Constitutive Expression of \( \text{phVEGF}_{165} \) After Intramuscular Gene Transfer Promotes Collateral Vessel Development in Patients With Critical Limb Ischemia

Iris Baumgartner, MD; Ann Pieczek, RN; Orit Manor, PhD; Richard Blair, MD; Marianne Kearney, BS; Kenneth Walsh, PhD; Jeffrey M. Isner, MD

**Background**—Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries, a concept called “therapeutic angiogenesis.” The objectives of this phase 1 clinical trial were (1) to document the safety and feasibility of intramuscular gene transfer by use of naked plasmid DNA encoding an endothelial cell mitogen and (2) to analyze potential therapeutic benefits in patients with critical limb ischemia.

**Methods and Results**—Gene transfer was performed in 10 limbs of 9 patients with nonhealing ischemic ulcers (\( n = 7/10 \)) and/or rest pain (\( n = 10/10 \)) due to peripheral arterial disease. A total dose of 4000 \( \mu \)g of naked plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor (\( \text{phVEGF}_{165} \)) was injected directly into the muscles of the ischemic limb. Gene expression was documented by a transient increase in serum levels of VEGF monitored by ELISA. The ankle-brachial index improved significantly (0.33 ± 0.05 to 0.48 ± 0.03, \( P = .02 \)); newly visible collateral blood vessels were directly documented by contrast angiography in 7 limbs; and magnetic resonance angiography showed qualitative evidence of improved distal flow in 8 limbs. Ischemic ulcers healed or markedly improved in 4 of 7 limbs, including successful limb salvage in 3 patients recommended for below-knee amputation. Tissue specimens obtained from an amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells by immunohistochemistry. PCR and Southern blot analyses indicated persistence of small amounts of plasmid DNA. Complications were limited to transient lower-extremity edema in 6 patients, consistent with VEGF enhancement of vascular permeability.

**Conclusions**—These findings may be cautiously interpreted to indicate that intramuscular injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with critical limb ischemia. (*Circulation*. 1998;97:1114-1123.)

Key Words: angiogenesis • genes • ischemia • growth substances

Critical limb ischemia is estimated to develop in \( \sim 500 \) to 1000 individuals per million per year. In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization, and the disease thus frequently follows an inexorable downhill course. Psychological testing of such patients has disclosed quality-of-life indices similar to those of patients with cancer in the terminal phase of their illness. As concluded in the Consensus Document of the European Working Group on Critical Limb Ischemia, no pharmacological treatment has been shown to favorably affect the natural history of critical limb ischemia. Indeed, amputation, despite its associated morbidity, mortality, and functional implications, is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain, of critical limb ischemia. A second major amputation will be required in nearly 10% of such patients. Despite the use of prosthetics and rehabilitation, reestablishment of full mobility is inconsistently achieved, particularly in the elderly. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling.

Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral and myocardial ischemia, a concept called therapeutic angiogenesis. Several of these studies have used VEGF, also known as vascular permeability factor, a secreted endothelial-cell mitogen with high-affinity binding sites limited to endothelial cells. Endothelial cell specificity has been considered to represent an important advantage of VEGF for therapeutic angiogenesis, because endothelial cells represent the critical cellular element responsible for new vessel formation.
We recently demonstrated angiographic and histological evidence of angiogenesis after intra-arterial gene transfer of naked plasmid DNA encoding human VEGF in a patient with critical limb ischemia. In this report, we present the results of intramuscular phVEGF165 gene transfer performed in an initial phase 1 clinical trial comprising 9 patients with 10 critically ischemic limbs.

**Methods**

**Patients**

Patients qualified for intramuscular gene therapy if they (1) had chronic critical limb ischemia including rest pain and/or nonhealing ischemic ulcers present for a minimum of 4 weeks without evidence of improvement in response to conventional therapies and (2) were not optimal candidates for surgical or percutaneous revascularization. Requisite hemodynamic deficit included a resting ABI <0.6 and/or TBI <0.3 in the affected limb on 2 consecutive examinations performed at least 1 week apart. Criteria used to describe a change in limb status were adapted from standards recommended by the Society for Vascular Surgery/North American Chapter and International Society for Cardiovascular Surgery. Patients were allowed to continue on aspirin and coumarin, provided that these therapies had been used for a minimum of 6 months before gene transfer. Vasoactive medications were discontinued unless prescribed for cardiac disease or systemic hypertension. All patients gave written informed consent for their participation. The study was designed as a phase 1, nonrandomized study to document the safety of intramuscular phVEGF165 gene transfer and to monitor patients as well for evidence of bioactivity. This study design was unanimously approved by the Recombinant DNA Advisory Committee of the National Institutes of Health, by the Human Institutional Review Board and Institutional Biosafety Committee of St Elizabeth’s Medical Center, and by the US Food and Drug Administration.

**Plasmid DNA (phVEGF165)**

All patients received a eukaryotic expression vector encoding the VEGF165 gene transcriptionally regulated by the cytomegalovirus promoter/enhancer. Preparation and purification of the plasmid from cultures of phVEGF165-transformed Esherichia coli were performed in the Human Gene Therapy Laboratory at St Elizabeth’s Medical Center by the column method (Qiagen Mega Kit, Qiagen, Inc). The purified plasmid was stored in vials and pooled for quality control analyses.

**Intramuscular phVEGF165 Transfer**

Aliquots of 500 μg of phVEGF165 plDNA were diluted in sterile saline, and 4 aliquots (total, 2000 μg) were administered into calf and/or distal thigh muscles of the patients by direct intramuscular injection into the ischemic limb. The injection sites were arbitrarily selected according to available muscle mass and included sites above as well as below the knee. The volume of each of the 4 injectates per limb was progressively increased during the course of the study from 0.75 mL (3 treatments) to 3 mL (6 treatments) to 5 mL (11 treatments). Four weeks after the first 2000-μg injection, a second 2000-μg injection was administered, increasing the total amount of plDNA to 4000 μg per patient. One patient was treated for bilateral critical limb ischemia with a total amount of 8000 μg pDNA (4000 μg per limb).

**Serum VEGF Levels**

ELISAs were performed at baseline and weekly up to 12 weeks after the initial treatment of 7 limbs to detect evidence of gene expression at the protein level. Samples were immediately centrifuged for 20 minutes at 3600 rpm at 4°C, and the serum was stored at −20°C until analysis. Serum VEGF was determined with an immunoassay according to the manufacturer’s instructions (R&D Systems). Results were compared with a standard curve of human VEGF with a lower detection limit of 5 pg/mL. Samples were checked by serial dilution and were performed at least in duplicate.

**Hemodynamic and Angiographic Assessment**

Patients were followed up on a weekly basis in the first 8 weeks after gene therapy and at monthly intervals thereafter. Ischemic ulcers were documented by color photography. Resting ABI and TBI were calculated by the quotient of absolute ankle or toe pressure to brachial pressure. Intra-arterial digital subtraction angiography and MRA were performed within 1 week before and 4 weeks after each treatment and 3 months after the latter of 2 intramuscular injections. Digital subtraction angiography was performed as a selective single-leg runoff study using undiluted nonionic contrast media (Isovue-370, Squibb Diagnostics). A minimum of 2 images (early and late frames) at the thigh, knee, calf, and ankle/foot levels were recorded by digital acquisition and hard copies in a 35 × 45-cm format. The diameter of newly visible collateral vessels was assessed by comparison with a 0.09-in-diameter reference wire taped to the skin. MRA was performed with a 1.0-T superconducting system (Impact, Siemens) by means of a transmit-receive extremity coil, a body coil, or both and commercially available pulse sequences. A multisection two-dimensional time-of-flight gradient echo sequence without intravenous contrast medium was used. All axial images were reconstructed by use of the maximum-pixel-intensity algorithm at intervals of 60°.

**Immunohistochemistry**

Double immunohistochemical staining for proliferating endothelial cells was performed as previously described. Bound antibody was then detected with an alkaline phosphatase kit (Biogenex Laboratories). Complexes were visualized with fast red substrate (Biogenex). A counterstain of 10% Gill hematoxylin was applied before coverslips were applied.

**DNA Analysis**

Skin specimens at the site of gene injection and muscle specimens near or remote from the site of gene injection were retrieved from 2 amputees 8 and 10 weeks after intramuscular phVEGF165 transfer, respectively (patients 4 and 10, Table). Tissue was processed with a genomic DNA isolation kit (A.S.A.P., Boehringer Mannheim). For PCR analysis, primer sets unique to the promoter and VEGF coding regions were used to identify gene expression. Total cellular DNA (30 μg) and purified phVEGF165 DNA (0.5 μg) were subjected to 0.8% agarose electrophoresis. The predicted sizes of EcoRI-digested plasmid fragments were 998 and 4703 bp. DNA blotted to a nylon membrane (Amersham, Life Science) was hybridized with two 32P-labeled phVEGF165-specific probes (ncol-digested 679-bp phVEGF165 fragment, position 389 to 1068; aval-digested 787-bp phVEGF165 fragment, position 991 to 1778), washed, and exposed to Hyperfilm MP (Amersham, Life Science).

**Statistical Analysis**

Data are reported as mean±SEM. Comparisons between paired variables were performed with the nonparametric Friedman test and Wilcoxon rank sum test. All statistical tests were two-tailed, with a significance level of $P<.05$. 

---

**Selected Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI</td>
<td>ankle-brachial index</td>
</tr>
<tr>
<td>MRA</td>
<td>magnetic resonance angiography</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>phVEGF165</td>
<td>plasmid encoding 165-amino-acid isoform of human VEGF</td>
</tr>
<tr>
<td>TBI</td>
<td>toe-brachial index</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>

---

Baumgartner et al March 31, 1998 1115
Clinical, Hemodynamic, Angiographic, Laboratory, and Molecular Findings Before and After Intramuscular phVEGF<sub>165</sub> Gene Transfer

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age, y</th>
<th>Cigs, pk/y</th>
<th>DM</th>
<th>Previous Treatment</th>
<th>Signs/Symptoms</th>
<th>Outcome After Gene Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>33†</td>
<td>30</td>
<td>0</td>
<td>4 bypass grafts, 3 rev., prostaglandins</td>
<td>Calf ulcer, toe gangrene (digit I)</td>
<td>ABI +0.24; complete healing—limb salvage</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>53</td>
<td>0</td>
<td>+</td>
<td>3 bypass-grafts, 1 PTA, prostaglandins</td>
<td>Toe gangrene (digit V)</td>
<td>ABI +0.12; complete healing</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>77</td>
<td>0</td>
<td>+</td>
<td>None</td>
<td>Toe gangrene (digits I, IV)</td>
<td>TBI +0.11; gangrene/ osteomyelitis—BKA</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>39†</td>
<td>20</td>
<td>0</td>
<td>Sympathectomy</td>
<td>Forefoot gangrene</td>
<td>ABI +0.27; forefoot necrosis—BKA</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>74</td>
<td>90</td>
<td>0</td>
<td>1 PTA</td>
<td>Rest pain</td>
<td>ABI +0.15; rest pain resolved</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>84</td>
<td>40</td>
<td>0</td>
<td>6 bypass grafts, 1 PTA</td>
<td>Toe gangrene (digits I–V)</td>
<td>ABI +0.22; toe amputation—limb salvage</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>80</td>
<td>20</td>
<td>0</td>
<td>1 bypass graft</td>
<td>Rest pain</td>
<td>ABI unchanged, rest pain resolved</td>
</tr>
<tr>
<td>8*</td>
<td>F</td>
<td>39</td>
<td>20</td>
<td>0</td>
<td>Sympathectomy</td>
<td>Heel ulcer, toe gangrene (digits I–IV)</td>
<td>ABI +0.22; toe amputation—limb salvage</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>54</td>
<td>30</td>
<td>0</td>
<td>4 bypass grafts, 2 rev., 1 PTA</td>
<td>Rest pain</td>
<td>TBI +0.18; rest pain resolved</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>54</td>
<td>70</td>
<td>0</td>
<td>6 bypass-grafts, 1 PTA</td>
<td>Toe gangrene (digits I, III, IV)</td>
<td>No change in ABI/TBI, BKA</td>
</tr>
</tbody>
</table>

Results

Demographic and clinical data for the 5 women and 4 men (mean age, 59±19 years) treated with phVEGF<sub>165</sub> are shown in the Table. Average length of follow-up at the time of this report was 6±3 months (range, 2 to 11 months). Local intramuscular gene transfer induced no or mild local discomfort up to 72 hours after the injection. Serial creatine phosphokinase measurements remained in the normal range, there were no signs of systemic or local inflammatory reactions, and no patient developed antibodies to VEGF. To date, neither loss of visual acuity nor change in funduscopic examination due to diabetic retinopathy<sup>33</sup> has been observed in any patient treated with phVEGF<sub>165</sub> gene transfer. Likewise, no development of a latent neoplasm<sup>34</sup> has been observed. The only complication seen was transient lower-extremity edema, consistent with VEGF enhancement of vascular permeability.<sup>35</sup>

Transgene Expression

Blood levels of VEGF transiently peaked 1 to 3 weeks after gene transfer in 7 patients in whom weekly blood samples were obtained (Fig 1). (In 2 patients, baseline and/or more than two follow-up blood samples were not obtained.) Indirect clinical evidence of VEGF overexpression was evident from the development of peripheral edema (+1 to +4 by gross inspection) in the 6 patients with ischemic ulcers. In 4 of these patients, edema was limited to the treated limb, whereas in 2 patients, the contralateral limb was affected as well, albeit less severely. The onset of edema corresponded temporally to the rise in serum VEGF levels.

Noninvasive Arterial Testing

Absolute systolic ankle or toe pressure increased in 8 limbs after gene transfer and was unchanged in 1 limb at the time of the most recent follow-up (53±4.8 at baseline, 66±4.6 most recent follow-up, P = .008). ABI and/or TBI increased from 0.33±0.05 (range, 0 to 0.58; n = 10) at baseline to 0.43±0.04 (0.22 to 0.57, P = .028; n = 10) at 4 weeks, to 0.45±0.04 (0.27 to 0.59, P = .016; n = 10) at 8 weeks, and to 0.48±0.03 (0.27 to 0.67, P = .017; n = 8) at 12 weeks (Fig 2). Improvement in the pressure index was sustained, but the increases in values obtained after the second (4-week) injection were not significantly different from measurements made 4 weeks after the initial injection. Exercise performance improved in all 5 patients with rest pain or ischemic ulcers who were able to perform graded treadmill exercise.<sup>36</sup> All patients experienced a significant increase in pain-free walking time (2.5±1.1 minutes before gene therapy versus 3.8±1.5 minutes at an average of 13 weeks after gene therapy, P < .043). A statistically significant increase in absolute, claudication-limited
walking time (4.2 ± 2.1 minutes before versus 6.7 ± 2.9 minutes after gene therapy, P = .018) was documented as well. Two patients reached the target end point of 10 minutes of exercise.

Angiography
Digital subtraction angiography showed newly visible collateral vessels at the knee, calf, and ankle levels in 7 of 10 treated ischemic limbs. The luminal diameter of the newly visible vessels ranged from 200 to >800 μm, although most were closer to 200 μm; the latter frequently appeared as a “blush” of innumerable collaterals (Fig 3A and 3B). Follow-up angiograms disclosed no evidence of collateral artery regression in any patients. Serial magnetic resonance angiograms of the ischemic limb disclosed qualitative evidence of improved distal blood flow in 8 limbs, including enhancement of signal intensity in previously identified vessels, and an increase in the number of newly visible collaterals (Fig 3C and 3D).

Change in Limb Status and Ischemic Rest Pain
Therapeutic benefit was demonstrated by regression of rest pain and/or improved tissue integrity in the ischemic limb. Limb salvage, for example, was achieved in a 33-year-old woman (patient 1, Table), who had undergone 7 unsuccessful surgical reconstructions at another hospital. She presented with a necrotic great toe and a 9 × 3-cm ischemic ulcer at the site of vein harvest in her distal left limb (Fig 4). The ulcer had failed to respond to 6 months of conservative measures, during the last 3 of which she had been treated with methadone, oxycodone/acetaminophen, amitriptyline hydrochloride, and a fentanyl patch. She had been advised by her vascular surgeons to undergo below-knee amputation. Within 8 weeks after gene transfer, her ABI had increased by 0.24, and the ulcer dimensions had diminished sufficiently to permit placement of a split-thickness skin graft. The graft healed successfully and remained healed at 9-month fol-

Figure 1. Serial levels of VEGF determined by ELISA disclosed a transient elevation 1 to 2 weeks after intramuscular phVEGF165 gene transfer. Baseline and/or follow-up venous blood samples, which were incomplete in 3 of 10 treated limbs (patients 2, 8, and 10 in the Table), are not shown.
closer to 200 m, and these frequently appeared as a blush of innumerable collaterals. C and D, MRA before and 8 weeks after gene therapy. After gene therapy, signal enhancement is clearly evident, consistent with improved flow in ischemic limb.

Figure 3. A and B, Newly visible collateral vessels at calf level 8 weeks after phVEGF165 gene transfer. Luminal diameter of newly visible vessels ranged from 200 to >800 μm (arrow); most were closer to 200 μm, and these frequently appeared as a blush of innumerable collaterals. C and D, MRA before and 8 weeks after gene therapy. After gene therapy, signal enhancement is clearly evident, consistent with improved flow in ischemic limb.

low-up (Fig 4). A second patient, a 39-year-old woman (patients 4 and 8, Table), presented with a 3-month history of gangrene of the distal half of her right foot. Although the ABI in her right limb improved by 0.27 after gene transfer, the footgangrene was not reversed, and she underwent right below-knee amputation. While she was being treated for the right limb, however, she developed gangrene in the left limb (Fig 5). After gene transfer to the left limb, the ABI in her left lower extremity increased by 0.22 in association with angiographically demonstrable, newly visible collateral vessels. Although she required amputation of the left great toe, the operative site healed promptly, and her remaining 4 toes and heel recovered completely, including restoration of normal nail growth (Fig 5). In 2 other patients, complete (patient 2, Table) or partial (patient 6, Table) healing of ischemic ulcers present for 12 and 2 months, respectively, avoided major limb amputation. In the 3 patients (patients 5, 7, and 9, Table) who presented with rest pain (of 6, 5, and 3 months’ duration, respectively) unassociated with loss of tissue integrity, rest pain resolved completely in all 3 patients after gene transfer and has remained so at the most recent follow-up.

For the total group of 10 limbs, frequency of ischemic rest pain expressed as afflicted nights per week decreased significantly (5.9±2.1 at baseline versus 1.5±2.8 at 8-week follow-up, P=.043). On the basis of criteria proposed by Rutherford et al., limb status improved in 9 of 10 extremities treated (Table). Moderate improvement, including both an upward shift in clinical category (≥1 clinical category in patients with rest pain and ≥2 categories in patients with tissue loss) and an increase of >0.1 in the ABI, was documented in 5 patients.

Immunohistochemistry and Molecular Analyses
Tissue specimens retrieved from 1 amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells (Fig 6A). This finding was particularly striking given that endothelial cell proliferation is nearly absent in normal arteries.37 PCR performed on these samples indicated persistence and anatomic redistribution of DNA fragments unique to phVEGF165. Noteworthy amplification of DNA fragments was shown in muscle and skin samples derived from the site of injection as well as in several muscle samples remote from the injection site (Fig 6B). Southern blot analysis confirmed persistence of intact plasmid DNA in muscle specimens derived from 2 amputee 8 and 10 weeks after gene therapy (patients 4 and 10, Table) (Fig 6C).

Discussion
The natural history of critical limb ischemia has been well documented to have an inexorable downhill course.3 The inclusion criteria for this study were drafted to restrict treatment to patients in whom the natural history of critical limb ischemia had been established previously. Seven of the 10 limbs had developed frank gangrene. Although 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 before 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 like these with critical limb ischemia has not to our knowledge been reported previously.1 Furthermore, because VEGF had not been administered previously 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 and the US Food and Drug Administration, was conducted as a nonrandomized, consecutive treatment series, similar to phase 1 oncology protocols used to study new chemotherapeutic agents administered to human subjects.

Analysis of gene expression at the protein level by use of an ELISA assay for VEGF documented a transient peak of the gene product in the systemic circulation 1 to 3 weeks after gene transfer in 7 patients. Further evidence of gene expression was observed in 6 patients, who developed temporally related peripheral edema, including 2 with bilateral edema. Parenthetically, the latter finding constitutes what is to the best of our knowledge 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 8 limbs after gene therapy \((P = .008)\). ABI and/or TBI increased from 0.33 ± 0.05 at baseline to 0.48 ± 0.03 at 12 weeks \((P = .017)\). To put this in perspective, an increase of 0.1 in the ABI is considered indicative of a successful surgical or percutaneous intervention. To the best of our knowledge, such improvement has not previously been achieved spontaneously or with medical therapy in patients with critical limb ischemia.

Similarly, angiographic demonstration of newly visible collateral vessels, accompanied here by noninvasive (MRA) evidence of improved blood flow, has to the best of our knowledge not been reported previously 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 \(\mu\)m in diameter; the spatial resolution of images obtained by MRA is even less. Using synchrotron radiation microangiography to assess collateral artery development after VEGF gene transfer in a rat model of hindlimb ischemia, Takeshita et al showed that neovascularization included a substantial contribution of vessels <180 \(\mu\)m in diameter. Thus, conventional angiographic techniques used in the present 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 (200 to 800 \(\mu\)m).

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. Rest pain resolved in all 3 of the patients who presented with rest pain alone. 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.

Beginning with the reports of Wolff et al, work from several laboratories convincingly demonstrated evidence of transgene expression after direct injection of nonviral, covalently closed pDNA into skeletal muscle. The conceptual basis for therapeutic angiogenesis after phVEGF \(_{165}\) gene transfer in particular has been established previously by our laboratory. The results of the present trial extend previous findings from studies performed in live animals to patients with advanced peripheral artery disease.

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 to obtain sustained expression.\textsuperscript{51} Those cases in which phVEGF\textsubscript{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 on 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\textsuperscript{52} have documented that genes that encode for secreted proteins, as opposed to proteins that remain intracellular, may yield meaningful biological outcomes because of paracrine effects of the secreted gene product. Second, heparin avidity of the VEGF\textsubscript{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, although endothelial cells were previously viewed solely as the target for VEGF, it is now clear that endothelial cells subjected to hypoxia can synthesize VEGF as well.\textsuperscript{53} This autocrine feature of VEGF creates the opportunity for amplifying the effects of even a small amount of exogenous VEGF, because endothelial cell proliferation in the ischemic territory creates additional potential cellular sources of VEGF synthesis and secretion. Fourth, VEGF inhibits apoptosis,\textsuperscript{54} in part by upregulating endothelial cell expression of fibronectin and \( \alpha_\text{v}\beta_3 \),\textsuperscript{54,55} thus preserving the survival signal generated by attachment of endothelial cells to their extracellular matrix. Such reduction in endothelial cell apoptosis would be expected to complement the mitogenic effect of VEGF, resulting in a further net increase in endothelial cell viability. Fifth, with regard to the target of gene therapy, it has been noted\textsuperscript{14,26,49} that VEGF-induced angiogenesis is not indiscriminate or widespread but rather is restricted to sites of ischemia. This appears to result from paracrine upregulation of the principal high-affinity VEGF receptor (\( \text{Kdr} \)) in response to factors released from hypoxic skeletal myocytes.\textsuperscript{56} Receptor upregulation on endothelial cells 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, because transfection efficiency is augmented when the injected skeletal muscle is ischemic.\textsuperscript{40,46}

Previous work from our laboratory established that phVEGF\textsubscript{165} transgene expression is limited to \( <30 \text{ days} \) in animal models of limb ischemia.\textsuperscript{26,46,49} Although Southern blot and PCR analyses indicated that small amounts of plasmid DNA were preserved in tissue specimens derived from 2 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 in both 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\textsubscript{165} gene therapy.
transfer that 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 gene transfer 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.34,57 It is also theoretically possible that VEGF may aggravate deteriorating eyesight due to diabetic retinopathy.33 To date, however, no change in visual acuity has been observed in any patient treated with phVEGF165 gene transfer. Nevertheless, these findings are preliminary and do not establish the long-term safety of VEGF, administered either as a gene or gene product.

Second, although it is conceivable that continuous, predominantly local production of VEGF resulting from the transgene may be preferable, from the standpoints of both 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 studies15,58 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 and 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.45,48,51,59

In summary, these preliminary data may be cautiously interpreted to support both the strategy of intramuscular gene therapy (limbs 4 and 10, Table) and the concept of therapeutic angiogenesis for treatment of selected patients with critical limb ischemia.

Acknowledgments

This study was supported in part by an Academic Award in Vascular Medicine (HL-02824) and grants HL-53354 and HL-57516 (Dr Isner) and AR-40197 and HL-50692 (Dr Walsh) from the National Institutes of Health, Bethesda, Md, and the E.L. Wiegand Founda-
tion, Reno, Nev. Dr Baumgartner is the recipient of a Swiss National Science Foundation grant.

References


Constitutive Expression of phVEGF_{165} After Intramuscular Gene Transfer Promotes Collateral Vessel Development in Patients With Critical Limb Ischemia

Iris Baumgartner, Ann Pieczek, Orit Manor, Richard Blair, Marianne Kearney, Kenneth Walsh and Jeffrey M. Isner

_Circulation_. 1998;97:1114-1123
doi: 10.1161/01.CIR.97.12.1114

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/97/12/1114

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/