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Vascular endothelial growth factor-induced angiogenic gene therapy in patients with peripheral artery disease

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Abbreviations: ABI, ankle-brachial index; ASO, arteriosclerosis obliterans; CT, computerized tomography; IE, immediate-early; PAD, peripheral artery disease; PEG, polyethylene glycol; TBI, toe-brachial index; VAS, visual analog scale

Abstract

This phase 1 clinical trial tested the safety of intramuscular gene transfer by using naked plasmid DNA encoding the gene for VEGF, and analyzed the potential therapeutic benefits in patients with severe peripheral arterial disease (PAD). This study was an open-labeled, dose-escalating, single-center trial on nine male patients with severe debilitating PAD who had not responded to conventional therapy. Seven had Buerger's disease and two had arteriosclerosis obliterans. Plasmid DNA (pCK) containing human *VEGF*$_{165}$ was given by eight intramuscular injections in and around the area in need of new blood vessels. The study evaluated three escalating total doses (2, 4, and 8 mg of pCK-*VEGF*$_{165}$), with half of each total dose given four weeks apart. The follow-up duration was nine months. The gene injections were well tolerated without significant side effects or laboratory abnormalities related to gene transfer. Three patients showed transient edema in their extremities. Ischemic pain of the affected limb was relieved or improved markedly in six of seven patients. Ischemic ulcers healed or improved in four of six patients. The mean ankle-brachial index (ABI) improved significantly. Six of nine patients showed an increase in collateral vessels around the injection sites demonstrated by digital subtraction angiography. However, there was no relationship between the degree of ABI improvement and the dose given. Mean plasma levels of VEGF did not increase significantly. In conclusion, intramuscular injections of pCK-VEGF$_{165}$ can be performed safely to induce therapeutic angiogenesis in patients with severe PAD.

Keywords: angiogenesis; gene therapy; peripheral vascular disease; vascular endothelial growth factor

Introduction

The age-adjusted prevalence of human peripheral artery disease (PAD) is approximately 12% (Criqui *et al*., 1985). Symptoms of PAD include pain on walking (claudication) or at rest, and ulcers and gangrene in distal limbs. The most common causes of PAD are arteriosclerosis obliterans (ASO) and Buerger's disease (thromboangiitis obliterans; TAO). Despite advances in the treatment of PAD, many patients cannot be managed adequately with either medical therapy or revascularization procedures (Beard, 2000; Hiatt, 2001).

Therapeutic angiogenesis has emerged as a promising investigational strategy for the treatment of patients with PAD (Baffour *et al*., 1992; Pu *et al*., 1993; Takeshita *et al*., 1994a, b). After more than a decade of preclinical studies and recent clinical trials, gene therapy has been established as a potential method for inducing therapeutic angiogenesis in patients with ischemic limb disease (Isner *et al*., 1996; Baumgartner *et al*., 1998; Isner *et al*., 1998; Rajago-

palan *et al*., 2001; Shyu *et al*., 2003).

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF) is specifically mitogenic for endothelial cells (Keck *et al*., 1989; Leung *et al*., 1989; Plouet *et al*., 1989). This is an important advantage of VEGF for gene therapy because endothelial cells are responsible for neovascularization. Among the vectors used for clinical trials in angiogenic gene therapy, naked DNA is probably the safest and the most convenient vector. However, its approach has been limited because of low levels of gene expression in target tissues (Verma and Somia, 1997; Jeong *et al*., 2002).

To develop a high-efficiency expression plasmid for naked *VEGF* DNA gene therapy, we have constructed an expression vector, pCK, that is able to drive high levels of gene expression in skeletal muscles (Lee *et al*., 2000). A study of the use of pCK-$VEGF_{165}$ in an ischemic hind limb model using rabbits showed that it significantly increased distal limb blood flow (Chea *et al*., 2001). We therefore performed a phase 1 clinical trial to evaluate the safety and potential efficacy of intramuscular gene transfer of pCK-$VEGF_{165}$ in Korean patients with PAD.

Materials and Methods

Patients

We included patients who had suffered chronic limb ischemia, including severe claudication, resting pain or nonhealing ischemic wounds (ulcers, gangrene) for a minimum of four weeks without evidence of improvement in response to conventional therapies, and who were not optimal candidates for surgical or percutaneous revascularization. Requisite hemodynamic deficits included a resting ankle-brachial index (ABI) of less than 0.6 and/or a toe-brachial index (TBI) of less than 0.3 in the affected limb on two consecutive examinations performed at least one week apart. All patients had angiographic evidence of superficial femoral artery or infrapopliteal disease in the affected limb. Criteria used to describe limb status were adapted from standards recommended by the Society for Vascular Surgery/North American Chapter and the International Society for Cardiovascular Surgery (Rutherford and Becker, 1991). We excluded patients with a history of malignancy, proliferative diabetic retinopathy, a history of alcohol or drug abuse, or any other significant medical condition. All patients were allowed to continue previous medication. The study was designed as a phase I, nonrandomized, dose-escalating study to document the safety of intramuscular pCK-$VEGF_{165}$ gene transfer and to monitor clinical effects. This study protocol was approved by the Human Institutional Review Board, by the Institutional Biosafety Committee of Samsung Medical Center, and by the Korean Food and Drug Administration. This is the clinical trial of the first human gene therapy approved by the Korean Food and Drug Administration. All patients gave written informed consent before participation.

Screening tests before gene therapy

All patients had undertaken screening tests prior to gene injection. Possible malignancies were excluded by blood tests, urinalysis, stool examination, tumor markers, chest X-ray, chest computerized tomography (CT), abdominal ultrasonography, abdomen and pelvis CT, and by gastric and colonic fiberscopy. These tests were repeated at the end of study (nine months after the initial gene injection) to exclude malignancy. Fundoscopic examinations were performed before and at the end of the study.

Plasmid vector

pCK, the expression vector into which the $VEGF_{165}$ was inserted, has been described previously (Lee *et al*., 2000). pCK contains not only the full-length immediate-early (IE) promoter of the human cytomegalovirus but also its entire 5' untranslated region upstream from the start codon of the IE gene. The plasmid DNA was produced to clinical grade according to a proprietary process established at Dong-A Pharmaceutical Co. (Seoul, Korea). Briefly, *E. coli* DH5α cells carrying the plasmids were grown in kanamycin-containing medium in a 15-l fermenter. The fermentation broth was subjected to a series of purification steps including centrifugation, alkaline lysis, ammonium acetate and polyethylene glycol (PEG) precipitation, anion exchange chromatography and gel filtration chromatography. The quality of purified plasmid was verified according to quality-control methods for clinical grade plasmid DNA. Finally, purified plasmid DNA was dissolved in saline (0.9% NaCl) at a concentration of 1 mg/ml and then dispensed into sterile vials.

Intramuscular injection regimen

The study evaluated an escalating set of total doses (2, 4, and 8 mg of pCK-$VEGF_{165}$) with half of each total dose given four weeks apart. Three patients were included for each total dose, and there was a three-month safety-check period before starting the next dose level in a new group of patients. Each dose was diluted with sterile saline to make the total volume 16 ml. A 2 ml aliquot of this was injected intramuscularly over two min at each site. We performed eight intramuscular injections using 23 gauge needles to the affected ischemic limb, mainly the calf and

thigh muscles, without using local anesthesia. After the injections, we observed the patients for 30 min to check for any immediate adverse reactions. Four weeks after the first gene injection, a second set of injections was given at the same sites.

Hemodynamic and angiographic assessment

Patients were followed on a weekly basis for the first three months after gene injection, and at three-month intervals thereafter, for a total follow-up of nine months after the first injection. Ischemic ulcers were documented by color photography. Shrinkage of the ulcer area to less than 20% of the baseline area was defined as an improvement. Improvement of resting pain was evaluated using a visual analog scale (VAS) of pain scores. Patient recorded their VAS every day and the mean VAS of the previous week was calculated at each visit. The amount of analgesics used was recorded by questionnaire. Resting ABI or TBI were calculated from the quotients of absolute ankle or toe pressure to brachial pressure, respectively. Digital subtraction angiography was performed within one week before, and three and nine months after the first gene injection. During angiographic follow up, we maintained the same amounts of contrast, the force of contrast injection and the position of the catheter tip. New collateral vessel formation was assessed at the time at which contrast flow in the main conducting arteries was most clearly visible. New collateral vessels were assessed as +0 (no collateral development), +1 (slight), +2 (moderate), or +3 (rich) (Tateishi-Yuyama *et al*., 2002).

Plasma VEGF concentrations

Plasma VEGF concentrations were measured at baseline and weekly up to 12 weeks after the initial gene injection. Samples were collected in sodium citrate tubes and immediately centrifuged for 20 min at 3,600 rpm at 4°C, and the plasma was stored at -20°C until analysis.

Plasma VEGF concentration was determined using an ELISA assay as follows. Human recombinant VEGF (Calbiochem, La Jolla, CA) was separated from the carrier protein by SDS-PAGE under reducing conditions and recovered by electroelution. The recovered VEGF was dialyzed against 50 mM carbonate buffer (pH 9.0), and the concentration of VEGF was adjusted to 0.5 μg/ml. Ninety six-well plates were coated with VEGF by incubating the plates with 100 μl/well of the VEGF solution for 16 h at 4°C. The VEGF solution was aspirated and the wells were blocked by incubating with 250 μl/well of 1% BSA in PBS for 1 h at room temperature. The wells were washed once with 1% sucrose and dried. The wells were incubated with 100 μl/well of patient plasma (diluted 1:10 in 30% BSA/PBS) for 1 h at 37°C. The wells were then washed five times with 0.05% Tween-20 in PBS, and bound antibody was detected by incubating the wells with 100 μl/well of anti-human IgG-horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL), (diluted 1:10,000 in 30% BSA/PBS) for 30 min at 37°C. After washing five times with 0.05% Tween-20/PBS, 100 μl/well of TMB solution was added for color development and allowed to react for 30 min in the dark. The color reaction was stopped by adding 100 μl/well of 1 M H_2SO_4. Light absorbance at 450 nm was read using a microwell plate reader (Tecan, Switzerland).

Detection of anti-VEGF antibody

Plasma antibodies to VEGF were detected by immunoblotting at baseline and weekly up to 12 weeks, and at six and nine months after the initial gene injection. Recombinant human VEGF (Calbiochem) was separated from the carrier protein by SDS-PAGE under reducing conditions and transferred from the gel onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA) by electroblotting. After washing with PBS, reactive sites on the nitrocellulose membranes were blocked by incubating the strips in 3% BSA/PBS for 1 h at room temperature. The membranes were washed with PBS, dried and cut into strips 4 mm wide. The amount of VEGF immobilized on each strip was 0.1 μg. After three washes in PBS, the strips were incubated in 2 ml of plasma from patients (diluted 1:100 in 1% BSA/PBS) for 1 h with gentle shaking to allow the anti-VEGF antibodies in the plasma to bind to the immobilized VEGF band. The strips were washed three times with PBS, and immune complexes were detected with a 1:5,000 dilution of anti-human IgG-alkaline phosphatase (Calbiochem). After washing three times with PBS, the strips were developed in BCIP/NBT for 30 min to visualize bound antibody. The reaction was stopped by washing the strips with 20 mM EDTA/PBS.

Statistical analysis

Data are presented as means ± SEMs. Comparisons between variables from baseline to three or nine months were performed using nonparametric paired *t*-tests. Two-way ANOVA was applied for the comparison of mean plasma levels of VEGF. All statistical tests were two-tailed with significance assumed at $P < 0.05$.

Results

Baseline clinical characteristics

All nine patients were male with a mean age of 51.0 ± 14.9 years. There were seven TAO patients and two with ASO. Demographic and clinical data of the patients are shown in Table 1.

Safety assessment

There was mild discomfort up to 72 h after the in-

Table 1. Clinical, hemodynamic, and angiographic findings before and after gene therapy.

Clinical history and findings before gene therapy							Outcomes after gene therapy	
No.	Age (years)	Total dose	Dx	Class	Previous treatment	Signs/ Symptoms	ABI (0/3/9 months) Limb status at 9 months	New collateral vessels (+3 to 0) (3/9 months)
1	40	2 mg	TAO	6	Sympathectomy Amputation	Claudication, Resting pain, Ulcer, Toe gangrene (digit III)	0.94/1.21/0.96 Resting pain improved Ulcer stationary	+2/+1
2	46	2 mg	TAO	5	Sympathectomy Amputation	Claudication Resting pain Ulcer (digit I) Toe gangrene (digit III)	0.5/1.09/0.87 Resting pain improved Ulcer improved	+2/+1
3	51	2 mg	TAO	5	Sympathectomy Amputation Beraprost	Claudication Resting pain Ulcer (digit I)	0.65/0.70/0.63 Resting pain and ulcer improved after bypass at six months	+2/+1
4	58	4 mg	TAO	5	Sympathectomy Amputation	Claudication Resting pain Ulcer (digit I) Toe gangrene (digit III, V)	0.40/0.58/0.64 Resting pain improved Ulcer improved	+3/+2
5	38	4 mg	TAO	5	1 Bypass Beraprost	Claudication Resting pain Ulcer (digit I)	0.57/0.60/0.70 Resting pain stationary Ulcer stationary	0/+1
6	72	4 mg	ASO	4	None	Resting pain	0.53/0.47/0.52 Resting pain improved	+1/0
7	41	8 mg	TAO	5	None	Claudication Resting pain Ulcer (digit I, V)	0.52/0.53/0.56 Resting pain improved Ulcer completely healed	0/0
8	76	8 mg	ASO	3	3 PTA, 1 bypass 2 Revision Beraprost	Claudication	0.00/0.31/0.38 Claucation improved	0/0
9	43	8 mg	TAO	5	Beraprost	Claudication Resting pain Ulcer (digit I)	0.59/0.88/0.83 Resting pain improved Ulcer completely healed	+2/+2

Dx, diagnosis; TAO, thromboangiitis obliterans; ASO, arteriosclerosis obliterans; Class, limb status adapted standards recommended by the Society for Vascular Surgery/North American Chapter and International Society for Cardiovascular Surgery (Rutherford *et al.,* 1991); PTA, percutaneous transluminal angioplasty.

jections; however, gene injection was well tolerated without the need for local anesthesia. During the trial, no patient developed significant laboratory abnormalities in tests including those for complete blood counts, electrolyte concentrations, chemistry and lipid profiles, tumor markers, and inflammatory markers. No changes in diabetic retinopathy were observed from fundoscopic examinations. Likewise, no development of a latent neoplasm has been observed. Three patients complained of transient lower extremity edema, consistent with VEGF enhancement of vascular permeability, but this was completely controlled by diuretic therapy. Sensorineuronal hearing loss was observed in one patient (number 6 in Table 1), however this was judged as being unrelated to the gene transfer, and it resolved spontaneously. This event was reported to Human Institutional Review Board of our Institute and the Korean Food and Drug Administration, which approved continuation of the study.

Change in ischemic limb status

Therapeutic benefit was demonstrated in most of the patients by regression of resting pain and/or improved tissue integrity in the ischemic limb. A 51-year-old man (patient number 3), who had undergone below-knee amputation of the contralateral limb presented with severe resting pain and a necrotic great toe. He had focal stenosis of the distal femoral artery and occlusion of the infrapopliteal arteries. Even though angiographic findings at three months after gene therapy showed improved collateral vessels in the calf area, the ulcer had failed to respond. He had undergone short distal bypass of a stenotic lesion of the distal femoral artery using a graft from the great saphenous vein six months after gene injections. After surgery, his ulcer improved. This patient was thus excluded from the final assessment and eight patients were evaluated for clinical signs at the end of the study protocol.

Limb pain

Ischemic pain in the affected limb was markedly decreased after therapy. The VAS was 51.5 ± 9.6 mm at baseline (n = 8); 23.8 ± 9.7 mm (n = 8) at a three- month follow-up and 25.1 ± 9.5 mm (n = 7) at a nine-month follow-up ($P < 0.05$) (Figure 1A). Analgesic use per week showed a similar decline (data not shown). This improvement in resting pain was distinct in the first three months, and it was maintained until nine months. In two patients, however, the amount of analgesics could not be reduced. A 58-year-old man (patient number 4) presented with resting pain and ulcers on his toes and fingers. After gene transfer into the affected leg, while the resting pain of his foot decreased from a VAS of 74 at baseline to 20 at three months, the resting pain in his fingers did not improve, and he could not decrease his analgesic dose. A 38-year-old man (patient number 5) had continued resting pain and also could not decrease his analgesic intake.

Ischemic ulcers

Seven patients had ischemic wounds (ulcer and/or gangrene). Patient number 3 was excluded from the final assessment. Two patients with ischemic ulcers were completely healed after gene transfer and two more showed improvements. Representative photos are shown in Figure 2. Patients who had relatively larger ulcers experienced more difficulty in healing than the patients with smaller ulcers. In two patients (numbers 1 and 2), the lesions had progressed rapidly before therapy. However, this progression slowed down after gene transfer. Before gene transfer, seven

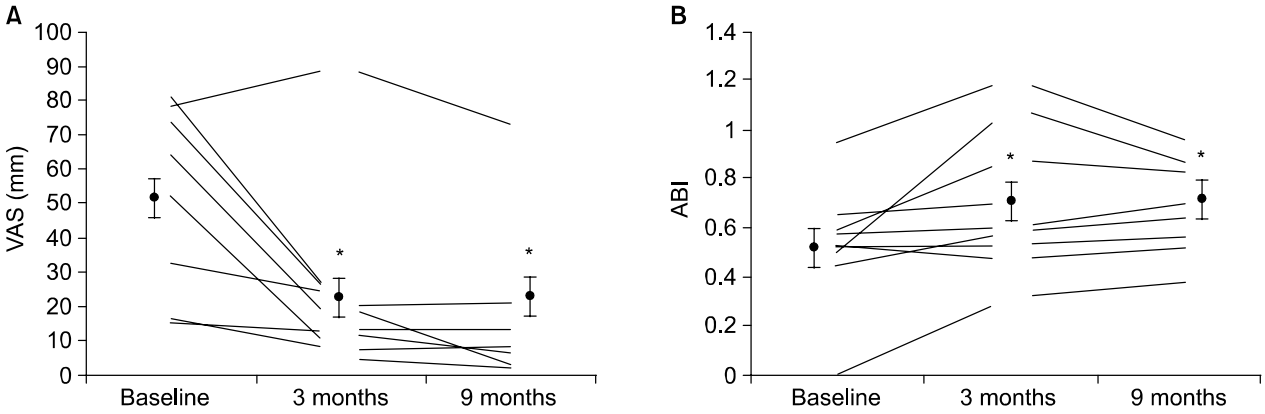

Figure 1. (A) Improvements in resting pain after gene therapy. VAS; visual analog scale. (B) Gain in ankle-brachial index (ABI) after gene therapy. The dot and bar indicate mean ± SEM. *$P < 0.05$ versus baseline levels.

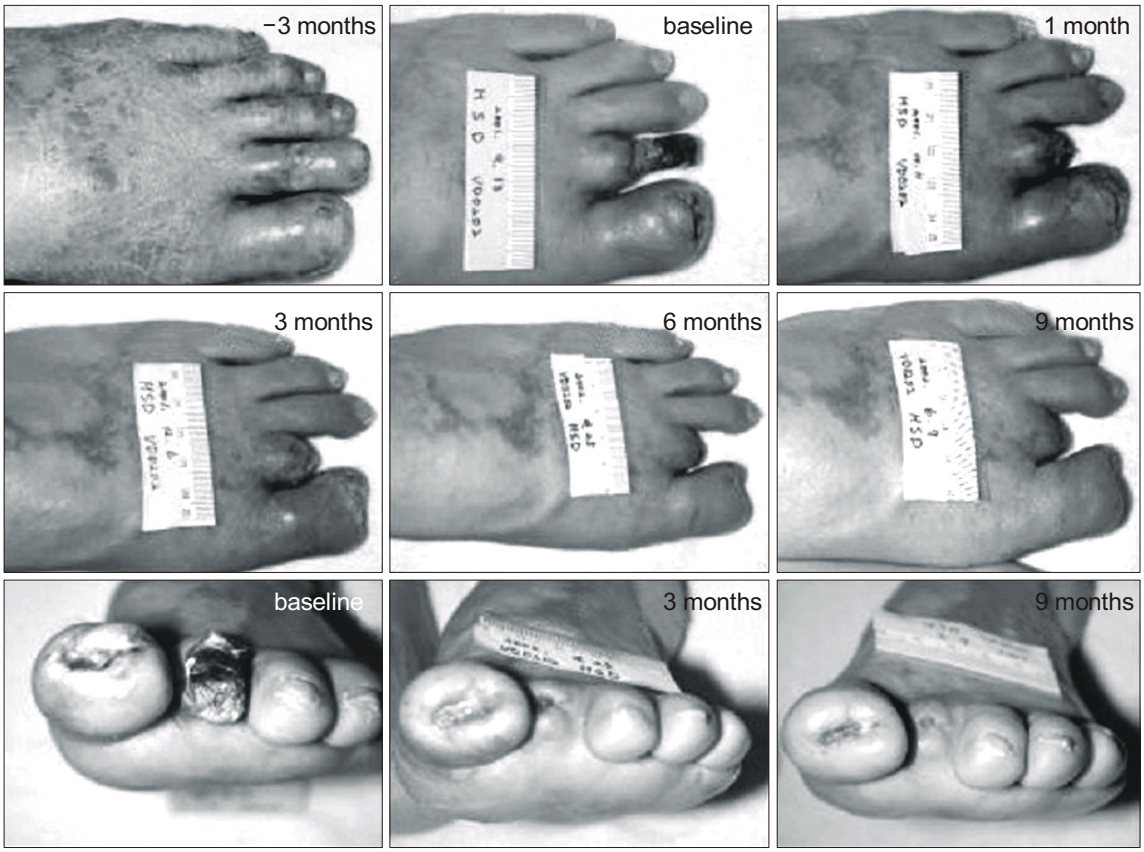

Figure 2. Limb salvage after gene therapy. Nonhealing ischemic wound of the great and second toes had been progressing rapidly before gene therapy. One month after gene injection, a second toe was self-amputated and its stump healed without ulcer progression. A nonhealing ulcer of the great toe also showed improvement after gene therapy. Before gene therapy, the patient was wheelchair bound and taking multiple analgesics. Nine months after gene transfer, he was freely walking and was free from resting pain after being successfully weaned from all analgesics.

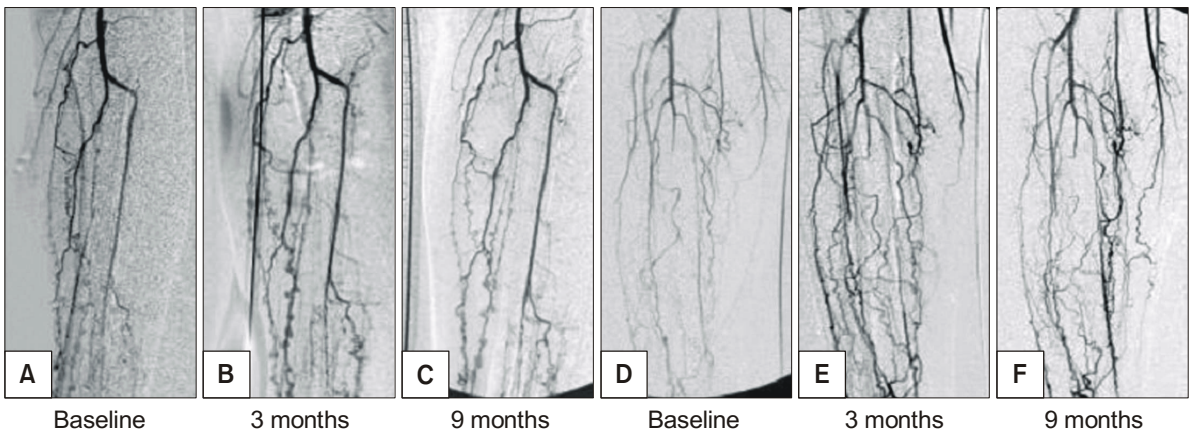

Figure 3. Subtraction angiographic analysis of collateral vessel formation. A, B and C from patient number 2. D, E and F from patient number 9. Compared with baseline angiography, newly developed collateral vessels were visible at the calf level three months after gene therapy. Increased collateral vessels remained at the nine-month follow-up. In patient number 2, semiquantitative analysis of new collateral vessels decreased from +2 at 3 month (3B) to +1 at 9 month (3C) after gene injection. In patient number 9, there was no regression of new collaterals.

patients had been identified as candidates for limb amputation. After the therapy, three patients no longer needed amputation. Two patients avoided major amputation but underwent toe amputation after the nine-month follow-up. Seven of the eight patients showed significant improvements in resting pain and/or the conditions of their ischemic wounds at nine months.

Change in the ankle-brachial index

The ABI increased from 0.52 ± 0.08 (n = 9) at baseline to 0.71 ± 0.10 (n = 9) at three months (P = 0.025), and 0.69 ± 0.07 (n = 8) at nine months (P = 0.016) (Figure 1B). However, the extent of improvement was not correlated with the dose used ($P > 0.05$). Improvement in the pressure index was sustained for up to nine months.

Angiographic findings

Three months after the beginning of gene transfer, lower extremity angiography showed newly visible collateral vessels at the knee, calf, and ankle level in six of the nine patients. Representative angiographs are shown in Figure 3. Contrast densities were similar suggesting that these were obtained under identical imaging conditions. The mean score of development of collateral vessels increased to 1.3 ± 0.4 at three months after commencing gene transfer ($P < 0.05$, n = 9) (Table 1). The newly developed collateral vessels persisted until nine months (mean score 0.9 ± 0.3, n = 8).

Blood levels of VEGF and VEGF antibody

Mean blood concentrations of VEGF did not increase from baseline level (9.8 ± 1.8 pg/ml). The measured amounts of VEGF after the initial gene injection were 8.0 ± 1.1, 6.5 ± 0.9, 8.4 ± 0.9, 7.6 ± 1.1, and 9.3 ± 1.7 pg/ml at 2, 4, 6, 8, and 12 weeks, respectively. No patient developed antibodies to VEGF.

Discussion

We have shown here that VEGF-induced angiogenic gene therapy is safe for human use and potentially effective as evidenced by substantial increases in ABI and by formation of new collateral vessel formation demonstrated on angiograms. pCK-$VEGF_{165}$ gene transfer not only improved resting pain in most of the patients, but also ischemic ulcers or gangrene in more than half of them.

The prognosis and quality of life for patients with chronic severe leg ischemia, as manifested by severe claudication, resting pain or ischemic ulcers, are poor. Patients who are not indicated for conventional revascularization therapy, called 'no-option patients', represent 10～15% of all PAD patients and usually receive only conservative management such as pain control and vasodilators (Wolfe 1986; Albers *et al*., 1992). However, no pharmacological treatment has been shown to improve the natural history of severe limb ischemia (Isner and Rosenfield, 1993). Consequently, alternative treatment strategies are needed for these patients.

Collateral vessels are important as an alternative source of blood flow in the case of arterial stenosis or occlusion. They reduce limb ischemia and greatly improve the clinical symptoms such as claudication, resting pain, ischemic wounds and prognosis. Therapeutic angiogenesis is a new treatment strategy to increase collateral vessels in ischemic area by the injection of angiogenic factor or genes. VEGF is a specific angiogenic protein for endothelial cells (Keck *et al*., 1989; Leung *et al*., 1989; Plouet *et al*., 1989). VEGF is induced by ischemia, and it induces endothelial cell proliferation and mobilization. Many experimental and clinical studies have reported that VEGF could be used as a method of treatment for patients with critical limb ischemia by the increase of the collateral vessel in ischemic areas (Takeshita *et al*., 1994a, b; Isner *et al*., 1996; Baumgartner *et al*., 1998; Isner *et al*., 1998; Shyu *et al*., 2003).

Previous studies showed that intramuscular gene therapy with plasmid encoding human $VEGF_{165}$ ($phVEGF_{165}$) was safe and effective in the treatment of patients with critical limb ischemia (Baumgartner *et al*., 1998; Isner *et al*., 1998; Shyu *et al*., 2003). In this phase I clinical trial, patients who received pCK-$VEGF_{165}$ gene transfers also showed improvements in resting pain and increases in collateral vessel growth. The resting pain improvements after gene transfer were more dramatic than the improvements in ABI or ischemic wounds. We speculate that the decrease in ischemic resting pain was caused not only by increased blood flow but also by recovery from ischemic neuropathy. Studies in animals show that constitutive over-expression of VEGF results in the restoration of large- and small-fiber peripheral nerve function in diabetic and ischemic neuropathy models (Schratzberger *et al*., 2000; Schratzberger *et al*., 2001; Veves and King, 2001). Preliminary clinical studies have also demonstrated improvement in signs and symptoms of sensory neuropathy in patients with PAD following intramuscular injection of ph$VEGF_{165}$ (Isner *et al*., 2001; Simovic *et al*., 2001).

In previous studies with ph$VEGF_{165}$, Baumgartner *et al*. and Shyu *et al*. reported that the gains in ABI scores three months after gene therapy were 0.15 and 0.14, respectively (Baumgartner *et al*., 1998; Shyu *et al*., 2003). In our study, the gain was 0.19; thus, even though the patient characteristics in these

trials differed, all trials showed similar ABI score improvements. In the study of Shyu *et al*., there appeared to be a dose-related response in terms of ABI and the minimal effective dose was a total of 2,400 μg (Shyu *et al*., 2003). In our study, however, we could not find a dose response in terms of the incidence of edema, or improvements in resting pain, ABI or collateral development. In our previous dose-response study (Lee *et al*., 2000), when pCK-*VEGF*$_{165}$ was injected into the tibialis anterior muscle of Balb/C mice, the level of local gene expression in the muscle reached a plateau at a dose of 125 μg, which corresponds to the dose injected at one site when using a total of 2 mg of plasmid in two sets of injections in this clinical trial. This could explain why we could not obtain a further gain at the higher dose rates. Thus, multiple injections with low doses (*i.e.* 125 μg/site) would be more effective than a few injections of high doses.

Another concern in our study is that we could not detect elevated levels of blood VEGF after gene transfer. Shyu *et al*. reported that mean plasma levels of VEGF increased significantly from a baseline of 26 ± 31 pg/ml to 63 ± 56 pg/ml two weeks after gene therapy (Shyu *et al*., 2003). However, Freedman *et al*. (2002) reported that baseline plasma VEGF was highly variable and was not normally distributed in a study of the kinetics of VEGF protein release into the systemic circulation after gene transfer for 34 patients with PAD. In their study, after intramuscular gene transfer, median plasma VEGF rose slightly, although significantly, by seven days (38 to 41 pg/ml, $P < 0.05$), but was not different from baseline at 14, 21, or 28 days, and day-7 plasma levels did not differ significantly as a function of dose despite an almost 10-fold difference. They concluded that interpretation of results for individual subjects is complicated by wide variations in baseline VEGF and low circulating levels compared with baseline after gene transfer. Our data are in line with the above observations that baseline plasma VEGF concentrations are highly variable and there would be a marginal or no increase after intramuscular transfer of pCK-*VEGF*$_{165}$.

Finally, current methods used to perform diagnostic contrast angiography cannot provide images of arteries measuring < 200 μm in diameter (Takeshita *et al*., 1997). Using synchrotron radiation microangiography, neovascularization after VEGF gene transfer in a rat model of hindlimb ischemia included a substantial contribution of vessels < 180 μm in diameter (Takeshita *et al*., 1997). Thus, conventional angiographic techniques used in the present study may have failed to depict the full extent of angiogenesis achieved after pCK-*VEGF*$_{165}$. In this sense, Laser doppler imaging (LDI) used to detect cutaneous blood flow and/or tissue oxymetry ($Tc0_2$) may give an additional clue to assess the improved perfusion in treated limbs.

Our study is a phase I clinical trial. Therefore, the safety of patients is a major aim of the study and it is not designed to draw conclusions regarding efficacy. However, clinical efficacy including resolution of resting pain, and healing of ischemic ulcers was associated with objective findings of improved ABI and blood flow on angiography.

In conclusion, this phase I, open-label, dose-escalating study showed that intramuscular *VEGF* gene transfer was safe and feasible in Korean patients with chronic severe leg ischemia. The procedure was well tolerated without complications directly related to gene expression except for transient leg edema. Even though the procedure was associated with clinical improvements, phase II and III trials will help determine the efficacy of this therapy in patients with severe PAD.

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