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Published in:
Human Gene Therapy

DOI:
10.1089/hum.2006.17.683

Publication date:
2006

HUMAN GENE THERAPY 17:683–691 (June 2006)

Treatment with Intramuscular Vascular Endothelial Growth Factor Gene Compared with Placebo for Patients with Diabetes Mellitus and Critical Limb Ischemia: A Double-Blind Randomized Trial

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ABSTRACT

Despite advances in revascularization techniques, limb salvage and relief of pain cannot be achieved in many diabetic patients with diffuse peripheral vascular disease. Our objective was to determine the effect of intramuscular administration of phVEGF$_{165}$ (vascular endothelial growth factor gene-carrying plasmid) on critical limb ischemia (CLI) compared with placebo (0.9% NaCl). A double-blind, placebo-controlled study was performed in 54 adult diabetic patients with CLI. The primary end point was the amputation rate at 100 days. Secondary end points were a 15% increase in pressure indices (ankle-to-brachial index and toe-to-brachial index), clinical improvement (skin, pain, and Quality of Life score), and safety. In patients ($n = 27$) treated with placebo versus phVEGF$_{165}$-treated patients ($n = 27$) the following results were found: 6 amputations versus 3 (p = not significant [NS]); hemodynamic improvement in 1 versus 7 ($p = 0.05$); improvement in skin ulcers, 0 versus 7 ($p = 0.01$); decrease in pain, 2 versus 5 (p = NS); and overall, 3 versus 14 responding patients ($p = 0.003$). No grade 3 or 4 adverse effects were seen in these patients. We conclude that this small, randomized gene therapy study failed to meet the primary objective of significant amputation reduction. However, significant and meaningful improvement was found in patients treated with a VEGF$_{165}$-containing plasmid. There were no substantial adverse events.

OVERVIEW SUMMARY

In patients with diabetes mellitus and critical limb ischemia (CLI) surgical revascularization is often impossible because of the predominance of microvascular occlusions, and these patients have limited options beyond amputation. Angiogenesis of the lower extremity remains, therefore, an important area of therapeutic investigation. Initial approaches in small studies showed beneficial clinical effects by using the VEGF$_{165}$ gene. This trial represents the first randomized placebo-versus-phVEGF$_{165}$ controlled gene therapy trial to be published on diabetic patients with CLI. These patients received phVEGF$_{165}$ injected intramuscularly into the most ischemic limb. This injection was repeated once at

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4 weeks, resulting in a total of 4000 μg of phVEGF$_{165}$. In a matching placebo procedure patients received 0.9% NaCl. In this study significant clinical and hemodynamic improvements were found in patients treated with phVEGF$_{165}$ without adverse events. However, the primary end point of the study, a significant reduction in amputation rate, was not met. This small randomized study contributes to the data on clinical gene therapy in CLI and could serve to restart interest in the angiogenic approach in patients with CLI.

INTRODUCTION

Critical limb ischemia (CLI) is a disease manifested by sharply diminished blood flow to the legs; it is the most common cause of nontraumatic amputation in diabetes. The condition is responsible for 70% of the 150 lower limb amputations per million population (Eskelinen *et al*., 2004). Although a combination of neuropathy, obstructive macrovascular disease, and/or microvascular changes is usually pivotal in the development of the diabetic foot, the contribution of microvascular occlusions is predominant in the diabetic subgroup with CLI and is not accessible for surgical revascularization. Amputation is unavoidable in 0.7 per 10,000 patients with diabetes mellitus (da Silva *et al*., 1996; Holstein *et al*., 2000). Among CLI patients who have already had all possible surgical revascularization done, amputation is inevitable in approximately half (da Silva *et al*., 1996; Klevsgard *et al*., 2001). The median survival of patients with CLI is approximately 3 years (ICAI Group, 1997; Cheng *et al*., 2000). The quality of life during this period is limited (Albers *et al*., 1992).

Newer methods of treatment of CLI have been explored. Preclinical studies have defined a role for vascular growth factors in neoangiogenesis in animal models of peripheral ischemia (Takeshita *et al*., 1994; Nikol *et al*., 2002). The most potent angiogenic factor affecting endothelial cell proliferation is vascular endothelial growth factor 165 (VEGF$_{165}$), an endothelial cell-specific mitogen from a family of six isoforms. However, as a protein its short half-life and its effects on vascular permeability have limited its clinical application (Kusumanto *et al*., 2003; Schratzberger *et al*., 2003). Its use in the form of gene therapy, either as naked plasmid or in a viral vector, has been reported in only small studies showing beneficial clinical effects in some but not all trials (Isner *et al*., 1996, 1998; Baumgartner *et al*., 1998; Makinen *et al*., 2002; Shyu *et al*., 2003; Kim *et al*., 2004).

If the clinical benefits originally seen in the studies by Isner and Baumgartner and coworkers, using intramuscular injections of naked VEGF$_{165}$ plasmid DNA (phVEGF$_{165}$), could be reproduced in a well-defined patient group in a randomized study, this would, in our opinion, redefine the place of this form of gene therapy for CLI.

The primary aim of our study was to assess the effects of phVEGF$_{165}$, in addition to maximal standard treatment, on the amputation rate in a placebo-controlled randomized study, in a group of diabetic patients with CLI. The secondary objectives were hemodynamic improvement, clinical improvement, and safety.

MATERIALS AND METHODS

Study design

This was a two-center, randomized, double-blind, controlled study comparing phVEGF$_{165}$ with placebo (0.9% NaCl), with limb survival and/or predefined changes in pressure indices as the primary measure of effect. The study was approved by the Centrale Commissie Mensgebonden Onderzoek (CCMO: Central Medical Ethics Committee) in the Netherlands and was performed in two centers (University Medical Center Groningen and Leiden University Medical Center, The Netherlands).

Patients received phVEGF$_{165}$ or placebo by computerized block randomization, without stratification or matching, performed by the pharmacy of the University Medical Center Groningen. Patients were assigned either to receive 2000 μg of phVEGF$_{165}$ or placebo on day 0 and day 28. Follow-up evaluation was performed on days 7, 14, 35, 42, 72, and 100, with registration of clinical symptoms, wound status, and hemodynamic condition. In addition, routine hematology, chemistry, urinalysis, anti-double-stranded DNA (dsDNA) antibodies, and circulating VEGF and phVEGF$_{165}$ levels were determined. Ophthalmologic examination was performed before treatment, on day 28, and at the conclusion of the study.

Patients

Patients were recruited from a large number of academic and nonacademic hospitals in the Netherlands by approaching their departments of vascular surgery.

Patients with either type I or type II diabetes mellitus established according to current American Diabetes Association (ADA) criteria were eligible. Evidence of critical limb ischemia had to be present, including rest pain and/or ulcers that had not healed for a minimum of 2 weeks despite conventional therapy. Patients with compressible vessels had to have a resting ankle systolic blood pressure $<$50 mmHg, or toe systolic blood pressure $<$30 mmHg in the affected limb. Patients had to be unsuitable candidates for surgical or percutaneous revascularization as judged after contrast angiography by the vascular surgeon and intervention radiologist. Further exclusion criteria included active proliferative diabetic retinopathy, a history of malignancy, severe comorbidity, and/or compromising comedication. Patients gave written informed consent for their participation.

Gene product and administration

The plasmid carrying the human VEGF$_{165}$ gene (GenBank accession no. AB021221), which is transcriptionally regulated by the cytomegalovirus promoter/enhancer, was manufactured under Good Manufacturing Practices guidelines according to Isner *et al*. (1996) and Sarkar *et al*. (2001). The plasmid was a gift from J.M. Isner, and was the same as has been used by his group. Patients received four aliquots, each containing 500 μg of phVEGF$_{165}$ diluted in a volume of 1.0 ml of 0.9% NaCl (total, 2000 μg) injected intramuscularly (26-gauge needle) into the thigh (two aliquots) and calf muscles (two aliquots) of the most ischemic limb. The injection sites were arbitrarily selected according to available muscle mass as described in the protocol from Baumgartner *et al*. (1998). This procedure was re-

peated once at 4 weeks, resulting in a total of 4000 μg of $phVEGF_{165}$ being administered into the ischemic leg.

In a matching placebo procedure patients received, four times, 1.0 ml of 0.9% NaCl. No difference between the $phVEGF_{165}$ and placebo could be seen or felt by the physician who performed the injection.

Procedures

Ischemic skin defects were copied onto a transparent sheet to calculate the ulcer surface area. In addition, ischemic skin defects were documented by color photography. Assessment of ischemic rest pain was performed on the basis of a visual analog scale (VAS) for pain scores and by documentation of the daily use of analgesics.

Ankle pressure was measured according to conventional procedures with the patient at rest in a semisupine position. Measurements were performed by an experienced vascular technician using an 8-MHz Doppler ultrasound system (Parks Medical Electronics, Aloha, OR) with the occluding cuff around the ankle, unless wounds extended to the proximal foot or ankle, in which case the cuff was placed around the upper leg. Toe pressures were measured after at least 10 min of warming, using a photoplethysmographic diode on the pulp area and a small occluding cuff at the base of the toe. The pressure at which pulsatile signals reappeared on cuff release was noted. If a skin defect was present in digit I or II, toe pressures were not measured. The ankle-to-brachial index (ABI) and toe-to-brachial index (TBI) were calculated as the quotient of the absolute ankle or toe pressures, and the simultaneously measured brachial pressure.

In accordance with the literature (Mätzke *et al*., 2003) our trained vascular technicians scored a coefficient of variation of ABI in patients with CLI of 3.2% and the interobserver difference did not exceed this.

QOL assessment using the RAND-36 questionnaire (Hays *et al*., 1993; Van der Zee and Sanderman, 1993) was performed to determine whether a clinical response had a positive effect on the QOL (Klevsgard *et al*., 2000).

Ophthalmologic examination at baseline and at each subsequent follow-up visit included best corrected visual acuity measurement and intraocular pressure, slitlamp biomicroscopy, indirect ophthalmoscopy, and fundus photography. Fluorescein angiography (with intravenous administration of 5 ml of 10% sodium fluoride) was performed at baseline and after 100 days of study. Diabetic retinopathy was classified as follows: no retinopathy, background retinopathy, preproliferative diabetic retinopathy, or proliferative diabetic retinopathy.

Systemic VEGF levels were determined by Quantikine human VEGF enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Whole blood (in a citrate–theophylline–adenosine–dipyridamole [CTAD] tube) was diluted three times with phosphate-buffered saline (PBS). To damage the membranes the cellular suspension was frozen and thawed twice. Serum (coagulation for at least 1 hr) samples were centrifuged for 15 min at $3000 \times g$ at room temperature. Samples were stored in aliquots at $-80°C$ until analysis. Results were compared with a standard curve for human VEGF, with a detection limit of 5 pg/ml.

Analysis of systemic $phVEGF_{165}$ in whole blood was performed by polymerase chain reaction (PCR) after isolation of

Table 1. Baseline Characteristics[a]

General	*Control (n = 27)*	*$phVEGF_{165}$ (n = 27)*
Age (years): mean (range)	68.4 (40–84)	68.7 (45–85)
Women	12	11
Diabetes type 1/2	4/23	5/22
ID	10	8
HbA1C (%): mean (range)	8.0 (5.8–9.8)	8.1 (6.4–12.2)
Diabetes duration (years): mean (range)	14.2 (0.67–55)	17.0 (0.08–14)
Pain	23	24
Skin ulcer[b]	17	21
Duration (months) of ulceration: median (range)	5.0 (1–12)	3.0 (1–12)
Concomitant cardiovascular		
Hypertension	18	15
Hypercholesterolemia	8	9
CAD	9	12
Duration of leg ischemia symptoms		
Months: mean (range)	9.5 (1–48)	8.6 (1–30)
Prior vascular reconstruction/ percutaneous angioplasty	10	10
Prior amputation	3	3

Abbreviations: CAD, coronary artery disease; ID, insulin dependent; HbA1c, hemoglobin A1c.

[a]Data represent numbers, unless otherwise stated. No significant differences were found (χ^2 corrected for continuity; for continuous variables the independent *t* test was performed).

[b]Surgical removal of debris occurred in 15% of the patients in both groups within the 4 weeks before treatment.

the DNA according to the Boom procedure (Boom *et al*., 1990). This method allowed a detection limit of 2.0 fg of $phVEGF_{165}$ per microliter of blood.

Definitions of measures of effect

Response was defined as limb survival, hemodynamic improvement of ABI or TBI at two different time points, or improvement of skin ulcers and rest pain. Limb survival was defined as the absence of a major amputation. A major amputation is an amputation proximal to the level of the ankle. A hemodynamic improvement is defined as an absolute increase of >15% in ABI or TBI. This increase is considered a significant clinically relevant improvement (Rutherford and Becker, 1991; Rutherford *et al*., 1997; Mätzke *et al*., 2003). Ischemic wound response was defined as a decrease in wound surface area of >60%. Improvement of pain was defined as >50% decrease in pain scores as assessed using the VAS at various time points (baseline to day 28, 72, and day 100).

Safety was assessed by incidence and severity of adverse events: vital signs (i.e., fever, or hypotension defined as systolic blood pressure <90 mmHg) during and after administration of intramuscular injections, diabetic retinopathy, edema, anti-dsDNA (Farr assay), proteinuria, telangiectasia, circulating $phVEGF_{165}$, and analysis of survival.

Statistical analysis

The target number of patients was determined on the basis of the expected incidence of amputations in the control group and the foreseen success rate of the intervention. The incidence of amputation in diabetic patients is 0.7/10,000. Data from studies by Klevsgard *et al*. (2001) and da Silva *et al*. (1996) give indirect indications of the incidence of amputation in end-stage CLI. In both studies the patients were still amenable to surgical intervention, and therefore constituted an earlier disease state than in our patients. Nevertheless, amputation occurred in 40% of the patients in the da Silva study and in at least 40% in the Klevsgard study. We therefore projected an amputation rate of 50% in the control group.

The expected success rate of the intervention was estimated from the available clinical data on VEGF gene therapy at that

Table 2. Overview of Responding Patients and Treatment Results

A. Overview Responding Patients

Responding patients[a]	*Improvement in ABI*[b]	*Improvement in skin ulcer*[c]	*Decrease in pain*[d]	*Hemodynamic responder*	*Clinical responder*
1	×			×	
2		×			×
3			×		×
4	×			×	
5	×		×	×	×
6	×			×	
7	×	×	×	×	×
8	×	×		×	×
9			×		×
10		×			×
11			×		×
12	×	×		×	×
13		×			×
14		×			×
A			×		×
B	×			×	
C			×		×

B. Treatment Results[e]

End point	*Control [n/total (%)]*	*phVEGF$_{165}$ [n/total (%)]*	p *Value*
Major amputations	6/27 (22%)	3/27 (11%)	NS
Hemodynamic improvement[b]	1/17 (6%)	7/21 (33%)	0.05
Improvement in skin ulcer[c]	0/17 (0%)	7/21 (33%)	0.01
Decrease in pain[d]	2/11 (18%)	5/21 (24%)	NS
Responding patients	**3/27 (11%)**	**14/27 (52%)**	**0.003**

[a]1–14, phVEGF×15S-treated patients; A–C, controls.
[b]×, absolute increase of >15% in ankle-to-brachial index or toe-to-brachial index.
[c]×, decrease in ulcer surface of >60%.
[d]×, >50% decrease in rest pain on the VAS.
[e]Data represent number of patients (percentage of evaluable patients).

time (Isner *et al*., 1996, 1998; Baumgartner *et al*., 1998; Simovic *et al*., 2001). About three-quarters of these patients were either rescued from imminent amputation or showed substantial improvement in parameters such as ABI, which can be considered directly relevant to the chance of avoiding amputation in the near future.

On the basis of this success rate, 54 patients were considered to be needed to be able to demonstrate the expected reduction from 50 to 25% in the amputation rate (power, 0.85; $p = 0.05$).

Baseline characteristics and response rate comparisons between the groups were analyzed by χ^2 test, corrected for continuity. For continuous variables the independent *t* test was used. For QOL analysis changes within the group between baseline, 28-day, and 100-day assessments were analyzed by Friedman test. Differences between the groups were measured by Mann–Whitney *U* test. The same tests were used for analysis of laboratory parameters. Survival analysis was calculated according to Kaplan–Meier test. A p value < 0.05 was considered statistically significant.

RESULTS

In the period between February 2000 and January 2004, 97 patients were screened: 54 were found to be eligible and were randomized. Patient refusal and surgical alternatives were the most common exclusion grounds. Five patients were excluded because of proliferative diabetic retinopathy. Basic demographic characteristics were similar in both groups (see Table 1).

Treatment results

All patients were evaluated for all end points (pressure, skin ulcers, and pain). Usually wounds precluded measuring of pressure. An overview of responding patients and treatment results are summarized in Table 2A and B.

A major amputation was performed in six of the control patients and in three of the phVEGF$_{165}$-treated patients (p = NS). The mean time to amputation was 78 days in the phVEGF$_{165}$-treated patients and 25.5 days in the control arm ($p = 0.11$). The amputation rate in the control group was therefore 22%.

For hemodynamic assessment 16 patients were not evaluable because of incompressible vessels or extensive ulceration that made ankle or toe pressure assessment not feasible. An absolute increase of >15% in ABI or TBI on at least two time points occurred in 7 of 21 evaluable phVEGF$_{165}$ patients. In the control group only one patient of the 17 evaluable patients showed a hemodynamic increase of >15%. phVEGF$_{165}$ treatment tended to improve pressure parameters ($p = 0.05$). Median time to improvement (day 0 to first increase of >15%) was 4 weeks and this improvement was still present on day 100.

Skin ulcers were evaluated in all 38 patients with ulcers (see Table 1). Of 21 evaluable phVEGF$_{165}$ patients there were 7 responders, whereas none of the control patients showed an improvement of ulceration ($p = 0.01$). Ulcer healing of more than 60% occurred after a median 5 weeks after injection and was still present on day 100 (Fig. 1).

One phVEGF$_{165}$-treated patient presented with ischemic ulceration in both legs. Skin ulcers in the injected leg, as well as the opposite leg, showed clinically relevant improvement with a >60% decrease in ulcer surface.

In seven patients there was no rest pain. Five patients were not evaluated for pain because of minor surgical intervention, that is, amputation of the toe or extensive debridement of skin defects, shortly before the first intramuscular injection. In 10 patients, who could not understand the VAS scale, pain data were incomplete. However, in five of these patients it could be

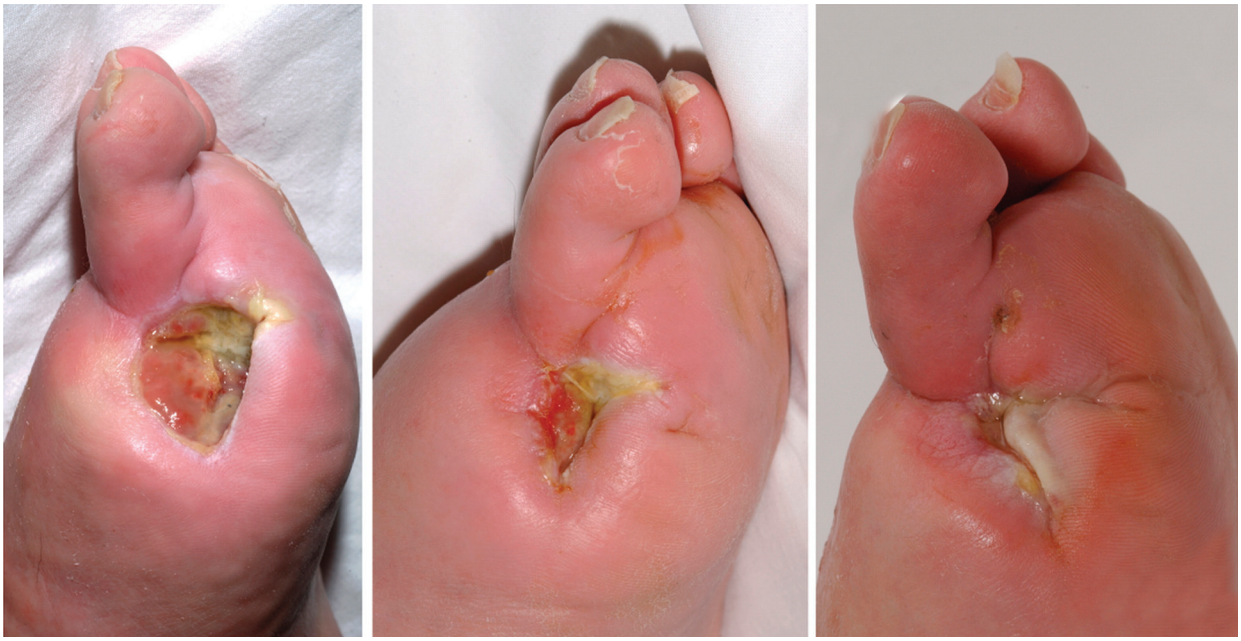

FIG. 1. Patient with CLI and an unclosed wound 2 months after surgery. Treatment with phVEGF$_{165}$ started 2 months after surgery. Pictures were taken on day 0 (*left*), day 28 (*middle*), and day 100 (*right*). On day 100 the wound is nearly closed.

Table 3. Quality of Life[a]

	Baseline		*100 days*	
	Control	*phVEGF$_{165}$*	*Nonresponders*	*Responders*[b]
Physical functioning	15.9 ±11.7	19.1 (±15.4)	16.1 (±10.3)[c]	31.6 (±14.5)[c]
Social functioning	40.6 ±19	42.3 (±22.0)	44.7 (±19.4)[d]	61.7 (±25.2)[d]
Physical role	4.3 ±16.3	7.0 (±10.8)	0 (0)	18.7 (±34.8)
Emotional role	22.7 ± 39.0	19.3 (±35.7)	25.6 (±43.5)	35.4 (±44.7)
Mental health	52.2 ± 11.9	51.4 (±11.0)	55.1 (±10.3)	55.0 (±14.3)
Vitality	42.1 ± 13.3	42.4 (±14.0)	44.8 (±10.9)	50.6 (±11.8)
Pain	24.9 ± 13.5	33.8 (±121.5)	37.6 (±19.9)[e]	58.9 (±17.0)[e]
Health experience	36.1 ± 19	42.6 (±19.5)	42.1 (±15.7)[f]	47.2 (±24.5)
Health change	33.3 ± 27.3	34.5 (±29.0)	36.6 (±28.4)	59.4 (±31.5)[f]

[a]Data represent means (SD). Score range, 0–100; higher scores indicate better quality of life.
[b]Responder, a patient with a response in any category (pain, ulcer, hemodynamic).
[c]$p = 0.002$ (Mann–Whitney test).
[d]$p = 0.045$ (Mann–Whitney test).
[e]$p = 0.073$ (Mann–Whitney test).
[f]$p = 0.05$ (Mann–Whitney test).

determined on the basis of anamnesis and use of pain medication that there was no change.

Two of 11 control patients had a >50% decrease in pain score versus 5 of 21 phVEGF$_{165}$ patients (p = NS).

Overall there were 17 responding patients (Table 2). As some patients responded in more than one category (hemodynamic, skin ulcers, or pain) there were a total of 22 responses. In the phVEGF$_{165}$-treated patients there were four patients with more than one category improved; in the control group, none. Three responders received placebo and 14 received phVEGF$_{165}$. The advantage for phVEGF$_{165}$ compared with placebo was significant ($p = 0.003$).

Quality of life assessment

Quality of life (QOL) assessment (see Table 3) was performed in 46 patients; 8 patients did not provide sufficient data. At baseline the only imbalance between the control and phVEGF$_{165}$-treated groups was in health experience (data not shown). Overall there was no improvement in QOL with phVEGF$_{165}$ treatment as compared with placebo treatment (data not shown). However, clinical and/or hemodynamic responders showed improved physical functioning, social functioning, and health change as compared with nonresponders ($p = 0.002$, $p = 0.045$, and $p = 0.05$, respectively).

VEGF blood levels

The median serum VEGF level at baseline was 321 pg/ml (range, 53–1677 pg/ml) in control patients and 275 pg/ml (range, 53–1103 pg/ml) in phVEGF$_{165}$-treated patients (p = NS). The median whole blood VEGF level were 846 pg/ml (range, 199–1963 pg/ml) in the control group compared with 911 pg/ml (range, 365–1843 pg/ml) in the phVEGF$_{165}$ patients (p = NS). There was no transient increase in circulating VEGF after intramuscular treatment in either group. In individual patients 10% showed a 50% increase in VEGF level within 14 days of intramuscular injection in both groups.

Table 4. Safety Analysis During Follow-up Period of 100 Days[a]

	Control (n = *27*)	*phVEGF$_{165}$* (n = *27*)
Hemodynamic complications	0	0
Ankle edema	11	10
Increase in proteinuria	0	0
Hypoglycemia	0	2
Telangiectasia	1	1
Anti-dsDNA (>2 IU/ml)	—	0
Circulating phVEGF$_{165}$[b]	—	4[b]
Deaths	2	2
Proliferative retinopathy	0	0
Diagnosis of cancer	0	0

[a]Data represent number of patients; no significant differences were detected (Fisher exact test).
[b]In 20 evaluable samples of 20 patients within days 1–3 of intramuscular administration.

Safety

phVEGF$_{165}$ was well tolerated (Table 4). No changes in systolic or diastolic blood pressure were observed. Edema was already present before injection in 11 patients of the control group and in 10 patients of the phVEGF$_{165}$ group. The edema increased in these 21 patients and new formation of edema occurred in three phVEGF$_{165}$-treated patients, and in four control patients (p = NS).

New teleangiectasias were found in two patients: in one phVEGF$_{165}$-treated patient and one control patient. They occurred within 14 days after intramuscular injection, and persisted thereafter.

Otherwise unexplained hypoglycemia (<3 mmol/liter) occurred in two phVEGF$_{165}$-treated patients in the first 2 to 3 weeks after intramuscular injection.

Microalbuminuria (30, to a maximum of 300, mg/day) was detected in the majority of the 37 patients measured, and remained stable during follow-up in both groups without significant variation. In the phVEGF$_{165}$-treated group no anti-dsDNA was detected. Analysis for the presence of phVEGF$_{165}$ in peripheral blood was possible in 20 phVEGF$_{165}$ patients; phVEGF$_{165}$ could actually be detected in 4 patients within the first 3 days after injection.

There were four deaths within the follow-up period of 100 days. These deaths were not related to the treatment: two patients died in the phVEGF$_{165}$-treated group and two patients in the placebo group. In the phVEGF$_{165}$-treated group one patient died of sepsis, 3 weeks after a major amputation of the leg, and one patient died in the postoperative period after a total hip replacement. In the control arm two patients died: one patient died 2 weeks after a major amputation, and the other patient died of protracted *Staphylococcus aureus* sepsis caused by an infected hip prosthesis.

One-year survival of nonamputated patients in the control and phVEGF$_{165}$-treated groups was, respectively, 60 and 84% (p = NS).

The progression from no diabetic retinopathy to minimal background retinopathy (fewer than 10 red dots) was 15% in both treatment arms; no progression to proliferative diabetic retinopathy was seen. Other ophthalmologic parameters remained stable during the whole study. There was no diagnosis of cancer.

DISCUSSION

Once the options for surgical intervention have been exhausted few treatment alternatives remain for patients with end-

Table 5. Studies with phVEGF$_{165}$ in Critical Limb Ischemia

		Treated limbs (CLI) (improvement/total)[b]					
Study design	*Treated limbs (CLI)*[a]	*Ulcer*	*Rest pain*	*ABI/TBI*	*Angio*	*Amputation*	*Systemic VEGF level (increase/total)*
IM	n = 1				1/1	1/1	
Phase I/II IM[d]	n = 10 limbs (9 pts) 6/9: ischemic ulcers 3/9: isolated rest pain	3/6	3/3	7/9	7/10	2/7	3/7
Phase I/II IM[e]	n = 7 (6 pts) 5/6: Ischemic ulcers 1/6: isolated rest pain	3/5	2/5	3/7	6/6	2/6	3/7
Phase I/II IM[f]	n = 24 (21 pts) 16/21: ischemic ulcers 21/21: rest pain	11/15	18/21	$p < 0.001$	19/24	2/21	8/21
Phase II[g]	n = 4 control	2/4	1/4	$p < 0.05$[h]	3/17	0/4	No increase in mean value
Intraarterial[i] Phase I IM[k]	n = 6 VEGF[j] n = 9[l] (9 pts)	3/6 4/6	0/6 6/7	$p < 0.05$[h]	10/16 6/9	0/6	No increase in mean value

Abbreviations: ABI/TBI, ankle-to-brachial index and toe-to-brachial index; angio, angiogenesis; CLI, critical limb ischemia; IM, intramuscular; pts, patients; VEGF, vascular endothelial growth factor.

[a]n, number of treated limbs with critical limb ischemia.

[b]Improvement of ischemic ulcers indicates complete or partial response; improvement in ABI/TBI indicates >10% absolute increase.

[c]Isner *et al.* (1996).

[d]Baumgartner *et al.* (1998).

[e]Isner *et al.* (1998).

[f]Shyu *et al.* (2003).

[g]Makiner *et al.* (2002).

[h]Patients with claudication and CLI.

[i]Only patients with CLI mentioned and treated with placebo or VEGF.

[j]VEGF-plasmid/liposome.

[k]Kim *et al.* (2004).

[l]One patient with claudication intermittens was not included in this report.

stage CLI. In this study we did not meet the primary end point of a reduced amputation rate. We did, however, demonstrate that intramuscular injections of the naked plasmid DNA encoding $VEGF_{165}$ ($phVEGF_{165}$) significantly improved wound healing and reduced hemodynamic insufficiency compared with placebo. Importantly, in the responders these clinical improvements resulted in improved physical functioning (mobility, and daily activities such as washing, dressing, and cleaning) and improved social functioning as detected by the RAND-36 questionnaire for QOL. Therefore "response" as defined in this study seems to be a meaningful notion.

Despite the rigorous entry criteria applied, a placebo effect, either a symptom of natural variation and fluctuation in the degree of ischemia, or an effect of intensified care in the study patients, is undeniable as three of the responders were among the placebo-treated patients. In contrast, significant clinical improvement was seen in 14 of the 27 patients treated with naked plasmid DNA. This 50% success rate is in agreement with the pioneering study of Baumgartner *et al*. (1998). Our results further confirm more recent data from studies in Chinese and Korean cohorts, in which even higher response rates were achieved (Shyu *et al*., 2003; Kim *et al*., 2004). On the other hand, no clinical improvement was found in a study by Makinen and coworkers, although angiography measurements suggested improvement (Makinen *et al*., 2002). However, their patient group was not well defined and the plasmid was given intraarterially instead of intramuscularly. Table 5 shows an overview of gene therapy studies in patients with CLI, treated with a plasmid carrying $VEGF_{165}$. The amputation rate in these series confirms ours (approximately 16%).

In a phase II randomized, double-blind, controlled study of patients with intermittent claudication, $VEGF_{121}$ in an adenoviral vector was administered as a single intramuscular injection, instead of $phVEGF_{165}$ (Rajagopalan *et al*., 2003). In contrast to our study, this trial was not associated with an increase in ABI/TBI, improved wound healing, or improved QOL. Besides the difference in study design, a difference in local VEGF concentration might be responsible for the observed difference in clinical results. There is a major difference between these VEGF proteins as $VEGF_{121}$ diffuses easily whereas $VEGF_{165}$ binds to matrix components.

Our primary aim was limb salvage, or a reduction in major amputation rate; however, our estimate of expected amputations (50%) proved to be too pessimistic. The small number of amputations (17%) that actually occurred ultimately precludes any conclusions about this form of gene therapy regarding potential treatment efficacy in terms of limb salvage. Although improvements in surgery have in the past influenced the need for amputation in this patient group (Ebskov *et al*., 1994), among our patients who were beyond surgical intervention this cannot have played a role. In a more recent Danish study, a further decline in amputations followed the institution of dedicated multidisciplinary foot clinics (Gottrup *et al*., 2001). As these clinics have also been instituted in the Netherlands, they may be partly responsible for the low incidence of amputations in our patient cohort. An alternative possibility would be that our patient cohort was included too early in the course of their disease. This explanation is unlikely in view of the rigorous entry criteria, the limited median survival of the whole group, and the severely affected QOL at the beginning of the study. In a cohort of ambulant diabetic patients in the same hospital area with early peripheral artery disease (Meijer *et al*., 2001), the QOL scores were between 50 and 90% in relevant domains compared with 5 to 50% in the present study.

In contrast to other studies, but in agreement with a phase I study in nondiabetic patients and the phase II study by Makinen and coworkers, we found no evidence of increased levels of systemic VEGF (Baumgartner *et al*., 1998; Isner *et al*., 1998; Makinen *et al*., 2002; Shyu *et al*., 2003; Kim *et al*., 2004). Therefore discussion concerning whether local or systemic effects, or both, contribute to the response remains open. There do not seem to be easy ways to clarify this problem in the clinical situation. Although amputation material is occasionally available for analysis, results would be unlikely to give information about successful interventions. Biopsies to clarify the mechanism in responding patients who have had severely compromised wound repair are undesirable.

Intramuscular $phVEGF_{165}$ was well tolerated. With the possible exception of hypoglycemia in two patients, no side effects occurred.

This small randomized study could serve to regenerate interest in the angiogenic approach in CLI. In a disease with an intrinsic placebo effect, the first priority obviously would be to confirm these results in a larger study. The number of patients to be studied should permit rather low numbers of amputations in the control group, between 10 and 15%. Also, the use of completely different end points, preferably of a noninvasive nature, should be considered. In such a study the theoretical possibility of a therapeutic effect of an empty plasmid could also be excluded. Improvements in the treatment scheme would thereafter include changes in the duration of treatment, the possible combined application of multiple effective genes, and the use of alternative transfection methods.

ACKNOWLEDGMENTS

This study was supported by a grant from Fornix BioSciences (Lelystad, The Netherlands). The authors thank Mrs. W.A. Dam, technician, for measuring circulating $VEGF_{165}$ and $phVEGF_{165}$ levels.

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Received for publication December 13, 2005; accepted after revision April 2, 2006.

Published online: May 2, 2006.