemic limbs of mice in which angiogenesis was impaired. Transendothelial migration of human T cells has been previously shown to be compromised in elderly versus young subjects, although the basis for this defect in transmigration remains enigmatic. The critical contribution of T cells to VEGF expression and collateral vessel growth has been reinforced by the finding of accelerated limb necrosis in athymic nude mice with operatively induced hindlimb ischemia.

Reduction in endogenous VEGF expression, however, was not the only factor contributing to impaired neovascularization in these animals; older, diabetic and hypercholesterolemic animals—like patients—also exhibit age-related endothelial dysfunction, manifest as reduced vasodilation and decreased production of NO in response to endothelium-dependent agonists. Endothelial dysfunction did not preclude a favorable response to cytokine replacement therapy: indeed the absolute magnitude by which blood pressure ratio, angiographic score, and capillary density were increased in response to supplemental administration of recombinant VEGF protein was similar for young and old animals. In older animals, however, these indices failed to reach the ultimate levels recorded in younger animals, apparently reflecting the inherent limitations imposed by a less responsive EC substrate. This clinical experience and these animal studies have two implications. First, the findings suggest that the fundamental mechanism by which therapeutic neovascularization augments collateral development is to provide cytokine supplements to individuals who—because of advanced age, diabetes, hypercholesterolemia, and/or other as yet undefined circumstances—are unable to appropriately upregulate cytokine expression in response to tissue ischemia. In this regard, ligand supplementation may be analogous to erythropoietin administration in patients with refractory anemia.

Second, cytokine administration clearly comprises only one aspect of the therapeutic intervention. Regardless of how much ligand is administered, the resident population of ECs that is competent to respond to an available level of angiogenic growth factors may also constitute a potentially limiting factor in strategies designed to promote neovascularization of ischemic tissues. A reasonable goal may therefore consist in developing a complementary strategy that would provide substrate together with ligand, a “supply side” version of therapeutic neovascularization.

**POSTNATAL VASCULOGENESIS**

The option of performing full-scale EC transplantation to optimize this therapeutic strategy is daunting if even feasible. Accordingly, we investigated an alternative strategy designed to exploit the conceptual notion that ECs and hematopoietic stem cells (HSCs) were ultimately derived from a common precursor, the putative hemangioblast. HSCs had been shown previously to be present in circulating blood, in quantities sufficient to permit their harvesting and readministration for autologous—in lieu of BM—transplantation. We therefore inferred that related descendents—EPCs—might be present along with HSCs in the peripheral circulation. Flk-1 and a second antigen, CD-34, shared by angioblasts and HSCs were used to isolate putative angioblasts from the leukocyte fraction of peripheral blood. In vitro, these cells differentiated into ECs. In animal models of ischemia, heterologous, homologous, and autologous EPCs were shown to incorporate into sites of active neovascularization.

More recently, we have utilized a BM transplant model to demonstrate incorporation of BM-derived EPCs into foci of neovascularization. Wild type mice were lethally irradiated with 9.0 Gy and were transplanted with BM harvested from transgenic mice of the same genetic background in which constitutive lacZ expression is
regulated by an EC-specific promoter, Flk-1 or Tie-2. Flk-1 (VEGFR-2) has been shown to be essential for EPC (angioblast) differentiation and blood vessel development during embryogenesis and postnatal neovascularization. The Tie-2 receptor has been shown to be expressed in endothelial lineage cells participating in angiogenesis, and in this regard is essential for blood vessel development and maturation. Consequently, β-galactosidase is constitutively overexpressed in the BM of the transplant recipient flk-1 or tie-2/lacZ mice, but not in any other somatic cells. Application of a solution of X-gal to the BM renders it blue, and any blue cells that are detected at remote tissue sites can thus be inferred to have been derived from BM and delivered to those sites via the peripheral circulation. After a period of 4 weeks post-transplant, by which time the BM of the recipient mice is reconstituted, a variety of surgical experiments may be performed, all of which are intended to provoke neovascularization. For example, preliminary experiments performed in a mouse model of corneal injury disclosed BM-derived cells incorporated into neovascular foci at the corneal limbus. A similar approach may be used to investigate the contribution of circulating, BM-derived EPCs to neovascularization of ischemic hindlimbs, injured corneas, and tumor vasculature.

Previous investigators have shown that wound trauma causes mobilization of hematopoietic cells, including pluripotent stem or progenitor cells in spleen, BM, and peripheral blood. Consistent with EPC/HSC common ancestry, data from our laboratory have shown that mobilization of BM-derived EPCs constitutes a natural response to tissue ischemia. In these experiments, we used the murine BM transplant model to establish direct evidence of enhanced BM-derived EPC incorporation into foci of corneal neovascularization following the development of hindlimb ischemia. Light microscopic examination of corneas excised 6 days after micropocket injury and concurrent surgery to establish hindlimb ischemia demonstrated a statistically significant increase in cells expressing β-galactosidase in the corneas of mice with versus those without an ischemic limb. This finding indicates that circulating EPCs are mobilized endogenously in response to tissue ischemia following which they may be incorporated into neovascular foci to promote tissue repair.

**THERAPEUTIC VASCULOGENESIS**

Having demonstrated the potential for endogenous mobilization of BM-derived EPCs, we considered that iatrogenic expansion and mobilization of this putative EC precursor population might represent an effective means to augment the resident population of ECs that is competent to respond to administered angiogenic cytokines. Such a program might thereby address the issue of endothelial dysfunction or depletion that may compromise strategies of therapeutic neovascularization in older, diabetic, and/or hypercholesterolemic animals and patients. Granulocyte-macrophage colony-stimulating factor (GM-CSF), which stimulates hematopoietic progenitor cells and myeloid lineage cells, as well as nonhematopoietic cells including BM stromal cells and ECs, was employed to test this notion. To effect GM-CSF–induced EPC mobilization while avoiding a direct effect on ECs, recombinant human GM-CSF (rhGM-CSF) was administered daily for 7 days prior to surgery to create hindlimb ischemia. GM-CSF pretreatment produced a statistically significant increase in the circulating population of EPCs and enhanced EPC differentiation versus controls. Moreover, capillary density analysis documented extensive neovascularization induced by GM-CSF pretreatment, and measurements of ischemic/normal hindlimb blood pressure ratio disclosed evidence of corresponding increase in hindlimb blood flow. These results thus indicate that GM-CSF exerts a potent stimulatory effect on EPC kinetics and that such cytokine-induced EPC mobilization can enhance neovascularization of severely ischemic tissues as well as de novo vascularization of previously avascular sites.

Differential expression of phenotypic markers that permit isolation of EPCs from not only HSCs, but ECs as well, will facilitate strategies of therapeutic vascu- logenesis. While VEGFR-2 is generally considered to distinguish EPCs from HSCs, there exists no epitope whose expression is restricted exclusively to either fully differentiated ECs or EPCs. There are at least three lines of evidence, however, that suggest that EPCs constitute the preponderance of such circulating BM-derived endothelial lineage cells. First, previous work has shown that freshly isolated CD34+ cells display a paucity of EC-specific markers, in contrast to plated cells cultured for 7 days. Second, recent work from our own laboratory has shown that, in contrast to EPCs, heterologously transplanted differentiated ECs rarely incorporate into foci of neovascularization. Third, previous work suggests that the number of differentiated ECs circulating in peripheral blood identified using P1H12 antibody, ranges between 2 to 3 per mL, whereas the population of circulating EPCs in normal individuals based on work from our own laboratory is in the range of 0.5 to 1x10^7 per mL of blood. These experimental findings call into question certain funda-
mental concepts regarding blood vessel growth and development in adult organisms. Postnatal neovascularization has been previously considered synonymous with proliferation and migration of preexisting, fully differentiated ECs resident within parent vessels, i.e., angiogenesis. The finding that circulating EPCs may home to sites of neovascularization and differentiate into ECs in situ is consistent with “vasculogenesis,” a critical paradigm for establishment of the primordial vascular network in the embryo. While the proportional contributions of angiogenesis and vasculogenesis to postnatal neovascularization remain to be clarified, our findings, together with the recent reports from other investigators, suggest that growth and development of new blood vessels in the adult is not restricted to angiogenesis, but encompasses both embryonic mechanisms. As a corollary, augmented or retarded neovascularization—whether endogenous or iatrogenic—likely includes enhancement or impairment of vasculogenesis.

Moreover, the observation that circulating EPCs home to foci of neovascularization suggests potential utility as autologous vectors for gene therapy. For treatment of regional ischemia, neovascularization could be amplified by transfection of EPCs to achieve highly localized constitutive expression of angiogenic cytokines and/or provisional matrix proteins. For antineoplastic therapies, EPCs could be transfected with or coupled to antitumor drugs or angiogenesis inhibitors.

**VEGF GENE TRANSFER AUGMENTS CIRCULATING ENDOTHELIAL PROGENITOR CELLS**

Preclinical studies in animal models and early studies performed in small numbers of patients with lower-limb ischemia support the notion that GTx of VEGF DNA may promote neovascularization of ischemic tissues. Such neovascularization has been attributed exclusively to sprout formation of ECs derived from preexisting vessels. We investigated the hypothesis that VEGF GTx may also augment the population of circulating EPCs.

In patients with critical limb ischemia receiving VEGF GTx, gene expression was documented by a transient increase in plasma levels of VEGF. A culture assay documented a significant increase in EPCs (219%, P < 0.001), while patients who received an empty vector had no change in circulating EPCs, as was the case for volunteers who received saline injections (VEGF vs empty vector, P < 0.001; VEGF vs saline, P < 0.005).

Fluorescence-activated cell sorter (FACS) analysis disclosed an overall increase of up to 30-fold in endothelial lineage markers KDR (VEGFR-2), VE-cadherin, CD34, αvβ3, and E-selectin following VEGF GTx. Constitutive overexpression of VEGF in patients with limb ischemia augments the population of circulating EPCs.

These findings support the notion that neovascularization of human ischemic tissues following angiogenic growth factor therapy is not limited to angiogenesis, but involves circulating endothelial precursors that may home to ischemic foci and differentiate in situ through a process of vasculogenesis. Moreover, consistent with previous reports that established that direct injection of phVEGF165 into muscle of the ischemic limb, as well as into ischemic myocardium, transiently elevates plasma VEGF levels in the systemic circulation, we observed a rise in plasma levels of VEGF associated with modulation of EPC kinetics following VEGF GTx. The increase in EPCs was statistically significant as early as 1 week post-GTx, and remained statistically significant at 2, 3, and 4 weeks follow-up. By comparison, EPC kinetics in the control subjects—including patients with or without critical limb ischemia, injected with empty vector or saline—were unchanged.

Due to limitations in the types of analyses that may be performed in human subjects, the origin and fate of the augmented population of circulating EPCs in these patients must be inferred from experiments performed previously in live animal models. Daily intraperitoneal injection of recombinant human VEGF165 (rhVEGF) to C57BL/6J mice for a period of 1 week increased the total number of circulating EPCs. These effects were abrogated by coincidental application of a neutralizing antibody prepared against rhVEGF.

When mice were pretreated with rhVEGF or control buffer for 7 days prior to cornea micropocket injury and then examined on day 7 post-injury (ie, 7 days following the last dose of rhVEGF), in situ BS-1 lectin staining disclosed enhanced corneal neovascularization in the rhVEGF group compared with controls. These findings were reproduced in mice transplanted with BM from transgenic mice constitutively expressing β-galactosidase encoded by lacZ under the transcriptional regulation of an EC-specific gene, tie-2, to establish direct evidence for incorporation of BM-derived EPCs into capillaries and stromal tissue of the corneal neovascularature.

Like fully differentiated ECs, EPCs express specific endothelial antigens including KDR (VEGFR-2), CD34, and VE-cadherin. While KDR and VE-cadherin are...
generally considered to distinguish EPCs from HSCs, there exists no epitope whose expression is restricted exclusively to EPCs versus fully differentiated ECs. There is, however, evidence that EPCs constitute the preponderance of such circulating, BM-derived endothelial lineage cells. First, the present work indicates that the population of circulating EPCs in normal individuals (3 to 5 x 10^3/mL) far exceeds the number of differentiated ECs circulating in peripheral blood (2 to 3/mL). Second, animal experiments from our own laboratory have suggested that the cellular population mobilized into the circulation and then incorporated into neovascular foci following VEGF administration is most consistent with BM-derived EPCs. These clinical findings call into question certain fundamental concepts regarding the mechanisms by which VEGF promotes blood vessel growth and development in adult organisms. The role of VEGF in postnatal neovascularization has been previously considered synonymous with proliferation and migration of preexisting, fully differentiated ECs resident within parent vessels, i.e., sprout formation or angiogenesis. The finding
that VEGF augments the number of circulating EPCs in human patients, together with the aforementioned murine experiments, implies that its impact on postnatal neovascularization is the combined result of vasculogenesis as well as angiogenesis. The proportional contributions of angiogenesis and vasculo-genesis to postnatal neovascularization, including the extent to which each is influenced by VEGF, remain to be clarified.

Finally, these findings have implications for the use of naked DNA in human gene therapy. Earlier studies suggested that the low transfection efficiency associated with the use of naked DNA might make it unsuitable for therapeutic applications in trials of human gene therapy. Subsequent experience in live animal models, however, demonstrated that transfer of genes encoding for secreted proteins, such as VEGF, could yield important biological effects due to the paracrine effects of the secreted gene product. The demonstration that VEGF gene therapy augments the compartment of circulating EPCs constitutes further evidence that GTx of naked DNA may indeed be sufficient to modulate the biology of human subjects.

CELL THERAPY TO PROMOTE ANGIOGENESIS

Animal studies and preliminary results in humans suggest that lower-extremity and myocardial ischemia can be attenuated by treatment with angiogenic cytokines. The resident population of endothelial cells that is competent to respond to an available level of angiogenic growth factors, however, may potentially limit the extent to which cytokine supplementation enhances tissue neovascularization (Figure 9, page 163). Accordingly, we transplanted human endothelial progenitor cells (hEPCs) to athymic nude mice with hindlimb ischemia. Blood flow recovery and capillary density in the ischemic hindlimb were markedly improved, and the rate of limb loss was significantly reduced (Figure 10).

These findings provide the first evidence that exogenously administered EPCs augment naturally impaired neovascularization in an animal model of experimentally induced limb ischemia. Not only did heterologous cell transplantation improve neovascularization and blood flow recovery, but important biological consequences—notably limb necrosis and auto-amputation—were reduced by 50% in comparison with two different types of controls.

Cell transplantation in this case is predicated upon ex vivo expansion of EPCs isolated from human peripheral blood mononuclear cells (hPBMCs) harvested from healthy adult human subjects. Incubation with endothelial mitogens, including VEGF, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and endothelial growth factor (EGF), for 7 to 10 days resulted in an 80- to 90-fold expansion of cells expressing the EC-specific antigens KDR, CD31, and VE-cadherin. This calculation is based on data from our lab-
oratory, which indicate that approximately 0.05% or
5 × 10^2 hEPCs can be isolated from 1 × 10^6 hPBMCs of
human subjects. The ex vivo culture strategy permits
expansion of the population of hEPCs to 4–5 × 10^4 cells
per 1 × 10^6 hPBMCs, yielding an 80– to 90-fold increase
in the original number of harvested cells. Previous anal-
yses of embryonic neovascularization suggest that co-
expression of Flk-1 (KDR) and VE-cadherin denote the
point of divergence of ECs from hematopoietic line-
ages. Moreover, a combination of monoclonal antibod-
ies prepared against Flk-1/KDR, VE-cadherin, CD31,

Tie-1, and Tie-2 have been interpreted to define most
intermediate stages during differentiation of embryonic
stromal cell–derived ECs. The capacity to take up acety-
lated low-density lipoprotein (acLDL) as well as Ulex
europaeus agglutinin–1 UEA-1 further characterize ECs.

Cultured EPCs, as opposed to freshly isolated CD34
antigen-positive (CD34+) EPCs,74 were used in these
experiments for three reasons. First, the number of
EPCs obtained by ex vivo expansion (3–5 × 10^4 from 1 mL
whole blood) exceed the number of CD34+ cells that
can be freshly isolated (0.5 × 10^4/mL blood). Second,
the purity and quality of EPCs in a cultured population
are superior to that of freshly isolated CD34+ cells, as
CD34+ was originally defined as the prototypical
antigen expressed by both HSCs and endothelial lineage
cells, hematopoietic cells may contaminate freshly
isolated CD34+ cells. Indeed, pilot studies demonstrat-
ed that the extent of neovascularization achieved fol-
lowing transplantation of freshly isolated CD34+ cells
was inferior to culture-expanded EPCs. Third, for ther-
aputic strategies designed to employ transplanted
cells that constitutively express pro- or anti-angiogenic
factors, GTx of EPCs is facilitated by the use of culture-
committed versus less differentiated CD34+ EPCs
(T. Asahara, unpublished data).

Under the described conditions, contamination by oth-
er cell lines, including lymphocytes, macrophages, and
dendritic cells, was minimized as indicated by limited
to absent expression of CD3, CD19, CD68, CD83, and
CD86. Incubation of similar mononuclear cell cultures
with other cytokines such as GM-CSF or TNF-α has
been reported to favor isolation of dendritic cells, in
contrast, VEGF appears to inhibit dendritic cell matura-
tion from CD34+ precursors. As cytokine composition
of the culture media may influence in vitro mononu-
clear cell differentiation (T. Kalka-Moll, unpublished
data), the cytokine mixture employed for EPC culture,
containing VEGF, bFGF, IGF, and EGF, appears to pref-
errentially promote endothelial lineage differentiation.
Isolation of circulating mononuclear cells for ex vivo
EPC expansion was carried out using peripheral blood
from human donors. Isolation of circulating EPCs from
human subjects thus appears realistic for harvesting
EPCs for therapeutic neovascularization in future clin-
ical applications. The feasibility of retrieving cells from
peripheral blood has been previously established.
Augmented mobilization of BM-derived EPCs may be
achieved using several cytokines, including GM-CSF,26
similar to the approach utilized in preparation for stem-
cell transplants. The potential value of this approach
is that it supplies substrate—ie, a source population
of robust ECs—that may complement current strategies
of ligand administration for patients in whom
depicted and/or dysfunctional ECs preclude an opti-
mal response to cytokine supplements. More recently,
a similar strategy has been shown to be successful for
preserving left ventricular function post–myocardial
infarction.78

**FUTURE PERSPECTIVES**

It is interesting to speculate that the role of angiogenic
growth factor receptors, their cognate ligands, and
BM-derived EC precursors may be assuming increased
importance in an era of increasing longevity and con-
currently compelling evolutionary selection pressures.
In the days when the life span of an average human
was limited to 30 years, trauma and infection led to
deaths well before individual ability to upregulate VEGF
expression and/or mobilize EC progenitors became an
issue. Several million years later, nature may begin to
favor survival of those best equipped to adapt to the
stresses and survival threats posed by tissue ischemia
unrelated to snake bites or elephant stampedes. Look-
ing forward to the long term, the genetic endowment
permitting one to appropriately upregulate cytokine
expression and mobilize EPCs in a fashion that is op-
timally suited to revascularize ischemic tissues may
constitute a distinct survival advantage. In the short
term, recognition of those elements that comprise the
genetic profile of such individuals may permit us to
identify those individuals who are least capable of
mounting a satisfactory response, and develop appro-
priate therapeutic interventions.
THREE KEY QUESTIONS

So what promises—or disillusions—does the stimulation of new blood vessel growth hold in the field of cardiovascular diseases? What can therapeutic angiogenesis treat, are there any risks attached to this type of treatment, and how long will its effect last? Three authors now turn their attention to these very points. Peter Carmeliet goes straight to the heart of the matter and asks the one question that affects all the others: “What are the candidate pathologies for therapeutic angiogenesis?” First used in the treatment of ischemic heart disease and lower-limb occlusion, therapeutic angiogenesis is now being considered in cardiac failure due to post–myocardial infarction, restenosis, diabetic neuropathy, stroke, and other cardiac diseases. However, as Peter Carmeliet points out, the real challenge lies in identifying safe indications. This is why Iris Baumgartner takes a closer look at the potential risks attached to the therapeutic use of angiogenic growth factors: “Angiogenesis and cardiovascular disease: what are the risks?” In so doing, she addresses two major concerns: (i) the risk that the angiogenic response per se might not work as well as expected, ie, that hypotension, edema, or vessel malformation might develop, and (ii) the risk that something very serious might go wrong, such as promoting the growth of dormant tumors or atherosclerotic plaques. The above two authors’ concerns over both risk and efficacy lead Sigrid Nikol to answer the question: “Angiogenesis and cardiovascular disease: how long will angiogenesis last and how can we stop it?” In other words, if the effect of treatment is beneficial, what are the ways to make it last longer, and if it causes unacceptable adverse effects, is there any way to turn the switch off? Her answer highlights the impressive sophistication of the methods used to modulate this exciting therapeutic approach.

REFERENCES

Adaptive hypoxic tolerance in the subterranean mole rat Spalax ehrenbergi: the role of vascular endothelial growth factor.

2. Vale PR, Losordo DW, Milliken CE, et al.
Left ventricular electromechanical mapping to assess efficacy of phVEGF165 gene transfer for therapeutic angiogenesis in chronic myocardial ischemia.

3. Folkman J.
Angiogenesis in cancer, vascular, rheumatoid and other disease.

Mouse model of angiogenesis.

Endogenous regulation of angiogenesis in the rat aorta model.

Early expression of angiogenesis factors in acute myocardial ischemia and infarction.

Expression of vascular endothelial growth factor in patients with acute myocardial infarction.

Acidosis inhibits endothelial cell apoptosis and function and induces basic fibroblast growth factor expression.

Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia.

Regulation of vascular endothelial growth factor in cardiac myocytes.

Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells.


Genetic heterogeneity of angiogenesis in mice.  

Interindividual heterogeneity in the hypoxic regulation of VEGF:  
significance for the development of the coronary artery collateral  

Impaired myocardial angiogenesis and ischemic cardiomyopathy  
in mice lacking the vascular endothelial growth factor isoforms  
VEGF<sub>164</sub> and VEGF<sub>188</sub>.  

Selective requirement for Src kinases during VEGF-induced angio-
genesis and vascular permeability  

Systemic hypoxia changes the organ specific distribution of vascular  
endothelial growth factor and its receptors.  

Enhanced angiogenesis and growth of collaterals by in vivo  
administration of recombinant basic fibroblast growth factor in a  
rabbit model of acute lower limb ischemia: dose-response effect of  
basic fibroblast growth factor.  

Therapeutic angiogenesis: a single intra-arterial bolus of vascular  
endothelial growth factor augments revascularization in a rabbit  
ischémic hindlimb model.  

42. Isner JM, Pieczek A, Schainfeld R, et al.  
Clinical evidence of angiogenesis following arterial gene transfer  
of phVEGF<sub>165</sub>.  

43. Tsurumi Y, Takeshita S, Chen D, et al.  
Direct intramuscular gene transfer of naked DNA encoding vascular  
endothelial growth factor augments collateral development and  
tissue perfusion.  

Constitutive expression of phVEGF<sub>165</sub> following intramuscular gene  
transfer promotes collateral vessel development in patients with  
critical limb ischemia.  

45. European Working Group on Critical Leg Ischemia.  
Second European consensus document on chronic critical leg  
ischemia.  

46. Rutherford RB, Becker GJ.  
Standards for evaluating and reporting the results of surgical and  
percutaneous therapy for peripheral arterial disease.  

47. Isner JM, Baumgartner I, Rauh G, et al.  
Treatment of thromboangiitis obliterans (Buerger’s disease) by  
intramuscular gene transfer of vascular endothelial growth factor:  
preliminary clinical results.  

Contribution of endothelial progenitor cells to neovascularization  
(vasculogenesis) is impaired with aging.  

49. Losordo DW, Pickering JG, Takeshita S, et al.  
Vascular endothelial growth factor inhibits endothelial cell apoptosis  
induced by tumor necrosis factor–alpha: balance between growth  
and death signals.  
*J Mol Cell Cardiol.* 1997;29:1321-1330.

Gene transfer of naked DNA encoding for three isoforms of vascular  
endothelial growth factor stimulates collateral development in vivo.  

51. Takeshita S, Ishi T, Sato T.  
Increased expression of direct gene transfer into skeletal muscles  
observed after acute ischemic injury in rats.  

VEGF improves myocardial blood flow but produces EDRF-mediated  
hypotension in porcine hearts.  

Vascular endothelial growth factor/vascular permeability factor  
produces nitric oxide–dependent hypotension.  


Isolation of putative progenitor endothelial cells for angiogenesis. 
*Science*. 1997;275:964-967.

75. Asahara T, Takahashi T, Masuda H, et al.  
VEGF contributes to postnatal neovascularization by mobilizing bone marrow–derived endothelial progenitor cells. 

Isolation and characterization of endothelial progenitor cells from mouse embryos. 

Vascular endothelial growth factor is a secreted angiogenic mitogen. 

Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. 
Angiogenesis

Expert Answers to Three Key Questions

1. What are the candidate pathologies for therapeutic angiogenesis?
   
   *P. Carmeliet*

2. Angiogenesis and cardiovascular disease: what are the risks?
   
   *I. Baumgartner*

3. Angiogenesis and cardiovascular disease: how long will angiogenesis last and how can we stop it?
   
   *S. Nikol*
What are the candidate pathologies for therapeutic angiogenesis?

Peter Carmeliet, MD, PhD
Center for Transgene Technology and Gene Therapy - Flanders Interuniversitary Institute for Biotechnology - KULeuven - Campus Gasthuisberg - Leuven - BELGIUM

Improvement in tissue oxygenation by therapeutic angiogenesis was initially applied to ischemic hearts and occluded limbs. Future applications extend to other cardiac diseases, cardiac failure due to post-myocardial infarction remodeling, restenosis, diabetic neuropathy, and stroke. However, the risk:benefit ratio must be carefully weighed with regard to candidate conditions (taking into account risks such as promotion of tumor or atherosclerotic plaque growth) and patients (not only will many eligible patients have additional disease, raising difficult, often contradictory therapeutic options, but angiogenomic profiling is required to optimize and tailor angiogenic therapies to account for variations in individual response). The current challenge is to establish a unified framework to develop effective therapeutic strategies.

**Keywords:** angiogenesis; fibroblast growth factor; vascular endothelial growth factor; myocardial ischemia; atherosclerosis; restenosis; limb ischemia; neural ischemia

**Address for correspondence:**
Peter Carmeliet, MD, PhD, Center for Transgene Technology and Gene Therapy, Flanders Interuniversitary Institute for Biotechnology, KULeuven, Campus Gasthuisberg, Herestraat 49, B-3000, Leuven, Belgium (e-mail: peter.carmeliet@med.kuleuven.ac.be)

There are great hopes that tissue oxygenation may be improved in the near future by stimulating the growth of new blood vessels, a process generally termed “therapeutic angiogenesis.” While most attention has been initially focused on stimulating angiogenesis in ischemic hearts or in occluded limbs, angiogenic strategies might have a broader application in the future. Candidate disease states for therapeutic angiogenesis could include other cardiac diseases, cardiac failure due to post-myocardial infarction remodeling, restenosis, diabetic neuropathy, and stroke. A challenge for the future will be to carefully select clinical conditions for safe treatment. For instance, angiogenic growth factors may alleviate myocardial ischemia by stimulating angiogenesis in the cardiac muscle, but, at the same time, also worsen myocardial ischemia by stimulating atherosclerotic plaque growth. Since a number of patients eligible for angiogenic treatments are likely to suffer additional pathologies such as cancer, diabetic retinopathy, pulmonary hypertension, etc., clinicians will be confronted with difficult, often contradictory therapeutic options. The challenge now is to establish a unified framework to develop effective therapeutic strategies. In addition, patients may greatly differ in their responsiveness to therapeutic angiogenesis because of their genetic makeup. Angiogenomics may allow angiogenic therapies to be optimized and tailored to individual needs in the future. This short commentary discusses candidate pathologies, which are already being considered today, and speculates on the possible addition of other disorders for the future to the list of candidates.

**THE ANGIOGENIC SWITCH AND ANGIOGENOMIC PROFILING**

Significant molecular and cellular insights into the complex process of how blood vessels form have been obtained. Angiogenesis occurs during development, as well as cyclically in women of reproductive age, but most of the vasculature in a normal adult is quiescent, with only 0.01% of all endothelial cells un-

---

**SELECTED ABBREVIATIONS AND ACRONYMS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aFGF</td>
<td>acid fibroblast growth factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemo-attractant protein-1</td>
</tr>
<tr>
<td>PIGF</td>
<td>placental growth factor</td>
</tr>
<tr>
<td>TSP-1</td>
<td>thrombospondin-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
dergoing division. Even though endothelial cells can remain quiescent for years, they exhibit a remarkable plasticity in forming new vessels in response to angiogenic stimuli. Extensive molecular analysis of angiogenesis in tumors has revealed that this remarkable plasticity of endothelial cells is dependent on an “angiogenic switch,” ie, endothelial cells divide and form vessels as soon as angiogenic activators are in excess of inhibitors. However, in contrast to our understanding of tumor angiogenesis, we know little about the molecular switch that allows endothelial and vascular smooth muscle cells in the heart or brain to form new or larger vessels during ischemia. It also remains to be determined why the angiogenic switch is insufficient in patients suffering tissue ischemia. With the advent of recent gene-discovery technologies (microarray genomics, proteomics, etc), “angiogenomic profiling” will make it possible to document the expression pattern of candidate genes responsible for stimulation or inhibition of angiogenesis in various cardiovascular disorders in different individuals. Once this information is available, we will be able to determine whether myocardial ischemia results from insufficient production of angiogenic stimulators or, alternatively, from excess production of angiogenic inhibitors, and, if so, which factors are the key players. We might also obtain deeper insights into why the response to angiogenic factors varies more than 10-fold among experimental animals and, likely, also in human individuals. Such information will be instrumental in tailoring optimal angiogenic treatment to the individual needs of patients. Indeed, a patient with increased levels of angiogenic inhibitors in the myocardium may be more refractory to current angiogenic treatment and require different or adjunctive angiogenic stimulation. Furthermore, there is an urgent need for both reliable and simple diagnostic markers of (myocardial) angiogenesis, not only to diagnose their decrease or absence in those patients with insufficient angiogenesis, but also to evaluate the possible success of angiogenic treatment.

**MYOCARDIAL ISCHEMIA**

Myocardial ischemia results from an imbalance between the supply and consumption of oxygen. Initially, the myocardium develops a protective response—hibernation—in order to preserve high-energy metabolites at the expense of contractile dysfunction. At this stage, the hibernating myocardium is still viable and able to restore its contractile function upon proper revascularization. However, when the ischemic insult becomes too severe, the hibernating myocardium may undergo irreversible structural changes and degenerate to become replaced by fibrotic scar tissue. Genetic studies performed in our laboratory on transgenic mice lacking two isoforms of the vascular endothelial growth factor (VEGF) showed that these animals had impaired myocardial angiogenesis and developed ischemic heart disease with signs of hibernation that, over time, progressed to cardiac failure. Not only the formation of endothelial-lined capillaries (angiogenesis), but also that of coronary arteries (arteriogenesis) was impaired. These studies provided genetic evidence that insufficient availability of an angiogenic growth factor results in ischemic heart disease. In addition, they also constituted a rationale for preventing or rescuing hibernating myocardium by stimulating myocardial angiogenesis via administration of angiogenic growth factors. Indeed, preclinical animal studies have documented favorable effects as a result of stimulating growth of myocardial capillaries or expansion of preexisting collaterals after administration of VEGF or basic fibroblast growth factor (bFGF), either as recombinant protein or by gene transfer. Initial clinical experience with therapeutic angiogenesis and arteriogenesis in ischemic heart disease patients also raises hope that such strategies may be useful in improving cardiac function.

Which patients with ischemic heart disease are eligible for therapeutic angiogenesis? At present, patients with severe angina refractory to medical therapy and not amenable to conventional revascularization are selected for clinical testing. However, angiogenic therapy might also be considered as an adjunct to coronary bypass surgery, and patients with incomplete surgical revascularization could also benefit from adjunctive therapeutic angiogenesis. In future, a larger patient population with less severe ischemic heart disease may become eligible. Selection of these patients will require careful consideration, as angiogenic factors may not only stimulate myocardial angiogenesis, but also promote atherosclerotic plaque growth (see below). When a localized coronary obstruction occurs due to atherothrombosis, the hypoperfused myocardial territories may be salvaged by stimulation of collateral growth from adjacent territories and capillary angiogenesis. Other forms of myocardial ischemia may not respond so well to therapeutic angiogenesis such as in the case of more diffuse myocardial ischemia due to rarefaction of distal arteri-oles in hypertension or diabetes or to insufficient vascular growth during cardiomyocyte hypertrophy. The impaired angiogenic response of some of these patients (diabetes, aging) may pose an additional
challenge. It remains to be determined whether additional or alternative strategies aimed at preventing regression or enlarging the distal intramyocardial vessels (and thus not only the large collateral epicardial arteries) will be beneficial in these situations. Recent transgenic studies indicate that acid fibroblast growth factor (aFGF) improves myocardial perfusion, in part by preventing regression of coronary vessels, while a combination of VEGF and angiopoietin-1 increases the number, size and, branching of the vessels in the skin. Initial data from our own laboratory suggest that VEGF and its homologue, placental growth factor (PlGF), synergistically stimulate myocardial angiogenesis in a mouse model of myocardial ischemia (unpublished observation). Therapeutic angiogenesis may not only be useful in preventing myocardial necrosis, it might benefit from coincident stimulation of angiogenesis, thus improving vascularization of the grafted tissue.

While expression of VEGF, its homologue PlGF, and their receptors is upregulated in acute myocardial ischemia, we still know very little about the expression of these and other angiogenic factors in most other cardiac diseases. A better understanding of the angiogenic profile will be instrumental in identifying candidate conditions for angiogenic therapy. For instance, impaired hypoxic upregulation of VEGF may predispose to impaired collateral formation, suggesting that such patients would be candidates for VEGF therapy. In a number of cardiac diseases, we don't know yet what a change in this angiogenic profile means. For instance, a recent study indicated that VEGF levels were downregulated in dilated cardiomyopathy, but whether they are cause or consequence of the disorder remains undetermined. It also remains to be determined whether the insufficient "angiogenic switch" in myocardial ischemia is mostly due to insufficient expression of angiogenic stimulators or excess production of angiogenic inhibitors. Answers to these questions are required in order to tailor angiogenic therapy to the candidate pathologies in an optimal way.

LIMB ISCHEMIA

Peripheral artery occlusion is a major cause of invalidity and threat of amputation. There is little conceptual doubt that therapeutic angiogenesis and arteriogenesis are a valuable therapeutic option to improve oxygenation of ischemic limbs—either as sole therapy or as an adjunct to surgical procedures. The recent finding that VEGF also augments angiogenesis in normally perfused skeletal muscle, eg, in the absence of ischemia or inflammation, suggests that patients with intermittent claudication, but normal resting blood flow, are also potential candidates for therapeutic angiogenesis. Preclinical studies indicate that ligation of the femoral artery not only stimulates the formation of new vessels, but perhaps more importantly—also the expansion of preexisting arterioles. Collateral growth is remarkable, since it results in a 20-fold increase in lumen diameter. Capillary angiogenesis and collateral arteriogene-
sis are distinct processes from a molecular and cellular point of view. An increase in shear stress in the collaterals after occlusion of the supply artery has been proposed to upregulate leukocyte adhesion receptors, making it possible for monocytes to infiltrate the collateral wall (Figure 2). Monocytes play an essential role in arteriogenesis, since they provide mitogenic cytokines for vascular cells and proteinases for vascular remodeling. This may explain why local intra-arterial administration of the cytokine monocyte chemoattractant protein–1 (MCP-1) was successful when tested in preclinical models. VEGF appears to affect both angiogenesis and arteriogenesis. The chemoattractant effect exerted by VEGF and its homologue PlGF on monocytes may stimulate arteriogenesis, possibly explaining why diabetic patients whose monocytes fail to migrate in response to VEGF because of defective VEGF signal transduction may have impaired collateral growth. In addition, VEGF upregulates leukocyte adhesion receptors, stimulates vascular permeability (allowing extravasation of plasma proteins such as fibronectin and...
other scaffolds for vascular cell migration), and induces the release of proteinases. Thus, angiogenic factors may affect the growth of pre-existing collaterals. The genetic mechanisms by which certain patients fail to increase their collaterals, and thus develop limb ischemia, need to be further defined. Determining the nature and expression profile of additional angiogenic and arteriogenic candidate genes involved in this process among large patient populations is an important challenge for the future.

**ATHEROSCLEROSIS AND RESTENOSIS**

A large number of patients develop ischemic heart disease because of coronary obstruction due to atherosclerosis. In addition, postangioplasty and in-stent restenosis or vein-graft thickening pose significant threats to tissue perfusion. Are those patients eligible for angiogenic treatment or—on the contrary—should they be treated with antiangiogenic drugs? This important question is at present largely unresolved (Figure 3). When growth of atherosclerotic plaques exceeds the critical distance of diffusion for oxygen, it has been hypothesized that resultant hypoxia in the plaque upregulates angiogenic factors such as VEGF and cause intraplaque neovascularization. These factors may not only stimulate plaque growth, but also make plaques more vulnerable to rupture and intraplaque hemorrhage. Angiogenic factors may, however, also enhance plaque growth via other mechanisms, i.e., by chemoreacting monocytes to the plaques and mediating their adhesion to the activated endothelium, by increasing extravasation of plasma proteins (including fibrinogen) in the plaques, and by stimulating fibrin formation via expression of tissue factor, the initiator of coagulation. Some of these hypothetical mechanisms have been confirmed by experimental evidence. For instance, an association between smooth muscle cell accumulation, lesion instability, and expression of fibroblast growth factors (FGFs) was found in human atherosclerotic plaques, while administration of VEGF or transgenic expression of aFGF exaggerated neointimal thickening in animal models. Even though no conclusive evidence that treatment of humans with angiogenic factors accelerates atherosclerotic plaque growth is available at present, Moulton et al showed that angiogenesis inhibitors slowed down plaque growth in a mouse model of atherosclerosis. However, there is also experimental evidence that angiogenic factors may have a protective action against atherosclerosis, restenosis, or vein-graft thickening. A common key element in the pathogenesis of restenosis is endothelial damage. Therefore, strategies to protect the endothelium or enhance endothelial regrowth may protect against the development of these proliferative vascular pathologies.

A particularly interesting candidate is VEGF and its homologue VEGF-C. By stimulating nitric oxide and release of prostaglandins from the endothelium, VEGF and VEGF-C stimulate endothelial repair and survival, prevent platelet aggregation, control smooth muscle cell proliferation, and stimulate vasodilatation. VEGF and VEGF-C both accelerate reendothelialization and attenuate neointima formation in balloon-injured arteries. TSP-1 antibodies also accelerated reendothelialization and reduced intimal thickening. These findings underscore that beneficial effects can be achieved not only by administering angiogenic stimulators, but also by antagonizing angiogenic inhibitors. Some of the lipid-lowering statins and angiotensin-converting enzyme inhibitors may also protect the vasculature.

Taken together, inconsistent, even opposite therapeutic effects have been ascribed to angiogenic treatment, necessitating more research to resolve whether angiogenic growth factors could be safely used to improve oxygenation in ischemic hearts without aggravating coronary atherosclerosis or restenosis.

**NEURAL ISCHEMIA**

The high energy requirements of neural tissue, compared with its low energy reserves, render it particularly vulnerable to hypoxic conditions. Cerebral artery occlusion produces a central infarct and surrounding regions of incomplete ischemia (penumbra), which are dysfunctional, yet potentially salvageable. In the absence of reflow, this region undergoes progressive deterioration, culminating in infarction. Restoration of perfusion in the penumbra may ameliorate neural damage. Even though angiogenesis generally only occurs after a few days, angiogenic factors may increase the perfusion of the penumbral area via vasodilatation, a well-known property of VEGF. Subsequent angiogenesis may constitute a second mechanism to increase vascular perfusion in the penumbral area, and has been correlated with increased survival of patients after ischemic stroke. A number of angiogenic factors, including VEGF-family members and receptors, angiopoietins, or FGFs are upreg-
ulated in neural tissue following acute ischemia. However, receptors for these angiogenic factors are not only confined to endothelial cells, but, remarkably, are also detectable on neural cells, suggesting that angiogenic factors may affect neural cells directly. Direct neuroprotective effects of VEGF have indeed been documented in vitro. Increased perfusion and neuroprotection may explain why intraventricular injection of VEGF reduces the size of brain infarcts and has a favorable effect on ischemic neuropathy. Protection against endothelial dysfunction may be another mechanism. Recent findings suggest that VEGF preserves the vasa nervorum, increases the vascularity and restores blood flow through the sciatic nerves, and attenuates the ischemic peripheral neuropathy in diabetic animals (Figure 4). Studies from our laboratory indicate that impaired hypoxic upregulation of VEGF in mice causes adult-onset progressive motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis in man. While all these findings appear to argue for a beneficial effect of angiogenic factors in various neurological disorders, caution is warranted since the same angiogenic factors might also cause injurious effects to neural tissue. Indeed, VEGF antagonists have been shown to reduce brain infarcts, presumably by preventing cerebral edema. This has been attributed to an increase in the permeability of the blood-brain barrier by VEGF. Thus, it remains to be determined whether VEGF or other angiogenic growth factors will be as safe to use to treat cerebral ischemia as they appear to be for disorders of the peripheral nervous system.

A CHALLENGE FOR THE FUTURE: IDENTIFYING SAFE INDICATIONS FOR THERAPEUTIC ANGIOGENESIS

Angiogenesis is impaired in numerous diseases (Table I). Whereas angiogenesis was initially implicated in cancer, diabetic retinal neovascularization, psoriasis, and arthritis, recent evidence indicates that it contributes to the pathogenesis of a much longer list of pathologies. This is not surprising in light of the fact that angiogenic factors have pleiotropic activities including vasodilatation, plasma extravasation, and growth of new blood vessels (Table I). As a consequence, diametrically opposed therapeutic paradigms may arise for the treatment of cardiovascular complications, which need to be resolved in the future. For instance, will therapeutic angiogenesis improve myocardial angiogenesis in ischemic hearts, without any risk of atherosclerotic plaque growth, restenosis, diabetic retinal neovascularization, or progression of occult tumors? Will angiogenic factors be useful in neurological disorders without causing intracranial hypertension? Careful selection of patients and therapeutic strategies tailored to the angiogenomic makeup of individuals may be advisable. It remains to be studied whether vessels in ischemic tissues express distinct specific “addresses,” similar to tumor-assOCI-
ed endothelial cells. If so, angiogenic therapy could be selectively targeted to the ischemic organ or cells. Findings that administration of angiogenic factors stimulate angiogenesis in ischemic, but not in normal, tissues indeed support this notion. Appropriate staging of the disease may also increase therapeutic applications. For instance, antiangiogenic factors that inhibit the formation of small capillaries may be considered for use in order to suppress development of early atherosclerotic lesions, whereas stimulation of large collaterals may be preferable after obstructive lesions cause ischemic disease.

### Table 1. Angiogenesis in neoplasms and other diseases. Selected examples.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Processes characterized by abnormal angiogenesis or vascular malfunction</th>
<th>Organ</th>
<th>Processes characterized by abnormal angiogenesis or vascular malfunction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessels</td>
<td>(+): Atherosclerosis, hemangioma, hemangioendothelioma</td>
<td>Bone, joints</td>
<td>(+): Rheumatoid arthritis, synovitis, bone and cartilage destruction, osteomyelitis, pannus growth, osteophyte formation, cancer</td>
</tr>
<tr>
<td></td>
<td>(N): Vascular malformations</td>
<td></td>
<td>(-): Aseptic necrosis, impaired healing of fractures</td>
</tr>
<tr>
<td>Skin</td>
<td>(+): Warts, pyogenic granulomas, hair growth, Kaposi’s sarcoma, scar keloids, allergic edema, neoplasms</td>
<td>Liver, kidney, lung, ear, and other epithelia</td>
<td>(+): Inflammatory and infectious processes (hepatitis, pneumonia, glomerulonephritis), asthma, nasal polyps, transplantation, liver regeneration, cancer</td>
</tr>
<tr>
<td></td>
<td>(N): Psoriasis (skin vessels enlarge and become tortuous)</td>
<td></td>
<td>(N): Pulmonary hypertension, diabetes</td>
</tr>
<tr>
<td></td>
<td>(-): Decubitus or stasis ulcers, gastrointestinal ulcers</td>
<td></td>
<td>(-): Pulmonary and systemic hypertension (vascular pruning)</td>
</tr>
<tr>
<td>Uterus, ovary, placenta</td>
<td>(+): Dysfunctional uterine bleeding (contraception), follicular cysts, ovarian hyperstimulation, endometriosis, neoplasms</td>
<td>Brain, nerves, eye</td>
<td>(+): Retinopathy of prematurity, diabetic retinopathy, choroidal and other intraocular disorders, leukomalaica, cancer</td>
</tr>
<tr>
<td></td>
<td>(N): Preeclampsia</td>
<td></td>
<td>(-): Stroke, vascular dementia, Alzheimer’s disease, CADASIL</td>
</tr>
<tr>
<td></td>
<td>(-): Placental insufficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peritoneum, pleura</td>
<td>(++) Respiratory distress, ascites, peritoneal sclerosis (dialysis patients), adhesion formation (abdominal surgery), metastatic spreading</td>
<td>Endocrine organs</td>
<td>(+): Thyroiditis, thyroid enlargement, pancreas transplantation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-): Thyroid pseudocyst</td>
</tr>
<tr>
<td>Heart, skeletal muscle</td>
<td>(+): Work overload</td>
<td>Lymph vessels</td>
<td>(+): Tumor metastasis, lymphoproliferative disorders</td>
</tr>
<tr>
<td></td>
<td>(-): Ischemic heart and limb disease</td>
<td></td>
<td>(-): Lymphedema</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>(+): Obesity</td>
<td>Hematopoiesis</td>
<td>(+): AIDS (Kaposi), hematologic malignancies</td>
</tr>
</tbody>
</table>

**Key to symbols:** (+): increased vascularization; (-): insufficient vascularization; (N): abnormal remodeling; (++) increased vascularization and/or permeability. **Abbreviations:** AIDS, acquired immunodeficiency syndrome; CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy.
REFERENCES

1. Carmeliet P, Jain RK.

2. Carmeliet P.


7. Isner JM, Asahara T.


11. Carmeliet P.


Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nat Med. 1999;5:1135-1142.


Selective downregulation of VEGF-A(165), VEGF-R(1), and decreased capillary density in patients with dilatative but not ischemic cardiomyopathy. Circ Res. 2000;87:644-647.


20. Ito WD, Arras M, Winkler B, Scholz D, Schaper J, Schaper W.


22. Waltenberger J, Lange J, Kranz A.


Local delivery of vascular endothelial
growth factor accelerates reendothelializa-
tion and attenuates intimal hyperplasia in
balloon-injured rat carotid artery.

26. Hiltunen MO, Laitinen M,
Turunen MP, et al.
Intravascular adenovirus-mediated VEGF-C
gene transfer reduces neointima formation
in balloon-denuded rabbit aorta.

27. Chen D, Asahara T, Krasinski K,
et al.
Antibody blockade of thrombospondin
accelerates reendothelialization and reduces
neointima formation in balloon-injured rat
carotid artery [see comments].

The HMG-CoA reductase inhibitor simvas-
tatin activates the protein kinase Akt and
promotes angiogenesis in normocholesterol-
emic animals [see comments].

29. Fabre JE, Rivard A, Magnier M,
Silver M, Isner JM.
Tissue inhibition of angiotensin-converting
enzyme activity stimulates angiogenesis in
vivo.

30. Krupinski J, Kaluza J, Kumar P,
Kumar S, Wang JM.
Role of angiogenesis in patients with
cerebral ischemic stroke.

31. Szpak GM, Lechowicz W,
Lewandowska E, Bertrand E,
Wierzb-Bobrowicz T, Dymecki J.
Border zone neovascularization in cerebral
ischemic infarct.

32. Beck H, Acker T, Wiessner C,
Allegri PR, Plate KH.
Expression of angiopoietin-1, angiopoietin-2,
and Tie receptors after middle cerebral artery
occlusion in the rat.

33. Chen HH, Chien CH, Liu HM.
Correlation between angiogenesis and basic
fibroblast growth factor expression in experi-
mental brain infarct.

34. Jin KL, Mao XO, Greenberg DA.
Vascular endothelial growth factor: direct
neuroprotective effect in in vitro ischemia.
Proc Natl Acad Sci USA. 2000;97:
10242-10247.

35. Hayashi T, Abe K, Itoyama Y.
Reduction of ischemic damage by applica-
tion of vascular endothelial growth factor
in rat brain after transient ischemia.
J Cereb Blood Flow Metab. 1998;18:
887-895.

36. Schratzberger P, Schratzberger G,
Silver M, et al.
Favorable effect of VEGF gene transfer on
ischemic peripheral neuropathy.
Nat Med. 2000;6:405-413.

37. Oosthuyse O, Moons L, Beck H,
et al.
Deletion of the hypoxia response element in
the VEGF promoter causes adult-onset motor
neuron degeneration.
Nat Genet. 2001;28:131-138. (see also
accompanying News & Views in
Nat Genet and Nat Med.)

38. van Bruggen N, Thibodeaux H,
Palmer JT, et al.
VEGF antagonism reduces edema formation
and tissue damage after ischemia/reperfusion
injury in the mouse brain.
Angiogenesis and cardiovascular disease: what are the risks?

Iris Baumgartner, MD
Swiss Cardiovascular Center Bern - Division of Angiology - University Hospital Bern - SWITZERLAND

The issue of the potential risks of treatment with angiogenic growth factors is an important one. Does therapeutic angiogenesis promote the growth of dormant tumors or atherosclerotic plaques? Is it possible to obtain a functional vasculature with vascular endothelial growth factor (VEGF) or fibroblast growth factors (FGFs) without causing hypotension, edema, or vessel malformation? The therapeutic window for angiogenic growth factors is not well defined. To some extent, this is due to failure of animal models to mimic human disease and differences in genetic susceptibility. Although there is no doubt that a collateral circulation can be developed using appropriate growth factor(s), genuine risks are involved, and at the present time, it is only once the growth factors have left the laboratory that we start getting an idea of what the associated risks may be.

The vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families of angiogenic cytokines have been studied in clinical trials (Table I). Delivery of recombinant proteins is achieved by direct intravenous (VEGF1651,2, FGF-2), intra-arterial (FGF-2) or intracoronary (VEGF1651,2, FGF-2) infusion, intramyocardial injection (FGF-1), or perivascular implantation of sustained-release heparin-alginate microcapsules (FGF-2). Gene therapeutic concepts are based on intracoronary infusion of adenoviral vectors (FGF-4), or intramyocardial/ intramuscular injection of plasmids (VEGF1651,2, FGF-1) and adenoviral vectors (VEGF1651,2) containing the transgene that encodes the angiogenic growth-promoting factor.

Clinical data from protein- and gene-delivery trials, although scarce, suggest that both approaches are safe. Serious adverse events recorded by the National Institutes of Health, and the US Food and Drug Administration (FDA) included one death related to malignant lymphoma (recombinant FGF-2), and progression of a lung tumor (plasmid DNA encoding VEGF-C). Whether or not a transient increase in circulating growth factors accelerates tumor growth remains uncertain. Hypotension was a common adverse event described with intravascular infusion of recombinant proteins (VEGF165, FGF-2) in higher-dose groups owing to nitric oxide release and vasodilation.1,3-5 Although none of the patients had a persistent adverse outcome, results imply that maximum dose limitation may restrict the efficiency of intravascular application of recombinant proteins. In accordance with preclinical toxicity data, there was a considerable proportion of patients with increased urinary protein excretion after exposure to recombinant FGF-2.4,5 In a pilot trial of high-dose intravenous FGF-2 infusions in patients with intermittent claudication, proteinuria reached up to 3 g per day (unpublished data). As a result, an expert panel stated “...renal insufficiency due to membranous nephropathy accom-
panied by proteinuria may be the most significant long-term side effect of FGF-2 administration..."17 Occasional adverse events following intravascular FGF-2 administration were mild transient decrease in platelet counts (bone marrow toxicity) and electrocardiographic changes.5,18 In patients with chronic critical limb ischemia, transient lower-extremity edema consistent with VEGF-enhancement of vascular permeability was observed in 60% of patients with gangrene following VEGF165 gene transfer.19 Strikingly, edema development was not reported in coronary artery trials using VEGF.13,14,16 Intravascular infusion of angiogenic growth factors is appealing because of its practicability and applicability to broad groups of patients. Its disadvantages include systemic exposure to growth factors with potential adverse effects and low uptake in ischemic target tissues.20 Local delivery of sustained-release prepa-

<table>
<thead>
<tr>
<th>Growth factor specification</th>
<th>Application</th>
<th>Dose</th>
<th>Disease</th>
<th>Patients</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA encoding VEGF165</td>
<td>intra-arterial</td>
<td>100 µg to 1000 µg</td>
<td>PAD</td>
<td>12</td>
<td>9, 10</td>
</tr>
<tr>
<td>Plasmid DNA encoding VEGF165</td>
<td>intramuscular</td>
<td>2000 µg to 4000 µg†</td>
<td>PAD</td>
<td>50</td>
<td>11, 19</td>
</tr>
<tr>
<td>Plasmid DNA encoding VEGF165</td>
<td>intramuscular</td>
<td>125 µg to 250 µg 250 µg to 500 µg</td>
<td>CAD</td>
<td>20</td>
<td>12-14</td>
</tr>
<tr>
<td>Adenoviral vector expressing VEGF165 cDNA</td>
<td>intramyocardial</td>
<td>4×10⁸ pfu to 4×10¹⁰ pfu</td>
<td>CAD</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td>Plasmid DNA encoding VEGF-C</td>
<td>intramuscular</td>
<td>2000 µg to 8000 µg</td>
<td>PAD</td>
<td>31 (43)</td>
<td><a href="http://www.4.od.nih.gov/oba/protocols.pdf">www.4.od.nih.gov/oba/protocols.pdf</a> <a href="http://www.4od.nih.gov/oba/Rdna.htm">www.4od.nih.gov/oba/Rdna.htm</a></td>
</tr>
<tr>
<td>Plasmid DNA encoding VEGF-C</td>
<td>intramyocardial</td>
<td>200 mg to 2000 mg</td>
<td>CAD</td>
<td>48 (55)</td>
<td><a href="http://www.4.od.nih.gov/oba/protocols.pdf">www.4.od.nih.gov/oba/protocols.pdf</a> <a href="http://www.4od.nih.gov/oba/Rdna.htm">www.4od.nih.gov/oba/Rdna.htm</a></td>
</tr>
<tr>
<td>Recombinant VEGF165 protein</td>
<td>intracoronary followed by IV</td>
<td>4×17 ng/kg/min to 4×50 ng/kg/min</td>
<td>CAD</td>
<td>119 (178)</td>
<td>1, 2</td>
</tr>
<tr>
<td>Plasmid DNA encoding FGF-1</td>
<td>intramuscular</td>
<td>500 µg to 4000 µg</td>
<td>PAD</td>
<td>48</td>
<td>15</td>
</tr>
<tr>
<td>Recombinant FGF-1</td>
<td>intramyocardial</td>
<td>0.01 mg/kg</td>
<td>CAD</td>
<td>20 (40)</td>
<td>6, 7</td>
</tr>
<tr>
<td>Recombinant FGF-2</td>
<td>intracoronary or IV</td>
<td>0.33 to 48 µg/kg 18 to 36 µg/kg</td>
<td>CAD</td>
<td>16 (24)</td>
<td>8</td>
</tr>
<tr>
<td>Recombinant FGF-2</td>
<td>perivascular (heparin-alginate microcapsules)</td>
<td>10 µg to 100 µg</td>
<td>CAD</td>
<td>17 (25)</td>
<td>5</td>
</tr>
<tr>
<td>Recombinant FGF-2</td>
<td>intracoronary</td>
<td>3 µg/kg to 100 µg/kg</td>
<td>CAD</td>
<td>13 (19)</td>
<td>4</td>
</tr>
<tr>
<td>Adenoviral vector expressing FGF-4 cDNA</td>
<td>intracoronary</td>
<td>?</td>
<td>CAD</td>
<td>? (79)</td>
<td></td>
</tr>
</tbody>
</table>

* Number of patients, including placebo controls (numbers in brackets).
† One patient with bilateral treatment (each limb was injected with 4000 µg plasmid DNA).

**Abbreviations:** CAD, coronary artery disease; FGF, fibroblast growth factor; IV, intravenous; PAD, peripheral arterial occlusive disease; VEGF, vascular endothelial growth factor.

*Table I. Therapeutic angiogenesis: clinical trials.*
rations such as heparin-alginate microcapsules may in part overcome the drawback of low uptake, albeit at the cost of more invasiveness. One argument in favor of the gene therapeutic approach is that it can overcome the inherent instability of angiogenic growth factors by ensuring sustained, local production. The drawbacks of this approach include the risk of immune and inflammatory responses to viral vectors, and introduction of foreign genetic material (for a review see 17 and 21).

**THEORETICAL CONSIDERATIONS**

More clinical experience and long-term follow up will be necessary to address theoretical safety issues such as local angioma-genesis and stimulation of pathological angiogenesis in angiogenesis-dependent diseases such as atherosclerosis, solid or hematopoietic tumors, various forms of retinopathy, rheumatoid arthritis, gynecological disorders, or inflammations.

The following discusses some of these issues.

**Angioma-genesis**

In animal embryo models, it was shown that the inactivation of a VEGF allele results in defective large vessel development and capillary sprouting in the early stage of vascular development, whereas VEGF overexpression results in hypervascularization. Cerebrovascular malformations or the hemangioblastoma of the von Hippel-Lindau syndrome are variants of unregulated VEGF overexpression in humans. Available data suggest that a critical threshold level and stringent dose-dependency are necessary to guarantee correct vascular assembly.

In the first case report of intra-arterial gene transfer, where 2000 µg plasmid DNA encoding VEGF165 was applied to a patient with chronic critical limb ischemia, Isner noted the development of three spider angiomas over the ankle and foot approximately 1 week after gene transfer, which regressed spontaneously. Light microscopy of a biopsy showed a marked increase in vascular lumen diameter similar to that observed in supernumerary vessels following overexpression of VEGF in the avian embryo model. Angioma-like vessel proliferation in heart and skeletal muscle was also reported in a mouse model of myoblast-mediated overexpression of VEGF. Continuous, constitutive overexpression of VEGF was achieved by ex vivo retroviral gene transfer to myoblasts, which were transplanted into nonischemic myocardium or skeletal muscle. Histology disclosed that unregulated
VEGF expression led to a highly localized formation of disorganized vessels that closely resembled hemangioma. Blood vessels were sinusoidal and dilated, much like those evidenced in tumors or during early vasculogenesis. Since angioma-like structures were rarely described after a transient exposure to augment collateral development in animal models of myocardial or hindlimb ischemia, it was suggested that VEGF had different effects depending on concentration and microenvironment. At low concentrations under tissue hypoxia conditions, angiogenesis may prevail, whereas at high concentrations under nonischemic conditions, a deleterious response resulting in an immature vascular network (angiogenesis) may predominate (Figures 1 and 2).

Inoue demonstrated VEGF protein and receptor expression in endothelial cells and macrophages of human coronary plaques, suggesting that VEGF is closely involved in plaque neovascularization, and that growth factors are released from the cells that make up the plaque. Consistent with the above, it was shown that plaque neovascularization is often found in areas rich in macrophages, T-cells, and mast cells—cell types that can activate angiogenesis by secretion of growth factors such as VEGF and FGFs. Compelling evidence that neovascularization is a prerequisite for the development of atherosclerotic plaques was provided by an animal model showing fewer plaques in apolipoprotein E-/- mice fed angiogenesis inhibitors (endostatin, TNP-470) compared with control mice.

In addition to its potential for inducing plaque angiogenesis, VEGF increases vessel wall permeability (vascular permeability factor), promotes release of von Willebrand factor and tissue factor, and induces monocyte activation and migration. Each of these events is recognized as being important in the early pathogenesis of atherosclerosis. FGFs are pleiotropic proteins that target a wide variety of cell types, including smooth muscle cells and fibroblasts. Activation is associated with the potential for accelerating atherosclerosis and smooth muscle cell response to vascular injury. Using an arterial injury model in pigs, Nabel et al showed that the introduction of a secreted form of FGF-1 using an eukaryotic expression vector induced intimal hyperplasia and neocapillary formation.

At present there is little or no evidence that a transient increase in circulating VEGF or FGFs stemming from treatments designed to augment angiogenesis in ischemic heart or limb disease poses a clinically relevant risk with regard to plaque angiogenesis and atherogenesis (Table I). Short half-life, which prevents plasma accumulation, low systemic concentration, and transient increase may have prevented the critical threshold level from being reached. Furthermore, studies have shown that angiogenic growth factors stimulate endothelial re-growth in denuded arteries, leading to inhibition of neointimal thickening, reduction in thrombogenicity, and restoration of endothelium-dependent vasomotor reactivity. A strong argument against a significant contribution of circulating growth factors is provided by...
data from patients with cancer who may have excessively high serum levels of VEGF and FGFs without evidence of accelerated plaque angiogenesis or atherogenesis.

**Tumor angiogenesis**

Most tumors in humans persist in situ for months to years without neovascularization. Change in biological behavior occurs when a subgroup of cells in the tumor switches to an angiogenic phenotype. Tumor invasion by neovessels is undoubtedly a critical stage in tumor development, in particular with respect to metastasis, and it is also likely that angiogenesis stimulates circumscribed tumors to proliferate (Figure 4). Hence, the fear that angiogenic growth factor therapy might trigger clinical manifestations of cancer is based on the consideration that a modified balance of angiogenic cytokines induced by exogenous application may stimulate tumor angiogenesis. This potential side effect is made more difficult to deal with by the fact that it may not become clinically detectable until months or even years after the original treatment.

Plate et al were the first to demonstrate that VEGF protein and receptors were upregulated in glioma tumor and glioma tumor endothelial cells and that VEGF was a potential tumor angiogenesis factor in vivo. In a more recent work, it has been shown that malignant hematopoietic cells produce and respond to various angiogenic factors, raising the possibility that VEGF and FGFs may also play a role in hematopoietic neoplasms. Two events at the genetic level are associated with tumor development and progression: activation of oncogenes and inactivation of tumor suppressor genes. Indeed, it is known that endogenous inhibitors of angiogenesis such as thrombospondin can be downregulated by inactivation of p53 mutations, and that proangiogenic growth factors such as VEGF can be upregulated by a mutant ras-oncogene. Release of angiogenic growth factors by activated tumor cell clones may be augmented by environmental conditions like hypoxia and hypoglycemia. Although the acquisition of an angiogenic phenotype marks the transition from hyperplasia to neoplasia, it has been demonstrated that angiogenic growth factor expression alone, although sufficient to promote a growth process, is not sufficient to induce sustained tumorigenicity. Whether transient exogenous administration can initiate an angiogenic switch within the tumor environment remains to be clarified.

A recently described mechanism that may facilitate metastatic spread is VEGF-C induced enlargement of lymphatics.

**Nonneoplastic disorders to which angiogenesis contributes**

VEGF has been postulated to be a major retinal angiogenic factor in diabetic retinopathy, because it is known to induce hyperpermeability to microvessels, and hyperpermeability is an early sign of diabetic retinopathy. Aiello has shown that VEGF concentrations are high in the aqueous and vitreous fluid in patients with proliferative diabetic retinopathy, suggesting that overexpression of VEGF protein and receptors in diabetic retinopathy may stimulate neovascularization in later stages of diabetic retinopathy. The hypothesis is supported by the observation that inhibition of VEGF by application of soluble receptor domains prevented neovascularization in a mouse model of retinopathy. There is also evidence that VEGF plays a crucial role in the formation of subretinal neovascularization, which is a serious complication of age-related macular degeneration, and in the preretinal neovascular formation that develops in the nonperfused peripheral retina in sickle cell retinopathy.

The involvement of angiogenesis in human diseases is known in the case of rheumatoid arthritis. Kaposi’s sarcoma in acquired immune deficiency syndrome (AIDS) is an example of a disease in which angiogenesis plays a critical role.
deficiency syndrome (AIDS), dys- 
tional uterine bleeding, polycys-
tic ovary syndrome or endometrio-
sis, and most inflammatory and 
fectious disorders, such as hepatis, pneumonia, or glomerulonephritis 30 All of these have to be exclud-
ted before, and looked for after, 
herapeutic stimulation of angio-

REFERENCES


Intracoronary and intravenous administration of basic fibroblast growth factor: myocardial and tissue distribution.
Drug Metab Dispos. 1999;27:821-826.

Somatic gene therapy in the cardiovascular system.

22. Carmeliet P.
VEGF gene therapy: stimulating angiogenesis or angioma-genesis?

23. Barger AC, Beeuwkes R, Lainey L, Silverman KJ.
Hypothesis: vasa vasorum and neovascularization of human coronary arteries.

Angiogenesis inhibitors endostatin or TNF-β470 reduce initial neovascularization and plaque growth in apolipoprotein E-deficient mice.

25. Folkman J.
Angiogenesis in cancer, vascular, rheumatoid and other diseases.

Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies.

Vascular endothelial growth factor in ocular fluids of patients with diabetic retinopathy and other retinal disorders.

Angiogenic factors in human proliferative sickle cell retinopathy.

Experimental subretinal neovascularization is inhibited by adenovirus-mediated soluble VEGF/flt-1 receptor gene transfection: a role of VEGF and possible treatment for SRN in age-related macular degeneration.

30. Carmeliet P, Jain RK.
Angiogenesis in cancer and other diseases.

Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele.

32. Flamme I, von Reutern M, Drexler HCA, Syed-Alli S, Risau W.
Overexpression of vascular endothelial growth factor in the avian embryo induces hypervascularization and increased vascular permeability without alterations of embryonic pattern formation.

33. Kilic T, Pamir MN, Kullu S, Eren F, Ozek MM, Black PM.
Expression of structural proteins and angiogenic factors in cerebrovascular abnormalities.

34. Witzigmann-Voos S, Breier G, Risau W, Plate KH.
Up-regulation of vascular endothelial growth factor and its receptors in Von Hippel-Lindau disease-associated and sporadic hemangioblastoma.

35. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM.
VEGF gene delivery to muscle: potential role for vasculogenesis in adults.

VEGF gene delivery to myocardium. Deleterious effects of unregulated expression.

Vascular endothelial growth factor induces dose-dependent revascularization in a rabbit model of persistent limb ischemia.

Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion.

39. Su H, Lu R, Kan YW.
Adeno-associated viral vector-mediated vascular endothelial growth factor gene transfer induces neovascular formation in ischemic heart.

40. Tenaglia AN, Peters KG, Sketch MH, Annex BH.
Neovascularization in atherectomy specimens from patients with unstable angina.

Angiogenesis and the atherosclerotic carotid plaque: an association between symptomatology and plaque morphology.

42. Inoue M, Itoh H, Ueda M, et al.
Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions. Possible pathophysiological significance of VEGF in progression of atherosclerosis.

43. Schwartz CJ, Valente AJ, Sprague EA, Kelly JL.
Atherosclerosis as an inflammatory process: the roles of the monocyte-macrophage.

44. Lindner V, Lappi DA, Baird A, Majack RA, Reedy MA.
Role of basic fibroblast growth factor in vascular lesion formation.
Recombinant fibroblast growth factor–I promotes intimal hyperplasia and angiogenesis in arteries in vivo.  

46. Isner JM.  
Cancer and atherosclerosis.  

47. Folkman J.  
Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis.  

48. Plate KH, Breier G, Weich HA, Risau W.  
Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo.  

49. Volpert OV, Dameron KM, Bouck N.  
Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity.  
*Oncogene*. 1997;14:1495-1502.

Impact of oncogenes in tumor angiogenesis: mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells.  

Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage in vivo to Chinese hamster ovary cells.  

52. Hanahan D, Weinberg RA.  
The hallmarks of cancer.  
*Cell*. 2000;100:57-70.

53. Plate KH.  
From angiogenesis to lymphangiogenesis.  
*Nat Med*. 2001;7:151-152.

Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization.  

55. Flore O, Rafli S, Ely S, O'Leary JJO, Hyjek EM, Cesaran E.  
Transformation of primary human endothelial cells by Kaposi's sarcoma–associated herpesvirus.  

Vascular endothelial growth factor is essential for corpus luteum angiogenesis.  

57. Polverini PJ, Cotran RS, Gimbrone MA, Unanue ER.  
Activated macrophages induce vascular proliferation.  

58. Flore O, Rafli S, Ely S, O'Leary JJO, Hyjek EM, Cesaran E.  
Transformation of primary human endothelial cells by Kaposi's sarcoma–associated herpesvirus.  
Angiogenesis and cardiovascular disease: how long will angiogenesis last and how can we stop it?

Sigrid Nikol, MD
Associated Professor of Molecular Medicine - Medical Clinic C - University Hospital - Westfälische Wilhelms University - Münster - GERMANY

The therapeutic use of proteins or of genes of naturally occurring growth factors may be potentially beneficial for controlling the growth of collaterals or the remodeling of arteries. Whether the mother vessels will regress or evolve into well-differentiated daughter vessels that continue to function indefinitely depends on the duration of therapy. A number of vector systems and sophisticated local drug delivery strategies may be of use in combination with protein or gene therapy to control the duration of therapy. Proteins and nonviral or viral gene transfer may induce transient new vessel formation. A number of antiangiogenic chemicals and proteins may be potentially used to stop angiogenesis. In terms of gene therapy, retroviral and adeno-associated vectors allow stable gene transfer, with the potential of long-lasting therapeutic effects that can be controlled by inducible promoters.

Keywords: angiogenesis; remodeling; protein; transfection; gene integration; viral vector; nonviral gene transfer; encapsulation; inducible promoter; local drug delivery

Address for correspondence:
Sigrid Nikol, Associated Professor of Molecular Medicine, Medical Clinic C, University Hospital, Westfälische Wilhelms University, Albert Schweitzer Straße 33, D-48129 Münster, Germany (e-mail: nikol@uni-muenster.de)

Therapeutic angiogenesis is a potential method of treating chronic ischemia. It may be directed at any of the numerous growth factors and cellular components that form the complex network involved in both true new vessel formation (the in situ growth of muscular collateral arteries) and vessel remodeling. On a conceptual basis, it remains unknown whether recombinant proteins for the relevant factors, or the genes for these, will provide the best and longest-lasting therapeutic effects in patients.

Using recombinant proteins may provide the advantage that the therapy will be easier to halt if necessary. If multiple angiogenic factors are required, it may be easier to administer the proteins together than combining the genes for different factors. In this connection, an early clinical trial has already been reported, using the protein vascular endothelial growth factor (VEGF). However, despite promising results from animal experiments, this trial (the VEGF in Ischemia for Vascular Angiogenesis [VIVA] trial), which was the biggest clinical angiogenesis trial to date using recombinant VEGF, comprising 178 patients, did not demonstrate the protein form of therapy to be efficient. Direct administration of the protein may not have been effective because the protein was rapidly degraded and thus gave only a short therapeutic response. It is also possible that the intracoronary and systemic infusions used may not have allowed sufficient uptake at the desired vessel site. One of the problems now identified is that the mother vessels induced by VEGF from preexisting microvessels after pericyte detachment and basement membrane degradation are transient structures, which do not persist for more than a few days. Such mother vessels may either regress or evolve into well-differentiated daughter vessels that continue to function indefinitely, depending on the duration of the therapy administered. Multiple instead of single-dose therapy may be one solution. Prolonged protein delivery has been more effective at inducing angiogenesis using fibroblast growth factor (FGF) when administered via sustained-release heparin-alginate microcapsules, thus avoiding multiple applications.

With the early results showing that protein therapy may be less efficient, perhaps gene therapy may provide some answers. As well as

SELECTED ABBREVIATIONS AND ACRONYMS

FGF fibroblast growth factor
VEGF vascular endothelial growth factor
VIVA VEGF in Ischemia for Vascular Angiogenesis
being more efficient, gene therapy is potentially more controllable, through several mechanisms.

Hence, this article will discuss the potential of gene therapy in the context of inducing and controlling angiogenesis.

Protein production through gene therapy may offer a better prospect of influencing the duration of therapy than encapsulation of protein. Successful gene therapy for angiogenesis requires a combination of adequate transport into specific cells, using a relevant vector system, together with a suitable method for introduction into the body with minimal side effects, and a valid therapeutic target. The use of new concepts, such as the use of sophisticated local drug delivery strategies may further increase the value of gene therapy. Thus, transfer efficiencies may be increased, with minimal side effects, and local deposition may result in longer-lasting therapeutic effects with minimal or no systemic distribution. One of the advantages of gene therapy over the use of proteins clearly is the potential long-term effect, ideally with a minimal immune reaction. The immune and inflammatory effects of certain delivery systems, particularly adenovirus, have been highlighted by the unexpected death of a patient in a phase 1 study of gene therapy for the inherited disorder ornithine transcarbamylase deficiency. Angiogenic gene therapy will clearly be a more localized treatment; however, one of the concerns is naturally the possible duration of any effects following gene delivery and whether it can be halted when desired. This will depend on the vector and delivery system used. These points will also be discussed.

**DURATION OF EXPRESSION OF GENE THERAPY DEPENDING ON THE VECTOR SYSTEM**

Two approaches can be used to transfer genes into selected cells, transient transduction without integration of DNA, called transfection, and stable transduction by chromosomal integration (Figure 1). Genetic material may be delivered as nucleic acid alone, nucleic acid complexed with various chemical compounds, such as calcium phosphate, diethylaminoethyldeextran, lipospermines, or liposomes, or incorporated into a suitable virus. Transfection describes delivery of a gene predominantly into the cytoplasm and nucleus, where the gene has a relatively short-lived effect, with practically no genomic incorporation. The effect is nonselective, and DNA is introduced into cells in...
both proliferating and quiescent states. Plasmid DNA or adenoviral vectors are mainly used for this approach. Gene expression using plasmid DNA is detectable in porcine arteries for at least 5 months following gene transfer (Figure 2).5,6 In mouse muscle tissue, expression of plasmid vectors has been observed for up to 19 months after transfer by some workers,7 although others in different models find that expression is limited to weeks. Gene expression in vascular cells following adenoviral gene transfer is generally only for a few weeks,8,9 and the toxicity and immunological reactions associated with these vectors may lead to worsening of the underlying disease and therefore promote vessel occlusion.10

In contrast, chromosomal integration allows insertion into the target cell genome with more persistent expression, at best as long as the cell survives.11 Gene integration usually takes place during DNA replication, which makes this method selective for proliferating cells. Gene transfer for integration is usually performed using vectors derived from retrovirus or adenovirus-associated virus. Only recently have certain retroviral vectors been developed for gene delivery and integration into quiescent cells. Most retroviruses allow gene integration only during DNA replication, which makes these a method of delivering therapeutic genes selective to proliferating cells. Therefore, only very limited retroviral integration takes place in uninjured arterial or myocardial tissues where there is minimal cell proliferation.

As an alternative to the direct use of genes, the cells involved in angiogenesis may be used as gene delivery packages. The use of ex vivo expanded endothelial progenitor cells reintroduced to animals has recently been demonstrated to be more effective compared with the use of endothelial cells alone.12 This approach mimics one of the functions of VEGF, the mobilization of endothelial progenitor cells, and may have clinical value in the future. This kind of use of angioblasts is in the early stages of research and no conclusions can yet be drawn about any potential effect on therapeutic angiogenesis. In theory, however, such cells represent another avenue of investigation, especially as the cells may be genetically modified to enhance therapeutic effects. The duration of therapeutic effect compared with the use of angiogenic genes administered directly has yet to be investigated, but could be predicted to be longer, if the treatment was successful in the first place.

**DURATION OF ANGIGENESIS DEPENDING ON THE DELIVERY SYSTEM**

In the cardiovascular field, interventional techniques are already carried out within vessels, hence it seems logical for safety and dosage reasons to deliver therapy, and particularly gene therapy, locally in vivo using catheters. To this end, many local drug delivery catheters have been designed for transluminal or even pericardial application and are currently being evaluated in animal and clinical studies (Figure 3). Intraluminal infusion commonly results in high loss of agent, unless sustained-release, biodegradable poly-lactic-polyglycolic acid copolymer nanoparticles are used to penetrate deeper layers.13 Delivery to deeper layers, including media, adventitia, or pericardium, via catheters could have several advantages over intraluminal delivery, including less intraluminal loss, the ability to deposit drugs for longer periods, and
the use of therapies that act on adventitial pathways of angiogenesis and remodelling. Gene expression with several genes for proteins with a potential therapeutic effect, including VEGF, has been demonstrated in all arterial layers in a pig model up to 5 months after liposomal delivery using the needle injection catheter (Figure 3). Thus, whereas certain delivery systems lead to an immediate high concentration of drug in arterial wall, others can effectively leave a long-lasting depot there, an effect that may further be enhanced by microencapsulation, which does not compromise viability of cells (Figure 4). The pharmacokinetics of any combination making up the system must be carefully evaluated.

**HOw Long Do We Need THE Therapeutic Effect IN ANGIOGENESIS?**

This is probably the question which has so far been least addressed by investigators. It is likely that angiogenic treatment would need to be long term or repeated if the effects are only of short duration, particularly as it is known that new vessels can regress in the absence of continued local activity of growth factors. In addition, the degree to which
new vessels take up arterial blood flow—and this will clearly depend on the anatomical development in the context of extensive vessel disease in surrounding preexisting native vessels—will influence how long they stay open without repeated treatments.

Adenoviral gene transfer of VEGF demonstrated formation of mother vessels, an effect that had already resolved after 3 weeks. In the long term, creating an effect that avoids vessel regression and allows the formation of a robust capillary network and intact collateral circulation may be important. Whether this long-term effect should be achieved by multiple- rather than single-dose therapy, stable gene integration, the use of combinations of several growth factors instead of using one single growth factor, and what the role of flow is in maintaining patent vessels remains open.

As discussed above, gene therapy may provide more of a depot effect, particularly if combined with optimized vector and local delivery systems, but in terms of gene therapy, it is in fact not yet known how many cells need to be transduced and for how long to achieve the desired therapeutic effect. There are widely varying reports of vector efficiency, for example, arteries in patients have been locally transfected with adenoviruses with efficiencies of between 0.4% and 5%, compared with up to 15% achieved by transfection when combined with liposomes and only about one in a thousand cells typically infected by retroviruses. However, the results quoted here are only relevant when we look at the clinical effect. The therapeutic effect may also depend on the method of delivery into the artery at the injury site, on whether or not high concentrations of genes are available at the site of therapy, with release and degradation perhaps being slowed down by gene integration, chemicals, microencapsulation, or the presence of the depot itself. The number of cells that need to be transduced is probably dependent on the particular gene, the expression efficiency achieved per cell (depending on the amount of DNA entering the nucleus), and on the duration of local levels of protein necessary for the intended biologic effect. If transfection efficiency is a limiting factor, then delivery strategies that augment the therapeutic effect by action on untransfected cells (bystander effect) will be preferable. Even low transfection efficiencies of 0.1% may lead to significant angiogenesis when the paracrine therapeutic gene VEGF165 is injected into the adventitia in a porcine model using optimized liposomal gene transfer and long-lasting gene expression.5

Most approaches in angiogenesis aim to induce endothelial cell proliferation. That remodeling may also be important has been demonstrated, and the factors involved may be targets for gene therapy, with the precise therapeutic goals yet to be elucidated. Possible novel approaches aiming to influence remodeling may be directed at any of the arterial layers, including the adventitia. If cell proliferation is proven to play a pivotal role in angiogenesis and remodeling, it is still unclear to what extent, and particularly for how long, it is preferable to induce cell replication without inducing further stenoses or benign or malignant tumors. This must be clarified in future studies using appropriate models.

The type of gene transfer that generally raises safety and ethical concerns is that which involves manipulation of the germ cell DNA, resulting in long-term effects and affecting future generations. Germ cell gene transfer is at present restricted to producing transgenic plants or animals for economic and research purposes. In contrast, gene therapy as discussed in this article involves transfer of the gene only into specific differentiated cells. This somatic gene therapy will, at best, last as long as the treated cells survive, provided gene integration takes place. Gene therapy strategies are currently aimed at combining maximum efficacy with optimum safety, including the control of duration of therapeutic effects. Results from preclinical and clinical investigations in the field of angiogenesis suggest that transient gene expression for a minimum of several weeks and a maximum of several months may be sufficient for effective therapy. Use of nonimmunogenic vectors allows multiple applications at different time points and sites if needed, and may therefore be preferred.

**HOW CAN WE STOP THE EFFECT OF THERAPEUTIC ANGIOGENESIS?**

There are a number of antiangiogenic agents known to inhibit angiogenesis. They comprise chemicals such as thalidomide or proteins that are natural inhibitors of angiogenesis. Such inhibitors of angiogenesis are predominantly investigated by oncologists who aim at inhibiting tumor angiogenesis. They may be applied directly or using microencapsulated producer cells. However, to what extent they may be used to halt angiogenic therapy has yet to be ascertained, and they remain an important avenue for further research.

Current lines of investigation in gene therapy include the development of more controllable promoters, as it has been recently shown that repeti-
tive angiogenic stimulation is needed to induce a stable new capillary network in animal models. Promoters to direct transcription of introduced genes can be permanently switched on or be inducible. By creating inducible promoters that allow gene transcription to be turned on within a limited time frame, there is more control over the synthesis of the therapeutic protein. Induction of the promoter can be through naturally occurring stimuli, such as hypoxia in the case of angiogenesis, or by the systemic administration of certain drugs (e.g., dexamethasone, tetracycline), which may be administered repeatedly. More locally directed induction is possible by the use of radiation-induced promoters.24,25

Therapeutic genes can be delivered by specific cells producing viral vectors called packaging cells. For slow release and protection against immunological reactions, such cells may be microencapsulated before implantation at the site of therapy. Packaging cells administered in vivo may contain a suicide gene to induce cell death at any time desired.17 The herpes simplex virus thymidine kinase gene is a suicide gene that encodes for an enzyme that allows phosphorylation and thus incorporation of nucleoside analogues into newly synthesized DNA, blocking DNA polymerase and premature DNA chain termination. When nucleoside analogues such as the drug ganciclovir are introduced intravenously, cell suicide occurs local to the gene therapy. Death of the packaging cells results in halting the effect of viral gene transfer.

**CONCLUSION**

Various aspects of protein or gene therapy are currently under investigation for clinical use to solve the problem of chronic ischemia through the creation of intact and robust new vessels by angiogenesis. Gene therapy may provide more regulation over the duration of therapy through several mechanisms, including via the selection of certain vectors, inducible promoters, encapsulation methods, local gene delivery catheters, or the use of ex vivo genetically modified cells or expanded progenitor cells. Therapy can be modified to take place over only few days or up to the life span of treated cells, and it may be halted using a number of antiangiogenic chemicals or proteins.

Sigrid Nikol is supported by grants from the Deutsche Forschungsgemeinschaft and the Ernst und Berta Grimmke-Stiftung, Germany. She would like to thank Tanya Y. Huehns, MD, for proofreading the manuscript.

**REFERENCES**


Plants and the heart

At the heart of the fuel supply: the cereal grasses

Anirban Banerjee, PhD

Department of Surgery (C-311) - University of Colorado Health Sciences Center - Denver - Colorado - USA

Some physiologists claim that the brain accesses the most energy in the human body. Others say it is the heart, witness the politicians or grant reviewers who must assuredly be “brain dead,” while remaining perfused... This year has marked the arrival of the 6 billionth person on the planet. Six billion hearts that must beat ceaselessly day and night—and the smallest discrepancy warrants a visit to your local cardiologist, provided you can afford it. How does the world support this tremendous energy expenditure? Biological energetics cannot use fossil fuels, so where is the energy feedstock to drive the mushrooming human population?

Unlike the brain, the heart is not picky about fuel. Most any carbon-hydrogen compound will do. From two-carbon acetate to the 58-carbon palmitoyl triglycerides, all are eventually turned to CO$_2$ and water, with the help of preparatory metabolic pathways routing to the oxygen-consuming mitochondria. As heat engines go, heart muscle is impressive in its efficiency, converting some 40% to 50% of the caloric energy of food into biochemically useful adenosine triphosphate (ATP). It is estimated that 2 to 5 µmoles of ATP are consumed by 1 gram of mammalian heart tissue in a single beat. With about 350 g for the average human heart, beating say, at 70 per minute, human hearts alone may consume some 126 million tons of ATP per day representing some 45 terajoules (= 45 x 10$^{12}$ joules) of energy feedstock (assuming 50% efficiency). This amount 

Of all dry foods, about 95% of calories consumed by humans derives from three grasses (rice, wheat, and maize). This includes calories obtained directly from cereals, and those obtained from animal products reared on these same cereals. J. D. Harlan believed that wheat (Triticum aestivum and T. turgidum) was cultivated in Anatolia (Turkey) by hand as long as 10 000 years ago. Rice (Oryza sativa) was cultivated in Indochina some 5000 years ago. Maize is totally manmade, derived from teosinte—from the Nahua!; the scientific term is Zea mexicana— in Central America 4000 years ago, the most commonly planted species is now Zea mays (Indian corn). These are the basins of the first human civilizations and attest to how the ready availability of energy for a settled people spurs population growth, leisure, and hence civilization.
plants have themselves been changed by their intimate associations with humans. Agricultural practices emphasizing yield have selected husks that cannot break spontaneously, requiring threshing by man. ¹

All vegetable fuels are derived from carbon fixation by the plant enzyme RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase). ³ Using the reduced nicotinamide adenine dinucleotide (NADH) and ATP generated by solar energy within photosynthetic systems, RuBisCO fixes CO₂ to form phosphoglycerate, a 3-carbon compound that is eventually metabolized into sugars, amino acids, etc. Seven enzymes (with subunits encoded by both nuclear and chloroplast DNA) are required to regenerate ribulose-1,5-bisphosphate. This is the C3 cycle of all plants that eventually produces both the fuels and the oxygen that will be gobbled up by energy-hungry higher animals. RuBisCo is a remarkably inefficient enzyme that either fixes CO₂ or oxidizes sugars to produce CO₂. While most mammalian enzymes hum along at several 1000 catalytic reactions per second (at minimum), the ponderous RuBisCO turns over a few times per minute. This is an ancient enzyme whose nine-sheeted beta basket and 11-alpha helical structures now adorn modern texts on cell biology. This enzyme’s structure may have been adequate when ancient global CO₂ levels were sufficiently high to drive the fixation reaction. Times have changed, but functional proteomics and genetics constrain any change on the primordial structure. As a result, plants are forced to synthesize prodigious amounts of RuBisCO and devise complex leaf shapes and branching structures to maximize its performance. RuBisCO is the most abundant enzyme on the planet and possibly the sole energy link between the sun and the biosphere.

Among C3 plants, the families of grasses are relative newcomers. Fossil evidence suggests that the common members of this family, including lawn grasses, rye, oats, millet, and bamboo evolved after the middle Eocene (25 million years, Myr). ¹ However, the first flowering plants, the angiosperms (magnolia, lotus), had begun blossoming in the middle Jurassic (190 Myr). The cone-bearing gymnosperms (cycads, sequoia, and gingko) are another 100 Myr older. Analysis of several mitochondrial genes (which evolve slowly in plants) as well as chloroplast and nuclear genes support this dendrology. Some grasses such as maize (Zea) and smooth crabgrass (its name, Digitaria ischaemum, an apparent cardiological faux pas) use a more sophisticated approach to CO₂ fixation. ¹ An outer layer of mesophyll cells in a double-layered leaf structure that pumps CO₂ into inner bundle cells using malate (C₄) as the CO₂ transporter. Because of the higher local CO₂ concentration, the bundle sheath cells can conduct CO₂ fixation by the traditional C₃ (Calvin cycle) to produce sugars, etc, more efficiently. This works...
well only if there is abundant sunlight and heat (as in the tropics). Genetic engineers are attempting to introduce C4 cycles into rice and wheat (two of the three most important fuel sources for humans). In these plants, in particular, we can appreciate how RuBisCO plays the "yin" to the "yang" of cardiac bioenergetics.

Although cereal grasses are not known (yet), to contain the flamboyant toxins, antioxidants, and healing drugs so prevalent in their flowering cousins, they have made themselves essential to human survival. They are the best at capturing the largest amount of energy and present it in dry matter year in year out in all parts of the world. Nigel Calder argues that a visitor from space might mistake grass as the dominant life form on the planet! It occupies vast areas of the globe and engages human diligence to reproduce. As a timely counterpoint to the 6 billionth human, this year also marks the complete sequencing of the rice genome!

REFERENCES

1. Chapman GP. 
   Grass Evolution and Domestication. 

2. Bloom F. 
   Rice, races, and riches. 

3. Mann CC. 
   Genetic engineers aim to soup up crop photosynthesis. 

4. Calder N. 

5. Normile D. 
   Agricultural biotechnology. Monsanto donates its share of golden rice. 


Dialogues in Cardiovascular Medicine - Vol 6 · No. 3 · 2001

Bibliography of One Hundred Key Papers


<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Title</th>
<th>Journal</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Title and Details</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicosia RF, Ottinetti A.</td>
<td>Modulation of microvascular growth and morphogenesis by reconstituted basement membrane gel in three-dimensional cultures of rat aorta: a comparative study of angiogenesis in matrigel, collagen, fibrin, and plasma clot.</td>
</tr>
<tr>
<td>Park JE, Chen HH, Winer J, Houck KA, Ferrara N.</td>
<td>Placenta growth factor: potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR.</td>
</tr>
<tr>
<td>Pearlman JD, Hibberd MG, Chuang ML, et al.</td>
<td>Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis.</td>
</tr>
<tr>
<td>Pepper MS, Montesano R.</td>
<td>Proteolytic balance and capillary morphogenesis.</td>
</tr>
<tr>
<td>Rivard A, Berthou-Soulie L, Principe N, et al.</td>
<td>Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity.</td>
</tr>
<tr>
<td></td>
<td>Circulation. 1999;99:111-120.</td>
</tr>
<tr>
<td>Schaper W, Brabander MD, Lewi P.</td>
<td>DNA synthesis and mitoses in coronary collateral vessels of the dog.</td>
</tr>
<tr>
<td>Schnurch H, Risau W.</td>
<td>Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage.</td>
</tr>
<tr>
<td></td>
<td>Nat Med. 2000;6:405-413.</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Title</th>
<th>Journal/Volume/Issue/Pages</th>
</tr>
</thead>
</table>


<table>
<thead>
<tr>
<th>Authors</th>
<th>Title</th>
<th>Journal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authors</td>
<td>Title</td>
<td>Journal and Volume</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Authors</td>
<td>Title</td>
<td>Journal/Source</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------</td>
<td>---------------------------------------</td>
</tr>
</tbody>
</table>
VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease.

Kawamoto A, Gwon HC, Iwaguro H, et al.
Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia.
*Circulation.* 2001;103:634-637.

Kureishi Y, Luo Z, Shiojima I.
The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals.

Laham RJ, Sellke FW, Edelman ER, et al.
Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial.

Lazarous DF, Unger EF, Epstein SE, et al.
Basic fibroblast growth factor in patients with intermittent claudication: results of a phase I trial.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N.
Vascular endothelial growth factor is a secreted angiogenic mitogen.

Levy PL, Levy NS, Goldberg MA.
Post-transcriptional regulation of vascular endothelial growth factor by hypoxia.

Li J, Brown LF, Hibberd MG, Grossmann JD, Morgan JP, Simons M.
VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis.

Losordo DW, Vale PR, Symes J, et al.
Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia.

Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart.

Maisonpierre P, Suri C, Jones PF, et al.
Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis.
*Science.* 1997;277:55-60.

High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis.

Nitric oxide synthase modulates angiogenesis in response to tissue ischemia.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Title</th>
<th>Journal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Reference</td>
<td>Title</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>-------</td>
</tr>
</tbody>
</table>


| Wang GL, Semenza GL.                                                                 | Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia.  |
|                                                                                   | *Cell.* 1998;93:741-753.                                                                          |
| Witzenbichler B, Asahara T, Murohara T, et al.                                   | Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. |