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Induction of Functional Neovascularization by Combined VEGF and Angiopoietin-1 Gene Transfer Using AAV Vectors

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Vectors based on the adeno-associated virus (AAV) deliver therapeutic genes to muscle and heart at high efficiency and maintain transgene expression for long periods of time. Here we report about the synergistic effect on blood vessel formation of AAV vectors expressing the 165 aa isoform of vascular endothelial growth factor (VEGF165), a powerful activator of endothelial cells, and of angiopoietin-1 (Ang-1), which is required for vessel maturation. High titer AAV-VEGF165 and AAV-Ang-1 vector preparations were injected either alone or in combination in the normoperfused tibialis anterior muscle of rats. Long term expression of VEGF165 determined massive cellular infiltration of the muscle tissues over time, with the formation of a large set of new vessels. Strikingly, some of the cells infiltrating the treated muscles were found positive for markers of activated endothelial precursors (VEGFR-2/KDR and Tie-2) and for c-kit, an antigen expressed by pluripotent bone marrow stem cells. Expression of VEGF165 eventually resulted in the formation of structured vessels surrounded by a layer of smooth muscle cells. Presence of these arteriolae correlated with significantly increased blood perfusion in the injected areas. Co-expression of VEGF165 with angiopoietin-1-which did not display angiogenic effect per se-remarkably reduced leakage of vessels produced by VEGF165 alone.

Key Words: adeno-associated vectors, angiogenesis, angiopoietin-1, gene therapy, muscle, stem cells, vascular endothelial growth factor

INTRODUCTION

Formation of new blood vessels in the adult (angiogenesis) occurs by sprouting from existing vessels, a process that requires proliferation, activation and migration of endothelial cells and remodeling of extracellular matrix. Subsequent maturation consists in structural organization of unstable vessels with the formation of a layer of smooth muscle cells surrounding the proliferated endothelium [1,2]. Members of the vascular endothelial growth factor (VEGF) family are powerful inducers of angiogenesis [3]. Transient VEGF-A expression in vivo results in potent angiogenic sprouting with the formation of immature and leaky vessels, associated with tissue swelling due to markedly increased vascular permeability [4,5]. One class of molecules required for later stages of angiogenesis, involving vessel remodeling, maturation and stabilization, consists of the angiopoietins and their receptors [6,7]. Studies in transgenic mice have indicated that overexpression of angiopoietin-1 mitigates leakiness of skin microvasculature resulting from VEGF expression [8]. Consistently, an adenoviral vector expressing angiopoietin-1 was shown to protect adult vasculature from leakiness, counteracting the permeabilization activity of VEGF and of inflammatory agents [9]. A distinct mechanism of blood vessel formation occurring during early embryonic development is vasculogenesis, a process involving the differentiation of angioblasts into blood islands that fuse to form the primitive vascular network [2]. Increasing evidence suggests that angioblast-like cell precursors derived from the bone marrow circulate in the adult vasculature and contribute to physiological and pathological neovascularization in the adult [10,11].

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These circulating stem cell precursors are able to invade tissues and differentiate into several cell types after ischemic damage of the heart or of the skeletal muscle [12], and might constitute a reservoir for tissue and vessel regeneration of important therapeutic value. Very little is currently known about the actual molecules triggering recruitment, activation and differentiation of these cells. Induction of new blood vessel formation by gene transfer remains a very attractive clinical possibility for the treatment of ischemia in tissues not susceptible to conventional revascularization procedures. However, significant technical barriers remain in terms of safe and effective local gene delivery to the ischemic muscle and heart. In this context, viral vectors based on the adeno-associated virus (AAV) offer the unique possibility of obtaining high level and persistent gene expression of the encoded factors in skeletal muscle and heart, in the absence of immune or inflammatory reaction [13–15]. In addition, since cell transduction with AAV vectors occurs at high multiplicity of infection [16,17], this vector system also allows simultaneous expression of different factors in the same cell or tissue in vivo. Given the patent biological complexity of the process of new blood vessel formation, combined growth factor administration appears to be an important requisite in therapeutic terms.

In this work we exploit the potential of AAV gene transfer to the skeletal muscle to understand the long term biological effect of VEGF-A and angiopoietin-1 expression.

RESULTS

Transduction of Rat Muscle using AAV-VEGF and AAV-Ang1

We constructed two AAV vectors expressing the coding regions of the 165 aa isoform of human VEGF-A (AAV-VEGF165) and of human angiopoietin-1 (AAV-Ang1) under the control of the constitutive cytomegalovirus (CMV) promoter (Figure 1A). Efficiency of transduction and expression of the transgenes using these vectors was initially demonstrated by infection of hamster CHO cells followed by immunocytochemistry using antibodies reacting with human VEGF-A and angiopoietin-1 (Figure 1B).

High titer AAV vector preparations were injected into the muscle tibialis anterior of the right leg of normoperfused Wistar rats. Each animal was also injected in the muscle tibialis anterior of its left leg with either AAV-LacZ or PBS.

Long-term expression of VEGF165 and angiopoietin-1 in muscle fibers was assessed by immunohistochemistry at 3 months after treatment. In both cases, muscle areas injected with these vectors showed a large number of fibers positive for expression of the respective factors (shown in Figure 1C for an animal injected with both vectors).

To quantitatively assess the extent of transduction of injected muscles, as well as the dissemination of AAV vectors to other body districts, a quantitative competitive PCR procedure was developed measuring the AAV-VEGF165 DNA copy number in comparison with an endogenous rat gene. Primer pairs were selected in the human VEGF165 cDNA and in the rat GAPDH gene (Figure 1D). For both targets, DNA competitors were obtained that were recognized by the same primer pairs and differed from the respective targets by a 20 bp deletion (for VEGF165) or a 20 bp insertion (for GAPDH; Figure 1E). Genomic DNA extracted from injected and mock-injected muscles, heart, lung and liver from two animals treated with AAV-VEGF165 were mixed with scalar amounts of competitors and submitted to competitive PCR amplification (an example of which is shown in Figure 1F). The results of quantification are shown in Figure 1G and are expressed as a ratio between VEGF165 and GAPDH copy numbers. In injected muscle areas, one AAV DNA molecule was found every 200 rat genomic DNA molecules. In mock-injected muscle, as well as in heart and lung, AAV DNA copy number was at least five orders of magnitude lower than that found in the injected muscle. In one animal, we found one AAV DNA molecule every 1×10^6 rat genomes in liver, while in the other one this amount was 10 times lower (Figure 1G). From these data, we conclude that AAV preparations locally injected in the skeletal muscle persist for long times in the sites of inoculation and have a minimal capacity to spread to other body districts.

Biological Effects of Long Term VEGF165 and Angiopoietin-1 Expression

When histological sections were examined, striking findings were noticed in AAV-injected animals. Muscles that received VEGF165 either alone or in combination with angiopoietin-1 showed remarkably increased cellularity as compared to mock or LacZ-injected controls (Figure 2A). By immunohistochemistry, most of the infiltrating cells scored positive for expression of the proliferating cell nuclear antigen (PCNA), a marker associated with cellular proliferation (Figure 6).

To quantify the number of these cells, three independent investigators blinded for experimental procedures observed 50 different sections from 4 animals per group; the results are expressed in Figure 2B as number of cells per muscle fiber. As compared to LacZ-injected muscles, cellularity was increased of over 5 times in animals treated with VEGF165, either alone or in combination with angiopoietin-1. In contrast, treatment with angiopoietin-1 alone only had a modest apparent effect.

To understand whether increased cellularity of injected muscles was paralleled by neoangiogenesis, we performed immunohistochemistry with an antibody against CD31, a marker of endothelial cells. The number of CD31-positive cells was greatly increased in muscles transduced with AAV-VEGF165 (Figure 2C). We also quantified the

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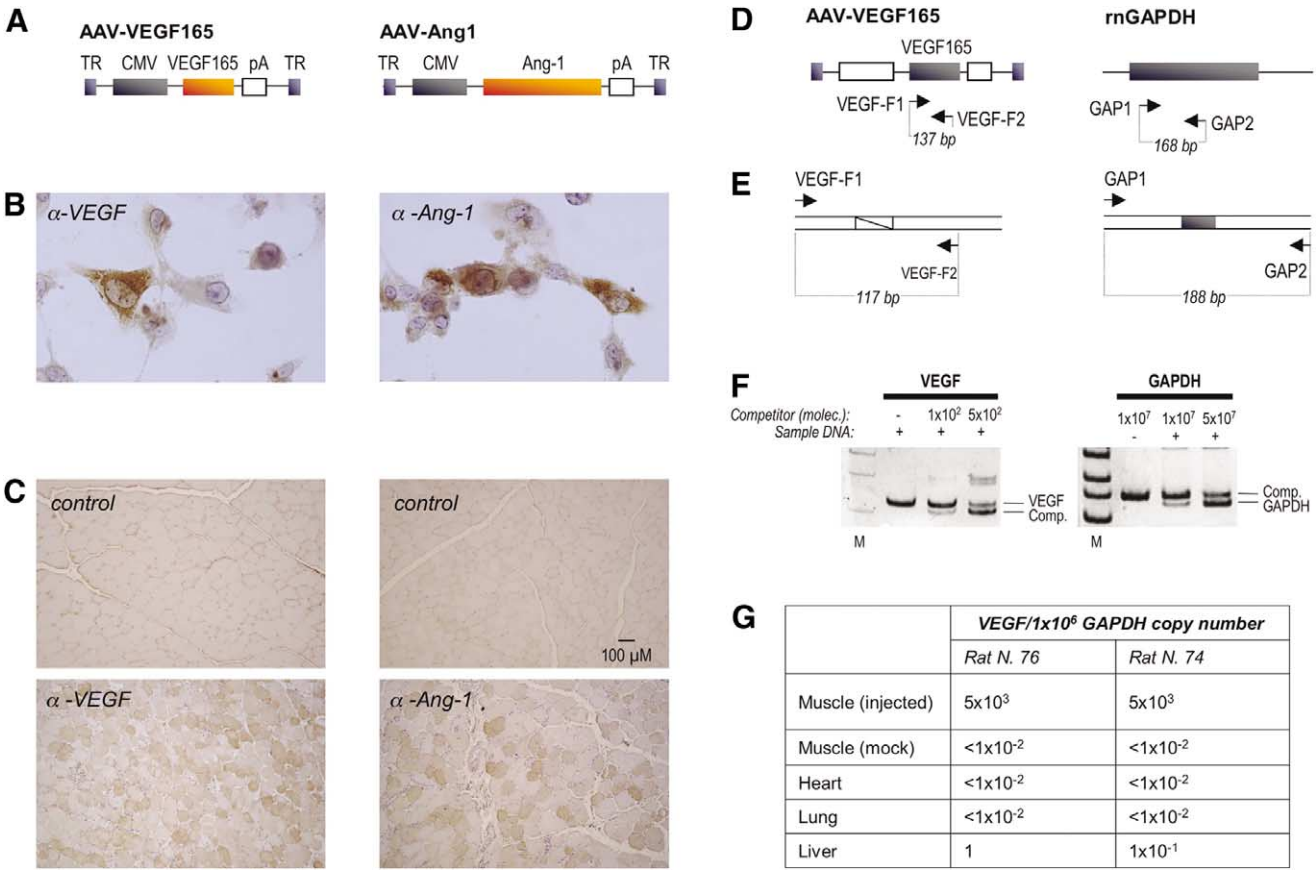

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	VEGF/1x10⁶ GAPDH copy number	
	Rat N. 76	*Rat N. 74*
Muscle (injected)	$5x10^{3}$	$5x10^{3}$
Muscle (mock)	$<1x10^{-2}$	$<1x10^{-2}$
Heart	$<1x10^{-2}$	$<1x10^{-2}$
Lung	$<1x10^{-2}$	$<1x10^{-2}$
Liver	1	$1x10^{-1}$

FIG. 1. Delivery of VEGF165 and angiopoietin-1 cDNAs to rat skeletal muscle using AAV vectors. A. Schematic representation of the AAV vectors used in this study. TR: terminal repeat sequences; CMV: cytomegalovirus immediate early promoter; pA: polyadenylation site B. Immunocytochemistry of CHO cells transduced with AAV-VEGF165 (left panel) and AAV-Ang1 (right panel). C. Immunostaining of muscle sections of mock treated rats (upper panels) and of rats treated with AAV-VEGF165 plus AAV-Ang1 at 3 months after injection (lower panels) using antibodies against VEGF-A (panels on the left side) and angiopoietin-1 (panels on the right side). Several muscle fibers in the injected areas show reactivity to the respective antibodies only in treated muscles. D. Primers for PCR amplification. Two primer pairs were selected in the AAV-VEGF165 construct (VEGF-F1 and VEGF-F2) and in the rat GAPDH gene (GAP1 and GAP2) amplifying 137 bp and 168 bp DNA fragments respectively. E. Schematic representation of DNA competitors for competitive PCR amplification of AAV-VEGF165 and rat genomic GAPDH DNAs. F. Example of competitive PCR amplification. Fixed amounts of sample DNA from different organs were mixed with scalar amounts of competitor DNA and PCR-amplified with the two primer pairs. After amplification, gels were stained with ethidium bromide and the competitor (Comp.), VEGF or GAPDH DNA bands were quantified. According to the principles of competitive PCR, the ratio between the amplification products in each reaction is linearly correlated with the input DNA amounts for the two species. M: molecular weight markers. molec.: DNA molecules. G. Results of VEGF165 DNA quantification in different organs. Two rats injected with AAV-VEGF165 were sacrificed 3 months after treatment and DNA extracted from the indicated organs. Results are expressed as ratios between VEGF165 and GAPDH DNA copy number.

number of capillaries in these sections-defined as small (5–10 μm diameter) vessels surrounded only by CD31-positive cells (shown by arrows in Figure 2C). The number of capillaries per muscle fiber was increased ~2 fold in samples treated with VEGF165 compared to controls and to muscles injected with angiopoietin-1 alone. No difference in the number of capillaries was observed in animals sacrificed at one month as compared to those sacrificed at three months post injection. However, an enlargement of the muscle area in which neovascularization was observed was clearly detectable in the latter group (not shown).

Increased formation of capillaries is an expected outcome of short term VEGF overexpression, given the very well known stimulating properties of VEGF on endothelial cell proliferation and migration [18]. In contrast, largely unexpected was the remarkable increase in larger and more mature vessels, surrounded by a thick layer of cells expressing smooth muscle α-actin (α-SMA), a marker of smooth muscle cells. These arteriolae with a diameter in the 20–120 μm range were ~8-fold more represented in AAV-VEGF expressing muscles, either alone or in combination with angiopoietin-1, as compared to AAV-LacZ or mock injected controls (Figure 3).

In our experiments we never observed formation of

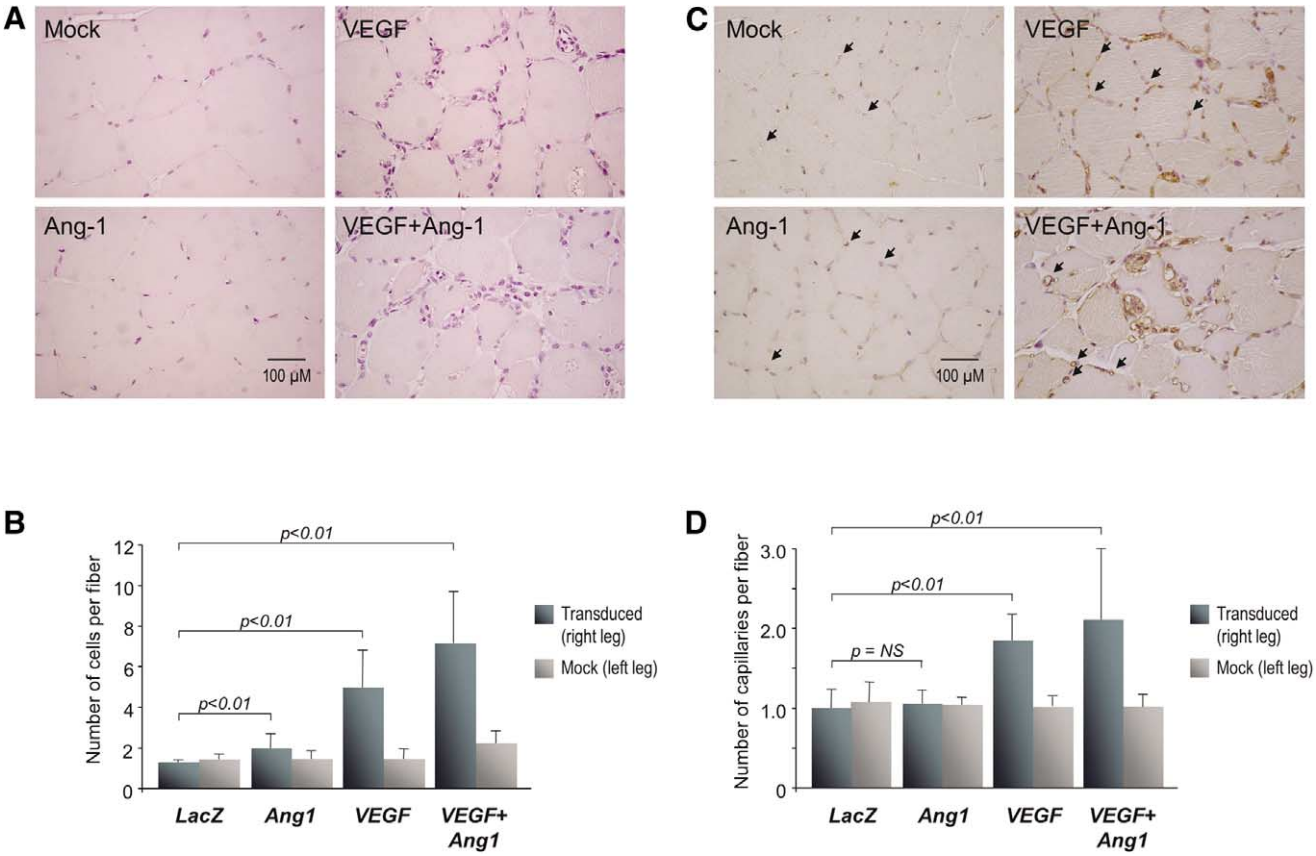

FIG. 2. Quantification of proliferating cells and capillaries in transduced muscles. A. Muscle sections injected with AAV-VEGF165, AAV-Ang1 or both at 3 months after treatment, stained with hematoxylin. B. Quantification of cells in treated muscles. For each animal, counts were obtained from both the leg injected with the angiogenic vector and the controlateral leg. Shown are means and standard deviations of counts, expressed as number of nuclei per muscle fiber. Values were analyzed using a two-tailed t test, considering unequal variance in groups and assuming statistical significance at $p < 0.01$. Large cellular infiltrations in the interstitial space (such as those shown in Figure 6) were not included in the counts. C. Immunostaining of muscle sections of animals treated with the different vector combinations, as indicated, at three months after injection using an antibody against the endothelial cell marker CD31. An increase in the number of cells positive to this antibody is visible in samples of animals injected with AAV-VEGF165. Arrows indicate capillaries, defined as 5–10 μm diameter vessels surrounded only by CD31-positive cells. D. Number of capillaries in injected muscles. Counts were obtained from both the leg injected with the angiogenic vector and the controlateral leg. Presentation of data and statistics are as in Panel 2B. NS: not significant.

hemangioma-like structures even after prolonged expression of VEGF165. However, in several histological sections of muscles expressing this factor we observed that PCNA-positive proliferating cells, several of which showed reactivity to α-SMA antibody, accumulated at the periphery of muscle fibers, with a tendency to invaginate into and invade them (Figure 4E and 4F). In addition, we also noticed a peculiar effect by which some muscle fibers were substituted by vascular lacunae filled of red blood cells (Figure 4A–D), as demonstrated by staining samples with 3,3′-diaminobenzidine (DAB), a chemical compound reacting with oxidized hemoglobin (Figure 4D).

Functional Analysis of Vasculature in Transduced Muscles

To investigate whether the observed histological effects were paralleled by increased blood flow in the injected areas, muscle perfusion was analyzed by using fluorescent microspheres. The results obtained, which are expressed in Figure 5A as a ratio between treated and control muscles, indicate that perfusion of muscles expressing VEGF165 was significantly increased both in the presence or absence of angiopoietin-1. In contrast, transduction of angiopoietin-1 had no effect on perfusion.

The results so far described in terms of cell proliferation, increase in the number of CD31- and α-SMA-positive cells, formation of new blood vessels and perfusion indicate that overexpression of angiopoietin-1 had no apparent role in promoting and sustaining these processes. However, it is still debated whether VEGF alone might be sufficient to determine the development of a mature and functional vascular network, one of the major concerns being the well documented permeabilization

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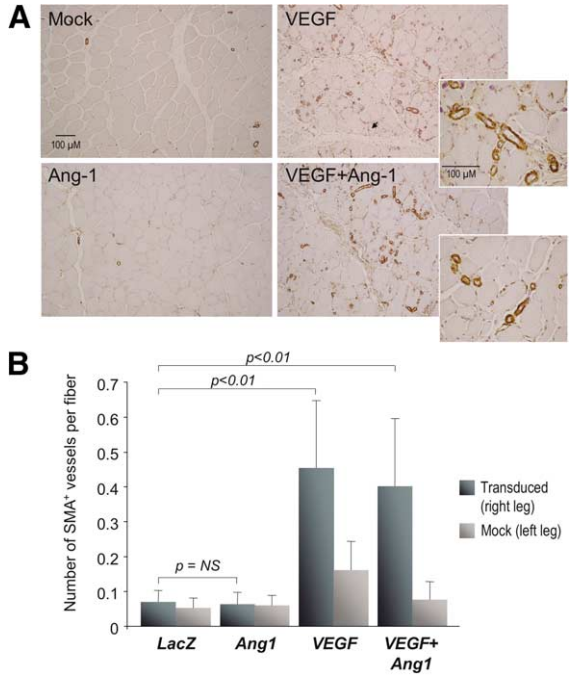

FIG. 3. Immunohistochemistry using an antibody against smooth muscle α-actin (α-SMA). A. Sections of muscles treated with AAV vectors expressing the factors indicated in the different panels were reacted to an antibody against smooth muscle α-actin, a marker of smooth muscle cells. Muscles treated with AAV-VEGF165 show a large number of newly formed vessels (enlarged in the insets) with the characteristics of arteriolae in the 20–120 μm diameter range. Shown are immunostainings from animals sacrificed at 3 months post vector injection. B. Quantification of α-SMA positive vessels. Presentation of data and statistics are as in Panel 2B.

effect exerted by this factor on the vascular wall [4]. In facts, when we analyzed vascular leakiness by injection of Evans blue in the femoral arteries supplying both control and transduced muscles (an adaptation of the Miles test), we found that vessels in muscles expressing VEGF alone were remarkably (~6 times) more leaky than controlateral controls (visually shown in Figure 5B and quantified by spectrophometric analysis of dye in Figure 5C). By contrast, leakiness of vessels produced by co-expression of VEGF with angiopoietin-1 was remarkably reduced and more similar to that of the normal muscle vasculature.

Characterization of Cells Infiltrating Muscles Treated with VEGF165

As described above, a peculiar feature of muscle areas expressing VEGF165 was the presence of a large number of PCNA-positive, proliferating cells. In several instances, the presence of these cells was massive, especially in the interstitial area surrounding large vessels (Figure 6A).

To start understanding the source of these cells, we attempted their phenotypic characterization using immunohistochemistry with different antibodies. As already discussed above, several of the infiltrating cells were found positive for the endothelial-specific CD31 and the smooth muscle cell-specific α-SMA markers. Relatively few cells were found reactive to an antibody against the pan-leukocyte CD45 marker, thus also ruling out a major role of inflammation as a process of cellular recruitment. Interestingly, some infiltrating cells expressed two markers of the activated and proliferating endothelium, namely Tie-2, the receptor for angiopoietin-1 the expression of which is restricted to the vascular endothelial lineage, and VEGFR-2/KDR, the major receptor for VEGF-A. These antigens start to be expressed by a bone marrow derived cell population comprising the early hematopoietic/endothelial cell precursors [19]. Consistent with a possible derivation of these cells from a stem cell precursor is the observation that several of them were found positive for the c-kit/CD117 marker, an antigen that commonly defines pluripotent bone marrow stem cells [19].

DISCUSSION

Prolonged expression of the 165 aa form of VEGF-A in the normoperfused skeletal muscle had several important bi-

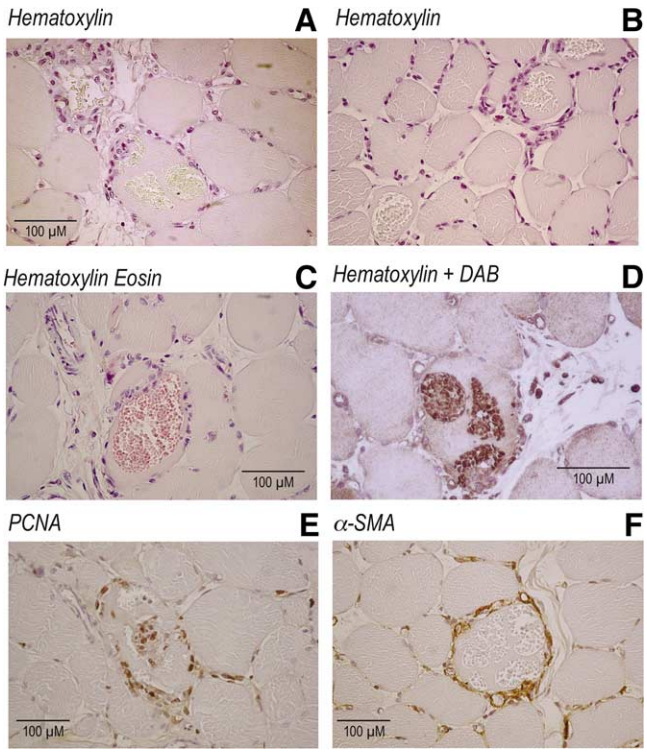

FIG. 4. Substitution of muscle fibers by vascular lacunae in muscles treated with AAV-VEGF165. A to F. Sections from treated muscles showing muscle fibers substituted with vascular lacunae at 3 months post vector injection. Sections were stained with hematoxylin (A and B), hematoxylin and eosin (C), hematoxylyin + 3,3′-diaminobenzidine (DAB) to reveal red blood cells (D), or immunostained with antibodies against PCNA (E) or α-SMA (F).

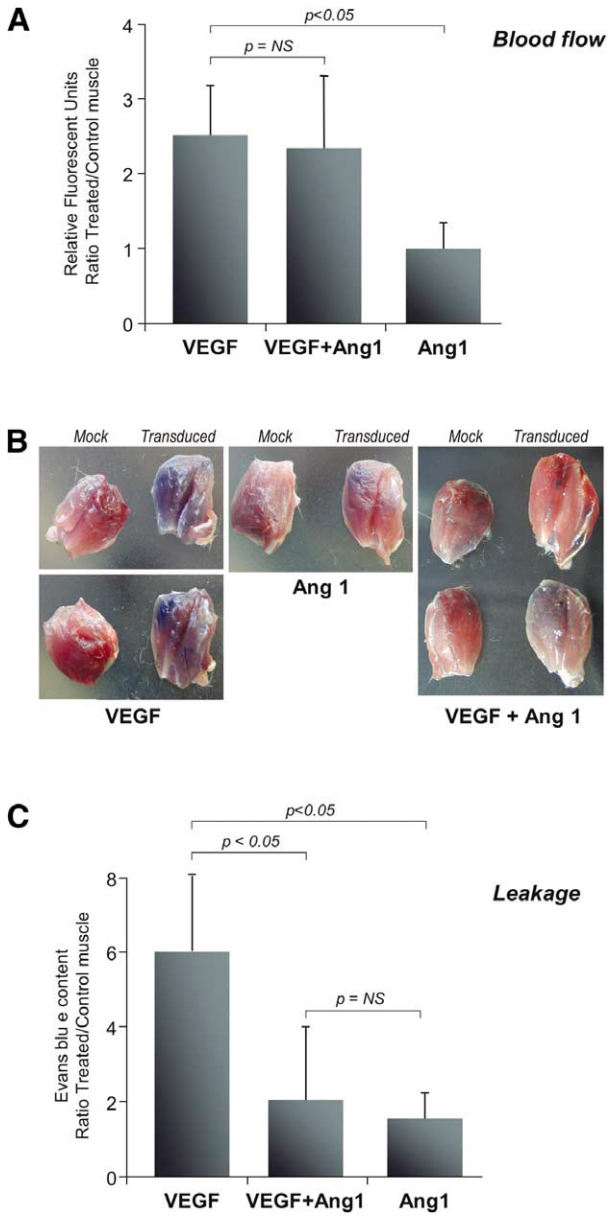

FIG. 5. Functional analysis of vasculature in treated and control muscles. A. Analysis of blood flow. Fluorescent microspheres injected in the abdominal aorta were recovered from isolated muscle tibialis anterior from both the right (treated) and left (mock) legs of each animal and quantified. Shown are mean and standard deviation of the ratios between the two measurements. Values were analyzed using a two-tailed t test, considering unequal variance in groups and assuming statistical significance at $p < 0.05$. Experiments were performed at 3 months after injection of AAV-VEGF165, AAV-Ang1 or both vectors as indicated. B and C. Miles assay for vascular leakage performed at 3 months after vector injection. Evans blue was injected in the femoral artery, and animals were sacrificed 30 minutes later. The muscle tibialis anterior was isolated from both the right (treated) and left (mock) legs of each animal. Panel B shows massive dye infiltration in muscles treated with AAV-VEGF165 alone. Panel C reports spectrophotometric quantification of the dye in muscle samples, expressed as a ratio between treated and mock-treated legs. Evans blue diffusion in muscles treated with VEGF165 is markedly reduced by co-expression of angiopoietin-1. Shown are means and standard deviations of 4 measurements. Statistical evaluation was performed as in Figure 5A.

ological consequences. The most striking phenotypic change we observed was the increase in the number of blood vessels present in the injected areas. This event was somehow expected as far as capillaries are concerned, given the very well known properties of VEGF on endothelial cell proliferation and migration [18] and according to studies that analyzed capillary formation after intramuscular injection of adenoviral vectors expressing VEGF [20,21] or of skeletal myoblasts engineered to secrete the factor [22,23]. In contrast, largely unexpected was the increase in larger vessels, surrounded by a layer of cells expressing α-smooth muscle actin, a marker of smooth muscle cells. The distribution of these arteriolae was irregular and massive close to the injection site and progressively more regular moving to the periphery of the injected area, suggesting a gradient effect correlated to VEGF dosage. The presence of these vessels demonstrates that prolonged angiogenic stimulation by VEGF not only determines activation, proliferation and migration of endothelial cells, but eventually turns out to trigger formation of larger blood vessels coated with α-SMA-positive cells. Besides the direct effect of VEGF itself on smooth muscle cells, vessel maturation is also most likely the consequence of the release of other cytokines from activated endothelial cells [2]. Experiments recently performed by implantation of myoblasts constitutively expressing VEGF-A also revealed formation of smooth muscle-coated vessels similar to arterioles directly adjacent to the implantation site, in perfect agreement with our results [24]. Finally, analogous findings have also been recently reported by studying the effect of long term VEGF overexpression in a transgenic mouse model [25].

While the size of the arteriolae formed after VEGF overexpression is too small to permit angiographic detection of the newly formed vascular network (not shown), they still determined an important increase in blood perfusion of the injected muscles, as detected by perfusion studies using fluorescent microspheres. In this respect, it is worth noticing that these studies most likely underestimate the actual perfusion rate of the VEGF-expressing areas, since they were performed on the whole tibialis anterior muscle, in which injected areas represent less than 20% of the total muscle volume. Injection of skeletal myoblasts engineered to deliver VEGF to the skeletal muscle [23] and to the myocardium [26] promotes a potent angiogenic response but also leads to the formation of vascular tumors resembling hemangiomas. In over 100 animals (including rats, some of which shown in this study, and mice) injected with AAV-VEGF165 we never observed the formation of such tumors. These differences are most likely related to AAV gene transfer biology, in which the onset of growth factor expression is progressive [13] and the peak levels of produced factor are lower than with other vector systems, while expression persists for much longer periods of time. In contrast, we observed a previously unnoticed event consisting of the substitution

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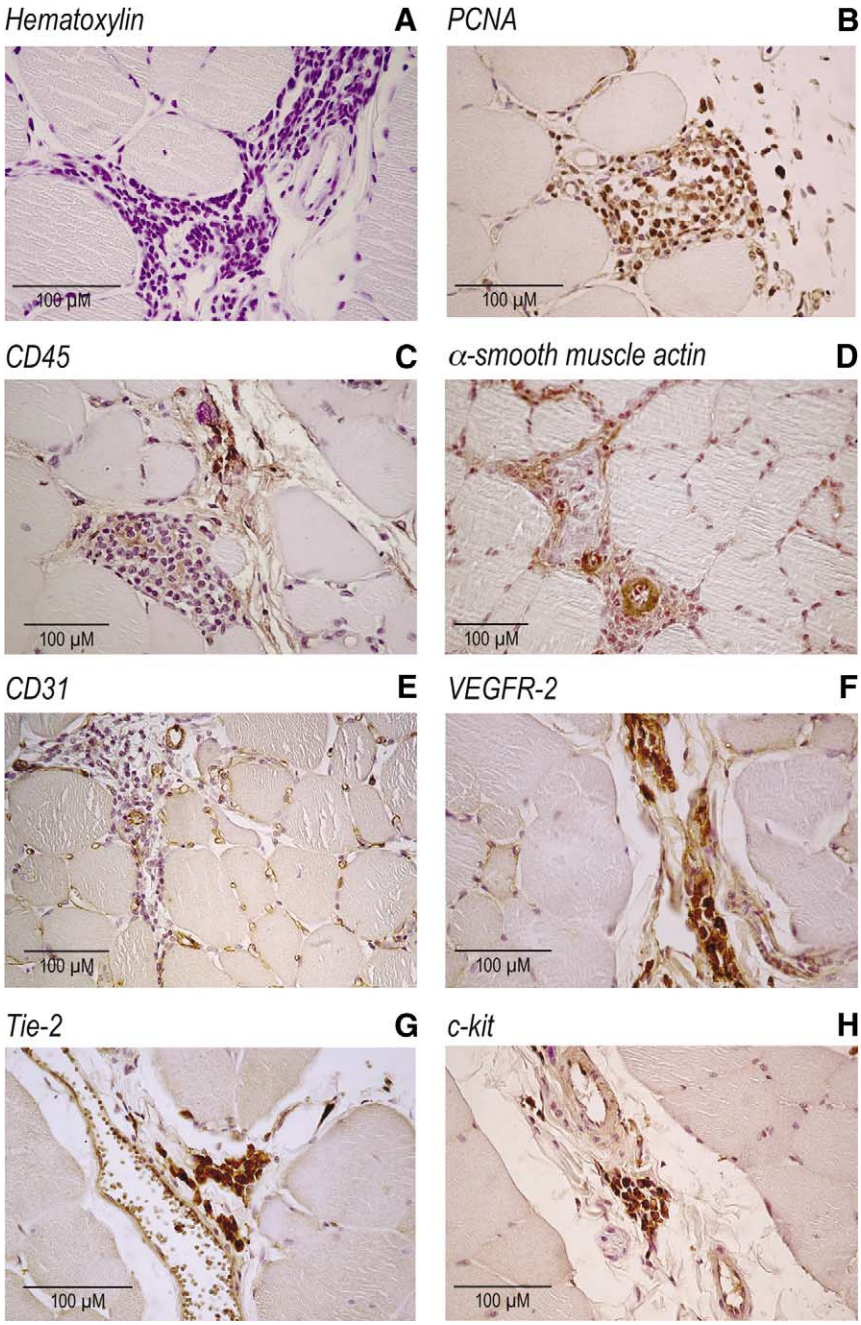

FIG. 6. Characterization of cells infiltrating muscles expressing VEGF165. A to F. Histological sections of muscles that were injected with AAV-VEGF165, analyzed at 3 months after treatment. Massive infiltrates of cells were found in the interstitial space surrounding large arteries (panel A). Most of these cells were expressing PCNA, indicating their active proliferation (B). Panels C to H show characterization of these cellular infiltrates by different antigenic markers, as indicated on top of each panel.

of several muscle fibers (1–5% of fibers in the injected areas) with vascular structures filled of red blood cells and thus connected to the general circulation. Staining of these areas with an anti-CD31 antibody reveled that, with few exceptions, these structures were lacking evident endothelial wall. These vascular structures might be cautiously interpreted as the effect of invagination of muscle cell membrane and its fusion to the membrane of endothelial cells, thus forming a vascular lacunae which is connected to the circulation. Alternatively, they might result from the invasion of muscular cells by locally proliferating cells eventually regressing and differentiating to form a vascular lacuna in which a thin endothelial layer remains undetected by immunohistochemistry. In contrast to the potent stimulatory effect of VEGF on endothelial cell proliferation and migration, and to its powerful vascular permeabilization activity [27], angiopoietin-1 has been shown to be essential for maturation of blood

vessels during embryonic development [28] and for the mitigation of vasal leakiness promoted by inflammation in the adult vasculature [9]. Consistent with these observations, in our experiments overexpression of VEGF alone determined highly increased vascular permeability. In contrast, leakiness of vessels produced by co-expression of VEGF with angiopoietin-1 was remarkably more similar to that of the normal muscle vasculature. This result underlines the notion that new blood vessel formation is a multistep process sustained by the sequential and coordinated activity of several factors, and that therapeutic angiogenesis will require not only increase in vessel number in the ischemic area but also their proper functional maturation. It is very likely that these and other functional and structural requirements will be met only by delivering a proper cocktail of regulated growth factor genes to the ischemic area, a demand that appear feasible using AAV vectors.

An interesting finding of our study was the observation that some of the proliferating cells found in muscles expressing VEGF were positive for markers of hematopoietic/endothelial cell precursors. Different investigators have recently reported that lineage negative, c-kit positive bone marrow cells are capable of infiltrating the infarcted myocardium (in which expression of VEGF is probably high) where they undergo alternate differentiation routes, including cardiomiocytes, endothelial and smooth muscle cells [12,29,30]. In addition, circulating endothelial progenitor cells of bone marrow origin have been isolated that are incorporated in sites of myocardial neovascularization [31]. Altogether these observations suggest that circulating stem cells might sense ischemic or damaged tissues and migrate to these areas, where they promote tissue formation by a mechanism that is probably different from angiogenic sprouting and might resemble embryonic vasculogenesis. Our results suggest that prolonged expression of VEGF, either directly or through the induction of other cytokines, might be one of the local triggers for progenitor cell accumulation and proliferation. Consistent with this conclusion are the results obtained by intravenous administration of an adenoviral vector expressing VEGF165, which determined rapid mobilization of hematopoietic stem cells and marked increase in circulating endothelial precursors [32]. Alternative to their bone marrow origin, the source of cells infiltrating VEGF-expressing tissues might be from a pool of resident progenitor cells, which are found in the heart and in the muscle [33–35] and might be triggered to proliferate in response to growth factor overexpression. Preliminary experiments performed by bone marrow transplantation from mouse male donors to lethally irradiated female recipients indicate that a large number of these infiltrating cells derive from the transplant (data not shown).

In conclusion, the results obtained by this study indicate that functional angiogenesis can be obtained by simultaneous expression of different cytokines for prolonged periods of time. While long-term expression of angiogenic growth factors is not required for the induction of angiogenesis in experimental models of acute or subacute ischemia, it may still turn out to be essential to attain therapeutic benefit for the treatment of human myocardial or limb chronic ischemia, in which new blood vessels formation is likely to require prolonged stimulation over time to promote maturation of larger collaterals. Furthermore, stable persistence of AAV vector genomes would permit the implementation of molecular systems for regulated control over transgene expression. This would have the additional advantage to overcome the concerns raised by the possibility that disregulated expression of angiogenic genes might favor the risk of pathological neovascularization at distant sites. Finally, the possibility of triggering progenitor cells accumulation and proliferation at specific sites by overexpressing VEGF-A has obvious important clinical implications, since it might permit tissue and blood vessel reconstitution through gene transfer to ischemic or damaged areas as a possible alternative to cellular transplantation.

MATERIALS AND METHODS

Production, purification and characterization of rAAV vectors. The rAAV vectors used in this study are based on the pTR-UF5 construct, kindly provided by N. Muzyczka (University of Florida, Gainesville, FL). The coding sequences of the VEGF165 and angiopoietin-1 cDNAs were obtained by RT-PCR amplification from HL60 and 293 cell RNA respectively and cloned in the vector under the control of the CMV promoter to substitute the GFP gene. Cloning and propagation of AAV plasmids was carried in the JC 8111 E. coli strain. Infectious AAV2 vector particles were generated in 293 cells, cultured in 150-mm-diameter Petri dishes, by co-transfecting each plate with 15 μg of each vector plasmid together with 45 μg of the packaging/helper plasmid, pDG (kindly provided by J.A. Kleinschmidt, Heidelberg, Germany), expressing AAV and adenovirus helper functions. Viral stocks were obtained by CsCl gradient centrifugation as already described [16,36]. rAAV titers were determined by measuring the copy number of viral genomes in pooled, dialyzed gradient fractions using a competitive PCR procedure with primers and competitors mapping in the CMV promoter region common to all vectors [16]. Viral preparations used in this work for animal transduction had titers between 1×10^{13} and 1×10^{14} viral genome particles per ml.

Competitive PCR quantification of vector and GAPDH genomic DNAs. Primers for PCR amplification of VEGF165 cDNA were VEGF-F1 (5′-GAGGGCAGAATCATCACGAAGT-3′) and VEGF-R1 (5′-TCCTATGTGCTGGCCTTGGTGA-3′). Primers for rat GAPDH gene amplification were GAP1 (5′-GACCTCAACTACATGGTCRA-3′) and GAP2 (5′-ATACTCAGCACCAGCATCAC-3′). Localization of these primers is schematically shown in Figure 1D. Competitors for both target DNAs were constructed by an established recombinant PCR procedure [16].

Animals and experimental protocols. Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, December 12th 1987). Adult male Wistar rats weighting 250-300 grams were housed one per cage and maintained under controlled environmental conditions. After general anesthesia, rats were injected through a small skin incision in the tibialis anterior muscle using a 0.5 ml tuberculin syringe with a 27.5 G needle. Each muscle received 3–5 injections for a total of 200 μl volume of purified AAV vector preparations or

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PBS (mock controls). A total of 48 rats were injected for this study. Each animal received in the tibialis anterior muscle of the right leg AAV-VEGF, AAV-Ang1, or the combination of both vectors (16 animals per group). In animals treated with both AAV-VEGF and AAV-Ang1, injection of the latter vector was performed 7 days after the first treatment. All animals received in their left legs mock injections of either AAV-LacZ or PBS. Animals were sacrificed after one (4 animals per group) and three months (12 animals per group) from AAV vector injection. Rats sacrificed one month post-injection were used only for histological characterization. Out of the three-month groups, 4 animals were used for blood flow tests, other 4 animals for the permeability tests and the last 4 animals for histological examination.

Histological evaluation and immunohistochemistry. For histological evaluation, muscle samples were fixed in 2% formaldehyde and embedded in paraffin. Three μm sections were stained with haematoxylin and eosin or haematoxylin only. Immunohistochemical detection of specific antigens was performed with the following antibodies: rabbit polyclonal against VEGF (sc-152, Santa Cruz Biotechnology, Santa Cruz, CA); goat polyclonal against angiopoietin-1 (sc-6320, Santa Cruz Biotechnology); mouse monoclonal against CD45 (OX30, Abcam Ltd, Cambridge, UK); mouse monoclonal against α-smooth muscle actin (clone 1A4, Sigma); rabbit polyclonal against Tie2 (sc-9026, Santa Cruz Biotechnology); goat polyclonal against PECAM1 (sc-1506, Santa Cruz Biotechnology, Inc), mouse monoclonal against Flk-1 (sc-6251, Santa Cruz Biotechnology); rabbit polyclonal against c-kit (sc-168, Santa Cruz Biotechnology); mouse monoclonal against PCNA (sc-56, Santa Cruz Biotechnology). Protocols for immunohistochemistry were according to the Vectastain Elite ABC Kit (universal or goat) from Vector Laboratories. After treatment, slides were rinsed in PBS and signals were developed using 3,3′-diaminobenzidine as substrate for the peroxidase chromogenic reaction (Lab Vision Corporation, Fremont, CA).

Blood flow assay. Blood flow was determined by injections of 2×10^5 15 μm yellow-green fluorescent microspheres (Molecular Probes Inc, Eugene, OR) in the abdominal aorta of animals, as described [37]. Recovery of microspheres from tissue and extraction of fluorescent dye was performed according to the manufacturer's instruction. Briefly, weighted muscles were digested in 10 ml of 2 M ethanolic KOH, 0.5% tween 80 (v/v) (Sigma) overnight at 60°C with periodic shaking. Homogenates were centrifuged at $2000 \times g$ for 20 min in a swinging rotor. The supernatant, with the exception of 1 ml, was removed and the pellet was resuspended in 9 ml 0.25% Tween 80 in water. After an additional centrifugation and washing in deionised water, microspheres were dissolved in 3 ml 2-etoxyethyl acetate (Sigma-Aldrich) for 4 hours at room temperature. Debris was removed by centrifugation and fluorescence was measured in the supernatant by the VersaFluor Fluorometer System (BioRad, Richmond, CA) using EX 490/10 and EM 510/10 (BioRad) as excitation and emission filters respectively. Values were normalised and results are expressed as ratios between fluorescence of treated and control muscles.

Vessel wall permeability assay. Analysis of vessel permeability was performed by an adaptation of the Miles assay [38] to rat muscle. Animals were injected in the femoral artery with 300 μl of 0.5% (w/v) Evans blue (Sigma) in PBS and sacrificed after 30 min. The muscle tibialis anterior was removed and weighted, and the dye was extracted by overnight incubation in 2 ml formamide at 55°C and quantified spectrophotometrically at 610 nm. Values of absorbance were converted, according to a standard curve, in Evans blue content. The results are shown in Figure 5C expressed as a ratio between treated and control muscles from the same animal, in order to reduce inter-animal variations.

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