Effect of intramuscular electro-acupuncture-mediated *VEGF* gene transfer on experimental limb ischemia

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Abstract To investigate the feasibility of intramuscular electro-acupuncture-mediated *VEGF* gene transfer on experimental limb ischemia, the transcription and expression of *VEGF* gene have been detected with RT-PCR and immunohistochemistry staining, and the biological effect of *VEGF* gene inside the nat limb has been observed through vascular angiography. Results from angiography show that the transfer of *VEGF* gene into the rat with experimental limb ischemia can stimulate the formation of neovessel, establish collateral circulation, augment blood perfusion, and ameliorate distal limb necrosis. These findings reveal that intramuscular electro-acupuncture-mediated *VEGF* gene transfer may present a new therapeutic modality for treating tissue ischemia.

Keywords: electro-acupuncture, gene transfer, vascular endothelial growth factor, occlusive angitis.

OCCLUSIVE peripheral arterial disease is a refractory disorder, and there is still no efficacious treatment for

this condition. At present, the salvage primarily relies on surgical and percutaneous revascularization techniques. While the high mortality and complications limit the use of them for treating these diseases, many of the patients with limb ischemia have to undergo limb amputation. It is estimated that the onset is 0.1‰—0.2‰ in the youth and 0.5‰—1‰ in the elder in China, 150 000 patients in total require lower limb amputation in the United States, and the prognosis after amputation is often poor[1]. Consequently, the need for alternative treatment strategies in such patients is compelling. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific angiogenic growth factor, 4 different homodimeric species of VEGF have been identified, each monomer having 121, 165, 189 or 206 amino acids, respectively. These 4 forms differ in their solubility characteristics. $VEGF_{121}$ and $VEGF_{165}$ are secreted but are bound to heparin-containing proteoglycans in the cell surface or basement membrane, and thus tend to remain cell associated[2]. All 4 forms of VEGF having mitogenic effect on vascular endothelial cells can stimulate the formation of focal neovessel and increase collateral circulation[3, 4]. Two delivery systems including medical suture[4] and hydrogel polymer balloon[5] have been applied to treating this disorder. Despite the fact that the biological functions of VEGF are relatively clear, both of them have d isadvantages which hinder the clinical application of VEGF. In the present study, a novel method for gene delivery, electro-acupuncture-mediated gene transfer which combined the Chinese traditional acupuncture with modern gene introduction, was developed. With an acupuncture needle carrying exogenous gene into muscle after electronic stimuli, efficient gene delivery was achieved.

1 Materials and methods

(i) Plasmid construction, preparation and purification. Eukaryotic expression vector pcD_2/$VEGF_{121}$ was constructed in our laboratory, including CMV promoter and polyA tail (fig. 1). Plasmid was prepared using alkaline lysis procedure, purified with PEG8000, characterized by restriction enzyme digestion and quantitated by OD 260/280 nm absorption[6].

(ii) Preparation of gene needle. Take the acupuncture needles with a length of 8 cm and diameter of 0.45 mm. Grind the fore 1/3 part of their tips to roughness. Sterilize and dry the needles, stick plasmid DNA to them several times, and blow them to dry for use. Each needle can be loaded with a maximum of 400 μg of plasmid. We termed these needles with exognous genes gene needles or gene electro-needles.

(iii) Animal model. In this study, we used a rat ischemic hindlimb model. All adult Wistar rats weighing 200—250 g, supplied by the Experimental Animal Center of Beijing Medical University, were anesthetized with intraperitoneal pentobarbital. Ischemia was induced in the left hind limb of rats by ligation of the distal extreme of external iliac artery and complete excision of the femoral artery.

(iv) Gene transfer. Gene transfer was performed by gene needle developed in our laboratory previously. A protective sheath was inserted into adductor

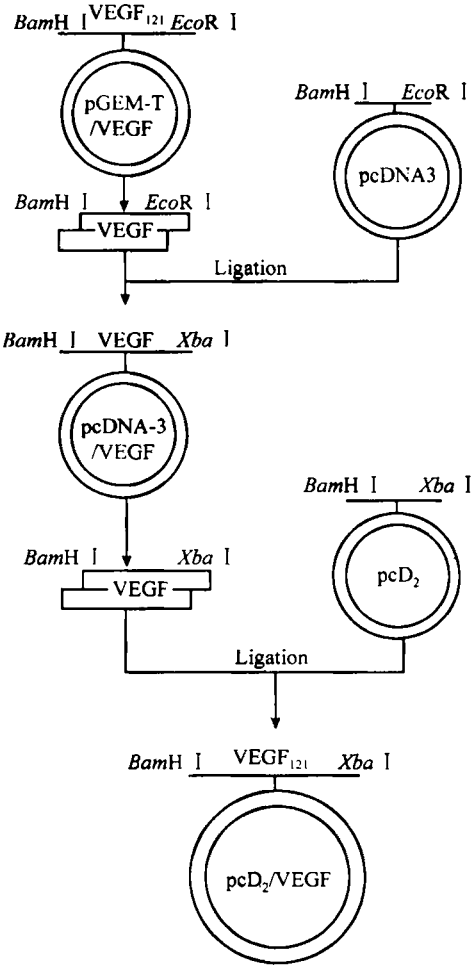

Fig. 1. The diagram of $pcD_2/VEGF_{121}$ construction.

longitudinally at the depth of 1.5 cm before the gene needles carrying pcD_2 as control group or pcD_2/VEGF as experimental group were inserted, the protective sheath then was withdrawn. Gene acupuncture was detained in muscle and then the needle was operated (twist, turn, pull and raise) for 3—5 min. A direct current pulse instrument was connected, with the negative pole to the gene needle and the positive pole to the opposite skin. Stimulating was kept on at the voltage of 0.2 mV, and current of 1 mA for 30 min.

(Ⅴ) Selective angiography. At 0, 7, 14, 28 d post operation a catheter was introduced into the left femoral artery through abdominal aorta. A total of 2 mL of contrast media was injected under pressure at a flow rate of 2 mL/s. Serial images of the ischemic hind limb were then recorded on X-ray films for the analysis of collateral vessel development.

(Ⅵ) Detection of VEGF expression. RT-PCR. At day 7 after therapy, total RNAs were extracted from the transfected muscle and remote tissues including heart, liver, lung and kidney by Trizol kit. RNA concentration was determined spectrometrically at 260 nm. PCR (94℃, 40 s; 55℃, 40 s; 72℃, 1 min; 40 cycles) was done. Primers were 5′-GGGGGATCCGCCTCCG-AAACCATGAACTT-3′ and 5′-CCCGAATTC-TCCTGGTGAGAGATCTGGTT-3′. GAPDH mRNA level served as an internal control for normalization.

Immunohistochemistry. The adductor muscle of the ischemic limb of each rat was harvested at day 7 and day 14 after transfection, fixed with methanol and paraffin embedded. Serial sections were made from each specimen with a cryostome. Cryosections were then developed with ABC-peroxidase kit (Hua Mei Inc.) as described by the manufacturer.

Reagents. All enzyme and nucleotides were from Promega. Immunohisochemical detection system was from Hua Mei Inc. Other reagents were from Sigma Chemical Co. or as listed in the text.

2 Results

(Ⅰ) Expression of *VEGF* gene in skeletal muscle. 7 d after initiation of therapy, total RNAs were prepared from the tissues (skeletal muscle, heart, liver, lung, and kidney) of experimental and control rats by Trizol kit. RT-PCR was then performed on these RNAs. As shown in fig. 2, the transcription of *VEGF* gene in skeletal muscles of ligated groups (lanes 1 and 3) was higher than that of the non-ligated group (lane 2). Among ligated groups, the gene transcription in skeletal muscles of *VEGF* transfected group was significantly higher than that of pcD_2 transfected animals, but the transcription in other tissues showed no significant differences between them.

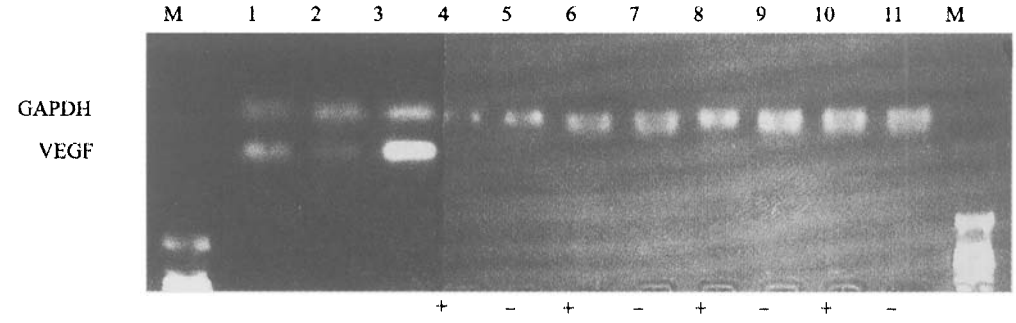

Fig. 2. Expression of $VEGF_{121}$ gene in rat skeletal and other tissues after gene transfer with electric acupuncture *in vivo*. 1, ligation + pcD_2; 2, non-transferred muscle without ischemic induction; 3, ligation + pcD_2/*VEGF*; 4, 5, heart; 6, 7, liver; 8, 9, lung; 10, 11, kidney, + pcD_2/*VEGF*, - pcD_2; M, lamda DNA *Hind* Ⅲ.

To determine if the plasmid in the skeletal muscle was being expressed, immunohistochemical analysis was carried out (fig. 3). There is strong positive yellow-brown peroxidase staining in *VEGF* transfected myocytes. In contrast, no evident *VEGF* immunostaining was seen in transfected myocytes.

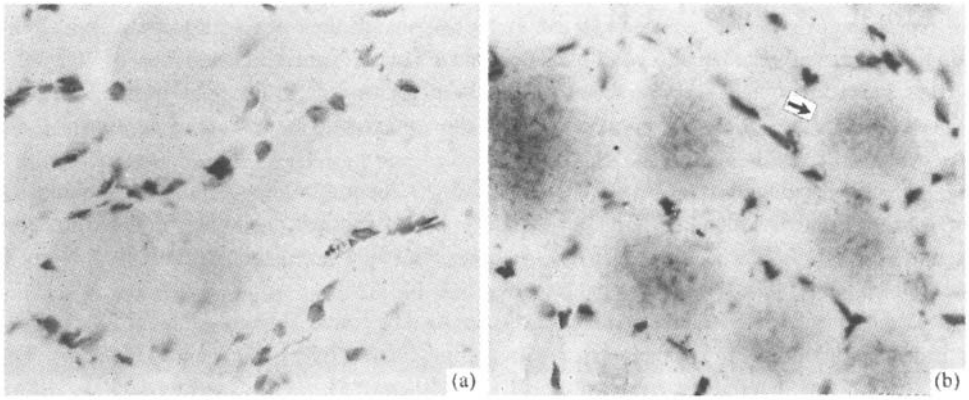

Fig. 3. Immunohistochemical staining of rat skeletal muscle after gene transfer with pcD_2 (a) or pcD_2/*VEGF* (b). The black arrowhead denotes positive staining.

(ii) Assessment of *VEGF* gene on angiogenesis of ischemic limbs. Selective angiograms recorded from both *VEGF*-treated and pcD_2-treated animals are shown in fig. 4. Neovessel calculation of collateral circulation was performed as the method described in ref. [7]. The neovessel of *VEGF* transfected animals improved significantly compared with that of pcD_2 transfected group at the same time (table 1).

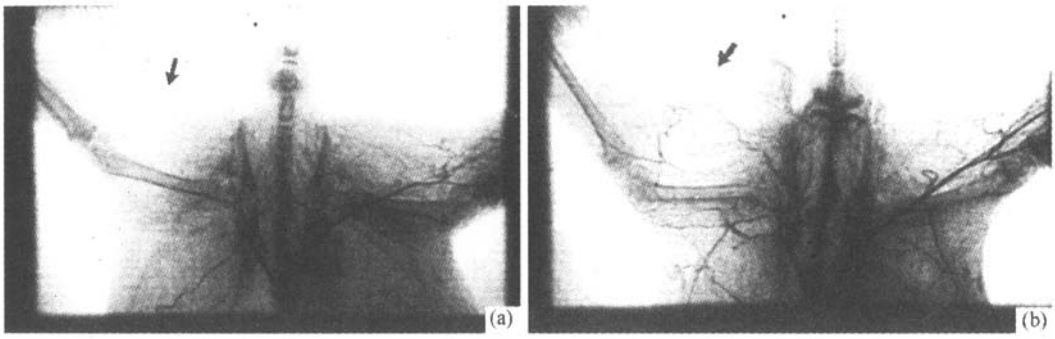

Fig. 4. Effect of $VEGF_{121}$ gene on revascularization and collateral circulation 28 d after gene transfer *in vivo*. (a) pcD_2; (b) *VEGF*. Artery reconstitution in the *VEGF* gene-treated group is readily seen (arrows).

Table 1 Angiographic evidence of angiogenesis ($\bar{x} \pm s$)

Ligation time/d	pcD_2/*VEGF* transfection		pcD_2 transfection	
	Rat number	Neovessel	Rat number	Neovessel
0	10	0	10	0
7	6	$7.33 \pm 0.82^*$	6	3.33 ± 1.03
14	11	$9.73 \pm 1.49^*$	7	4.71 ± 1.38
28	6	$10.57 \pm 1.62^*$	8	6.50 ± 1.78

* $P < 0.01$, t test.

At 7, 14 and 28 d after initiation of therapy, all rats were clinically evaluated for incidence of distal necrosis. Obvious ischemic limb necrosis was observed in 4/21 pcD_2 treated animals, no evidence of ischemic limb necrosis was found in *VEGF* treated animals (figure 5).

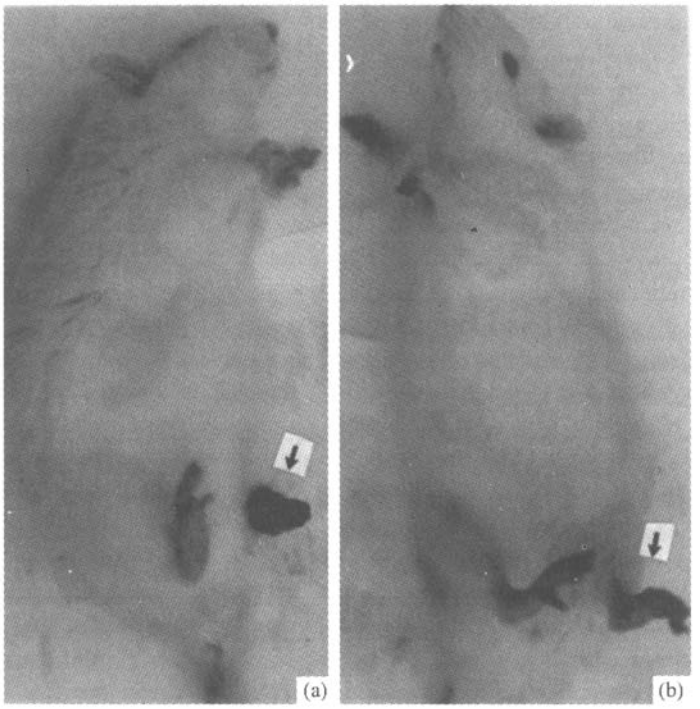

Fig. 5. A representative photograph shows clinical assessment of the incidence of muscle atrophy and distal limb necrosis in the rat ischemic hindlimb after gene transfer. (a) pcD_2; (b) $pcD_2/VEGF$. The black arrows indicate limb necrosis.

3 Discussion

Among the growth factors that promote angiogenesis, *VEGF* is specifically mitogenic on endothelial cells, and the first exon of the *VEGF* gene includes a secretary signal sequence that permits the protein to be secreted naturally from intact cells[2]. Much attention has been paid to *VEGF* in recent years. Evidence that it stimulates neovessel formation and inhibits restenosis *in vivo* has been confirmed in many studies. Directly intramyocardium *VEGF* gene injection enhances the development of small coronary arteries supplying ischemic myocardium, resulting in marked augmentation of maximal collateral blood flow delivery[8]. Artery gene transfer of *VEGF* gene with balloon successfully inhibits the incidence of restenosis by accelerating endothelialization, thereby reducing thrombosis and obstruction due to intimal thickening[9]. Preliminary clinical application of *VEGF* genes to patients with critical ischemia of distal portion of one lower extremity was performed by Isner *et al*.[5] and angiographic and histological evidence of angiogenesis was achieved. The results of the present study document the successful transfer and expression of *VEGF* genes in skeletal muscles of the ischemic limb, with evidence of increased collateral vessel development and consequent amelioration in physiological deficits induced by ischemia.

The gene delivery system is the key factor in gene therapy. We have used medical suture coated by *VEGF* gene as delivering device and produced statistically significant augmentation of angiographically visible collateral vessels[4]. The approach, however, needs skin incision, which easily produces infection and is not convenient for clinical treatment repeatedly. Although the same result can be achieved with hydrogel polymer balloon catheter, it has the problems of requiring complex techniques and expensive device. All these disadvantages limit them being used extensively. As a medium, muscles have many advantages such as easy manipulation, safety, large volume, rich blood supply and immediate access of cell-expressed protein into blood. The plasmid DNA, compared with other vectors, has several advan-

tages. Gene transfer can be repeated without apparent immunologic response to the DNA vector. There is less likelihood of recombination events with the cellular genome, eliminating the risk of the insertional mutagenesis that is associated with the use of viral vectors. However, application of this method has been limited by the relatively low expression level of the transferred gene. Therefore, how to improve the efficiency of intramuscular naked foreign NDA transfer becomes a new research point. Zhang *et al*.[10] demonstrated that the efficiency of electro-acupuncture-mediated LacZ gene transfer is 2.6-fold higher than that in the injection group. Work by Hiroyuki Asihara *et al*.[11] has shown that the transfection efficiency of electroporation is increased from 20- to 100-fold that of intramuscular injection. A survey of non-nuclear membrane-associated DNA binding protein from both skeletal and cardiac muscle revealed several sarcoplasmic reticulum restricted DNA binding species[12], the mechanism for that striated muscle internalizing plasmid DNA is unknown.

The mechanism of electro-acupuncture-mediated gene transfer is unknown. Several factors likely contributed to the success of *VEGF* gene transfer. First, the acupuncture needle is stuck directly into the target cells, it exerts slight local injury and the electric pulse stimuli promote exogenous DNA entering focal myocytes; second, under special electric field, the plasmid with negative charge is repelled into myocytes and dispersed into focal muscles; the third factor is, because the muscle cells are oblong in shape arranged longitudinally, the number of cells between electrodes would be much larger in a transverse orientation than in a longitudinal orientation. Therefore, more cells are likely to be transfected with a transverse orientation[11]. Finally, the ischemic *milieu* of the transfected hindlimb muscle contributes to the success of the therapeutic angiogenesis by stimulating myoblast proliferation and muscle regeneration which promote gene transfection[13].

Intramuscular electro-acupuncture-mediated gene transfer has been proved to be simple and easy, efficient and safe with slight injury, thus suitable for application. However, it should be pointed out that efficiency of gene transfer by electro-acupuncture is affected by many factors, such as preparation of gene needle, amount of genes loaded, preparation of acupuncture, intensity of current and length of time. Therefore, further improvement on this technique should be made.

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