Improvement in Chronic Ischemic Neuropathy After Intramuscular phVEGF165 Gene Transfer in Patients With Critical Limb Ischemia

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Objective: To investigate the effects of vascular endothelial growth factor gene therapy on ischemic neuropathy in patients with critical limb ischemia.

Design: An open-label, dose-escalating trial. Patients with angiographically proven critical leg ischemia received injections of phVEGF165 human plasmid in the muscles of the ischemic limb. Testing before treatment and at 3 and 6 months included (1) symptom severity score, (2) clinical examination score, and (3) electrophysiologic studies. Clinical and electrophysiologic examiners were masked to each other's findings.

Setting: A tertiary care referral hospital and a major teaching affiliate of Tufts University School of Medicine, Boston, Mass.

Results: Of 29 consecutive patients enrolled, 17 (19 limbs) completed the 6 months of study. Six patients had diabetes. Compared with baseline studies, treated patients had significant clinical improvements in the symptom score (P < .01), sensory examination score (P < .01), total examination score (P = .01), peroneal motor amplitude (P = .03), and quantitative vibration threshold (P = .04). Improvement in the vascular ankle-brachial index in treated legs (P < .01) corresponded to improvement in neuropathy in the same limb. Neurologic improvement was seen in 4 of 6 patients with diabetes who completed the study. No clinical, electrophysiologic, or vascular improvements were observed in untreated legs.

Conclusions: Ischemic neuropathy might be a reversible condition, and therapeutic angiogenesis might be an effective treatment. The presence of diabetes does not preclude a response to this therapy.

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CHRONIC AND severe arterial occlusive disease damages peripheral nerves and results in a regional neuropathy in the ischemic limb.1-11 Clinical and electrophysiologic features of this chronic ischemic neuropathy have been reported previously.1,3,5,6,9,11 The symptoms are predominantly sensory and might be easily obscured by ischemic limb pain.

Animal models of hind limb ischemia have established that certain angiogenic growth factors can be used to elicit angiogenesis in vivo.12-15 This is consistent with the paradigm of angiogenesis established by Folkman that endothelial cells can migrate, proliferate, and remodel in response to certain growth factors and in doing so form new sprouts from parent vessels that constitute angiogenesis. Vascular endothelial growth factor (VEGF) was identified as an endothelial cell–specific mitogen that plays a critical role in regulating endogenous neovascularization in response to tissue ischemia.12-15,17-22 Preclinical studies have documented that VEGF promotes angiogenesis in vivo, and recent preliminary clinical trials of gene therapy have established that the results of these animal studies can be extrapolated to humans with ischemic limbs.23-26

The present prospective study of 29 consecutive patients undergoing phVEGF165 gene transfer was undertaken to investigate the impact of therapeutic angiogenesis on peripheral neuropathy caused by critical limb ischemia.

RESULTS

Twenty-nine consecutive patients undergoing phVEGF165 gene therapy were enrolled for neurologic study; 7 did not complete the 3-month follow-up, yielding 22 patients for analysis at that point (50% women; mean age, 54 years; age range, 23-77 years). Of the 7 excluded patients, 4 had limb amputations, 1 was lost to fol-
PATIENTS AND METHODS

PATIENT COHORT

Patients qualified for a phase 1, open-label, dose-escalating trial of direct intramuscular gene transfer of naked DNA encoding VEGF165 (phVEGF165) if they (1) had chronic critical limb ischemia, including rest pain or nonhealing ulcers, for a minimum of 4 weeks; (2) had angiographically documented severe vascular occlusive disease in the affected extremity; and (3) were not candidates for surgical or percutaneous revascularization based on usual practice standards.23,27,28 The cohort for this study consisted of sequential patients meeting the selection criteria. This study was a component of the clinical trial entitled Intramuscular Gene Therapy for Therapeutic Angiogenesis (FDA IND57777) as approved by the Recombinant DNA Advisory Committee of the National Institutes of Health, the Human Institutional Review Board and Institutional Biosafety Review Board of St Elizabeth’s Medical Center (Boston, Mass), and the US Food and Drug Administration.

INTRAMUSCULAR phVEGF165 TRANSFER

Preparation and purification of the plasmid from cultures of phVEGF165-transformed Escherichia coli were performed in the Human Gene Therapy Laboratory at St Elizabeth’s Medical Center as described previously.29 The plasmid into which the VEGF complementary DNA has been inserted, phVEGF165, is a simple eukaryotic expression plasmid that uses the 763-base pair (bp) cytomegalovirus promoter/enhancer to drive VEGF expression. This promoter/enhancer has been used to express reporter genes in a variety of cell types and can be considered to be constitutive. Downstream from the VEGF complementary DNA is the SV40 polyadenylation sequence. Also included in this plasmid is a fragment containing the SV40 origin of replication, which is the SV40 origin of replication and the β-lactamase gene for ampicillin resistance.

Each treated limb was injected with a total of 3000 to 9000 µg of phVEGF165 in 2 or 3 intramuscular injection sessions 4 weeks apart using a 27-gauge needle. At each treatment session, 8 injections were made into readily palpable muscles below the knee.21 In most patients, 1 of the 8 injections was performed into a foot muscle.

NEUROLOGIC ASSESSMENT

Patients were prospectively evaluated by 2 neurologists, one performing the clinical assessment (D.H.W.) and the other performing the electrophysiologic testing (D.S.). The examiners were masked to each other’s results and to the findings of patients’ vascular examinations. Both lower extremities were studied, except in patients whose contralateral limb had been amputated. Because this was an open-label study with no sham injections, there were no true control limbs. We tested the contralateral leg to assess the natural course of the disease. Each patient was evaluated before treatment and 3 and 6 months after phVEGF165 gene transfer.

Neurologic Scales

Semi quantitative scales (modified from Notermans et al30) were used to assess changes in neurologic function before and after treatment and to compare the treated and untreated legs.

A symptom score (SS) encompassed 5 neuropathy-related symptoms: (1) distal leg weakness, (2) proximal leg weakness, (3) numbness, (4) paresthesias, and (5) pain (excluding pain at the site of skin ulceration or from vascular claudication). Each symptom was graded on a scale from 0 to 3 (0 indicates none; 1, mild; 2, moderate; and 3, severe). The maximum possible SS (indicating the most severe symptoms) was 15.

Lower extremity sensory testing was graded by a sensory examination score (SES), which evaluated (1) intensity of sensory deficit for pinprick and light touch compared with a proximal, normal region using a scale from 0 to 4 (0 indicates normal; 1, >75% of normal; 2, 50%-74% of normal; 3, 25%-49% of normal; and 4, <25% of normal); (2) distribution of sensory symptoms for light touch and pinprick graded on a scale from 0 to 4 (0 indicates normal; 1, abnormal to toes; 2, abnormal to ankle; 3, abnormal to middle of calf; and 4, abnormal above middle of calf); (3) sensation at the toes and ankle using a scale from 0 to 4 (0 indicates normal; 1, mild loss; 2, moderate loss; 3, severe loss; and 4, absent), and (4) proprioception at the great toe (6 trials) graded on a scale from 0 to 4.

Table 1

Table 2

Figure 1

Figure 2

CLINICAL SCORES

Mean SSs and SESs in treated limbs improved significantly at 3 months (Table 1), and these changes persisted at 6 months. Mean TESs significantly improved at 6 months as well (Table 2 and Figure 1 and Figure 2).

low-up, 1 refused testing, and 1 had lower limb bypass surgery. Because of rapid progression of vascular insufficiency in the contralateral leg, 2 patients had both legs treated with phVEGF165; thus, the total number of treated limbs analyzed at 3 months was 24. Five additional patients did not complete the 6-month follow-up (2 refused testing, 1 had lower limb bypass surgery, 1 had a limb amputation, and 1 accidental death occurred), leaving a total of 19 limbs (17 patients) for analysis at that point.

TRANSGENE EXPRESSION

Enzyme-linked immunosorbent assay analysis of serially obtained venous blood samples provided evidence that gene transfer was successful in achieving constitutive overexpression. In patients who completed 6 months of study, mean±SEM values for VEGF increased from 18±5 pg/mL at baseline to peak levels of 68±20 pg/mL and then returned to 22±6 pg/mL by 5 weeks. The VEGF levels before and after gene therapy were significantly different from peak values (P<.05). There was no relation between the magnitude of clinical improvement and circulating VEGF levels.
At 6 months, the SS was improved in 12 (63%) of 19 limbs and the TES was improved in 9 (47%) of 19 limbs. Neither the SS (P<.001, treated vs untreated) nor the TES (P=.004, treated vs untreated) improved in any of the untreated limbs.

**ELECTROPHYSIOLOGIC MEASURES**

At 6 months, mean peroneal CMAP amplitudes (P=.03) and vibration thresholds (P=.04) significantly improved in treated limbs (Table 2). Eleven (58%) of 19 limbs had absent sural responses. The mean (SD) sural sensory nerve action potential amplitude in treated legs was 4.48 (6.05) µV at baseline and 5.87 (7.30) µV at 6 months, which was not a statistically significant change (P=.06). Ten (53%) of 19 limbs showed improvement in electrophysiologic measures at 6 months (7 limbs had increased peroneal and summed CMAP amplitudes, 2 had an increased peroneal CMAP amplitude, and 1 had an increased summed CMAP amplitude), whereas no improvements were seen in any of the contralateral, untreated limbs (P=.002, treated vs untreated). Table 3 summarizes the comparison of amplitude and conduction velocity changes between treated and untreated legs during the 6 months of study. The mean percentage increase for the peroneal CMAP amplitude was 50% in the treated legs, whereas in the untreated legs it decreased by 11% (P=.02). Similarly, the mean summed CMAP amplitude increased 16% in treated legs vs a decrease of 9% in untreated legs (P=.04). The percentage change in the

**VASCULAR TESTING**

Each patient underwent vascular testing that included measurement of the ankle-brachial index (ABI) to document the severity of the vaso-occlusive disease and response to therapeutic angiogenesis.33 Change in the ABI for individual limbs was assessed to evaluate the relation between vascular and neurologic outcomes. Improvement in the vascular outcome measure for each individual limb was defined as an ABI increase of at least 0.1, the magnitude of improvement that has been previously suggested to constitute a successful outcome of surgical or catheter-based revascularization.37 Because the principal inclusion criterion for this study was clinical and angiographic evidence of critical limb ischemia, patients with noncompressible arteries were not excluded, although the ABI was not meaningful.

**SERUM VEGF LEVELS**

Venous blood samples were analyzed using an enzyme-linked immunosorbent assay at baseline and weekly after the initial gene transfer. 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 concentration was determined with an immunoassay according to the manufacturer’s instructions (R&D Systems, Minneapolis, Minn). Results were compared with the standard curve of human VEGF concentrations, with a lowered detection limit of 5 pg/mL. Samples were checked using serial dilution at least in duplicate.

**STATISTICAL ANALYSIS**

Changes among baseline, 3-month, and 6-month assessments of SS, SES, TES, and electrophysiologic measures were analyzed. To compare clinical and electrodiagnostic data with the ABI in individual patients, we defined overall neurologic improvement at 6 months as fulfilling at least 2 of the 3 following measures: (1) 2-point or greater decrease in SS, (2) 4-point or greater decrease in TES, and (3) 30% or greater improvement in peroneal or tibial CMAP amplitude or summed motor amplitude. A 30% amplitude increase was selected to reduce the possibility of improvement reflecting test-to-test variability. The t test, Wilcoxon rank sum test, and Fisher exact test were used for all comparisons. P<.05 was considered statistically significant.
peroneal CMAP and summed CMAP amplitudes for each individual limb is presented in Figure 3 and Figure 4.

There were no differences in the neurologic improvement rates among patients injected with different doses (3000–9000 µg) of phVEGF165.

**ANKLE-BRACHIAL INDEX**

The mean ABI improved in treated limbs at 3 and 6 months (Tables 1 and 2). The ABI improved in 8 of 10 limbs that satisfied at least 2 of 3 neurologic improvement criteria for individual limbs. Of the 2 remaining limbs, 1 had noncompressible arteries and 1 had no improvement. The ABI significantly improved in only 1 of 10 limbs, 1 had an amputation), and 2 had no improvement. Three of the 4 improved limbs in patients with diabetes also had a significant improvement in the ABI (Table 4).

**COMMENT**

Chronic occlusive arterial disease has a recognized adverse effect on peripheral nerves, although there have been few studies detailing the clinical, electrophysiologic, and histologic features of this chronic ischemic neuropathy. The symptoms generally include numbness, painful paresthesias, aching, and burning, and the signs are reduced appreciation of pinprick, light touch, and vibration sensation; mild distal weakness; and depressed or absent deep tendon reflexes. An asymmetric neuropathy develops in many patients, particularly in those without diabetes. The main electrophysiologic features of ischemic neuropathy are consistent with a sensorimotor polyneuropathy with axonal features. Two studies examined electrophysiologic changes after surgical revascularization, one demonstrating improvement only in the peroneal motor conduction velocity and the other with no improvement in multiple electrophysiologic outcome measures. Hunter and colleagues con-
cluded that ischemic neuropathy is an irreversible condition on the basis of these data.

Use of phVEGF165 gene transfer for therapeutic angiogenesis was established in rabbit and murine models of hind limb ischemia and ultimately led to the first clinical trial of cardiovascular gene therapy in patients with critical limb ischemia.12-15,23-25,40-45 Preliminary studies23-25 have suggested that increased limb vascularity and tissue perfusion after phVEGF165 gene therapy might improve the clinical features of limb ischemia (ie, restoration of tissue integrity and consequent limb salvage).

In our study of the effect of intramuscular phVEGF165 gene transfer on chronic ischemic neuropathy, patients had reduced symptoms, decreased neurologic disability, and improved electrophysiologic measures in treated limbs. In contrast, none of the clinical or electrophysiologic measures improved in contralateral, untreated limbs.

The clinical improvement in treated limbs was evident at the 3-month examination as decreased neuropathic symptoms and improved SES. At 6 months, the TES also improved. When individual patients were followed up longitudinally, more than half of the treated limbs demonstrated significant improvement in contrast to none of the untreated limbs.

Two electrophysiologic measures also improved in treated limbs after 6 months: mean peroneal CMAP amplitude and the quantified vibratory threshold. There was no change in the motor or sensory conduction velocities, distal latencies, or sural sensory nerve action potential amplitudes. This latter observation is not surprising because 58% of limbs had absent sural nerve sensory potentials at entry into the study, indicating severe sural nerve damage that was unlikely to be reversible. Furthermore, mean percentage improvements in the peroneal and summed motor amplitudes

![Figure 1](http://archneur.jamanetwork.com/pdfaccess.ashx?url=/data/journals/neur/6665/)

![Figure 2](http://archneur.jamanetwork.com/pdfaccess.ashx?url=/data/journals/neur/6665/)
at 6 months were significantly greater in treated vs untreated limbs.

The vascular outcome measure (ABI) also improved at 3 months and to a greater extent at 6 months in treated limbs only. The ABI was improved in 8 of 10 limbs that had shown neurologic improvement. Both of the remaining limbs had noncompressible arteries, precluding demonstration of improvement. Thus, the neurologic and vascular improvements were linked.

Table 3. Comparison of Mean Percentage Changes at 6-Month Follow-up

<table>
<thead>
<tr>
<th>Outcome Measure</th>
<th>Treated Legs</th>
<th>Untreated Legs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Limbs, No.</td>
<td>Mean (SD)†‡</td>
</tr>
<tr>
<td>Tibial CMAP amplitude</td>
<td>18</td>
<td>4.22 (33.09)</td>
</tr>
<tr>
<td>Peroneal CMAP amplitude</td>
<td>18</td>
<td>50.28 (88.92)</td>
</tr>
<tr>
<td>Summed motor CMAP amplitudes</td>
<td>19</td>
<td>16.47 (37.75)</td>
</tr>
<tr>
<td>Average CMAP conduction velocity</td>
<td>19</td>
<td>−2.68 (9.81)</td>
</tr>
</tbody>
</table>

*CMAP indicates compound muscle action potential.
†Percentage change from baseline to 6 months.
‡Treated vs untreated legs.

Figure 3. Changes in peroneal motor amplitude at 6 months. A indicates mean percentage change; vertical line, SD; and solid circle, improvement from 0 to 160 µV (unable to calculate percentage). P=.02, treated vs untreated legs.

Figure 4. Changes in summed motor amplitudes at 6 months. A indicates mean percentage change; vertical line, SD. P=.04, treated vs untreated legs.
and postmortem studies have suggested that neovascularization through the vasa nervorum, the nutrient arteries that constitute the microcirculation of the peripheral nerves. In vivo and postmortem studies have suggested that neovascularization from angiogenic cytokines principally involves small vessels of a dimension that would include the vasa nervorum (180 µm). Alternatively, our findings might reflect a direct effect of phVEGF165 on neural elements. Soker and colleagues, for example, reported that the neuropilin-1 receptor, a cell surface glycoprotein associated with axonal guidance in the developing nervous system, binds phVEGF165.

The effects of ischemia on muscle could be a confounding variable in our evaluation of muscle strength and motor neurophysiologic measures. Severe acute ischemia has been reported to have a profound effect on muscle in animal studies, inducing early muscle necrosis. In fact, the changes can precede and be more severe in muscle than in peripheral nerve. In chronic ischemia in humans, however, the situation seems different. In a comprehensive assessment of muscle histopathologic specimens in 40 patients with severe peripheral vascular disease, Farinon and colleagues concluded that the major muscle alterations were the result of peripheral nerve damage. Other studies have reached similar conclusions. Furthermore, the acute ischemic mononeuropathy induced by surgical vascular shunting or acute large-vessel occlusions has been demonstrated to represent multiple distal mononeuropathies with no evidence of a significant primary muscle component. We therefore believe that the clinical and electrophysiologic improvements in this study reflect effects on motor nerves and not on muscle.

In summary, our findings establish that ischemic neuropathy might be a reversible condition and that therapeutic angiogenesis might be a novel and effective treatment for ischemic neuropathy. We are aware of the limitations of an open-label study with no placebo control group. However, the substantial clinical improvement in treated limbs and the absence of improvement in untreated contralateral limbs constitute strong data regarding the biological efficacy of phVEGF165 gene therapy. Finally, our experience with the subset of patients with diabetes, although small, demonstrates a degree of reversibility that may, on further study, have significant clinical and pathophysiologic implications. Our findings need to be confirmed in a larger controlled clinical trial.

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