



Angiogenesis and cardiovascular disease

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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 I 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.

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

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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 up-regulated 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 "biochemical splinting," in which case metabolic functions are converted from primarily aerobic to predominantly anaerobic. In the extreme, reduced blood flow may be associated with literal splinting or impaired wall motion characteristic of "hibernating myocardium."² Alternatively, "behavioral splinting" may be employed. For cardiac muscle, this may be achieved by reduced activity, and if necessary pharmacologic therapies that reduce myocardial wall stress. For skeletal muscle, pharmacologic interventions are so limited that reduced levels of activity constitute the typical response to claudication. These adjustments are only necessary, however, if natural reparative mechanisms have failed to address the problem of ischemia successfully by restoring blood flow to the affected muscle group. Our current notions concerning the means by which this is achieved, and those factors that may selectively obviate this adaptive response are the subject of this *Lead Article*.

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.³ 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,⁴ 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.⁵ 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.^{6,7}

More recently, D'Arcangelo et al⁸ have suggested that tissue acidosis may constitute an independent stimulus to upregulated VEGF expression. While the mechanism for upregulated VEGF expression in response to acidosis 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.⁶

SELECTED ABBREVIATIONS AND ACRONYMS

5'UTR	5'-untranslated region
Ang-1; -2	angiopoietin-1; -2
BM	bone marrow
EC	endothelial cell
EMM	electromechanical mapping
EPCs	endothelial progenitor cells
FACS	fluorescence-activated cell sorter
FGF	fibroblast growth factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTx	gene transfer
HIF-1	hypoxia-inducible factor-1
hPBMCs	human peripheral blood mononuclear cells
HPCs	hematopoietic stem cells
HRE	hypoxia response element
IPN	ischemic peripheral neuropathy
LLS	linear local shortening
NO	nitric oxide
NOD	nonobese diabetic (model of diabetes)
PDGF-A; -B	platelet-derived growth factor isoforms A and B
PF-4	platelet factor-4
phVEGF₁₆₅	plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor
rhVEGF₁₆₅	recombinant human 165-amino-acid isoform of vascular endothelial growth factor
TNF-α	tumor necrosis factor α
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor



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 al¹⁴; 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 skeletal^{16,17} or cardiac¹⁸ muscle. The consequent 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 neovasculature 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 neovascularity in response to myocardial or lower-extremity ischemia.

Cytokines	Protein	Gene ph	Gene ad	EC specific	Pleiotropic	Secretory sequence
VEGF ₁₆₅	✓	✓		✓		✓
VEGF ₁₂₁			✓	✓		✓
VEGF-2 (VEGF-C)		✓		✓		✓
HIF-1 α			✓			
FGF-1 (aFGF)	✓				✓	
FGF-1 modified		✓			✓	✓
FGF-2 (bFGF)	✓				✓	
FGF-4			✓		✓	✓

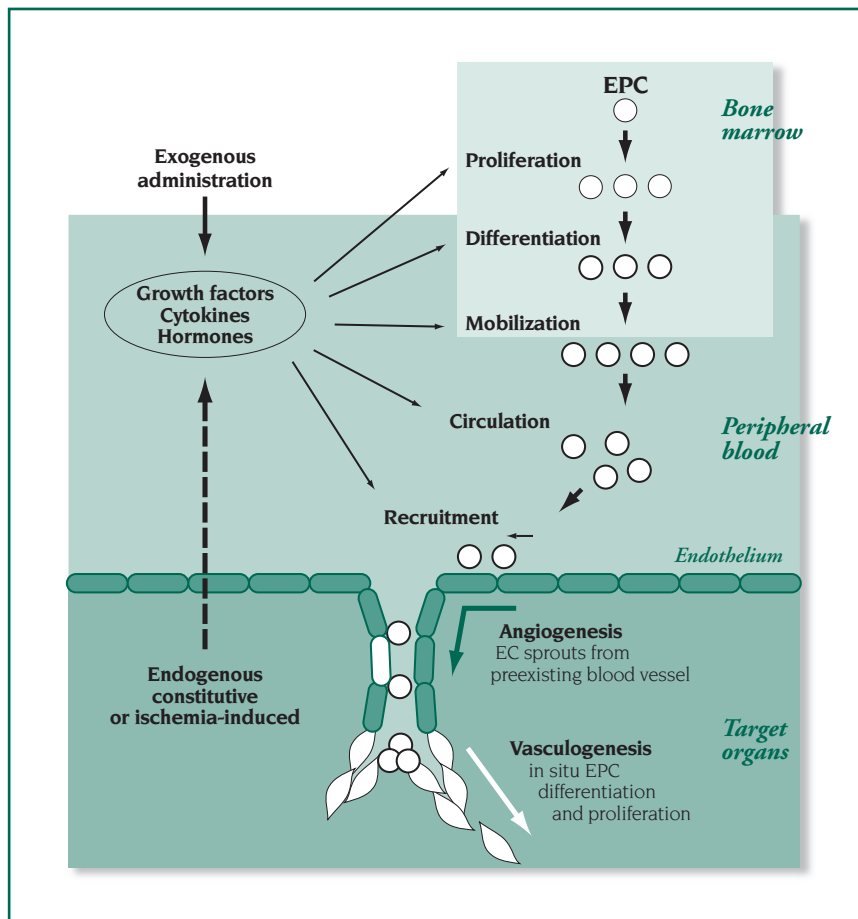
Legend: a, acidic; ad, adenoviral vector; b, basic; FGF, fibroblast growth factor; HIF, hypoxia-inducible factor; ph, plasmid human; VEGF, vascular endothelial growth factor.

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

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.

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).²⁴ 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 neovasculature 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, in-

cluding 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).²⁵ Experimental hindlimb ischemia in mice, for example, increases the frequency of the EPC-enriched population in the circulation by >400%.²⁶ 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 trans-

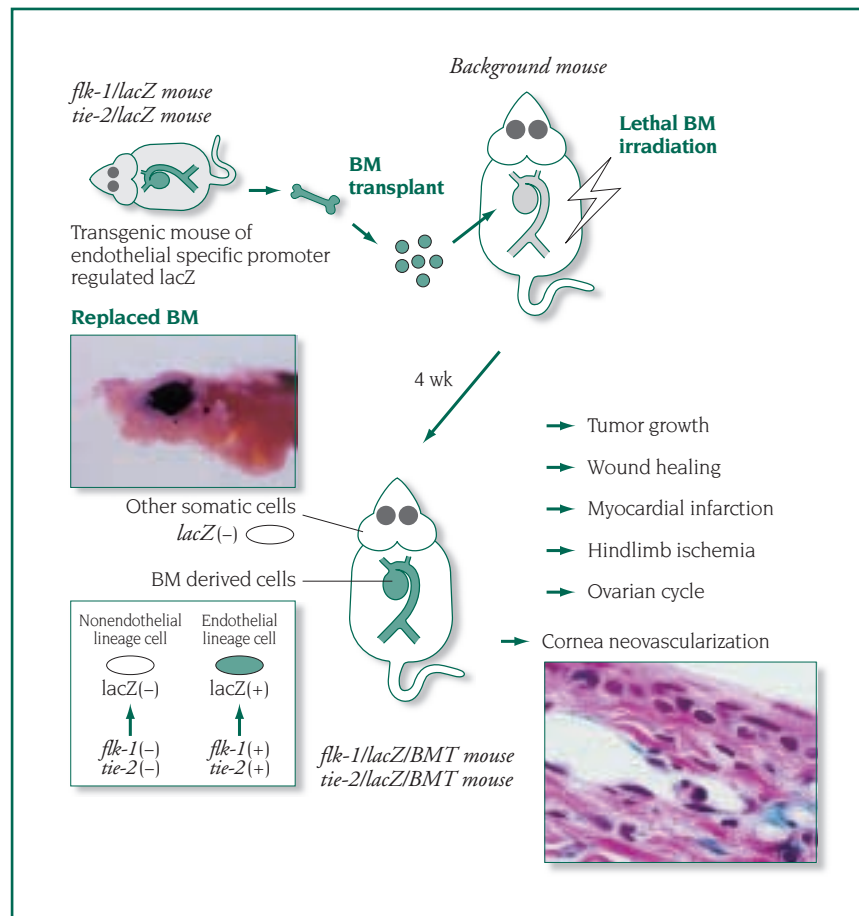


Figure 2. Bone marrow (BM) transplantation (T) model employed to study the contribution of postnatal vasculogenesis to neovascularization of ischemic tissues. Transgenic mouse constitutively expressing lacZ gene transcriptionally regulated by an endothelial cell (EC)-specific promoter, flk-1 or tie-2, 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.²⁷ 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-1 α protein. In vitro studies from our laboratory²⁸ and others²⁹ of HIF-1 α protein expression under hypoxic conditions suggest that the stabilization of HIF-1 α 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.²⁸ 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.³⁰

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-1 α /VP16 naked DNA hybrid.³² 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,³³ this finding has been recently confirmed in studies of human coronary collateral development.³⁴ Reconstitution of hindlimb perfusion in the NOD mouse by VEGF GTx³³ 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 al³⁵ have documented a 10-fold range of response in growth factor-stimulated angiogenesis



among different strains of inbred mice. This extended to a differential sensitivity to angiogenesis inhibitors, with at least one strain demonstrating complete resistance to such therapy.

In human subjects, it has long been appreciated that certain patients—without a distinctive phenotype—are “good collateral formers,” while other patients are “poor collateral formers.” Indeed, it is not at all uncommon to see patients with peripheral artery disease who, in spite of extensive lower-extremity arterial occlusions, remain nearly asymptomatic as a result of a naturally robust collateral network. Past investigations 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.

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 alluded 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 diabetics 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 vectorial 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 I 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 7 vascular surgical reconstructions. 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 I trial, unani-mously 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 I oncology protocols employed to study new chemotherapeutic agents administered to human subjects.

A total dose of 4000 μ g phVEGF₁₆₅ was injected directly into the muscles of the ischemic limb. Analysis of gene expression at the protein level using an enzyme-

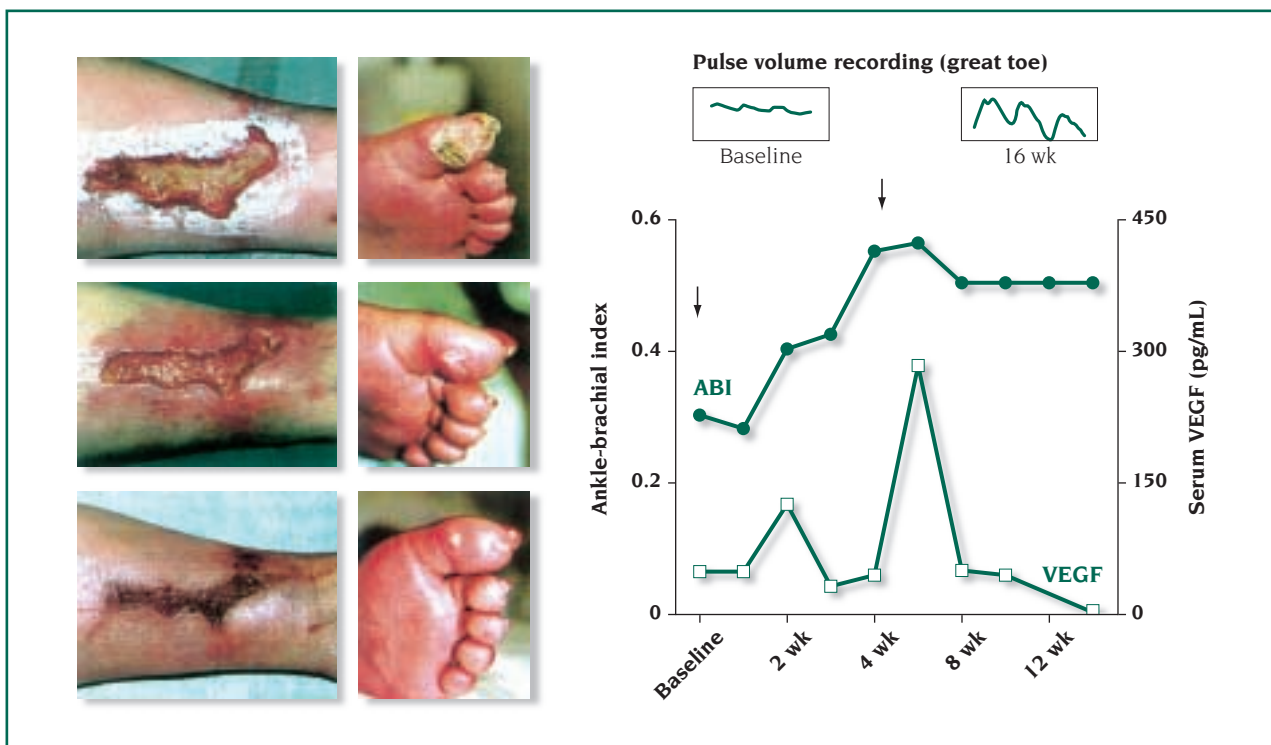


Figure 3. Limb salvage after gene therapy in a 33-year-old woman. **Left top:** Nonhealing wound on the medial aspect of the calf and ischemic necrosis involving the great toe. **Left middle:** Ingrowth of granulation tissue in calf wound, healing of great toe. **Left bottom:** Three months after gene transfer, healing of split-thickness skin graft at wound site, and full resolution of great toe necrosis. Prior to gene therapy, patient was wheelchair-bound on multiple analgesics including methadone, amitriptyline hydrochloride, clonidine, oxycodone/acetaminophen, and a fentanyl patch. Three months after gene transfer, she was freely ambulatory and had successfully weaned from all analgesics. **Right:** Evidence of plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor (phVEGF₁₆₅) bioavailability documented by an increase in venous VEGF blood levels, and bioactivity expressed as an increase in ankle-brachial index (ABI). ABI progressively increased from 0.28 before to 0.56 after gene therapy (weeks [wk] refer to time post-transfection). This was associated with the development of a phasic pulse volume recording (PVR) compared with the nonphasic tracing recorded at baseline. Vertical arrows indicate timing of each of the two intramuscular phVEGF₁₆₅ injections.

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.⁴⁶ 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 $<200\ \mu\text{m}$ in diameter²³; 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 al²³ 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 phVEGF₁₆₅ 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-1 α /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 ischemia⁴⁷ consistent with the finding of an age-dependent angiogenesis in animals.^{28,48}

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 phVEGF₁₆₅ 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 laboratory⁴⁹ 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 VEGF₁₆₅ 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.¹¹ 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,⁵⁰ in part by up-regulating EC expression of fibronectin and $\alpha_v\beta_3$, 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 (phVEGF₁₆₅) gene transfer.

crease in EC viability. Fourth, with regard to the target of gene therapy, it has been noted^{41,42,51} 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 from hypoxic skeletal myocytes.¹⁶ 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.^{43,52}

Previous work from our laboratory established that phVEGF₁₆₅ transgene expression is limited to less than 30 days in animal models of limb ischemia.^{42,43,51} 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 phVEGF₁₆₅ 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 phVEGF₁₆₅ 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^{53,54} 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.

THE THERAPEUTIC ANGIOGENESIS FOR MYOCARDIAL ISCHEMIA

Preliminary clinical trials established that the results obtained in human subjects with critical limb ischemia^{42,44,47} may extend to patients with myocardial ischemia.⁵⁵⁻⁵⁸ 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 imaging⁵⁷; 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 electromechanical assessment of myocardial perfusion (NOGA™ system, Biosense-Webster, J&J, 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 on-line 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 (48.1±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.

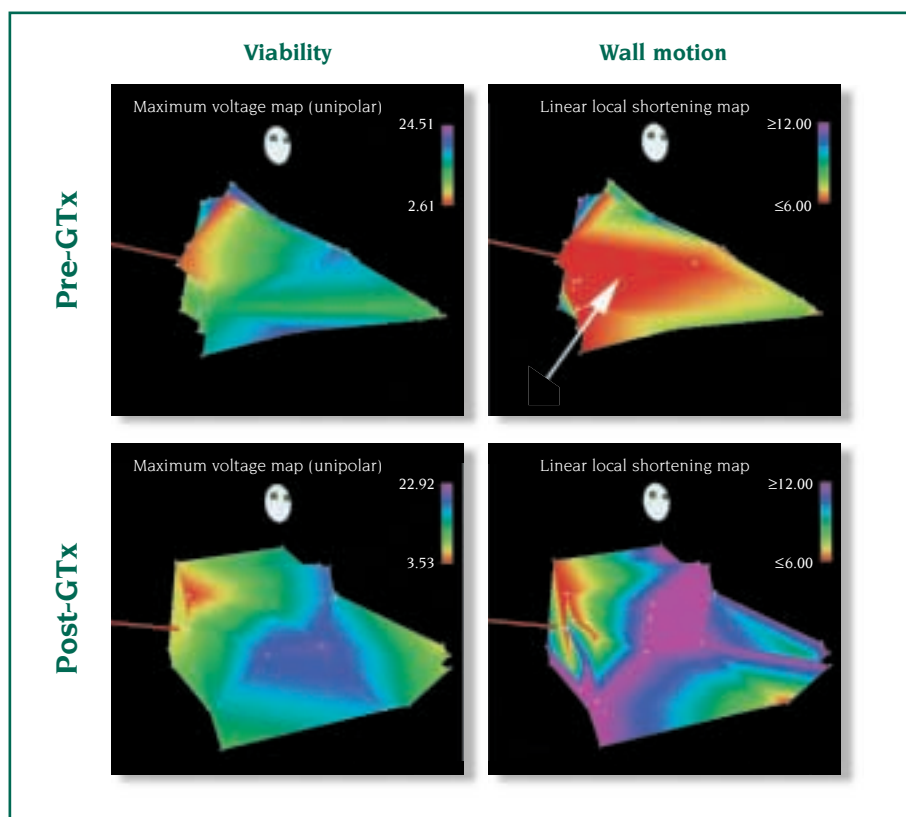


Figure 5. NOGA™ left ventricular (LV) electromechanical mapping (EMM) performed in a 48-year-old male. NOGA™ images in the right anterior oblique (RAO) projection prior to gene transfer (GTx) show the unipolar voltage (UpV) map (**upper left panel**) with normal voltages suggestive of viable myocardium (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.

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 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 (phVEGF₁₆₅ 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.

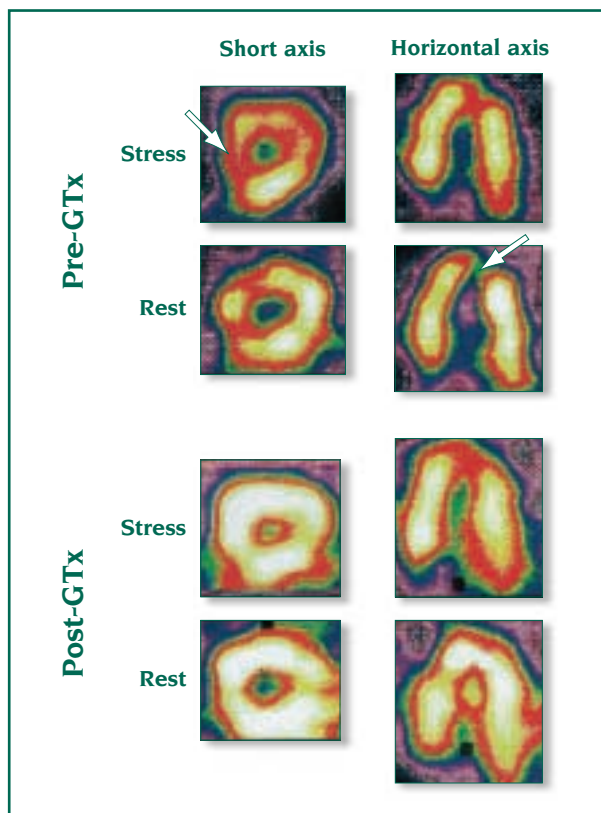
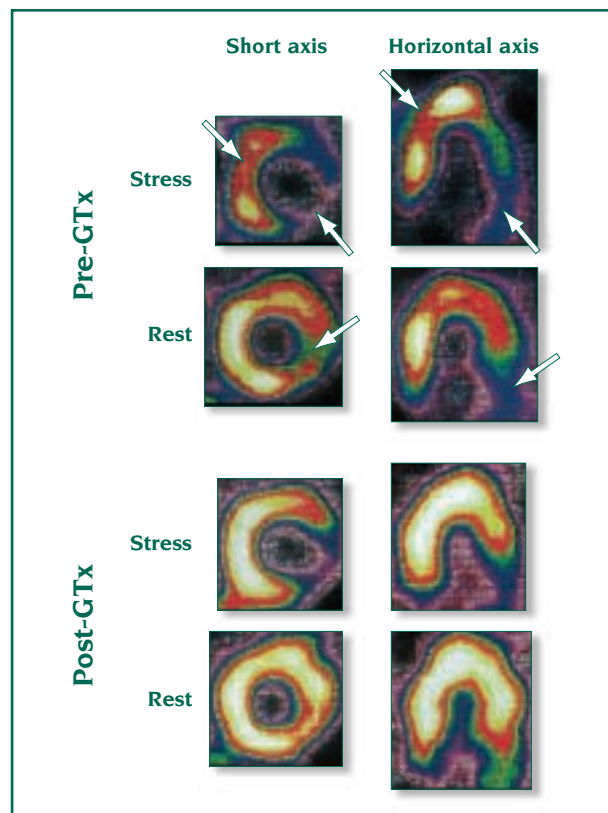
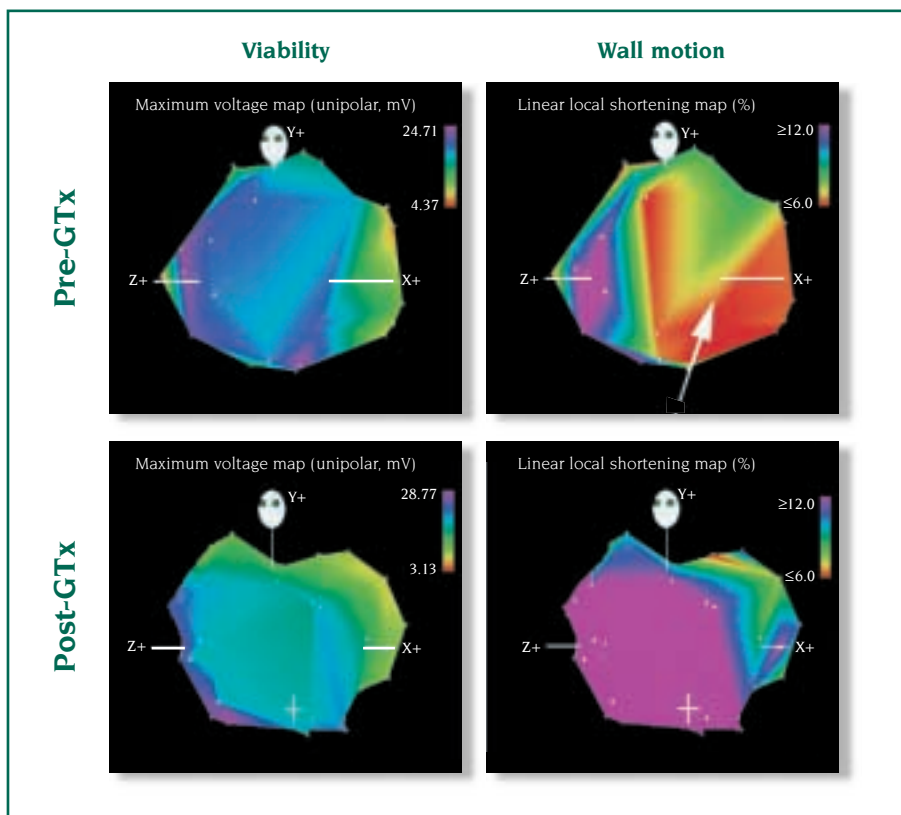




Figure 7. NOGA™ left ventricular (LV) electromechanical mapping (EMM). UpV and linear local shortening (LLS) NOGA™ images in the left anterior oblique (LAO) projection (upper left and right panels, respectively) of a 53-year-old male prior to plasmid DNA encoding the 165-amino-acid isoform of human vascular 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).



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 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.⁶⁴

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 NOGA™ 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.⁴⁴ 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.⁶⁵ 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.^{27,41} 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.⁶⁷

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 phVEGF₁₆₅ 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*^{23,68} and *postmortem*^{69,70} 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 Cavanagh⁷¹ 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..."⁷²; VEGF, originally known as vascular permeability factor (VPF)⁷³ 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.⁴⁷ 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.²⁷ 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.²⁸ Similarly retarded neovascularization and reduced VEGF expression was observed in diabetic (NOD)³³ and hypercholesterolemic (ApoE^{-/-})¹² 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.¹²

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 im-

posed 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 descendants—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.⁷⁴ 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.²⁶ 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.²⁶ 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.²⁶ 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 vasculogenesis. 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.⁷⁴ 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 PIH12 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 1 × 10³ 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, ie, 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 models⁵¹ and early studies performed in small numbers of patients with lower-limb⁴⁷ and myocardial^{56,57} 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.³⁰

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, $\alpha_v\beta_3$, 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,^{44,47} as well as into ischemic myocardium,⁵⁷ 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.⁷⁵ 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 neovasculature.

Like fully differentiated ECs, EPCs express specific endothelial antigens including KDR (VEGFR-2), CD34, and VE-cadherin.^{25,74,76} While KDR and VE-cadherin are

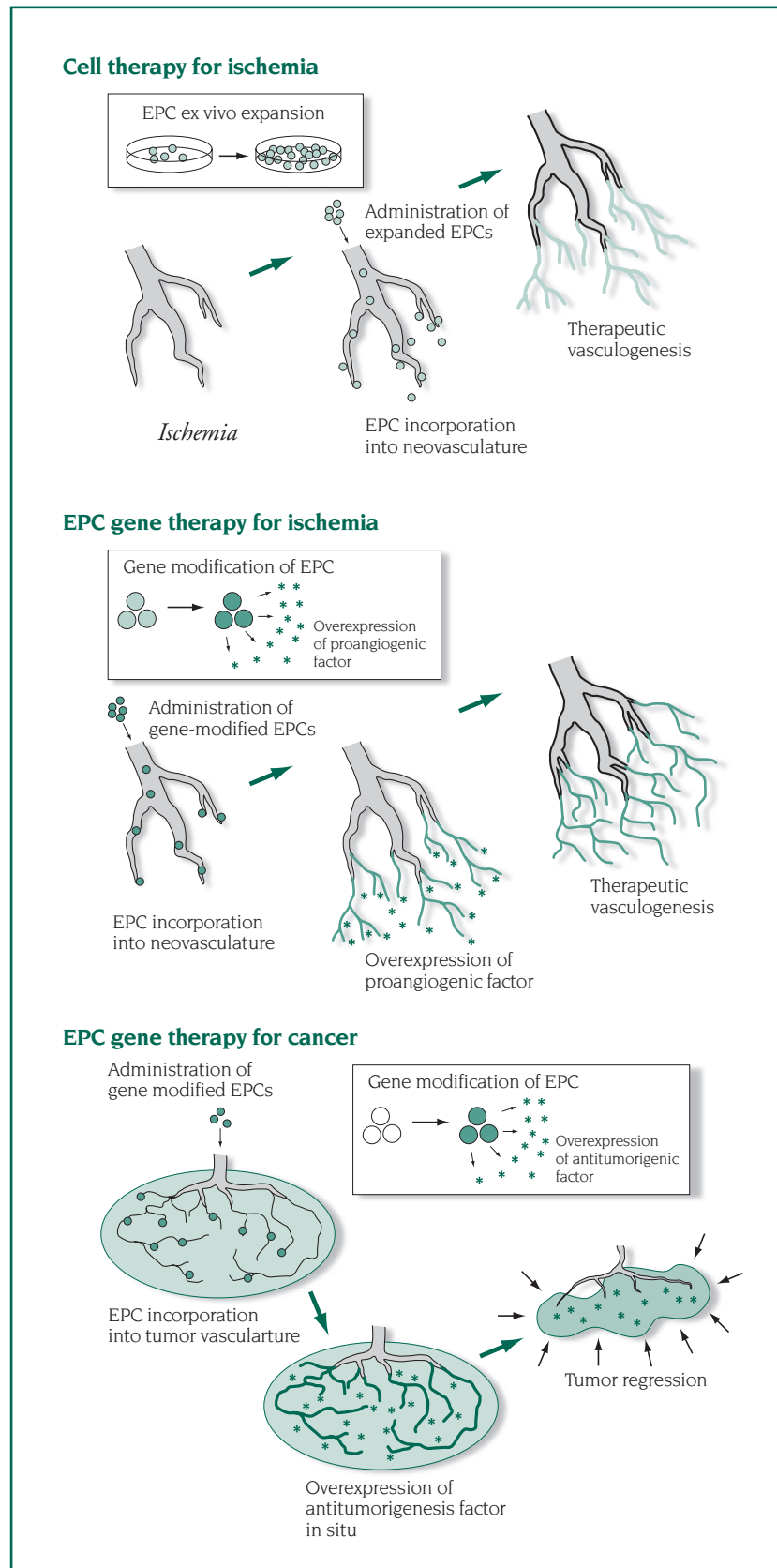


Figure 9. Therapeutic vasculogenesis.

Top: Circulating endothelial progenitor cells (EPCs) may be harvested in analogous fashion to methods currently established for harvesting hematopoietic stem cells (HSCs) for autologous marrow transplantation. EPCs, purified and expanded ex vivo, may be then readministered, with or without angiogenic growth factors, to optimize therapeutic neovascularization. **Middle:** Harvested EPCs may be transfected ex vivo with genes encoding for proangiogenic factors; administered EPCs, when incorporated into nascent vasculature, express growth-promoting factors directly at site of neovascularization and thereby potentially augment neovascularization. **Bottom:** If EPCs are transfected ex vivo with transgene encoding antiangiogenic factors, administered EPCs home to vascular infrastructure of developing neoplasm where they act as a "Trojan horse" to express antiangiogenic factors that sabotage tumor growth and metastasis.

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 $5 \times 10^3/\text{mL}$) far exceeds the number of differentiated ECs circulating in peripheral blood (2 to $3/\text{mL}$). Second, animal experiments from our own laboratory have suggested that the majority of the cellular population mobilized into the circulation and then incorporated into neovascular foci following VEGF administration is most consistent with BM-derived EPCs.⁷⁵

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, ie, sprout formation or angiogenesis. The finding

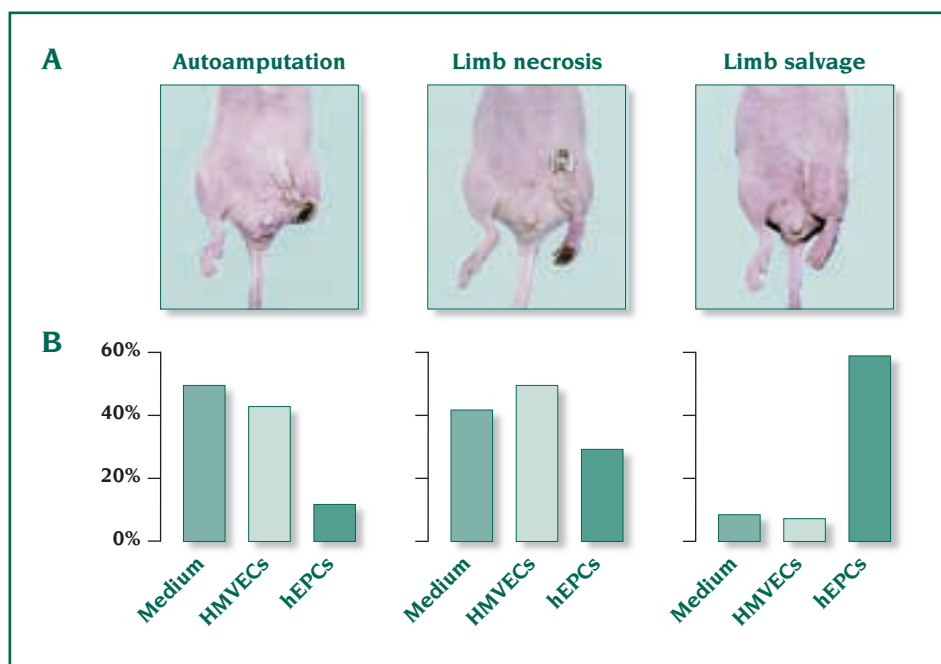


Figure 10. Administration of human endothelial progenitor cells (hEPCs) leads to reduced limb loss and increased limb salvage.

A: Representative macroscopic photographs of mice showing three different outcomes observed in the study. **Left panel** illustrates autoamputation, characterized by loss of the ischemic hindlimb; **middle panel** illustrates severe foot necrosis; **right panel** illustrates most favorable outcome, complete salvage of ischemic hindlimb with intact function.

B: Percent distribution of above outcomes among mice receiving control media, human microvascular endothelial cells (HMVECs), and hEPCs. The differences in outcome were statistically significant (hEPCs vs HMVECs, $P=0.006$; hEPCs vs culture media, $P=0.003$, HMVECs vs culture media, $P=NS$).

that VEGF augments the number of circulating EPCs in human patients, together with the aforementioned murine experiments,⁷⁵ 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.⁷⁷ 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.¹⁵ 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 $4 \cdot 5 \times 10^4$ cells per 1×10^6 hPBMCs, yielding an 80- to 90-fold increase in the original number of harvested cells. Previous analyses of embryonic neovascularization suggest that co-expression of Flk-1 (KDR) and VE-cadherin denote the point of divergence of ECs from hematopoietic lineages. Moreover, a combination of monoclonal antibodies 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 stem cell-derived ECs. The capacity to take up acetylated 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 described as the prototypical antigen expressed by both HSCs and endothelial lineage cells, hematopoietic cells may contaminate freshly isolated CD34⁺ cells. Indeed, pilot studies demonstrated that the extent of neovascularization achieved following transplantation of freshly isolated CD34⁺ cells was inferior to culture-expanded EPCs. Third, for therapeutic 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 other 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 maturation from CD34⁺ precursors. As cytokine composition of the culture media may influence in vitro mononuclear cell differentiation (W. Kalka-Moll, unpublished data), the cytokine mixture employed for EPC culture, containing VEGF, bFGF, IGF, and EGF, appears to preferentially 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 clinical 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 depleted and/or dysfunctional ECs preclude an optimal 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 concurrently 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. Looking forward to the long term, the genetic endowment permitting one to appropriately upregulate cytokine expression and mobilize EPCs in a fashion that is optimally 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 appropriate therapeutic interventions.

THREE KEY QUESTIONS

So what promises—or disillusion—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.

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