Therapeutic angiogenesis following intramuscular gene transfer of vascular endothelialgrowth factor 121 in a dog model of hindlimb ischemia

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Abbreviations:

AP: alkaline phosphatase
ATCC: American tissue culture collection
CMV: cytomegalovirus
D-MEM: Dulbecco's modified eagle medium
ELISA: enzyme-linked immunosorbent assay
FCS: fetal calf serum
GFP: green fluorescent protein
GOT: glutamic-oxaloacetic transaminase

GPT: glutamic-pyruvic transaminase
HMEC: human microvascular endothelial cells
PCR: polymerase chain reaction
PEI: polyethylenimine
SD: standard deviation
SV40t SS/pA: SV40t splicing/polyadenylation signals
TFF: tangential flow filtration
VEGF: vascular endothelial growth factor

Vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen, has been shown to promote therapeutic angiogenesis in animal models of critical limb ischemia. Ischemic skeletal muscle is advantageous for taking up and expressing foreign genes transferred as naked plasmid DNA. Accordingly, we investigated the hypothesis that intramuscular administration of naked plasmid DNA encoding the 121-amino acid isoform of VEGF could augment collateral development and tissue perfusion in a dog

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hindlimb ischemia model. Unilateral hindlimb ischemia was surgically induced in Beagle dogs. Ten days later, animals received intramuscular injections of pVEGF$_{121}$ plasmid directly in the ischemic muscles. Angiogenic effects were evaluated by angiography, calf blood pressure ratio and vasomotor reserve analyses. Thirty days after gene transfer, angiographically recognizable collateral vessels were increased in pVEGF$_{121}$-treated animals compared with controls. Improvement in perfusion to the ischemic limb was documented by a significantly higher calf blood pressure ratio for pVEGF$_{121}$ (0.79 ± 0.05) versus controls (0.56 ± 0.14, P<0.01). Vasomotor reserve assay suggested amelioration in blood availability at the microcirculation level in pVEGF$_{121}$-treated animals. Hematological variables showed no significant modification due to the treatment. Our results suggest that intramuscular gene transfer of VEGF$_{121}$ may promote therapeutic angiogenesis in critical limb vascular insufficiency.

Critical limb ischemia is estimated to develop in ~500 to 1000 individuals per million per year (Creager, 2001). Conventional drug therapy is of no proven benefit for these patients (Nikol and Huehns, 2001). Despite major advances in both percutaneous and surgical techniques, the disease frequently follows an inexorable down-hill course (Gardner and Killewich, 2001). Indeed, many of these patients face amputation (Dormandy et al. 1999), which is associated to high morbidity and mortality, and patient's life quality becomes remarkably affected. Consequently, the need for alternative strategies for the treatment of patients with critical limb ischemia is compelling.

Therapeutic angiogenesis is a novel concept consisting in the use of angiogenic growth factors to expedite and/or augment collateral artery development in ischemic tissues. It constitutes a potential alternative approach for treating vascular insufficiencies. Vascular endothelial growth factor (VEGF) (Leung et al. 1989), a heparin-binding dimeric glycoprotein, is among the various growth factors that have been shown to promote angiogenesis. VEGF is an endothelial cell specific mitogen (Neufeld et al. 1999) that is upregulated under hypoxia (Semenza, 2001).

Preclinical studies using recombinant VEGF have proven its efficacy in animal models of lower limb (Bauters et al. 1995; Walder et al. 1996) and myocardial ischemia (Hariawala et al. 1996; Lopez et al. 1998). However, recombinant protein therapy has several limitations for therapeutic angiogenesis. First, a single or multiple bolus doses of recombinant protein are not effective to maintain an optimally high and local concentration over time. Second, protein therapy requires expensive manufacturing faculties and years of scale-up effort. Third, rhVEGF has been shown to produce systemic hypotension (Hariawala et al. 1996; Horowitz et al. 1997).

Gene therapy presents a solution to the dosing dilemma, since it can be considered a sophisticated form of a sustained delivery system. Moreover, gene therapy is cheaper to develop, implement, and reimburse. Striking advantages of VEGF gene transfer in particular are its specificity for the target cells, with minimal transgene expression in other tissues; and its low systemic effect due to a short half life (29-77 min) in the circulation (Eppler et al. 2000). Several gene transfer studies have demonstrated that VEGF expression can stimulate the development of collateral vessels in a rabbit hindlimb ischemia model, using arterial (Takeshita et al. 1996b; Takeshita et al. 1996c) or intramuscular (Tsurumi et al. 1996; Tsurumi et al. 1997) administration. The results of clinical trials (Isner et al. 1996; Baumgartner et al. 1998; Isner et al. 1998) document that gene transfer of naked plasmid DNA encoding VEGF promotes collateral vessel development in patients with critical limb ischemia.

Adenovirus-mediated gene transfer of VEGF$_{121}$ has been shown to improve lower limb flow reserve and endothelial function in patients with peripheral arterial disease (Rajagopalan et al. 2001). Several studies with VEGF$_{121}$ have used the same approach to promote angiogenesis in the ischemic myocardium of animals (Patel et al. 1999; Lee et al. 2000), and patients (Rosengart et al. 1999a; Rosengart et al. 1999b). However, virus administration can lead to immune responses that could cause serious immunopathology. The use of naked plasmid DNA obviates these immunological concerns and clearly simplifies the transfection protocol. The present study was designed to test the hypothesis that intramuscular administration of naked plasmid DNA encoding the 121-amino acid isoform of VEGF could augment collateral development and tissue perfusion in a dog hindlimb ischemia model.

MATERIALS AND METHODS

Cloning VEGF$_{121}$ gene

The DNA sequence encoding human VEGF$_{121}$ was amplified from the plasmid hVEGF$_{121}$ (Plate et al. 1992) by polymerase chain reaction (PCR) and ligated into the expression vector pAEC-D2 (Herrera et al. 2000) digested with *Pvu*II and *Bam*HI. This vector contains the immediate-early promoter/enhancer from the human cytomegalovirus (CMV) to drive VEGF$_{121}$ expression. Downstream from the VEGF$_{121}$ gene are the SV40t splicing/polyadenylation signals (SV40t SS/pA). The kanamycin resistance marker (transposon 903) is also included in this plasmid. These fragments occur in the pUC19 vector, which includes the *Escherichia coli ColE*1 origin of replication. The recombinant plasmid was named pVEGF$_{121}$.

Bacterial culture and purification of pVEGF$_{121}$

E. coli DH10B cells [mcr A, mcr B (a), mrr (b), hsd R$_{17}$(c),

deoR (rec A1) end A1, lac ZD M15 (a) deletion of mcrBC (b) deletion of hsd R and hsd M] transformed with $pVEGF_{121}$ plasmid were grown in a fermentor containing 5 L of Terrific Broth medium with 50 mg/mL kanamycin at 37ºC, 500 rpm. agitation, and an air flow rate of 5 L/h. For increased copy number, the temperature was raised to 42ºC at mid-log phase (Lahijani et al. 1996). After bacterial alkaline lysis, the mixture was neutralized and clarified by tangential flow filtration (TFF) using a nominal molecular weight cutoff TFF polyethersulfone membrane (1,000,000 Da). Subsequent chromatography purification steps were performed as follows: Q-sepharose (Amersham Pharmacia, UK) fast flow anion exchange chromatography (AEC) and Sephacryl S-1000 (Amersham Pharmacia, UK) gel filtration chromatography.

In vitro transfection and expression of $VEGF_{121}$

CHO-K1 cells (ATCC CCL-61) were transfected using 1 mg $pVEGF_{121}$ plasmid by the polyethylenimine (PEI) (Aldrich, USA) method. In order to control transfection, cells were co-transfected with 5 mg pAGFP plasmid, carrying the green fluorescent protein (GFP) gene under the control of the CMV promoter. Transient co-expression of $VEGF_{121}$ and GFP was achieved in D-MEM (Sigma, USA) containing 10% FCS and 5 mg/mL gentamycin. To confirm $VEGF_{121}$ expression, 48-h culture supernatants from transfected CHO-K1 cells were assayed by ELISA (Quantikine, R&D Systems, USA).

Matrigel angiogenic activity assay

Terasaki plates (Nunc, USA) were coated with Matrigel basement membrane matrix (Becton Dickinson, USA) (Benelli and Albini, 1999). Approximately 10^3 human microvascular endothelial cells (HMEC), from E Ades (CDC, USA), were added per well. HMECs were grown for 16-20 h in the presence of 48-h culture supernatants from CHO-K1 cells transfected with $pVEGF_{121}$. A supernatant from cells transfected with pAEC-D2 vector was used as negative control, and $VEGF_{165}$ protein (0.5 ng/mL) was used as positive control. Finally, the wells were photographed under the inverted microscope.

Animal model

Beagle dogs with surgically induced unilateral hindlimb ischemia were used for the experiments. All protocols were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Health Guide for the Care and Use of Laboratory Animals. Twelve Beagle dogs, weighing 11-13 kg, were anesthetized with thiopental (20 mg/kg). A longitudinal incision was then performed, extending from 3 cm over the crural region to the pre-patellar region of one limb. Through this incision, the femoral artery was dissected free, along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries, were also dissected free.

After further dissecting the popliteal and saphenous arteries distally, the external iliac artery and all of the above arteries were ligated (3.0 silk; Ethicon, Sommerville, NJ). Finally, the femoral artery was completely excised from its proximal origin as a branch of the external iliac artery, to the point distally where it bifurcates to form the saphenous and popliteal arteries. Postoperation, all animals were closely monitored. Subcutaneous penicillin (10^6 units daily, during 7 days) was administered for prophylaxis.

Intramuscular gene transfer

Ten days after surgical induction of ischemia, animals received intramuscular injections with $pVEGF_{121}$ plasmid (n=6) or placebo (n=6), using 1-mL syringes and 26 G needles. Injections were performed slowly to prevent fluid loss from the epimysium. Each dog was injected at four different sites in the ischemic muscles. One $pVEGF_{121}$ dose contained 2 mg / 2 mL; each dose was divided in four 0.5 mg / 0.5 mL injections. Placebo consisted of sterile saline (0.9% NaCl), and it was administered in the same way as $pVEGF_{121}$ (four 0.5 mL injections).

Angiography

After palpation of the aorta beat, a long 18 G needle was introduced into the aortic artery and 20 mL of contrast media (sodium diatrizoate; Chinoin, China) were injected in a single puncture. Images of the ischemic hindlimb were recorded on 11x14 inches films. This procedure was carried out using the aforementioned anesthetic. Angiography was performed on day 10 (baseline), and again on day 40.

Calf blood pressure ratio

Ischemic/normal limb blood pressure ratio was measured by infrared photopletismography. Pressures were obtained by placing an appropriate pneumatic cull above the knee-joint of the rear legs. The infrared photosensor was placed on the rear foot. The systolic blood pressure was determined by slowly deflating the cuff pressure from the supra-systolic values and recording the first inflow with the photocell used as a pulse sensor.

Vasomotor reserve

Vasodilator response was assessed by intravenous infusion of 2 mg nitroglycerine. Systolic amplitude of the peripheral pulse wave was measured before and every five seconds after the nitroglycerine infusion during 10 min. Vasodilator responses were classified as no response, partial (for less than 10 min.) or total (for 10 min. or more).

Hematological assays

Animals were evaluated according to a set of hematological variables including hemoglobin, hematocrit, glycemia, creatinin, total proteins, albumin, alkaline phosphatase (AP), glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), coagulation time,

bleeding time, and platelet counting. Measurements were performed at days 10 (baseline), 20 and 40.

Statistical analysis

Calf blood pressure ratio results were expressed as mean ± SD. The Shapiro-Wilk test was used to analyze normal distribution. Statistical significance was evaluated using a Student's *t* test for equal variances, two-tailed distribution. To analyze vasomotor reserve experiment, a Fisher's exact test was used. Data from hematological variables were expressed as mean ± SD, and evaluated by the Mann Whitney's *U* test. Values of $P<0.05$ and $P<0.01$ were interpreted to denote statistically significant (*) and very significant (**) differences, respectively.

RESULTS

Cloning VEGF$_{121}$ gene

The human VEGF$_{121}$ gene was inserted into the expression vector pAEC-D2 (Herrera et al. 2000). The resultant plasmid, named pVEGF$_{121}$, with a size of 4039 bp, contains the VEGF$_{121}$ gene under control of the immediate-early promoter/enhancer from the human CMV. The kanamycin resistance gene was used for appropriate selection in bacterial systems without undesirable side effects in gene therapy. The insert was confirmed by restriction analysis and DNA sequencing (data not shown).

Purification of pVEGF$_{121}$

A process combining alkaline lysis, TFF, anion exchange and size exclusion chromatography, yielded a 99% pure, >95% supercoiled plasmid DNA, at a final concentration of 1 mg/mL in saline (0.9% NaCl) formulation. The average yield from 4 different lots was 252.6 ± 26 mg of plasmid DNA per 5 L culture.Final preparations of plasmid DNA were characterized by analytical quality control procedures. Data were consistently reproduced in the 4 lots (data not shown).

Expression and biological activity of VEGF$_{121}$

VEGF$_{121}$ concentration in supernatants from CHO-K1 cells transfected with four different lots of pVEGF$_{121}$ ranged between 401 and 683 (541±116) pg/mL of medium. The expressed VEGF$_{121}$ was shown to be biologically active when assayed in an *in vitro* model of angiogenesis. Human microvascular endothelial cells grown in the presence of supernatant from pAEC-D2-transfected cells exhibited a small round shape and did not spread. In contrast, treatment with VEGF$_{121}$-containing supernatants resulted in drastic morphological changes. The cells became elongated, forming thin cords of interconnecting cells (Figure 1). Similar effects were observed with VEGF$_{165}$ protein. These data demonstrate that VEGF$_{121}$ is able to mediate dramatic cell reorganization, which would be necessary *in vivo* for endothelial cell sprouting and tube formation.

Angiogenic stimulation in vivo

pVEGF$_{121}$ was assayed for *in vivo* angiogenesis in a dog hindlimb ischemia model. Animals were subjected to a surgical procedure in order to induce unilateral ischemia. An interval of 10 days was permitted for spontaneous development of collateral vessels. Then, animals received intramuscular injections of pVEGF$_{121}$ directly in the ischemic muscles. Anatomic and physiological effects of VEGF$_{121}$ gene transfer were evaluated at different time points up to day 40 (30 days after transfection).

Angiography. Representative angiograms recorded from both control and pVEGF$_{121}$-treated animals are shown in Figure 2. In control animals, collateral artery development in the medial thigh appeared unchanged in serial angiograms recorded at days 10 and 40 (Figure 2, A and B). In contrast, in the pVEGF$_{121}$-treated group, improvement of collateral artery development was typically observed in angiograms at day 40 when compared with the baseline (day 10) angiogram (Figure 2, C and D).

Calf blood pressure ratio. At day 10 after induction of ischemia (immediately before gene transfer), calf blood pressure ratio was similar in both groups (pVEGF$_{121}$=0.52±0.09, control=0.54±0.13; P=NS). By day 25, a slight improvement in blood pressure ratio was observed in both groups of animals, but differences between groups were not significant (pVEGF$_{121}$=0.67±0.09 versus control=0.60±0.13; P=NS). However, at day 40, the blood pressure ratio for animals in the pVEGF$_{121}$-treated group was significantly higher than that for animals in the control group (pVEGF$_{121}$=0.79±0.05 versus control=0.56±0.14; P<0.01) (Figure 3). Moreover, when compared with baseline, a significant increase in calf blood pressure ratio was found at day 40 in pVEGF$_{121}$ group (P<0.01), but not in the control group.

Vasomotor reserve. Animal responses to an intravenous infusion of nitroglycerin are shown in Figure 4. At day 10 postoperation no animal showed response. At day 25, 50% of control animals partially responded, while 66.7% of pVEGF$_{121}$-treated animals were partial or total responders. By day 40, responder animals remained 50% for the control group. In contrast, 100% of pVEGF$_{121}$-treated animals showed partial (33.3%) or total (66.7%) responses. Statistical analysis showed no significant differences (P>0.05) between groups at both, day 25 and day 40. Nevertheless, the biological significance of these results is discussed below.

Hematological assays. An important set of hematological variables were measured in order to evaluate possible side effects of pVEGF$_{121}$ on animal homeostasis (Table 1). The statistical analysis showed not significant differences between groups for all variables at all measurement points. In addition, no statistical differences were found at days 20 and 40 with respect to day 10 (baseline). Moreover, all