Adenovirus-Mediated VEGF121 Gene Transfer Stimulates Angiogenesis in Normoperfused Skeletal Muscle and Preserves Tissue Perfusion After Induction of Ischemia

Luis Henrique W. Gowdak, MD; Lioubov Poliakova, MD; Xiaotong Wang, MD; Imre Kovess, PhD; Kenneth W. Fishbein, PhD; Antonella Zacheo, BS; Roberta Palumbo, PhD; Stefania Straino, BS; Costanza Emanueli, PhD; Massimiliano Marrocco-Trischitta, MD; Edward G. Lakatta, MD; Piero Anversa, MD; Richard G.S. Spencer, MD, PhD; Mark Talan, MD, PhD; Maurizio C. Capogrossi, MD

Background—Administration of angiogenic factors stimulates neovascularization in ischemic tissues. However, there is no evidence that angiogenesis can be induced in normoperfused skeletal muscles. We tested the hypothesis that adenovirus-mediated intramuscular (IM) gene transfer of the 121-amino-acid form of vascular endothelial growth factor (AdCMV.VEGF121) could stimulate neovascularization in nonischemic skeletal muscle and consequently attenuate the hemodynamic deficit secondary to surgically induced ischemia.

Methods and Results—Rabbits and rats received IM injections of AdCMV.VEGF121, AdCMV.Null, or saline in the thigh, 4 weeks (rabbits) or 2 weeks (rats) before femoral artery removal in the injected limb. In unoperated rats, at the site of injection of AdCMV.VEGF121, we found 96% and 29% increases in length density of arterioles and capillaries, respectively. Increased tissue perfusion (TP) to the ischemic limb in the AdCMV.VEGF121 group was documented, as early as day 1 after surgery, by improved blood flow to the ischemic gastrocnemius muscle measured by radioactive microspheres (AdCMV.VEGF121 = 5.69 ± 0.40, AdCMV.Null = 2.97 ± 0.50, and saline = 2.78 ± 0.43 mL · min⁻¹ · 100 g⁻¹, P < 0.001), more angiographically recognizable collateral vessels (angioscore) (AdCMV.VEGF121 = 50.58 ± 1.48, AdCMV.Null = 29.08 ± 4.22, and saline = 11.83 ± 1.90, P < 0.0001), and improvement of the bioenergetic reserve of the gastrocnemius muscle as assessed by 31P NMR spectroscopy. Follow-up studies showed that superior TP to the ischemic limb in the AdCMV.VEGF121 group persisted until it was equalized by spontaneous collateral vessel development in untreated animals.

Conclusions—IM administration of AdCMV.VEGF121 stimulates angiogenesis in normoperfused skeletal muscles, and the newly formed vessels preserve TP after induction of ischemia. (Circulation. 2000;102:565-571.)

Key Words: angiogenesis ■ endothelium-derived factors ■ genes ■ ischemia ■ peripheral vascular disease

Treatment of patients suffering from peripheral arterial occlusive disease remains a considerable clinical challenge despite advances in both surgical and percutaneous revascularization techniques. Many patients cannot benefit from these therapies because of the anatomic extent and the distribution of arterial occlusion. In such patients, new therapeutic strategies have been sought to prevent the development of disabling symptoms related to ischemia, eg, claudication, resting pain, and loss of tissue integrity in the distal limbs.

Recently, therapeutic angiogenesis has been successfully applied in animal models of limb or myocardial ischemia. Phase 1 clinical trials of angiogenesis are under way for treatment of patients with peripheral vascular obstruction or coronary artery disease.

The presence of persistent tissue ischemia at the time of administration of an angiogenic factor has been considered an essential precondition for angiogenic effects. Whether an angiogenic factor delivered to a normoperfused skeletal muscle before the occurrence of ischemia could stimulate neovascularization and preserve blood perfusion once ischemia develops remains an unsolved issue. In addition to its importance for understanding the basic mechanisms of ther-

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apeutic angiogenesis, this question is of substantial clinical significance because the majority of patients with peripheral arterial disease do not have chronic ischemia but rather recurrent episodes of ischemia during physical activity.11

The present study was designed to determine whether prophylactic, adenovirus-mediated gene transfer of the 121-amino-acid form of vascular endothelial growth factor (AdCMV.VEGF121) could stimulate angiogenesis in a normoperfused limb and preserve blood flow to the skeletal muscles after the induction of ischemia, thereby ameliorating the hemodynamic deficit in the acutely ischemic limb.

Methods

Subjects

The care of animals in this study complied with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC: National Academy Press, 1996), and all protocols were approved by the Gerontology Research Center and by the Istituto Dermopatico dell’Immacolata Animal Care and Use Committee. Six-month-old male New Zealand White rabbits, weight 3.5 to 4.5 kg, and 10-month-old male Wistar rats, weight 500 to 600 g, were used.

Experimental Design

Adenovirus Vectors

Replication-deficient recombinant Ad vectors containing cDNA for VEGF121 were engineered according to a technique previously described15 and were supplied by GenVec, Inc. Briefly, the AdCMV.VEGF121 is an E1a , partial E1b , partial E3 Ad vector that carries, in the E1 position, an expression cassette containing the cytomegalovirus (CMV) immediate early promoter/enhancer driving the cDNA for the 121-residue form of human VEGF. AdCMV.Null, used as a control vector, is similar to AdCMV.VEGF121, but has no gene in the expression cassette.

Intramuscular Administration of AdCMV.VEGF121

Four weeks before the induction of ischemia, rabbits were randomly assigned to receive AdCMV.VEGF121 (107 or 108 pfu/mL), AdCMV.Null (107 pfu/mL), or saline. Rats received injections of AdCMV.VEGF121 (2×107 pfu/mL) or AdCMV.Null (2×107 pfu/mL) 2 weeks before surgery. The Ad vectors were stored in dialysis buffer solutions at −70°C. Each solution for injection was prepared immediately before use and given intramuscularly in 4 different sites in the thigh (250 μL/injection, 1 mL total volume in rabbits; 125 μL/injection, 0.5 mL total volume in rats) along the projection of the femoral artery.

Animal Model of Hindlimb Ischemia

Both species underwent surgical procedures to induce unilateral hindlimb ischemia as previously described. In rats, surgery was performed under intraperitoneal anesthesia with ketamine (60 mg/kg) and xylazine (10 mg/kg). Rats were preanesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg), intubated, and mechanically ventilated with a mixture of 1.5% isoflurane and oxygen. The femoral artery was then 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.

Study Parameters in Rabbits

Calf Blood Pressure Ratio

For 12 weeks after surgery, calf blood pressure was measured weekly with a Doppler flowmeter (Vascular Mini-Laboratory III, Parks Medical Electronics) in both hindlimbs of 32 rabbits. All measurements were performed by a single observer blinded to the treatment regimen. The calf blood pressure ratio (BPR) was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Blood Flow Measurements

Regional blood flow (RBF) to skeletal muscles in both hindlimbs of 64 rabbits was measured in the resting state by use of radioactive microspheres at day 1 and then at weeks 1, 4, and 12 after surgery. After animals were placed under anesthesia with pentobarbital (10 to 20 mg/kg IV), the chest was opened, and 3.3×105 radioactive microspheres (15.5 μm in diameter) labeled with 51Cr (NEN Life Science Products) were injected into the left ventricle. A blood reference sample was withdrawn from a catheter (18 gauge, Abbocath-T) advanced through the left carotid artery into the descending aorta at a constant rate of 2 mL/min starting 30 seconds before and continuing for 90 seconds after the injection was completed. Animals were killed, and the adductor and gastrocnemius muscles of both limbs were removed. Tissue samples and reference blood samples were digested with potassium hydroxide and then filtered with glass microfiber filters with 1.6-μm-diameter pores (Whatman International Ltd). The radioactivity of each sample of filtered wrapped microspheres was determined with a liquid scintillation counter (model LS5801, Beckman Coulter, Inc). The RBF (mL·min−1·100 g−1) was calculated by the formula ϕB = 100×(withdrawal rate/tissue weight)×(cpm sample−cpm reference blood), where cpm is counts per minute.

Postmortem Contrast Angiograms

Postmortem contrast angiograms of the ischemic limbs were obtained at day 1 after surgery in 15 rabbits. A total of 5 mL contrast medium (Hypaque sodium 50%, diatrizoate sodium, Nycomed, Inc) was injected into the right common iliac artery at a constant rate of 20 mL/min. Serial images of the ischemic hindlimb were recorded (Digimax MP4000 Series III Workstation, Acroma Medical Imaging, Inc). Collateral vessel development in the thigh was assessed by use of a grid overlay with 2-mm squares. The angiographic score (angioscore) was defined as the total number of contrast-opacified vessels crossing the squares divided by the total number of squares in the ischemic thigh multiplied by 100. All countings were performed by a single observer blinded to the treatment regimen. We also performed a qualitative assessment by observation of the arterial filling in the distal leg (saphenous and popliteal arteries). For purposes of comparison among different treatment groups, the arterial filling was noted as present or absent.

Study Parameters in Rats

Western Blot Analysis and ELISA

VEGF expression in rat skeletal muscles was examined by Western blot analysis and ELISA (R&D Systems, Inc).19

3P NMR Spectroscopy

3P NMR spectroscopy was used to determine bioenergetic characteristics of the gastrocnemius muscles at rest and during electrical stimulation. Studies were conducted in 11 rats on days 1, 7, and 14 after surgery. After sedation with ketamine (60 mg/kg) and xylazine (10 mg/kg), platinum stimulation electrodes (Grass Instruments Manufacturing) were inserted into the proximal head of the gastrocnemius and in the Achilles’ tendon and connected to a programmable stimulator (model S-10, Grass Instruments Manufacturing) with an isolation transformer via a low-pass filter. An elliptical radiofrequency surface coil tuned to the 3P resonance was positioned on the gastrocnemius. The foot of the stimulated leg was tied to a strain-gauge force transducer (Grass Instruments Manufacturing) with a 3-0 silk suture. The transducer was connected to a strain-gauge conditioner, preamplifier, and chart recorder (Gould Instrument Systems, Inc), allowing continuous monitoring of muscle contraction force. Electrical stimulation was applied as train of pulse pairs, each pulse 200 μs long, with a 200-ms interval separating the elements of each pair. This was repeated every 2 seconds. The stimulation voltage was determined by observation of the voltage beyond which there was no further increase in contraction force.
NMR data were acquired on a 1.9-T/31-cm NMR spectrometer (Biospec, Bruker Medizintechnik GmbH). The proton NMR signal from the 31P-tuned coil was detected and used for shimming.16 Adiabatic excitation pulses were used to compensate for radiofrequency inhomogeneity. Pulses were applied every 2 seconds, so that the 64 transients collected for each spectrum required 2 minutes. In each NMR experiment, 1 spectrum was collected immediately before stimulation, 3 spectra were collected during stimulation, and 6 spectra were collected immediately after cessation of stimulation. This protocol was then repeated for the contralateral leg.

After line broadening and Fourier transformation, each spectrum was manually phased and its baseline corrected with a spline fit. The frequency inhomogeneity. Pulses were applied every 2 seconds, so that the 64 transients collected for each spectrum required 2 minutes. In each NMR experiment, 1 spectrum was collected immediately before stimulation, 3 spectra were collected during stimulation, and 6 spectra were collected immediately after cessation of stimulation. This protocol was then repeated for the contralateral leg.

Histological and Morphometric Analysis
A histological and morphometric analysis was performed on 14 rats to evaluate the angiogenic effect of AdCMV.VEGF121 in the absence of ischemia. Fifteen days after injection of the viral vector, both legs were perfused via the abdominal aorta with 10% buffered formalin at 100 mm Hg for 15 minutes. Subsequently, the adductor and gastrocnemius muscles were immersion-fixed in formalin for 48 hours. From each sample, sections were cut with the muscle fibers oriented transversely and were stained with smooth muscle α-actin antibody to identify arterioles and differentiate them from capillaries and veins. Sections were incubated with mouse monoclonal anti-α-smooth muscle actin (clone 1A4, Sigma Chemical Co) diluted 1:30 in PBS and subsequently with anti-mouse rhodamine-labeled antibody.

For the morphometric analysis, the total area of the muscle present in each section was examined at ×200 magnification. Arteriole and capillary length density were measured as previously described.5,17 The measurements were performed by a single observer blinded to the treatment regimen.

Statistical Analysis
Results are expressed as mean±SEM. Statistical comparisons were performed by 2-factor ANOVA (treatment groups and time points after surgery) (BMDP Statistical Software). For NMR data analysis, 1-way ANOVA (treatment groups) for repeated measurements (repeated recorded spectra) was performed. All data were analyzed for main effects and interaction; simple effects were calculated when appropriate. The qualitative angiographic data were evaluated with Pearson’s χ² test. A value of P<0.05 was considered statistically significant for main effects and P<0.01 for interaction and simple effects.

Results
Expression Kinetics of AdCMV.VEGF121
In AdCMV.VEGF121-infected adductor muscles, transgene expression was elevated 3 days after infection and returned to control levels at day 14 (Figure 1). ELISA showed that with AdCMV.VEGF121, VEGF levels were 3.7±1.1 ng/mg protein 3 days after infection (n=3) and were undetectable at day 14 (n=3). In AdCMV.Null-infected muscles, VEGF levels were undetectable at days 3 (n=5) and 14 (n=3).

These results are consistent with those of previous in vivo studies that have shown that Ad-mediated transgene expression is transient and ceases within a few days after infection.5,12,18

Calf BPR
Calf BPR was similar in all groups 1 week after surgery and improved in all groups afterward until week 12 (P<0.0001) (Figure 2). However, animals treated with AdCMV.VEGF121 at 10⁶ pfu/mL showed a faster rate of recovery between weeks 1 and 4 than both controls (P<0.0001) and animals treated with AdCMV.VEGF121 at 10⁷ pfu/mL (P<0.001). The higher BPR in the AdCMV.VEGF121 at 10⁶ pfu/mL group remained significant until week 8. In animals treated with AdCMV.VEGF121 at 10⁸ pfu/mL, the rate of recovery was not different from that of controls.

**Table 1.** Transgene expression in rat adductor muscles. At day 3 after infection, Western analysis shows evidence of VEGF expression in muscles injected with AdCMV.VEGF121, whereas at day 14, VEGF levels are back to control and comparable to those in muscles injected with AdCMV.Null. Each point represents a different animal, and similar results were obtained in 3 separate experiments.

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**Figure 1.** Transgene expression in rat adductor muscles. At day 3 after infection, Western analysis shows evidence of VEGF expression in muscles injected with AdCMV.VEGF121, whereas at day 14, VEGF levels are back to control and comparable to those in muscles injected with AdCMV.Null. Each point represents a different animal, and similar results were obtained in 3 separate experiments.

**Figure 2.** BPR as a function of time after surgery. Animals treated with a lower concentration of AdCMV.VEGF121 (10⁶ pfu/mL) had a rate of recovery similar to both control groups. However, BPR in AdCMV-VEGF121 at 10⁷ pfu/mL group was significantly higher than AdCMV.VEGF121 at 10⁸ pfu/mL and both control groups until week 8. Pretreatment with AdCMV.VEGF121 at 10⁸ pfu/mL clearly accelerated tissue perfusion recovery, especially between weeks 1 and 4 after induction of ischemia. *P<0.0001 vs all other groups.
Blood Flow Measurements

There were no significant differences between treatment groups or time points in RBF to nonischemic limbs (Figure 3). RBF in the ischemic limb exhibited a 2-fold increase in AdCMV.VEGF121-treated animals relative to controls as early as day 1 after surgery (P<0.001). This difference remained significantly higher in the AdCMV.VEGF121 groups at all subsequent time points. The calculated ratio of RBF in the ischemic to nonischemic gastrocnemius muscles at day 1 after surgery was significantly higher in AdCMV.VEGF121-treated animals than in controls (data not shown). By week 1, in AdCMV.VEGF121-treated animals, the ratio reached 1, thus indicating a complete restoration of tissue perfusion.

Contrast Angiography

Representative postmortem angiograms obtained at day 1 after surgery are shown in Figure 4. At 24 hours after femoral artery removal, there was an increase in the number of vessels in AdCMV.VEGF121-treated animals compared with controls. In the saline group, there was no visible collateral development in the thigh. In contrast, in the AdCMV.VEGF121 (10⁶ pfu/mL) group, a network of newly formed vessels developed, sprouting mainly from the internal iliac artery toward the medial thigh. The resulting angioscore was significantly higher for AdCMV.VEGF121-treated animals, showing a 4-fold increase in the number of vessels compared with animals that received saline (AdCMV.VEGF121 = 50.58±1.48, saline = 11.83±1.90, P<0.0001). Animals treated with AdCMV.Null also had a significantly higher angioscore than the saline group (Null = 29.08±4.22, P<0.05), yet lower than the AdCMV.VEGF121-treated group (P<0.001).

The qualitative angiographic assessment showed that the increased vasculature in AdCMV.VEGF121-treated animals was functional, reestablishing flow to the more distal arteries in the leg (5 of 5 animals). Among animals that received AdCMV.Null, we documented distal arterial filling in the ischemic leg in 2 of 5 animals, whereas none of the animals in the saline group exhibited similar findings. Statistically, the AdCMV.VEGF121 group differed significantly from the saline (P<0.002) and AdCMV.Null (P<0.05) groups.

Figure 3. RBF to gastrocnemius muscles as a function of time after surgery. There was no difference in RBF to ischemic limb between controls. Both concentrations of AdCMV.VEGF121 were equally effective in increasing blood flow to ischemic gastrocnemius muscle as early as day 1 after surgery. Each time point represents average of results in 4 different rabbits for each condition. *P<0.0001, †P<0.001, and ‡P<0.01 vs controls.

Figure 4. Representative postmortem angiograms of ischemic limbs of rabbits treated with either saline (A) or AdCMV.VEGF121 (B) obtained at day 1 after surgery. AdCMV.VEGF121-treated animals show extensive collateral artery formation in contrast to saline-injected animals. Note that in AdCMV.VEGF121-treated animals, distal arterial filling of ischemic limb (arrow) was restored, whereas in saline group, there was no angiographic evidence of distal perfusion.
whereas controls were not different from each other \( (P > 0.05) \).

\[ \text{31P NMR Spectroscopy} \]

AdCMV.VEGF\(_{121}\)-treated animals showed markedly improved bioenergetic reserve after femoral artery removal compared with controls (Figure 5). At day 1 after surgery (A), AdCMV.VEGF\(_{121}\)-treated animals had less reduction of the PCr/(PCr + P\(_i\)) ratio during electrical stimulation of the gastrocnemius muscle and faster and more complete restoration of that ratio toward the baseline level in the recovery phase \( (P < 0.0001) \). The improved pattern of bioenergetic reserve in AdCMV.VEGF\(_{121}\)-treated animals persevered to day 7 \( (P < 0.004) \) (B) but not day 14 \( (P > 0.1) \) (C) after surgery, because controls eventually recovered enough to make this difference nonsignificant.

\[ \text{Histology and Morphometric Analysis} \]

Morphometric analysis of the muscle sections of the ischemic limbs revealed that 15 days after injection, there was a 29% increase in the capillary length density in the adductor muscles injected with AdCMV.VEGF\(_{121}\) \( (P < 0.03) \) (Figure 6A) and a 96% increase in the length density of arterioles 4 to 41 \( \mu \)m in diameter \( (P < 0.008) \) (B) in the same muscles. In the limbs treated with AdCMV.VEGF\(_{121}\), the angiogenic effect was limited to the muscle tissue directly injected with the adenoviral vector.

\[ \text{Discussion} \]

Our results show that in skeletal muscle, adenovirus-mediated VEGF\(_{121}\) gene transfer can evoke a significant angiogenic response in the absence of ischemia and that this response attenuates the hemodynamic deficit of subsequent surgically induced ischemia.

Other studies have demonstrated that angiogenic factors given in vivo were effective in improving blood flow to an ischemic limb. In contrast, Pu et al.,\(^{10}\) studying the angiogenic effects of endothelial cell growth factor in the rabbit hindlimb model, failed to find any significant effect on vessel growth in nonischemic tissue, whereas endothelial cell growth factor was effective on ischemic tissue. To the best of our knowledge, the results of the present study are the first to demonstrate the successful induction of angiogenesis in nonischemic skeletal muscle.

The morphometric analysis revealed that 15 days after administration of AdCMV.VEGF\(_{121}\), there was evidence of neovascularization in the absence of tissue ischemia. Neovascularization was restricted to the tissues that had been directly exposed to the viral vector and did not occur at a distance despite short-term systemic circulating levels of VEGF\(_{121}\).\(^{18}\)

Anatomic evidence of neovascularization was also obtained from angiograms performed at day 1 after surgery in rabbits treated 4 weeks earlier with AdCMV.VEGF\(_{121}\). In addition, the filling of distal arteries in the ischemic limbs of AdCMV.VEGF\(_{121}\)-treated animals showed that the development of new vessels induced by AdCMV.VEGF\(_{121}\) allowed the restoration of the flow in the distal limb, although angiogenesis was limited to the site of injection.

Evidence of enhanced perfusion was obtained from blood flow measurements, and at day 1 after surgery, animals treated with AdCMV.VEGF\(_{121}\) had a 2-fold increase in blood flow.
flow to the ischemic limbs relative to controls. This early evidence of improvement strongly suggests that functional blood vessels developed before the occurrence of ischemia. These results were consistent with angiographic data showing distal arterial filling in the ischemic limb, which also suggests that the recently formed vessels were patent and played a critical role in the amelioration of the surgically induced hemodynamic deficit.

$^{31}$P NMR spectroscopy is an established method for the noninvasive assessment of metabolic correlates of tissue perfusion.$^{23-24}$ In the present study, muscle metabolism in the gastrocnemius muscle, distal to the site of induced ischemia, was assessed. Our results demonstrated markedly improved bioenergetic capacity of the ischemic gastrocnemius muscle in AdCMV.VEGF$_{121}$-treated animals compared with controls. Consistent with the other outcome measures presented here, treated animals showed this improvement at day 1 after surgery. Thus, not only was perfusion partially restored to the ischemic hindlimb, but clear metabolic improvements resulted from this restoration. Previous investigators have used $^{31}$P NMR spectroscopy extensively to assess angiogenesis and blood flow in tumors. However, to the best of our knowledge, this is the first noninvasive $^{31}$P-NMR demonstration of improvement in tissue bioenergetics resulting from therapeutic angiogenesis.

An unexpected finding of our study was that animals treated with AdCMV.Null had a significantly higher angioscore than animals treated with saline, although lower than those treated with AdCMV.VEGF$_{121}$. This finding was in contrast to both blood pressure and blood flow data, suggesting that newly developed vessels induced by AdCMV.Null in rabbits, possibly secondary to a local inflammatory response, were insufficient to preserve total tissue perfusion distal to the site of injection. The distal arterial filling analysis performed in the angiographic studies further corroborates this hypothesis, because there was no statistically significant difference between animals treated with either saline or the null vector alone. However, it cannot be excluded that viral proteins or the injection injury itself may have acted in synergy with VEGF to induce angiogenesis in response to AdCMV.VEGF$_{121}$.

If therapeutic angiogenesis could be induced only in chronically ischemic tissues, patients with intermittent claudication but normal resting blood flow would not be candidates for therapeutic angiogenesis. Recent evidence suggests that intermittent claudication sustains an inflammatory state of the muscle.$^{25}$ However, it is unknown whether such an inflammatory state creates the conditions for cytokines to exert an angiogenic effect, how long after exercise the inflammatory state persists, and whether it occurs in proximity to occluded arteries, in which neovascularization may be beneficial, or in tissues remote and downstream from diseased arteries. Thus, it is important that angiogenesis may be induced in normoperfused skeletal muscles, in the absence of an inflammatory state that may result from intermittent episodes of arterial insufficiency.

In conclusion, our findings in 2 animal models of limb ischemia support the hypotheses that (1) therapeutic angiogenesis can be induced in skeletal muscle in the absence of ischemia; (2) the newly formed vessels preserve tissue perfusion and act to normalize muscle bioenergetic capacity on the subsequent induction of ischemia; (3) the angiogenic effect evoked by AdCMV.VEGF$_{121}$ persists until it is equalized by spontaneous development of collateral vessels in untreated animals; (4) adenovirus-mediated VEGF$_{121}$ gene transfer is highly effective in inducing angiogenesis in both species studied; and (5) AdCMV.Null may have angiogenic properties whose underlying mechanisms will require further investigation. These observations are in agreement with a previous study from our laboratory on adenovirus-mediated recombinant, secreted, acidic fibroblast growth factor gene transfer to the nonischemic rabbit heart.$^5$ Thus, our results widen the field of therapeutic angiogenesis to potential early intervention in patients with intermittent claudication in whom blood flow at rest is preserved and who represent the majority of patients with peripheral arterial disease.

References


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