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(54) Title: METHOD FOR TREATING ISCHEMIC TISSUE

(57) Abstract

The present invention provides a method for treating ischemic tissue in a mammal which comprises injecting said tissue with an effective amount of a nucleic acid capable of expressing an angiogenic protein. The method of the present invention may be used to treat any ischemic tissue, i.e., a tissue having a deficiency in blood as the result of an ischemic disease. Such tissues can include, for example, muscle, brain, kidney and lung. Ischemic diseases include, for example, cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

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METHOD FOR TREATING ISCHEMIC TISSUE

FIELD OF THE INVENTION

The present invention is directed to a method for enhancing blood vessel development in ischemic tissues.

BACKGROUND OF THE INVENTION

The therapeutic implications of angiogenic growth factors were first described by Folkman and colleagues over two decades ago (Folkman, *N Engl J Med*, 285:1182-1186 (1971)). Recent investigations have established the feasibility of using recombinant angiogenic growth factors, such as fibroblast growth factor (FGF) family (Yanagisawa-Miwa, et al., *Science*, 257:1401-1403 (1992) and Baffour, et al., *J Vasc Surg*, 16:181-91 (1992)), endothelial cell growth factor (ECGF)(Pu, et al., *J Surg Res*, 54:575-83 (1993)), and more recently, vascular endothelial growth factor (VEGF) to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia (Takeshita, et al., *Circulation*, 90:228-234 (1994) and Takeshita, et al., *J Clin Invest*, 93:662-70 (1994)). In studies with recombinant angiogenic growth factors, intra-muscular administration of the growth factor was repeated over a range of 10 to 14 days. Thus, one major limitation of recombinant protein therapy is its potential requirement to maintain an optimally high and local concentration over time.

Gene delivery systems employed to date have been characterized by two principal components: a macro delivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and microdelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macrodelivery has typically been achieved using one of two catheters

initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., *Lancet* 1:1106-1108 (1989); Wolinsky, et al., *J. Am. Coll. Cardiol.*, 15:475-485 (1990); March et al., *Cardio Intervention*, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., *supra*).

Recently, the feasibility of intra-arterial gene therapy for treatment of ischemia was demonstrated in a rabbit model with VEGF using another gene delivery system, a Hydrogel-coated angioplasty balloon. Successful transfer and sustained expression of the VEGF gene in the vessel wall subsequently augmented neovascularization in the ischemic limb (Takeshita, et al., *Proc Natl Acad Sci USA (In Press)*). However, alternative methods for inducing angiogenesis are still desirable for a number of reasons. First, use of catheter based gene delivery systems may bring out unpredictable abrupt closure or severe damage at the site of ballooning. The consequence may be more serious if the damaged artery is the major donor of the present collaterals or the only patent vessel supplying ischemic tissue. Second, it may be difficult to deliver a catheter to the distal lesion especially in cases of diffuse vascular disease. Finally, despite major advances in both surgical and percutaneous revascularization techniques, limb salvage and relief if ischemic pain cannot be achieved in many patients with diffuse peripheral vascular disease. Isner et al., *Circulation* 88:1534-1557 (1993)).

Striated animal muscle has been shown to take up and express injected foreign marker genes transferred in the form of plasmid DNA (Wolff, et al., *Science*, 247:1465-1468 (1990)). Therapeutic gene transfection in the form of naked plasmid DNA injected directly into muscles has advantages over techniques using viral vectors and catheter based delivery systems. Mainly, it is free from immunological reactions associated with viral proteins (Miller, *Nature*, 357:455-60 (1992)), and avoids possible vascular injuries due to catheter delivery or ballooning procedures. However, direct gene transfer is considered to have insufficient expression to be considered for use in human gene therapy trials (Wolff, et al., *supra*, and Jiao, et al., *Hum Gene Ther*, 3:21-33

(1992)).

SUMMARY OF THE INVENTION

It has now been discovered that surprisingly nucleic acid (DNA or RNA) capable of expressing an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), when injected into ischemic tissue induces angiogenesis, providing the ischemic tissue with increased blood vessels. This allows for the treatment of ischemic tissue associated with ischemic diseases, while avoiding the use of other gene delivery systems.

The present invention provides a method for treating ischemic tissue in a mammal which comprises injecting said tissue with an effective amount of a nucleic acid capable of expressing an angiogenic protein.

The method of the present invention may be used to treat any ischemic tissue, i.e., a tissue having a deficiency in blood as the result of an ischemic disease. Such tissues can include, for example, muscle, brain, kidney and lung. Ischemic diseases include, for example, cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

The ischemic tissue may be injected with the nucleic acid by any injection means. One preferred means is a hypodermic needle. Other means include an externally applied local injection apparatus, such as that used to inject antigens for allergy testing; or a transcutaneous "patch" capable of delivery to subcutaneous muscle. The nucleic acid may be injected at more than one site in the ischemic tissue. If

necessary, the nucleic acid may be reinjected to provide additional expression of the angiogenic protein.

The method of the present invention does not require a microdelivery vehicle such as cationic liposomes and adenoviral vectors, however, the nucleic acid may be carried by such vehicles. Nucleic acid encoding different angiogenic proteins may be used separately or simultaneously.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion that is capable of, directly or indirectly, inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor α and β (TGF-α and TFG-β), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNF-α), hepatocyte growth factor (HGF), insulin like growth factor (IGF), erythropoietin, colony stimulating factor (CSF), macrophage-CSF (M-CSF), granulocyte/macrophage CSF (GM-CSF) and nitric oxidesynthase (NOS). Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein. VEGF is a preferred protein.

The term "effective amount" means a sufficient amount of nucleic acid delivered to the cells of the ischemic tissue to produce an adequate level of the angiogenic protein, i.e., levels capable of inducing angiogenesis. Thus, the important aspect is the level of protein expressed. Accordingly, one can use multiple transcripts or one can have the gene under the control of a promoter that will result in high levels of expression. In an alternative embodiment, the gene would be

under the control of a factor that results in extremely high levels of expression, e.g., *tat* and the corresponding *tar* element.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B sets forth representative angiograms recorded from both control (1B) and VEGF-treated (1A) animals at day 30.

Fig. 2A and 2B illustrates the angiographic score seen in the acute (2A) and chronic ischemia models (2B).

Fig. 3A and 3B illustrates the favorable effect of intramuscular VEGF gene transfer upon revascularization at the capillary level (Fig. 3A VEGF, Fig. 3B control).

Fig. 4A and 4B illustrates capillary density and capillary/myocyte ratio in both the acute ischemia (4A) and chronic model (4B) of limb ischemia.

Fig. 5A and 5B illustrates reduction of the hemodynamic deficit in the ischemic limb in both the acute ischemia (5A) and chronic model (5B) following intramuscular VEGF-transfection as confirmed by measurement of calf blood pressure.

Fig. 6 illustrates the time course of VEGF expression. Lane 1: marker, Lane 2: positive control, Lane 3: nontransfected muscle, Lane 4: on RT, Lanes 5, 6, 7 and 8 are 3,7, 14 and 30 days after transfection, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for treating ischemic tissue in a mammal which comprises injecting said tissue with an effective amount of a nucleic acid encoding an angiogenic protein

operably linked to a promoter (nucleic acid cassette) to result in expression of the protein when delivered to the ischemic tissue. The resulting expression of the angiogenic protein results in increased blood vessel formation throughout the ischemic tissue. The methods of the present invention may be used to treat the ischemic tissue that results from ischemic diseases such as cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

The nucleic acid may be any nucleic acid (DNA or RNA) including genomic DNA, cDNA and mRNA, encoding an angiogenic protein i.e., a protein, polypeptide, mutein or portion that is capable of inducing the formation of new blood vessels. Such proteins include, for example, any protein, polypeptide, mutein or portion thereof that is capable of inducing, either directly or indirectly, the formation of new blood vessels. Folkman, et al., *Science*, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor α and β (TGF-α and TGF-β), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor α (TNF-α), hepatocyte growth factor (HGF), insulin like growth factor (IGF) erythropoietin, colony stimulating factor (CSF), macrophage-CSF (M-CSF), granulocyte/macrophage CSF (GM-CSF) and nitric oxide synthase (NOS). See, Klagsbrun, et al., *Annu. Rev. Physiol.*, 53:217-239 (1991); Folkman, et al., *J. Biol. Chem.*, 267:10931-10934 (1992) and Symeş, et al., *Current Opinion in Lipidology*, 5:305-312 (1994). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., *supra* (bFGF); Pu, et al, *Circulation*, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., *supra* (bFGF); Ferrara, et al., *Biochem. Biophys. Res. Commun.*, 161:851-855 (1989) (VEGF).

In addition, therapeutic angiogenesis has been achieved in the same or closely related models following administration of recombinant endothelial cell growth factor (ECGF) (Pu, et al., *Circulation*, 88:208-215 (1993)) and VEGF (Takeshita, et al., *Circulation*, 90:228-234 (1994) *supra*). Previous studies, employing the animal model of chronic limb ischemia, demonstrated an efficacy of intra-muscular endothelial cell growth factor (ECGF) (Pu, et al., *Circulation*, 88:208-215 (1993)) or VEGF (Takeshita, et al., *Circulation*, 90:228-234 (1994) *supra*) administration.

VEGF was purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, *Science*, 246:1309-1342 (1989) and Connolly, et al., *J. Biol. Chem.*, 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH_2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors *Flt-1* and *Flt-1*/KDR are present on endothelial cells. Ferrara, et al., *supra*, and Conn, et al., *Proc Natl Acad Sci USA*, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been

shown to induce mononuclear phagocyte chemotaxis). Shen, et al., *Blood*, 81:2767-2773 (1993) and Clauss, et al., *J. Exp. Med.*, 172:1535-1545 (1990). The DNA encoding VEGF is disclosed in U.S. Patent No. 5,332,671, the disclosure of which is herein incorporated by reference.

Evidence that VEGF stimulates angiogenesis *in vivo* had been developed in experiments performed on rat and rabbit cornea (Levy, et al., *Growth Factors*, 2:9-19 (1989) and Connolly, et al., *J. Clin. Invest.*, 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., *supra*), and the rabbit bone graft model. Connolly, et al., *J. Clin. Invest.*, 84:1470-1478 (1989) *supra*.

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., *Nature*, 362:844 (1993).

The nucleotide sequence of numerous angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the nucleic acid encoding the protein, the nucleic acid is preferably inserted into a cassette where it is operably linked to a promoter. The promoter must

be capable of driving expression of the protein in cells of the desired target tissue. The selection of appropriate promoters can readily be accomplished. Preferably, one would use a high expression promoter. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. The Rous sarcoma virus (RSV) (Davis, et al., *Hum Gene Ther* 4:151 (1993)) and MMT promoters may also be used. Certain proteins can expressed using their native promoter. Other elements that can enhance expression can also be included such as an enhancer or a system that results in high levels of expression such as a *tat* gene and *tar* element. This cassette can then be inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an *E. coli* origin of replication. See, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the β-lactamase gene for ampicillin resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. The cassette can also be bound to a nucleic acid binding moiety in a synthetic delivery system, such as the system disclosed in WO 95/22618.

If desired, the DNA may also be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, *BioTechniques*, 6:682 (1988). See also, Felgner and Holm, *Bethesda Res. Lab. Focus*, 11(2):21 (1989) and Maurer, R.A., *Bethesda Res. Lab. Focus*, 11(2):25 (1989).

Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al.,

Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricadet, et al., *J. Clin. Invest.*, 90:626-630 (1992); and Rosenfeld, et al., *Cell*, 68:143-155 (1992).

The effective dose of the nucleic acid will be a function of the particular expressed protein, the target tissue, the patient and his or her clinical condition. Effective amount of DNA are between about 1 and 4000μg, more preferably about 1000 and 2000, most preferably between about 2000 and 4000.

In certain situations, it may be desirable to use nucleic acid's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-arginine, fibronectin, urokinase, plasminogen activator and heparin.

In order to facilitate injection, the nucleic is formulated with a pharmaceutically acceptable carrier. Examples of suitable carriers include, saline, albumin, dextrose and sterile water. The nucleic acid is injected into the ischemic tissue using standard injection techniques by use of, for example, a hypodermic needle. Hypodermic needle sizes between no. 29 to no. 16 are preferred.

The nucleic acid may also be injected by an externally applied local injection apparatus, such as that used to inject antigens for allergy testing; or a transcutaneous "patch" capable of delivery to subcutaneous

muscle.

The nucleic acid can be injected at multiple sites throughout the ischemic tissue.

Once injected, the nucleic acid capable of expressing the desired angiogenic protein is taken up and expressed by the cells of the tissue. Because the vectors containing the nucleic acid of interest are not normally incorporated into the genome of the cells, expression of the protein of interest takes place for only a limited time. Typically, the angiogenic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reinjection of the DNA can be utilized to provide additional periods of expression of the angiogenic protein. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

Expression of the angiogenic protein and its secretion from the tissue cells induces angiogenesis, allowing for the treatment of ischemia and thus diseases such as limb ischemia, cerebrovascular ischemia, renal ischemia, pulmonary ischemia, ischemic cardiomyopathy and myocardial ischemia.

All documents mentioned herein are incorporated by reference herein in their entirety.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Induction of Angiogenesis in Ischemic Tissue By Direct Injection of DNA

METHODS

Plasmids

Complementary DNA clones for recombinant human $VEFG_{165}$, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a simple eukaryotic expression plasmid that utilizes 736 bp cytomegalovirus promoter/enhancer to drive VEGF expression. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. Also included in this plasmid is a fragment containing the SV40 origin of replication that includes the 72 bp repeat, but this sequence is not functionally relevant (for autonomous replication) in the absence of SV40 T antigen. These fragments occur in the pUC118 vector which includes an *E. coli* origin of replication and the β-galactosidase gene for ampicillin resistance. The biological activity of $VEGF_{165}$ secreted from cells transfected with this construct ($phVEGF_{165}$) was previously confirmed by evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells (Leung, et al., *Science*, 246:1306-9 (1989)).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted β-galactosidase sequence coupled to the simian virus 40 early promoters (Bonnerot, et al., *Proc Natl Acad Sci, U.S.A.*, 84:6795-9 (1987)) was used for all the control transfection experiments.

Animal model

New Zealand white rabbits with operatively induced unilateral hindlimb vascular insufficiency, (Takeshita, et al., *Circulation*, 90:228-

234 (1994) *supra*; Takeshita, et al., *J. Clin. Invest.* 93:662-70 (1994), *supra*; Pu, et al., *Circulation*, 88:208-215 (1993) *supra*, were used to model both acute and chronic ischemia. All protocols were approved by the Institutional Animal Care and Use Committee. The care of animals complied with the guidelines of the Canadian Council of Animal Care, the Principles of Laboratory Animal Care, and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, revised 1985). Fifty-nine male New Zealand White rabbits (mean weight = 3 kg) were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). Through a longitudinal incision performed in a medial thigh, the femoral artery was dissected free along its entire length, as were all major branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries. After further dissecting the popliteal and saphenous arteries distally, the external iliac artery as well as all of the above arteries were ligated. 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 into the saphenous and popliteal arteries.

Intramuscular (IM) gene transfer.

Acute limb ischemia. Twenty-eight rabbits were used to study the impact of IM gene transfer on acute hindlimb ischemia. Immediately following femoral artery excision as outlined above, five different sites in three major thigh muscles were injected directly with plasmid DNA using a 3 ml syringe and 27-gauge needle advanced through a small skin incision. For each injection, the tip of the needle was inserted into the adductor (2 sites), medial large (2 sites), and semimembranous muscles; care was taken, by directly visualizing each muscle during the injection, to avoid penetrating the muscle with the injectate. To the same end, the rate of injection was in each case slowed to approximately 5 sec so that

injected solution would not leak through the epimysium. This injection technique was used to administer a total volume of 2.5 ml of a) a 500 μg phVEGF$_{165}$ in saline (n = 8; b) 500 μg phVEGF$_{165}$ in 0.75% bupivacaine, previously shown to enhance transgene uptake by striated muscle (n = 10) (Danko I, Gene Therapy, 1:114-21 (1994); or c) 500 μg pGSVLacZ encoding nuclear targeted β-galactosidase (n = 10). After completing 5 injections (0.5 ml @ for each animal), the skin was then closed using 4.0 nylon.

Chronic limb ischemia. Thirty-one rabbits were used to study the effects of IM gene therapy for chronic hindlimb ischemia. The sole distinction between the chronic ischemia model and model of acute limb ischemia described above, is that an interval of 10 days was permitted for post-operative recovery, including development of endogenous collateral vessels. Accordingly, 10 days following femoral artery excision, the rabbits were returned to the catherization laboratory. After completing baseline physiological measurements described below, IM gene transfer using the identical technique described above was performed with a) 500 μg phVEGF$_{165}$ diluted in 2.5 ml of saline (n = 8); b) 500 μg phVEGF$_{165}$ diluted in 0.75% bupivacaine (n = 8); c) 500 μg of pGSVLacZ diluted in 2.5 ml of saline; or d) 500 μg of pGSVLacZ diluted in 2.5 ml of 0.75% bupivacaine (n = 8). In each case, after completing 5 all injections, the skin was closed as above.

Anatomic Assessment.

Selective angiography. Selective internal iliac arteriography was performed as previously described (Doucette, et al., *Circulation*, 85:1899-1911 (1992)). Briefly, a 3 Fr. infusion catheter (Tracker-18, Target Therapeutic, San Jose CA) was introduced into the common carotid artery and advanced to the internal iliac artery of the ischemic

limb using 0.014 in. guidewire (Hi-Torque Floppy II, Advanced Cardiac System, San Diego, CA) under fluoroscopic guidance. The tip of catheter was positioned in the internal iliac artery at the level of the interspace between the seventh lumber and the first sacral vertebrae. Following intraarterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, IL), a total of 5 ml of non-ionic contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, NJ) was then injected using an automated angiographic injector (Medrad, Pittsburgh, PA) programmed to reproducibly deliver a flow rate of 1 ml/sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film/sec for 10 sec.

Morphometric angiographic analysis of collateral vessel development was performed using a grid overlay comprised of 2.5 mm-diameter circles arranged in rows spaced 5 mm apart. This overlay was placed over the 4-sec angiogram recorded at the level of the medial thigh. A defined area was chosen in which the number of contrast-opacified arteries crossing over circles as well as the total number of circles encompassing the medial thigh area were counted in single blind fashion. An angiographic score was calculated for each film as the ratio of crossing opacified arteries divided by the total number of circles in the defined area of the ischemic thigh.

Capillary Density and Capillary/Myocyte Ratio. Anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs (Takeshita, et al., *J. Clin. Invest.*, 93:662-70 (1994) *supra*). Tissue specimens were obtained as transverse sections from the adductor muscle, the major muscle of the medial thigh, of the ischemic limb at the time of sacrifice (day 30). Muscle samples

were embedded in O.C.T. compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 μm in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion. Tissue sections were stained for alkaline phosphatase using an indoxyl-tetrazolium method to detect capillary endothelial cells as previously described, and were then counterstained with eosin. A total of 20 different fields from one muscle section were randomly selected, and the number of capillaries was counted under a 20X objective to determine the capillary density (mean number of capillaries/mm^2). To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of muscle fibers in the histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Physiologic Assessment.

Calf blood pressure. Calf blood pressure was measured using a Doppler Flowmeter (Model 1059, Parks Medical Electronics, Aloha, OR). The pulse of the posterior tibial artery was identified using a Doppler probe, and the systolic blood pressure in both limbs was determined using standard techniques (Takeshita, et al., *J. Clin Invest*, 93:662-70 (1994)). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to that of the normal limb.

Intra-arterial Doppler guidewire measurement of flow velocity. Intra-arterial Doppler assessment was also performed on day 0 and 30 before selective internal iliac angiography. The tip of the 3 Fr. infusion catheter was positioned 2 cm above the aortic bifurcation. A total of 5 ml of non-ionic contrast media (Isovue-370, Squibb Diagnostics, New

Brunswick, NJ) was injected using an automated angiographic injector (Medrad, Pittsburgh, PA) at a rate of 1 ml/sec. Serial images of the aorta-iliac bifurcation were then recorded on 105-mm spot film at a rate of 1 film/sec for 5 sec. The 0.018 in. Doppler guidewire (Cardiometrics, Inc., Mountain View, CA) was then used to measure blood flow velocity as previously described (Doucette, et al., *supra*). The wire was advanced via the 3 Fr. infusion catheter positioned at the origin of the common iliac artery, to the proximal segment of the internal iliac artery supplying the ischemic limb. The Doppler wire records a real-time, spectral analysis of the Doppler signal, from which the average peak velocity (APV, the temporal average of the instanteous peak velocity waveform) is calculated and displayed on line. We required a stabilized velocity for 2 min before recording resting APV. Maximum APV was recorded after bolus injection of papaverine (Sigma, St. Louis, MO), 2 mg in 0.4 ml saline, via the infusion catheter. The Doppler wire was then pulled back from the internal iliac artery and readvanced to the common iliac artery of the normal limb; the distal tip of the 3 Fr. infusion catheter was repositioned at the origin of the common iliac artery. Blood flow velocity was again recorded at rest and after papaverine injection. After completing all Doppler measurements, the 3 Fr. infusion catheter was redirected to the promixal segment of the internal iliac artery of the ischemic limb, and selective internal iliac angiography was performed as described above.

Quantitative Analysis of Angiography and Flow Calculation. The angiographic luminal diameter of the internal iliac artery in the ischemic limb and of the external iliac artery in the normal limb were determined using an automated edge-detection system (Quantum 2000I; QCS, Ann Arbor, MI). The film selected for analysis was scanned with a high resolution video camera; the signal produced by the video camera was

digitized and displayed on a video monitor (Laser Scan; ImageComm, Santa Clara, CA). Center-lines were traced manually for a 10-mm long segment beginning immediately distal to the tip of the Doppler wire. The contours were subsequently detected automatically on the basis of the weighted sum of first and second derivative functions applied to the digitized brightness information. The vascular diameter was then measured at the site of the Doppler sample volume (5 mm distal to the wire tip). Cross-sectional area was calculated assuming a circular lumen.

Doppler-derived flow was calculated as $Q_D = (\pi d^2 / 4)(0.5 \times APV)$ where Q_D = Doppler-derived time average flow, d = vessel diameter, and APV = time average of the spectral peak velocity. The mean velocity was estimated as 0.5 x APV by assuming a time-averaged parabolic velocity profile across the vessel. The Doppler-derived flow calculated in this fashion has been shown to correlate with flow measurements determined by electromagnetic flow meters both *in vitro* and *in vivo* (Doucette, et al., *supra*). As 2 mg of papaverine had no effect on vessel diameter, we thus used the diameter measurements from the angiogram recorded immediately before the Doppler measurements for the calculation of both rest and maximum flow.

Regional Blood Flow to Limb Muscle. Regional tissue perfusion of animals with chronic hindlimb ischemia was determined using colored microspheres, 15μm in diameter (Kowalik, *Circulation* 83, 974-82 (1991). After the completion of the invasive measurements described above, $3X10^6$ of Dye-Trak colored microspheres (Triton Technology, Inc., San Diego, CA) were injected through a 3 Fr. Teflon catheter into the left ventricle via the common carotid artery. To collect blood samples for a reference flow, a second catheter was inserted into lower abdominal aorta via the common carotid artery and connected to a syringe

withdrawal pump (Sage 351, Orion Research, Boston, MA). Through this catheter, the blood sample was withdrawn 10 sec prior to the microsphere injection over 3 min at a rate of 1.2 ml/min. The animals were then sacrificed and tissue samples (weight = 2g) of 2 different muscles (transfected medial thigh (adductor) muscle, and lower limb (gastrocnemius) muscle) of each hindlimb (ischemic and non-ischemic) were retrieved. The tissue samples and reference blood samples were digested with potassium hydroxide, following which microspheres were retrieved by vacuum filtering. After dyes were extracted from microspheres using dimethyl-formamide, the photometric absorption of each sample was determined using a conventional spectrophotometer (Model 8452A, Hewlett Packard, Palo Alto, CA.) Regional blood flow to muscle was calculated as follows:

Tissue blood flow = (withdrawal rate / tissue weight) X (ODtissue / ODreference blood), where OD = optical density.

VEGF Gene Expression in Skeletal Muscle.

To evaluate expression of phVEGF$_{165}$ gene in skeletal muscle, sixteen additional male New Zealand white rabbits from both acute and chronic ischemia models (2 rabbits at each time point) were sacrificed at 3, 7, 14 and 30 days post-transfection. The presence of human VEGF mRNA was detected using reverse transcription-polymerase chain reaction (RT-PCR) as previously described (Takeshita, et al., *Proc Natl Acad Sci (In press), supra*). To ensure specificity and avoid amplification of endogenous rabbit VEGF, primers were selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense)(SEQ ID NO:1); 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense)(SEQ ID NO:2). RT-PCR products were analyzed by 2% agarose gel electrophoresis. DNA bands were visualized under UV illumination after staining with ethidium

bromide.

Statistical analysis.

Results were expressed as mean ± standard deviation (SD). Statistical significance was evaluated using ANOVA followed by Scheffe's test. A value of $p<0.05$ was interpreted to denote statistical significance.

RESULTS

Anatomic Assessment.

Angiography. Representative angiograms recorded from both control and VEGF-treated animals at day 30 are illustrated in Fig. 1. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiograms recorded at days 0 and 30. In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 0 and 30. As illustrated in Fig. 2, at baseline (day 0), there were no significant differences in angiographic score among groups (C I: 0.47 ± 0.10, C II: 0.44 ± 0.10, C III: 0.43 ± 0.06, C IV: 0.42 ± 0.10). By day 30, however, the angiographic score of each VEGF-transfected group was significantly improved compared to that of control (C I: 0.76 ± 0.05, C II: 0.72 ± 0.05, C III: 0.52 ± 0.06, C IV: 0.58 ± 0.09, $p<.01$) as well as the acute model of limb ischemia (A I: 0.72 ± 0.06, A II: 0.71 ± 0.03, A III 0.48 ± 0.10, $p<.01$). Administration of bupivacaine had no observable effect.

Capillary density and capillary/myocyte ratio. A favorable effect of intramuscular VEGF gene transfer upon revascularization was also apparent at the capillary level (Fig. 3A and 3B). The adductor muscle of ischemic limbs was histologically examined at day 30 in both models.

As illustrated in Fig. 4, VEGF-transfected animals had more capillaries around muscle fibers, in acute ischemia (A I: 248 ± 37, A II: 228 ± 22, A III: 188 ± 32/mm^2, $p < .01$) as well as the chronic model of limb ischemia (C I: 259 ± 24, C II: 256 ± 31, C III: 197 ± 18, C IV: 192 ± 31 /mm^2, $p < .01$). Analysis of capillary/myocyte ratio was also higher in VEGF-transfected animals in both the acute and chronic of hindlimb ischemia (acute model: A I: 0.73 ± 0.07, A II: 0.70 ± 0.06, A III: 0.61 ± 0.09, $p < .01$; chronic model: C I: 0.78 ± 0.08, C II: 0.76 ± 0.05, C III: 0.51 ± 0.06, C IV: 0.55 ± 0.09, $p < .01$). No differences were observed between animals receiving bupivacaine versus those injected with saline.

Physiologic Assessment.

Calf Blood Pressure. Reduction of the hemodynamic deficit in the ischemic limb following intramuscular VEGF-transfection was confirmed by measurement of calf blood pressure. As illustrated in Fig. 5, in animals with acute ischemia, the blood pressure ratio measured on day 30 post-transfection was significantly higher in VEGF-transfected groups than controls (A I: 0.80 ± 0.09; A II: 0.76 ± 0.11; A III: 0.56 ± 0.10, $p < .01$). There was no difference between bupivacaine and saline treated animals. In chronic model, 10 days after induction of ischemia (immediately prior to transfection), the calf blood pressure ratio was virtually identical in all groups (C I: 0.36 ± 0.05; C II: 0.36 ± 0.04; C III: 0.36 ± 0.05; C IV: 0.32 ± 0.03). By day 30 post-transfection, the blood pressure ratio for the VEGF-transfected groups was significantly higher than for the control (C I: 0.84 ± 0.09; C II: 0.82 ± 0.06; C III: 0.67 ± 0.06; C IV: 0.66 ± 0.10, $p < .01$). There was no difference between bupivacaine and saline treated animals.

Intro-arterial Doppler guidewire measurement (**Table 1**). In acute ischemia model, VEGF-transfected animals revealed significantly higher

flow to the ischemic limb at rest (A I: 21.6±5.2, A II: 19.5±3.2, A III: 13.9±3.7 ml/min. $p<.01$) and at papaverine induced hyperemic status (A I: 59.1±16.9, A II: 55.2±5.1, A III: 39.0±11.5, ml/min, $p<.01$). In chronic model, the rest blood flow to ischemic limb as well as papaverine stimulated flow to ischemic limb were identical in all groups at day 0 (rest flow; C I: 13.7±1.5; C II: 15.5±1.4; C III: 13.7±2.2; C IV: 13.4±1.9 ml/min, hyperemic flow; C I: 28.9±3.6, C II: 30.6±3.0, C III: 31.3±3.7, C IV: 28.1±1.7 ml/min). On day 30, VEGF transfected animals revealed significantly higher flow to the ischemic limb at rest (C I:22.7±4.6, C II: 19.9±2.8, C III: 14.9±1.7, C IV: 14.1±1.5 ml/min, $p<.01$) and at papaverine induced hyperemic status (C I: 52.5±12.6, C II: 56.0±12.0, C III: 38.4±4.3, C IV: 35.8±5.6 ml/min, $p<.05$). The baseline and hyperemic flow to the non-ischemic limb was identical in all groups t day 0 as well as at day 30.

TABLE 1 Blood flow to ischemic limb

	acute ischemia model	
	baseline	hyperemic
VEGF	21.6 ± 5.2*	59.1 ± 16.9*
VEGF + Bup	19.5 ± 3.2*	55.2 ± 5.1**
β-gal	13.2 ± 3.7	39.0 ± 11.5
β-gal + Bup	NA	NA

	chronic ischemia model			
	day 0		day 30	
	baseline	hyperemic	baseline	hyperemic
VEGF	13.7 ± 1.5	28.9 ± 3.6	22.7 ± 4.6*	52.5 ± 12.6**
VEGF + Bup	15.5 ± 1.4	30.6 ± 3.0	19.9 ± 2.8*	56.0 ± 12.0**
β-gal	13.7 ± 2.2	31.3 ± 3.7	14.9 ± 1.7	38.4 ± 4.3
β-gal + Bup	13.4 ± 1.9	28.1 ± 1.7	14.1 ± 1.5	35.8 ± 5.6

values are described as means ± SD (ml/min)
* $p<0.1$, ** $p<.05$ vs. β-gal or β-gal + Bup by ANOVA

Regional Blood Flow to Limb Muscle (**Table 2**). Regional blood flow to ischemic limb muscles was analyzed using colored microsphere technique in chronic ischemia model. Regional blood flow at adductor muscle, transfected medial thigh muscle (C I: 4.3±0.5, C II: 4.6±1.2, C III: 2.9±0.6, C IV: 3.1±0.4 ml/min/100g tissue, $p<.05$), as well as distal lower limb muscle, gastrocnemius (C I: 3.9±0.8, C II: 4.2±0.7, C III: 2.8±0.9, C IV: 2.6±0.7, ml/min/100g tissue, $p<.05$) was 1.5 folds greater in VEGF transfected animals. No significant differences owing to injected solution were observed. There were no differences in the regional flow to the non-ischemic muscles in all groups (flow to adductor: C I: 5.2±0.5, C II: 5.6±1.0, C III: 4.9±0.6, C IV: 5.6±1.0 ml/min/100g tissue, flow to gastrocnemius: C I: 4.4±1.0, C II: 4.7±1.0; C III: 4.6±1.2, C IV: 5.0±1.1 ml/min/100g tissue).

TABLE 2 Regional blood flow to limb muscle

chronic ischemia model

	adductor		gastrocnemius	
	ischemic	normal	ischemic	normal
VEGF	4.3±0.5*	5.2±0.5	3.9±0.8*	4.4±1.0
VEGF+Bup	4.6±1.2*	5.6±1.0	4.2±0.7*	4.7±1.0
β-gal	2.9±0.6	4.9±0.6	2.8±0.9	4.6±1.2
β-gal+Bup	3.1±0.4	5.6±1.0	2.6±0.7	5.0±1.1

values are described as means±SD (ml/min/100g tissue)
* $p<0.5$ vs. β-gal or β-gal+Bup by ANOVA

Human VEGF Gene Expression in Muscle.

To confirm expression of *human* VEGF gene in transfected *rabbit* limb muscle *in vivo*, we analyzed transfected arteries for the presence of *human* VEGF mRNA by RT-PCR. As indicated above, to ensure the

specificity of RT-PCR for *human* VEGF mRNA resulting from successful transfection (versus endogenous *rabbit* VEGF mRNA), primers employed were selected from a region which is not conserved among different specie. Adductor muscles were harvested 3, 7, 14 and 30 days after VEGF gene injection. The presence of *human* VEGF mRNA was readily detected adductor muscles with phVEGF$_{165}$ from day 3 to day 14 in both acute and chronic models. Rabbit adductor muscles injected with pGSVLacZ gene were negative for *human* VEGF mRNA (Figure 6).

We have demonstrated that a gene encoding an angiogenic protein can be successfully transferred into ischemic muscle where the gene is expressed and induces angiogenesis, providing the ischemic tissue with an increase in blood vessels.

What is claimed is:

1. A kit for use in the treatment of ischemic tissue comprising a pharmaceutical composition comprising a nucleic acid capable of expressing an angiogenic protein having a secretory signal sequence in solution in a pharmaceutically acceptable injectable carrier and a syringe.

2. The kit of claim 1, wherein the nucleic acid is DNA.

3. The kit of claim 1, wherein the angiogenic protein is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β, platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α, hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, granulocyte/macrophage CSF and nitric oxidesynthase.

4. The kit of claim 1, wherein the angiogenic protein is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β, platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α, hepatocyte growth factor, insulin like growth factor, and nitric oxidesynthase.

5. The kit of claim 3, wherein said angiogenic protein is vascular endothelial growth factor.

6. The kit of claim 2, wherein the DNA is carried by a cationic liposome.

7. Use of a nucleic acid capable of expressing an angiogenic protein having a secretory signal sequence in the preparation of a pharmaceutical composition for injection into ischemic tissue to cause new blood vessel development in said tissue.

8. Use according to claim 7, wherein said angiogenic protein is vascular endothelial growth factor and said pharmaceutical composition is for the treatment of limb ischemia.

9. A pharmaceutical composition for the treatment of ischemic tissue, comprising a nucleic acid capable of expressing an angiogenic protein having a secretory signal sequence in solution in a pharmaceutically acceptable injectable carrier.

10. The composition of claim 9, wherein the nucleic acid is DNA.

11. The composition of claim 9, wherein the angiogenic protein is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β, platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α, hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, granulocyte/macrophage CSF and nitric oxidesynthase.

12. The composition of claim 9, wherein the angiogenic protein is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β, platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α,

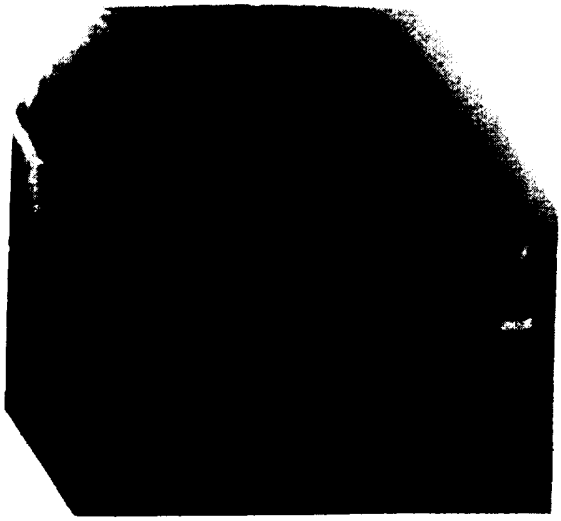
hepatocyte growth factor, insulin like growth factor and nitric oxidesynthase.

13. The composition of claim 12, wherein said angiogenic protein is vascular endothelial growth factor.

FIG. 1A



FIG. 1B



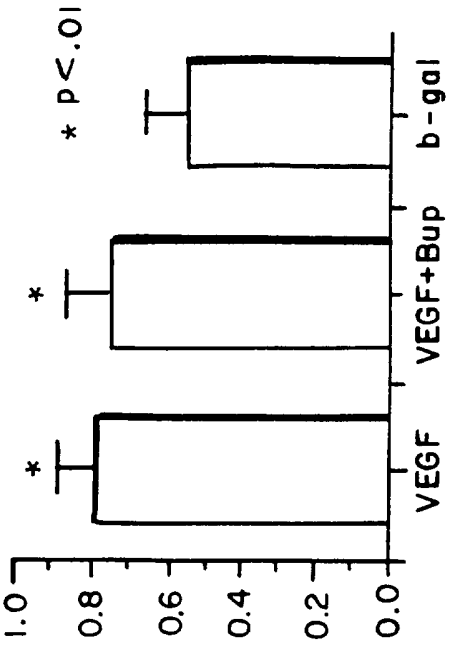

1.0
0.8
0.6
0.4
0.2
0.0
*
*
* p<.01
VEGF
VEGF+Bup
b-gal

FIG. 2A

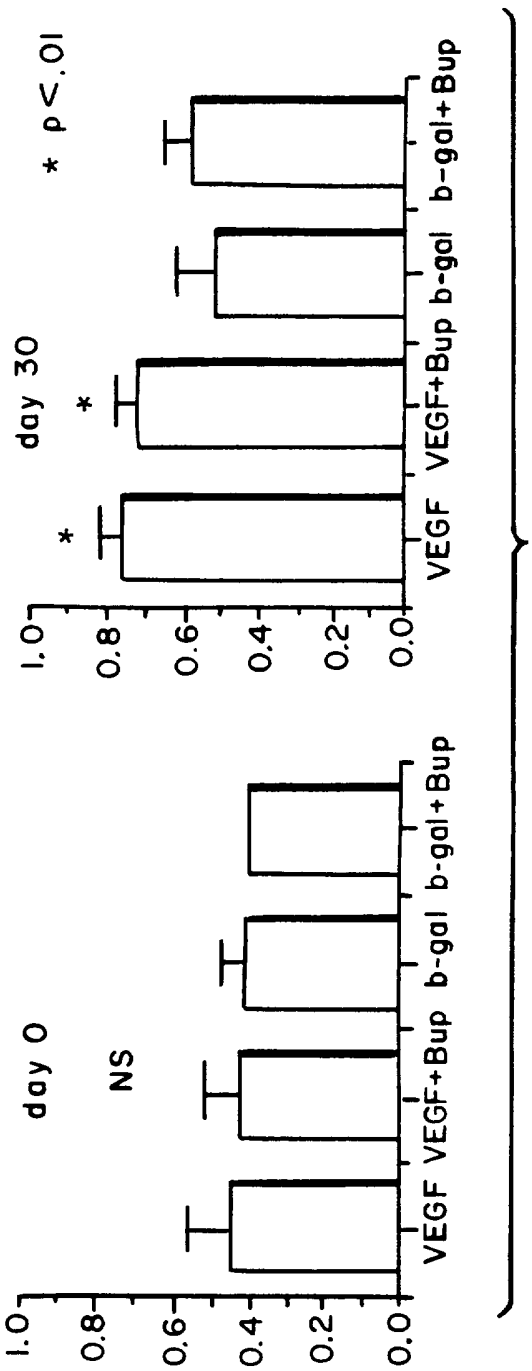

day 0
NS
day 30
*
*
* p<.01
1.0
0.8
0.6
0.4
0.2
0.0
VEGF
VEGF+Bup
b-gal
b-gal+Bup

FIG. 2B

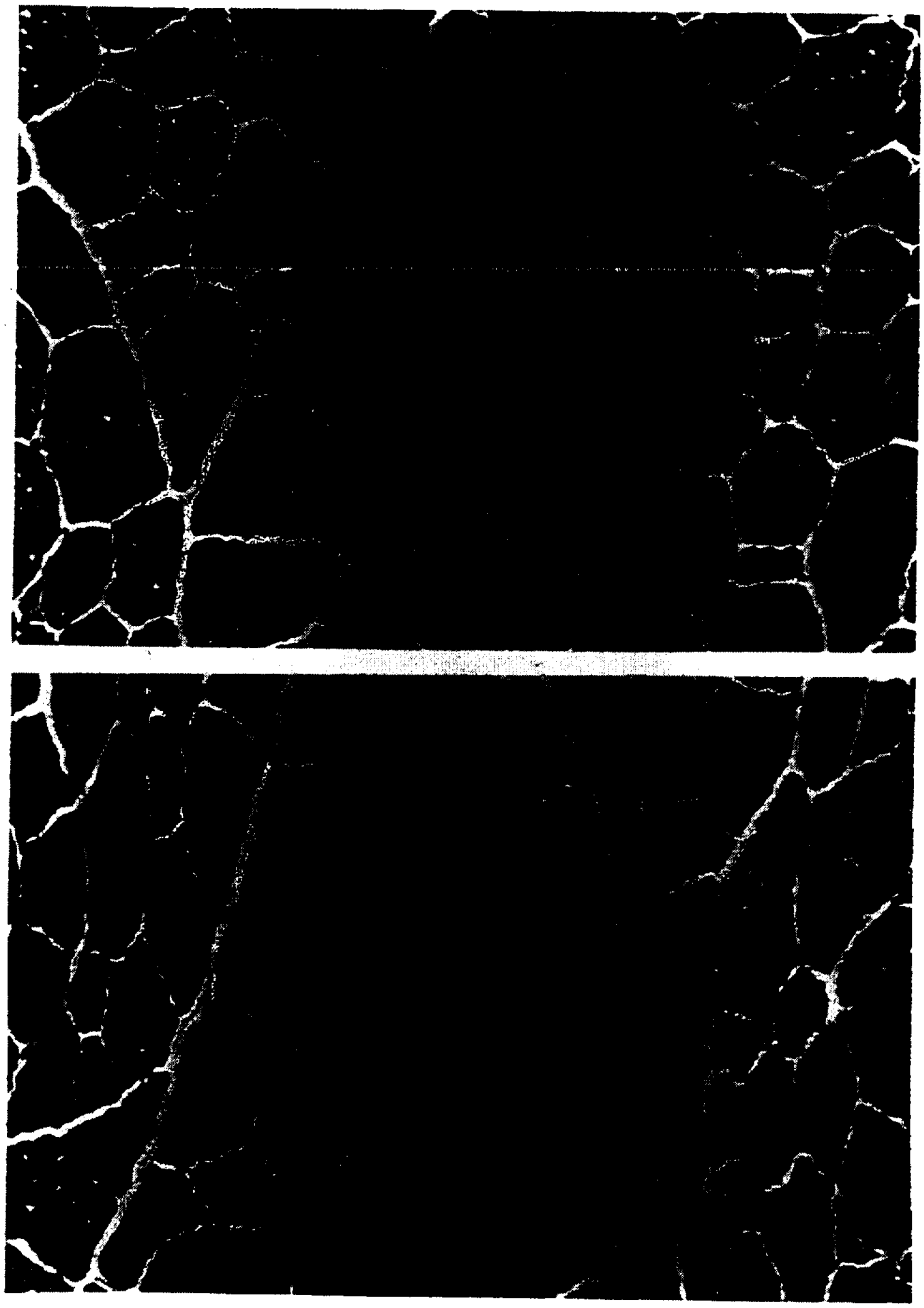


FIG. 3B

FIG. 3A

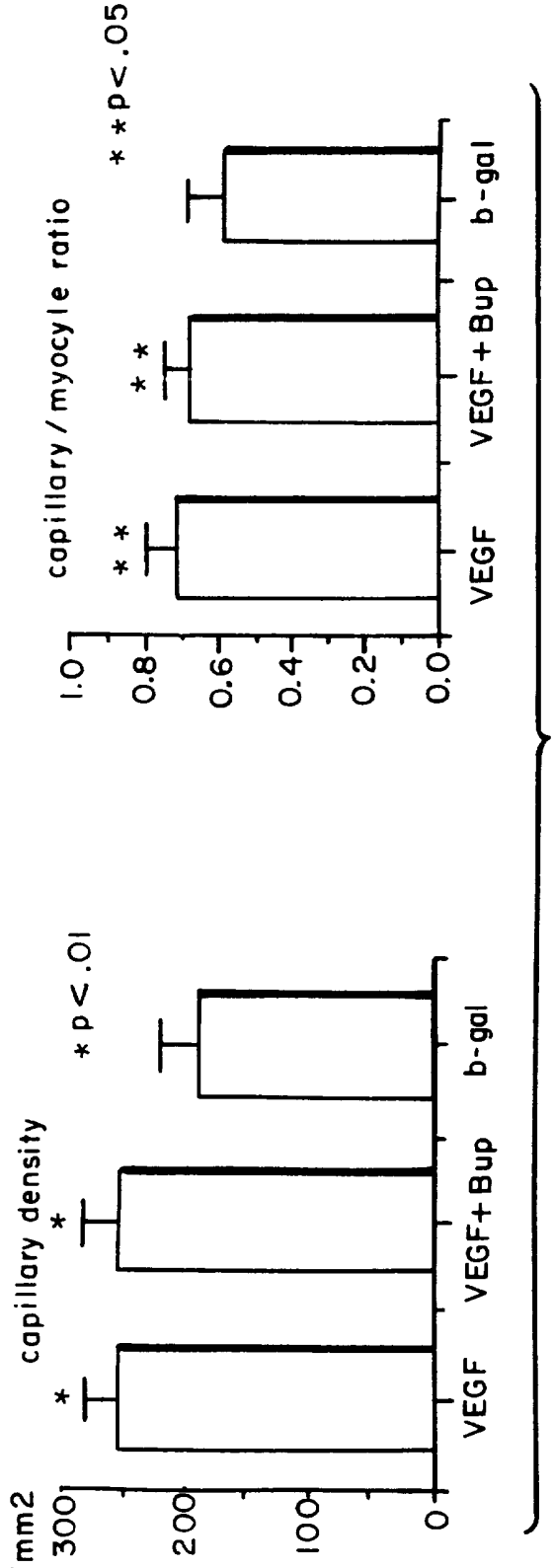

capillary density
/mm2
300
200
100
0
*
*
* p < .01
VEGF
VEGF+Bup
b-gal
capillary / myocyle ratio
1.0
0.8
0.6
0.4
0.2
0.0
* *
* *
* * p < .05
VEGF
VEGF+Bup
b-gal

FIG.4A

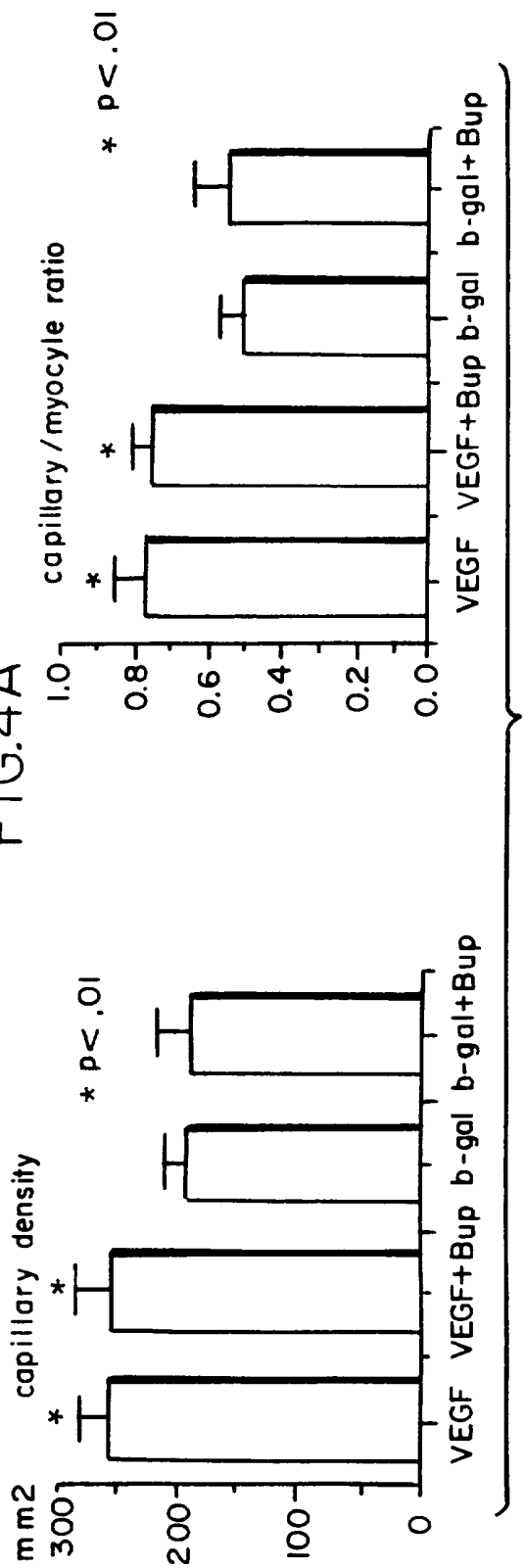

capillary density
/mm2
300
200
100
0
*
*
* p < .01
VEGF
VEGF+Bup
b-gal
b-gal+Bup
capillary / myocyle ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
*
* p < .01
VEGF
VEGF+Bup
b-gal
b-gal+Bup

FIG.4B

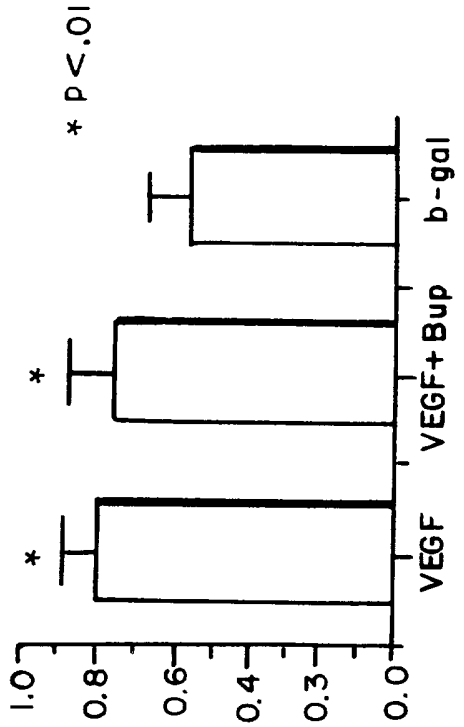

* p<.01
*
*
1.0
0.8
0.6
0.4
0.3
0.0
VEGF
VEGF+Bup
b-gal

FIG. 5A

* p<.01
* day 30
*
day 0
NS
1.0
0.8
0.6
0.4
0.2
0.0
VEGF
VEGF+Bup
b-gal
b-gal+Bup

FIG. 5B



FIG. 6

INTERNATIONAL SEARCH REPORT

Inte. ..ional application No.
PCT/US96/16723

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04; A61K 31/70
US CL :514/44; 435/ 69.1, 69.6, 69.8, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/ 69.1, 69.6, 69.8, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS, USPATFULL, APS
search terms: ischemic tissue, angiogenic protein, vascular endothelial growth factor, VEGF, fibroblast growth factor, FGF

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FELDMAN et al. Prevention of restenosis after coronary angioplasty: towards a molecular approach? Fundamentals of Clinical Pharmacology. 1995, Vol. 9, pages 8-16, especially pages 12-13.	1-13
A, P	SIMONS et al. Food for starving hearts. Nature Medicine. May 1996, Vol. 2, No. 5, pages 519-520, especially page 519.	1-13
A	ISNER et al. Arterial gene therapy for therapeutic angiogenesis in patients with peripheral artery disease. Circulation. 01 June 1995, Vol. 91, No. 11, pages 2687-2692, especially page 2691.	1-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 NOVEMBER 1996	Date of mailing of the international search report 02 DEC 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JILL SCHMUCK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inter. ..onal application No.
PCT/US96/16723

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MULLIGAN. The basic science of gene therapy. Science. 14 May 1993, Vol. 260, pages 926-930, especially page 928.	1-13
A	AFIONE et al. Gene therapy vectors as drug delivery systems. Clinical Pharmacokinetics. 1995, Vol. 28, No. 3, pages 181-189, especially page 185.	1-13
A	RIESSEN et al. Prospects for site-specific delivery of pharmacologic and molecular therapies. Journal of the American College of Cardiology. April 1994, Vol. 23, No. 5, pages 1234-1244, especially page 1238.	1-13
A	MARSHALL. Gene therapy's growing pains. Science. 25 August 1995, Vol. 269, No. 2005, pages 1050-1055, especially page 1050.	1-13
A, P	COGHLAN. Gene dream fades away. Focus. 25 November 1995, Vol. 148, pages 14-15.	1-13
A, P	BROWN. News Media, Researchers 'Oversold' Gene Therapy, Advisory Panel Says. The Washington Post. 08 December 1995, A1, see entire article.	1-13
X	SETOGUCHI et al. Stimulation of erythropoiesis by in vivo gene therapy: Physiologic Consequences of Transfer of the Human Erythropoietin Gene to Experimental Animals Using an Adenovirus Vector. Blood. 01 November 1994, Vol. 84, pages 2946-2953, especially page 2948.	1-3
X	NABEL et al. Recombinant fibroblast growth factor-1 promotes intimal hyperplasia and angiogenesis in arteries *in vivo*. Nature. 29 April 1993, Vol. 362, pages 844-846, especially page 844.	1-4, 7, 9-12
Y	TAKESHITA et al. *In vivo* evidence of enhanced angiogenesis following direct arterial gene transfer of the plasmid encoding vascular endothelial growth factor. Circulation. October 1993, Vol. 88, No. 4, Part 2, page I-476, column 2, abstract no. 2565, see entire abstract.	1-13
Y	PU et al. Enhanced revascularization of the ischemic limb by angiogenic therapy. Circulation. July 1993, Vol. 88, No. 1, pages 208-215, especially page 575.	1-13