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ORIGINAL ARTICLE

Repeated, but not single, VEGF gene transfer affords protection against ischemic muscle lesions in rabbits with hindlimb ischemia

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Vascular endothelial growth factor (VEGF) gene transfer-mediated angiogenesis has been proposed for peripheral artery disease. However, protocols using single administration have shown little benefit. Given that the transient nature of VEGF gene expression provokes instability of neovasculature, we hypothesized that repeated administration would provide efficient tissue protection. We thus compared single vs repeated transfection in a rabbit model of hindlimb ischemia by injecting a plasmid encoding human $VEGF_{165}$ ($pVEGF_{165}$) at 7 (GI, n = 10) or 7 and 21 (GII, n = 10) days after surgery. Placebo animals (GIII, n = 10) received empty plasmid. Fifty days after surgery, single and repeated administration similarly increased saphenous peak flow velocity and quantity of angiographically visible collaterals. However, microvasculature increased only with repeated transfection: capillary density was 49.4 ± 15.4 capillaries per 100 myocytes in GI, 84.6 ± 14.7 in GII ($P < 0.01$ vs GI and GIII) and 49.3 ± 13.6 in GIII, and arteriolar density was 1.9 ± 0.6 arterioles per mm^2 in GI, 3.0 ± 0.9 in GII ($P < 0.01$ vs GI and GIII) and 1.5 ± 0.6 in GIII. Muscle lesions were reduced only within repeated transfection. With single administration, gene expression peaked at 7 days and declined rapidly, but with repeated administration, it remained positive at 50 days. At 90 days of repeated transfection (additional animals), gene expression decreased significantly, but neovessel densities did not. Thus, repeated, but not single, VEGF gene transfection resulted in increased microvasculature, which, in turn, afforded effective protection against ischemic muscle damage.

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Introduction

Peripheral arterial disease (PAD) is a condition for which there is no effective pharmacological treatment. Its progression leads to critical lower limb ischemia, often resulting in amputation.[1,2] The formation of collateral vessels induced by hypoxia is generally insufficient, even to meet resting metabolic requirements.

Therapeutic angiogenesis by administering genes encoding pro-angiogenic growth factors has been proposed as an alternative,[3] especially based on the encouraging results observed not only in pre-clinical studies, but also in patients.[4–9]

Results from the few randomized, placebo-controlled clinical trials so far conducted have yielded controversial results. Although some authors have reported improvements in clinical and hemodynamic end points after gene transfection,[10–13] others have found no beneficial effect.[13]

In earlier studies of plasmid-mediated vascular endothelial growth factor (*VEGF*) gene transfer in pigs and sheep with myocardial ischemia and infarction, we observed that *VEGF* gene expression is transient, VEGF mRNA being undetectable 35 days after transfection.[14–16] Moreover, when studying the transfected sheep at different follow-up times, we observed that the myocardial angiogenic response present at 7 days post-transfection vanished in specimens studied later on. This agrees with the observations that the microvascular network induced by pro-angiogenic interventions lacks stability[17] or that it regresses in the absence of VEGF.[18] Although this may not represent a drawback for acute diseases (such as myocardial infarction) in which the interventions are expected to salvage viable tissue at the early phases of a rapidly evolving process, it may be significantly disadvantageous for chronic diseases, such as peripheral artery disease, in which the neoformed vessels should protect from ischemia in the context of a process of slow, insidious progression.

On these bases, we hypothesized that the scarcity of positive and long-lasting results observed in clinical trials of gene transfer-induced angiogenesis in peripheral artery disease is due, in part, to the transient nature of gene expression, and, consequently, gene transfer should be repeated to attain therapeutic benefit.

To test our hypothesis, we examined the time course of p*VEGF* expression and compared the therapeutic

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efficacy (hindlimb hemodynamics, microvessel density and extent of ischemic muscle damage) of single vs repeated administration of p*VEGF* in rabbits at 50 days after complete extirpation of the left superficial femoral artery and ligation of the deep femoral artery. Moreover, we assessed if repeated administration resulted in persistent gene expression over time in a group of additional rabbits studied at 90 days of follow-up.

Results

Ischemic hindlimb hemodynamics

Rabbits with hindlimb ischemia received 10 intramuscular injections of a plasmid encoding human $VEGF_{165}$ (total dose 3.8 mg) at 7 days (VEGF × 1, $n=10$) or at 7 and 21 days (VEGF × 2, $n=10$) after surgery. Control animals received empty plasmid (placebo, $n=10$). Following surgery, the left-to-right hindlimb blood pressure ratio decreased in all groups (placebo: 0.25 ± 0.26, VEGF × 1: 0.35 ± 0.36 and VEGF × 2: 0.23 ± 0.2; $P=$NS). Fifty days later, blood pressure ratio was 1.02 ± 0.34 in the placebo group, 0.9 ± 0.22 in the VEGF × 1 group and 1.11 ± 0.35 in the VEGF × 2 group ($P=$NS).

Peak systolic flow velocity in the ischemic hindlimb was similarly low after femoral extirpation, (placebo: 8.8 ± 1.7, VEGF × 1: 10.1 ± 1.7 and VEGF × 2: 9.9 ± 1.1 cm s^{-1}; $P=$NS), and at the end of follow-up, it improved equally in VEGF × 1 (22.8 ± 2.7 cm s^{-1}) and VEGF × 2 (22.7 ± 3 cm s^{-1}) groups, both values being significantly higher ($P<0.05$) than those of the control group (16.3 ± 1.8 cm s^{-1}).

Angioscore

At 50 days after surgery, internal iliac artery angiography was performed. Single and repeated p$VEGF_{165}$ administration induced similar increases in the density of angiographically visible collaterals. The angioscore was 209.5 ± 13.9 collaterals per cm^2 in the control group, 295.7 ± 13.5 in the VEGF × 1 group ($P<0.01$ vs control) and 297.4 ± 20.8 in VEGF × 2 group ($P<0.01$ vs control). Figure 1 shows representative angiograms.

Histology

Muscle lesions. The lesions found in ischemic muscle samples were of three types: (1) diminished number of muscle fibers and adipose tissue replacement; (2) muscle fiber atrophy and lipomatosis; and (3) areas of necrosis, muscle fiber replacement with scar connective tissue, inflammatory infiltrate (myositis) and reparation attempt with myoblastic proliferation. The diagnostic coincidence between two independent observers was 95%. Abnormal muscle was 60% in the control group, 47% in the VEGF × 1 group ($P=$NS vs control, χ^2–Yates) and 25% in the VEGF × 2 group ($P=0.04$ vs control and VEGF × 1). The remaining muscle, as well as that from the non-ischemic limbs, was normal.

Microvessels. Unlike angiographically visible collaterals, the microvasculature was increased only in the group receiving repeated administration of p$VEGF_{165}$ (Figure 2). Capillary density was 49.3 ± 13.6 capillaries per 100 myocytes in control group, 49.4 ± 15.4 in the VEGF × 1 group ($P=$NS vs control) and 84.6 ± 14.7 in the VEGF × 2 group ($P<0.01$ vs control and VEGF × 1).

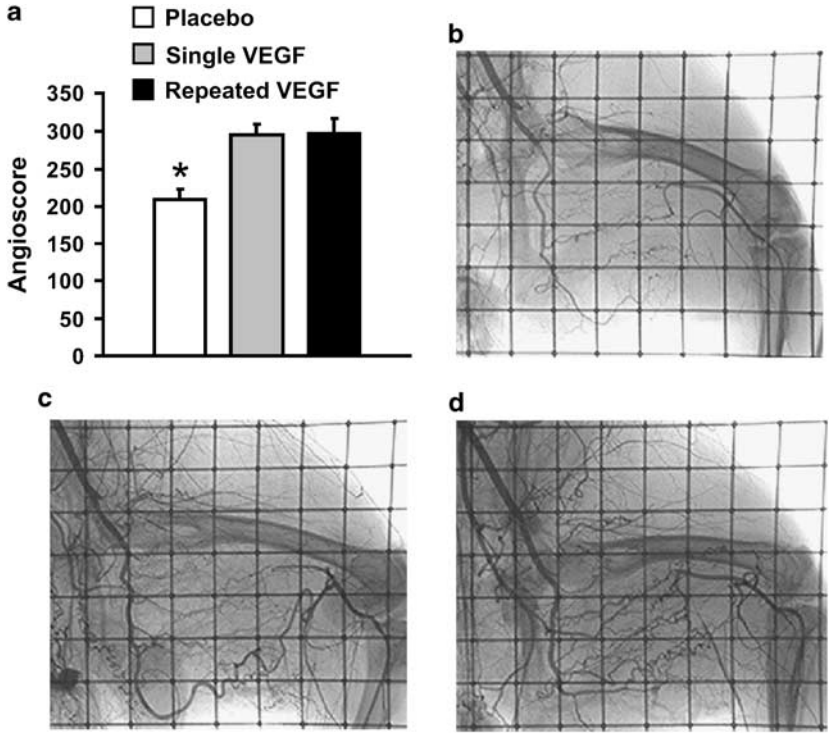

Figure 1 Angioscore and representative angiograms. (**a**) Angiographically visible collaterals were similarly increased in the single and repeated p$VEGF_{165}$ administration groups with regard to placebo; *$P<0.01$. (**b–d**) Representative angiograms of one rabbit from each of the three experimental groups (placebo, single *VEGF* administration and repeated *VEGF* administration, respectively).

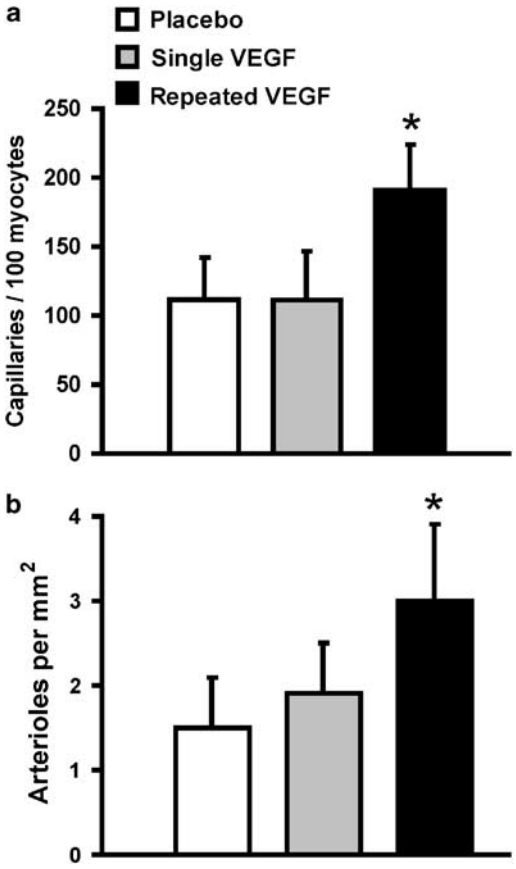

Figure 2 Microvessels. Capillary (**a**) and arteriolar (**b**) densities were significantly increased only in the group receiving repeated administration of p*VEGF*$_{165}$. * $P<0.01$ with regard to placebo and single administration groups.

Arteriolar density was 1.5 ± 0.6 arterioles per mm^2 in the control group, 1.9 ± 0.6 in the VEGF $\times$ 1 group ($P =$ NS vs control) and 3 ± 0.9 in the VEGF $\times$ 2 group ($P<0.01$ vs control and VEGF $\times$ 1). The differences between control and repeated administration groups remained stable at a longer follow-up time, as observed in additional animals operated to assess gene expression 3 months after treatment.

Detection of pVEGF$_{165}$, VEGF mRNA and VEGF protein

At 50 days, the human *VEGF*$_{165}$ gene (PCR), its mRNA (reverse transcriptase PCR, RT-PCR) and the VEGF protein were present only in the animals undergoing repeated p*VEGF* administration (Figure 3a). Immunohistochemistry revealed that the protein was located in the sarcolemma and the cytoplasm of muscle fibers and in satellite cells (Figures 3b–d).

To see if repeated administration resulted in persistent gene expression over time, we performed RT-PCR studies and immunohistochemistry for the VEGF protein in a group of 12 additional rabbits (placebo, $n=6$; VEGF $\times$ 2 = 6) killed at 90 days of follow-up. Figure 4 shows that VEGF mRNA was still present, though at a significantly lower level than at 50 days (0.01 ± 0.005 relative optical density units at 90 days vs 0.12 ± 0.03 at 50 days, $P<0.0001$). In addition, muscle biopsies taken at 50 days from these rabbits confirmed the presence of VEGF mRNA (as already observed, at that time point, in the two animals of the preceding protocol). At 3 months after repeated administration, the human VEGF protein was present in very few myocytes of four out of six *VEGF*-treated rabbits, located within the cytoplasm (Figure 4c). Importantly, despite the weakness of gene

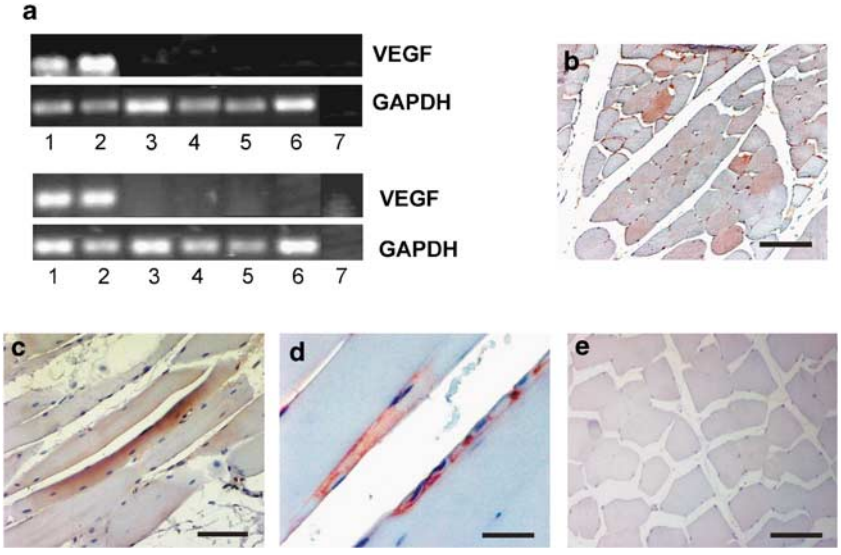

Figure 3 Transgene expression in transfected skeletal muscle at 50 days. (**a**) Human VEGF mRNA (reverse transcriptase PCR, upper half) and DNA (PCR, lower half) detection for the two rabbits of the repeated p*VEGF*$_{165}$ administration group (lanes 1 and 2), single p*VEGF*$_{165}$ administration group (lanes 3 and 4), placebo group (lanes 5 and 6) and negative control of reaction (lane 7). Transgene expression was positive only in rabbits belonging to the repeated administration group. (**b–d**) Positive immunohistochemistry for the human VEGF$_{165}$ protein in the cytoplasm and sarcolemma (**b** and **c**), and satellite cells (**d**) of skeletal muscle samples of rabbits from the repeated p*VEGF*$_{165}$ administration group. (**e**) Negative reaction in a rabbit belonging to the single p*VEGF*$_{165}$ administration group. All rabbits of this and the control groups consistently displayed negative reactions. Bars = 100 (**b**), 50 (**c**), 20 (**d**) and 80 μm (**e**).

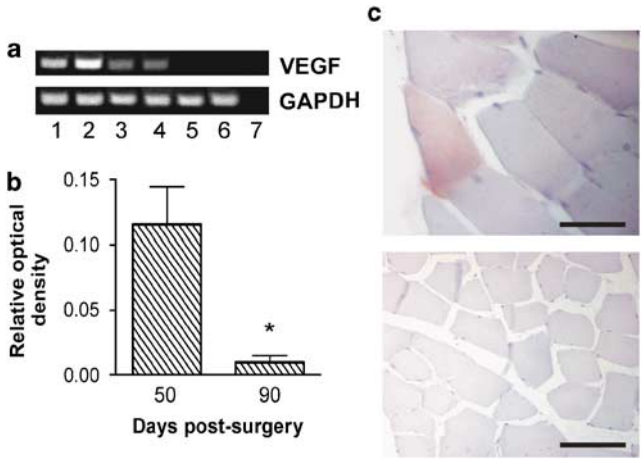

Figure 4 Transgene expression in the skeletal muscle of additional rabbits receiving repeated p*VEGF*$_{165}$ administration at 90 days of follow-up. (**a**) Human VEGF mRNA (reverse transcriptase PCR, RT-PCR) at 90 days (lanes 3 and 4). Lanes 1 and 2 show positive reactions for VEGF mRNA in muscle biopsies taken from these rabbits at 50 days of follow-up. Lanes 5 and 6 show negative reactions in placebo animals. Lane 7: negative control of reaction. RT-PCR for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as internal loading control. (**b**) Semiquantitative assessment of human VEGF mRNA at 90 and 50 days of follow-up. *$P<0.0001$. (**c**) Positive immunohistochemistry for human VEGF protein in a skeletal myocyte of a rabbit receiving repeated p*VEGF* administration at 90 days of follow-up (upper panel). Negative reaction in a control rabbit at 90 days of follow-up (lower panel). Bars = 40 and 80 μm, respectively.

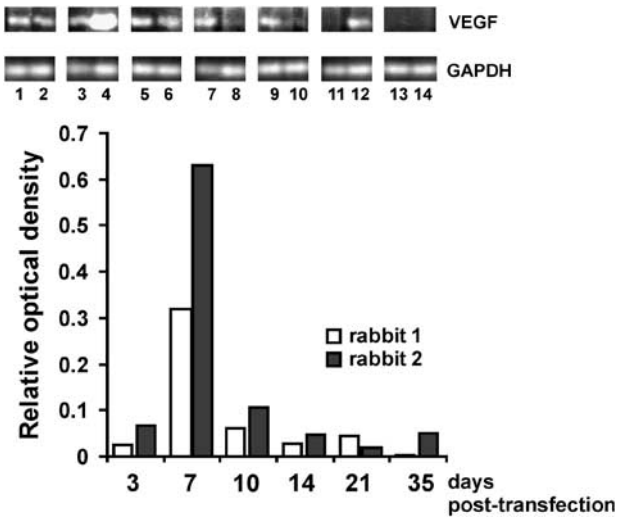

Figure 5 Time course of transgene expression. Human VEGF$_{165}$ mRNA (reverse transcriptase PCR, RT-PCR) was assessed in transfected muscle samples of two animals at six time points after single administration of p*VEGF*$_{165}$ (3 days: lanes 1 and 2; 7 days: lanes 3 and 4; 10 days: lanes 5 and 6; 14 days: lanes 7 and 8; 21 days: lanes 9 and 10; 35 days: lanes 11 and 12). Lanes 13 and 14 show negative reactions in two placebo-treated rabbits 7 days after injection. Transgene expression peaked at 7 days and decreased abruptly thereafter. At 35 days after transfection, it was indetectable in one animal and extremely low in the other. RT-PCR for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal loading control.

expression, the differences in capillary and arteriolar densities between control and VEGF × 2 groups did not decrease with regard to those observed at 50 days (52.9 ± 36.2 in control vs 87.8 ± 17.1 capillaries per 100 myocytes in VEGF × 2, $P<0.01$; and 1.2 ± 0.9 in control vs 3.2 ± 2.4 arterioles per mm^2 in VEGF × 2, $P<0.03$). Moreover, in two rabbits of the VEGF × 2 group in which the VEGF protein was undetectable, the capillary and arteriolar densities were well within the mean group values.

Time course of VEGF gene expression

With single p*VEGF*$_{165}$ administration, gene expression was positive at 3, 7, 10, 14 and 21 days and was maximal at 7 days after transfection (Figure 5). As shown, after this peak, expression diminished until day 35, when mRNA detection was positive in only one of two samples.

Discussion

Our results show that in a rabbit model of peripheral artery disease, single and repeated administration of a plasmid encoding human VEGF$_{165}$ induces similar increases in angiographically visible collaterals at 50 days after transfection. However, only when the plasmid is re-injected 2 weeks after the first administration, also capillary and arteriolar density are increased, and a significant reduction of ischemia-induced muscle damage is attained, implying that preservation of the skeletal muscle from ischemic injury is critically dependent upon the microvascular network supplying the tissue.

On the other hand, our results also show that the expression of the injected human *VEGF*$_{165}$ gene in rabbit skeletal muscles is transient, suggesting that the pro-angiogenic stimulus of a single administration is not persistent enough to prevent regression of the neoformed capillaries and arterioles. Contrastingly, repeated administration results in neovascularization persisting for at least 90 days, when gene expression, though present, is markedly lower.

Although stabilization of the VEGF-induced neovascularization is the most likely reason for the observed muscle protection, on account of earlier results showing that VEGF plays a role in myoblast migration and survival and that adenovirus-mediated *VEGF*$_{165}$ gene transfer inhibits ischemia-induced apoptosis in rat skeletal muscle cells,[19] a direct effect of VEGF on skeletal muscle repair in our rabbits cannot be ruled out.

Earlier studies have shown that the microvessels resulting from angiogenic gene therapy lack stability and tend to vanish.[17,18] In rabbits, Gounis *et al*.[17] showed that angiogenesis in the ischemic hindlimb in response to adenovirus *VEGF*$_{165}$ injection is confined to the period of *VEGF* expression that occurs in the first week of gene delivery. In transgenic mice devised with a genetic switch that allowed for the reversible induction of high levels of VEGF in the liver and myocardium, Dor *et al*.[20] observed that when *VEGF* expression was withdrawn 2 weeks after being induced, the acquired vessels regressed and disappeared. In contrast, delaying the off switch until 4 weeks after its onset, the neovasculature became refractory to VEGF withdrawal and persisted for many months. They concluded that premature cessation of the VEGF stimulus leads to regression of most

acquired vessels, thus challenging the utility of therapeutic approaches relying on short stimulus duration.

In rats with severe chronic hindlimb ischemia, Leong-Poi *et al*.[21] used, as gene delivery method, ultrasound-mediated destruction of microbubbles bearing $VEGF_{165}$ plasmid DNA. They observed that perfusion peaked at 14 days after delivery, followed by a partial regression of neovascularization at 6 weeks, when the presence of VEGF mRNA was undetectable.

Collectively, these results suggest that after a single administration of an angiogenic gene, increases in tissue perfusion and hence protection of the ischemic muscle may be expected in the short term but not necessarily at longer times. In effect, the follow-up duration of most pre-clinical studies reporting benefits of gene therapy strategies on hindlimb ischemia has ranged from 1 week to 30 days.[22–27] Beneficial effects at longer follow-up times (up to 12 weeks) of Ad$VEGF_{121}$ have been reported by Gowdak *et al*.[28] in rats and rabbits with hindlimb ischemia. However, their results are not strictly comparable with ours because the gene was injected between 2 and 4 weeks before the induction of hindlimb ischemia.

Interestingly, in our rabbits, a single administration of p$VEGF_{165}$ sufficed to induce a stable, long-lasting enhancement of larger, angiographically visible collaterals. A similar phenomenon (increased angioscore with no increase in capillary density) was reported by Pelisek *et al*.[29] in pigs with experimental peripheral artery disease at 3 months after liposome-mediated $VEGF_{165}$ gene transfer. They observed that the tissue surrounding peripheral arteries showed no increase of capillary density but an enhancement in angiographic score.

In humans, repeated p$VEGF_{165}$ gene administration has been tested only in studies using non-randomized, uncontrolled, open-label protocols.

After their first pioneering study in one patient with severe PAD,[4] Isner's group published three studies on intramuscular p$VEGF_{165}$ gene transfer in small groups of patients with atherosclerotic limb ischemia,[5] Buerger's disease[6] and ischemic neuropathy of diabetic and non-diabetic origin.[7] In all the three studies, the p$VEGF_{165}$ injections were repeated 4 weeks after the first administration and in all of them beneficial effects were reported. Although the high doses used may account for these results, the possibility exists that repetitive administration has contributed to the 3–6 months persistency of the therapeutic actions observed.

In a more recent study, Kim *et al*.[8] used three escalating doses in nine patients suffering critical leg ischemia, with half of each total dose given 4 weeks apart. Three months after the initial procedure, they found significant improvements in resting pain, ankle–brachial index and collateral development, which were still present at 9 months of follow-up. The authors state that these effects are not dose related, but they do not speculate about the prolonged duration of the effect being related to the repetition of the procedure.

In another study, Shyu *et al*.[30] injected p$VEGF_{165}$ at five different doses in 21 patients. In all of them, the injection was repeated 4 weeks after the initial procedure, and in some, an additional booster injection was given later on. At 6 months follow-up, significant improvements in resting pain, ankle–brachial index and magnetic resonance angiographic score were found. The authors attributed these results to the high total dose received rather than to the reiteration of the procedure.

However, in a placebo-controlled double-blind trial studying the effect of Ad$VEGF_{121}$ in a similar patient population, Rajagopalan *et al*.[13] showed that neither high nor low doses of the compound yielded any therapeutic actions at 26 weeks of follow-up, suggesting that the likely reason for Shyu's results was the repetition of gene transfer rather than (or in addition to) the total dose received.

The most recently published double-blind, placebo-controlled trial of gene therapy in human PAD is a study by Nikol *et al*.[12] addressing the effect of plasmid *FGF1* given every 15 days (four times, 4 mg each time). Although the primary end point (ulcer healing) was similar for treatment and placebo, the use of plasmid *FGF1* significantly reduced (by twofold) the risk of all amputations and, most importantly, major amputations. In addition, there was a trend toward a decrease in mortality. The authors suggest that the benefit may be primarily because of FGF1-induced effects at the microvascular level and that multiple administrations may allow sustained local exposure to the expressed FGF-1 at the site of administration. Despite the fact that the growth factor used in Nikol's study differs from ours, both arguments may apply to our own results.

Specifically regarding the *VEGF* gene, to the best of our knowledge, no randomized, controlled, double-blind studies of repeated vs single administration in human critical PAD have been so far conducted. The results of this study, as well as those from Nikol's study, support the possibility that such trials may be clinically relevant.

It must be pointed out that factors other than repeated administration may be involved in the long-term preservation of VEGF-induced neovessels. One of them is the local growth factor concentration. Adenoviral vectors have a high transfection efficiency, which favors microvascular growth, but its short-term expression may not permit maintenance of VEGF levels that would prevent microvascular regression. In this regard, lentiviruses and adeno-associated viruses would be preferable.[31] Plasmids, on the other hand, show lower transfection efficiency but a longer-term expression than adenoviruses. Another factor is the VEGF isoform employed. $VEGF_{121}$ has lower biological efficacy than $VEGF_{165}$, and, as correctly observed by Ylä-Herttuala *et al*.,[31] the RAVE trial[13] used a dose of Ad$VEGF_{121}$ much lower than that shown to have significant angiogenic action in pre-clinical studies.[32] A third issue that may account for the tendency of neovessels to regress is the use of monogenic therapies.[3] If our understanding of the process leading to neovessel formation is limited, even less would we know about the cascade of genes and signaling pathways involved in neovasculature stabilization. In the future, we will probably need to associate diverse genes in a certain sequence to achieve a long-lasting angiogenic effect. Likewise, future studies addressing issues, such as optimization of dose and treatment regime, should be conducted.

Finally, it must be noticed that we did not assess whether the neovasculature regresses or persists beyond complete extinction of gene expression in animals undergoing repeated p$VEGF_{165}$ administration. However, it is interesting to observe that although gene

expression at 90 days was much lower than that at 50 days, and the VEGF protein barely detectable in the tissue sections, the density of capillaries and arterioles did not at all decrease, suggesting that the stability of the acquired neovascular network became, at least in part, independent of *VEGF* gene expression.

In conclusion, our study shows that in rabbits with experimental peripheral artery disease repeated, but not single, transfection with p$VEGF_{165}$ results in a significant enhancement of the microvasculature, which, in turn, affords muscle protection from ischemic damage, suggesting a direct relationship between the transient nature of the $VEGF_{165}$ gene expression and the vanishing of neoformed capillaries and arterioles. This result may be of clinical relevance when designing gene therapy-based strategies in patients with peripheral artery disease.

Materials and methods

Surgical preparation

All procedures were conducted following the Guidelines for Use and Care of Laboratory Animals published by the NIH (NIH publication no. 85–23, revised 1996). The study protocols were approved by the Laboratory Animal Care and Use Committee of the Favaloro University.

A total of 56 New Zealand White rabbits of either sex and weighing 3.5 ± 0.7 kg were operated on. Thirty animals were used for the treatment efficacy protocol and 26 for gene expression assessment (14 for studying the time course of expression after single administration and 12 to assess long-term persistent expression after repeated administration).

Anesthesia was induced with xylazine (7 mg kg^{-1}) and ketamine (25 mg kg^{-1}), and maintained with intravenous 0.1-ml injections of xylazine–ketamine (1:9 dilution) every 3 min. Topical lidocaine hydrochloride 2 g%, 1–2 ml, was also administered. All animals spontaneously breathed oxygen-enriched room air during surgery. Heart rate and arterial oxygen saturation were monitored with a pulse oxymeter (Novametrix 515A; Wallingford, CT, USA).

The animal model has been described earlier.[33] Briefly, through a longitudinal incision in the left hindlimb extending from the inguinal ligament to the knee, the superficial femoral artery was dissected along its whole length. All its collaterals and terminal branches, including the inferior epigastric, lateral circumflex and superficial epigastric arteries, were also dissected and ligated. Subsequently, the superficial femoral artery was entirely excised from its origin at the inguinal ligament up to its bifurcation into the saphenous and popliteal arteries. The deep femoral artery was ligated at its origin and left in place. Finally, the surgical wound was closed and a single prophylactic dose of cephalexine 50 mg kg^{-1} was administered intravenously.

Therapeutic efficacy protocol

Seven and 21 days after surgery, the animals were anesthetized, and a 4-cm incision was performed parallel and medially to the scar of the earlier wound. In each of both operations, a solution (2 ml), containing 1.9 mg ml^{-1} p$VEGF_{165}$ or plasmid devoid of gene (placebo) was administered intramuscularly in 10 aliquots, (200 µl, 0.38 mg each) distributed between the quadriceps (four injections) and the adductor muscles (six injections). According to the injectates received in both operations, the following three groups resulted: placebo–placebo (control group); p$VEGF_{165}$–placebo (VEGF × 1); p$VEGF_{165}$–p$VEGF_{165}$ (VEGF × 2). The animals were randomly assigned to the treatment groups and the content of the vials was kept blind for all the investigators until the end of data processing. At the end of each operation, the skin was closed and the animals were allowed to recover.

Assessment of systolic pressure and peak flow velocity. Before the initial surgery, before the first and second reoperations for plasmid injection and before killing, peak systolic flow velocity and systolic blood pressure were measured in the ischemic and non-ischemic hindlimbs. Under deep sedation (xylazine 1 mg kg^{-1} and ketamine 20 mg kg^{-1}), peak systolic flow velocity of the saphenous artery flow was determined using pulsed Doppler ultrasound and a high-definition 5- to 12-MHz linear transducer (Philips ATL 5000; Bothell, WA, USA). The color Doppler facility of the equipment was used to identify the artery. Care was taken to use a 60° angle and the least sample volume in all four sessions.

For systolic pressure determination, an inflatable cuff, 9 cm in length and 2 cm in width (Hokanson, Bellevue, WA, USA), was positioned surrounding the thigh. Employing a Hokanson E20 Rapid Cuff Inflator & AG101 Air Source, the cuff was inflated beyond peak systolic pressure and slowly deflated until an audible signal was detected (Parks 811 Pulsed Doppler Ultrasound Detector and standard 10 MHz pencil probe; Aloha, OR, USA). The procedure was repeated three times with 5 min intervals and systolic blood pressure was considered to be the average of the three determinations. This value was used to calculate the blood pressure ratio, defined as the ratio of systolic pressure (in mm Hg) in the ischemic left hindlimb to systolic pressure (in mm Hg) in the normoperfused right hindlimb.

Angiography. On day 50, immediately before euthanasia, the animals were subjected to general anesthesia and an internal iliac artery angiography was performed. A 5F sheath was introduced in the aortic bifurcation by direct abdominal aortic exposure. A squared grid was positioned between the rabbit and the table. Two injections of 20 ml of iopamidol were performed (10 ml per second) at 450 psi using an injector (Medrad Mark V; Medrad Inc., Indianola, IA, USA). Images were obtained with DSA technique in 9-inch fields at 2.5 frames per second with an angiographer (Philips Integris 3000; Philips Medical System BV, Best, The Netherlands). Collateral vessel count was performed separately by two operators who were blinded from the treatment received by the animal, and the angioscore (number of collateral vessels observed in each space of the grid divided by the number of spaces, multiplied by 100) was calculated.

Histology and immunohistochemistry. The animals were killed 50 days after surgery and skeletal muscle

samples (left quadriceps, adductor and gastrocnemius) from both hindlimbs were fixed in 10% formalin. Samples were cut in halves and embedded in paraffin. Sections measuring 3 μm in thickness were stained with hematoxylin–eosin and Masson's trichrome. Two independent observers blindly analyzed the samples and characterized the lesions. The muscle samples were classified as normal or abnormal (independently from the kind of lesion found), and the number of normal and abnormal samples in each group were expressed as a percent of the total quantity of samples analyzed.

Study of vessel density was performed on the whole transversal section of the three muscles. The size of the studied area was digitally scanned at a magnification of $\times 2$, and the area was determined by a digital analyzer (Image Pro Plus; Media Systems Corp., Silver Spring, MD, USA). Capillaries were identified by immunohistochemistry, using an anti-CD34 antibody (Bio Genex, San Ramon, CA, USA). Capillary density was expressed as the number of capillaries per 100 myocytes. Arteriolar density (number of arterioles measuring 8–50 μm in diameter per mm^2) was measured after arterioles were identified with demonstration of smooth muscle cells in the vessel wall with anti-smooth muscle actin immunohistochemistry (Bio Genex).

These studies of vessel density were also performed in 12 additional animals (placebo, $n=6$; VEGF $\times 2=6$) killed at 90 days of follow-up.

To detect human VEGF protein in the muscle, deparaffinized tissue sections measuring 5 μm in thickness, from rabbits killed at 50 and at 90 days of follow-up, were incubated with a rabbit anti-human VEGF polyclonal antibody (Bio Genex) and post-treated with a biotinylated MultiLink antibody (Bio Genex). The tissue sections were incubated with peroxidase-labeled streptavidin, revealed with 3-amino-9-ethylcarbazole (AEC) as chromogen and stained with hematoxylin. To rule out false-positive results, we used two controls: muscle sections of placebo-treated rabbits and serial samples incubated with a control rabbit antibody.

Molecular biology. In two animals per group, and in 12 additional rabbits (placebo, $n=6$; VEGF $\times 2=6$) killed at 90 days, PCR and RT-PCR analysis were performed in samples taken from the adductor of the transfected limb and deep-frozen in liquid nitrogen. Besides, in these additional animals, RT-PCR analysis was made on biopsies of the transfected adductor taken at 50 days of follow-up.

PCR: Total DNA was isolated (Qiagen, Hilden, Germany) and quantitated (A260 nm spectrophotometry). Amplification was performed using GeneAmp PCR core kit (Perkin-Elmer, Boston, MA, USA) and specific primers reported earlier.[34]

RT-PCR: Total RNA was extracted (Trizol reagent; Gibco BRL, Grand Island, NY, USA), treated with DNAse I (Promega, Madison, WI, USA), quantitated and reverse-transcribed (random hexamers, Perkin-Elmer). PCRs were carried out using specific primers.

Time course of VEGF gene expression

The 14 rabbits employed for gene expression assessment underwent only the first reoperation (that performed at 7 days after the initial surgery). Twelve of them received p$VEGF_{165}$ and two received placebo. The injection procedure was the same used for the efficacy protocol. The 12 animals receiving p$VEGF_{165}$ were killed at 3, 7, 10, 14, 21 and 35 days after injection ($n=2$ at each time point), and the two placebo animals at 7 days after the injection. Immediately after killing, accomplished through an intravenous overdose of sodium thiopental followed by potassium chloride, samples of the adductor from the transfected limb were obtained and frozen in liquid nitrogen. Total RNA was extracted and subjected to semiquantitative RT-PCR. PCRs were carried out using specific primers and an optimized number of PCR cycles. Ethidium bromide-stained gels were scanned (Fotodyne Incorporated, Hartland, WI, USA) and analyzed using Gel-Pro Analyzer 3.1 software (Media Cybernetics, Bethesda, MD, USA). The ratio of rhVEGF mRNA to GAPDH mRNA was quantified. Results were expressed in relative optical density units by densitometry.

Plasmid construct

The eukaryotic expression vector consisted of a 4.8-kb plasmid that includes the human $VEGF_{165}$ gene, transcriptionally regulated by the cytomegalovirus promoter/enhancer, and a poli-A. An SV40 terminator is located at the 3′-end. Preparation, purification and quality control analyses of the plasmid from transformed *E. coli* cultures were performed under GMP conditions (Bio Sidus, Buenos Aires, Argentina). The purified plasmid was stored in vials at -70 °C.

Statistics

Data were analyzed with one-way analysis of variance, followed by a Newman–Keuls test. Results are expressed as mean ± s.d. Ischemic muscle damage was analyzed using a χ^2 test with Yates correction for discrete variables and expressed as lesion percentage. Differences were considered significant at $P<0.05$.

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