VEGF Gene Delivery to Muscle: Potential Role for Vasculogenesis in Adults

Matthew L. Springer,* Aileen S. Chen,* Peggy E. Kraft,* Mark Bednarski,† and Helen M. Blau*‡

*Department of Molecular Pharmacology
† Department of Radiology
Stanford University School of Medicine
Stanford, CA 94305-5332

Summary

Constitutive expression of VEGF after implantation of genetically engineered myoblasts into non-ischemic muscle led to an increase in vascular structures. Previously, effects of VEGF delivery to adult muscle have only been reported in ischemic tissues. The resulting vascular structures were reminiscent of those formed during embryonic vasculogenesis, rather than angiogenesis, sprouting from preexisting vessels. Initially, VEGF caused an accumulation of endothelial cells and macrophages, followed by networks of vascular channels and hemangiomas with locally high serum VEGF levels. No effects were evident in adjacent tissue or contralateral legs, where low serum VEGF was detected. These data suggest that the induction by VEGF of angiogenesis or vasculogenesis may be dose-dependent. Furthermore, VEGF expression must be carefully modulated, as overexpression is deleterious.

Introduction

Skeletal muscle possesses unique properties that make it an attractive target tissue for the delivery of genes encoding therapeutic circulatory proteins (for review see Blau and Springer, 1995). Muscle is highly accessible and vascularized and comprises a large percentage of the body mass. Skeletal muscle is also unique in that its precursor cells, myoblasts, which fuse to each other to form multinucleate muscle fibers during embryonic development, can fuse to preexisting adult muscle fibers upon intramuscular implantation (Watt et al., 1982; Dhawan et al., 1991; Hughes and Blau, 1992). Because primary myoblasts are readily transduced with retroviruses in culture (Springer and Blau, 1997) and can continue to express appropriate viral promoters for months to years after implantation into muscle (Dai et al., 1992; Rando and Blau, 1994), myoblasts have been widely used to transfer recombinant genes into mice, especially genes encoding secreted circulatory proteins (for example Barr and Leiden, 1991; Dhawan et al., 1991; Bohl et al., 1997). Experiments in monkeys and humans have shown that, as in mice, implanted myoblasts enter host fibers and no toxicity is observed, suggesting that this approach shows promise clinically for the delivery of therapeutic proteins (Kinoshita et al., 1996; Gussoni et al., 1997).

Regardless of the mode of gene introduction, the efficacious delivery of circulatory proteins via gene therapy requires that the proteins made in the muscle gain access to the circulation. Because vascular permeability in skeletal muscle is lower than that of secretory tissues (Joyner and Kern, 1990), muscle-mediated delivery of circulatory proteins by any of the above strategies would benefit greatly from increased blood flow to the site of protein production or from increased vascular permeability. One strategy is the delivery of genes encoding angiogenic or permeability enhancing proteins, which could increase the efficacy of codelivered therapeutic genes or may be therapeutic in their own right in diseases characterized by vascular insufficiency in the muscle, such as diabetes.

One such protein is vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), which is an angiogenic growth factor that was isolated by virtue of its ability to stimulate growth of endothelial cells and to increase permeability in vascular endothelium (Lobb et al., 1985; Leung et al., 1989; Plouet et al., 1989; Conn et al., 1990; Senger et al., 1990). Injection of VEGF protein has resulted in angiogenic sprouting of vessels in muscle that was deprived of blood and oxygen and therefore ischemic (Takeshita et al., 1994a, 1994b; Bauters et al., 1995); however, bolus injections of the protein have been reported to be deleterious, causing hypotension (Hariawala et al., 1996; Horowitz and Helen M. Blau* ³

³ To whom correspondence should be addressed (e-mail: hblau@ cmgm.stanford.edu).
every mouse that received VEGF-producing cells. At day 11, mice appeared outwardly normal and no differences were observed between VEGF and control muscles upon dissection. However, histological analysis of frozen sections revealed that control injected muscles contained the expected needle tracks of blue β-gal-expressing muscle fibers upon staining with X-gal (Rando and Blau, 1994; data not shown, see Figure 5). VEGF-cell-injected muscles also contained blue fibers, but these fibers were invariably associated with and frequently surrounded by regions of mononuclear cells visualized by hematoxylin and eosin staining (H/E; Figures 2A–2C). These cells did not stain blue with X-gal, confirming that they were not derived from the implanted myoblasts. The use of SCID mice ensured that these cells did not represent a cytotoxic T cell response; this was confirmed by the lack of immunostaining with antibodies against CD4 and CD8 (data not shown). Neutrophils, which can be readily identified based on nuclear morphology, were not observed in the cellular regions (Figure 2D). Similar sections were immunofluorescently stained with antibodies against von Willebrand factor (vWF), which is present in the blood vessel basement membrane produced by mature vascular endothelial cells (Rand et al., 1980), and antibodies against platelet-endothelial cell adhesion molecule (PECAM-1; CD31), which is present on all endothelial cells and on monocytes but not on tissue macrophages (Muller et al., 1989; DeLisser et al., 1994). PECAM-1-positive cells were observed throughout these regions both individually and in clusters, the vast majority of which did not produce detectable amounts of vWF (Figure 2E). Additional double immunostaining revealed that the PECAM-1-positive cells also expressed the endothelial markers VE-cadherin (CD144) and CD34 (Fina et al., 1990; Lampugnani et al., 1992; Figures 2F and 2G). These endothelial cells were accompanied by macrophages, identified by immunostaining with the macrophage-specific antibody F4/80 (Austyn and Gordon, 1981). That the PECAM-1-expressing cells and the F4/80-expressing cells were distinct populations is clearly shown (Figure 2H). No such regions were observed in contralateral uninjected legs of the same animals. In addition, vWF immunostaining of muscle showed no obvious difference in the density of blood vessels from legs injected with VEGF myoblasts, legs injected with control myoblasts, and contralateral uninjected legs (data not shown). Thus, at day 11, the most notable effect was the presence of macrophages and markedly increased presence of masses of individual endothelial cells in VEGF-expressing muscles.

Formation of Hemangiomas and Large Vessels

By day 24, the VEGF-cell-injected legs had begun to swell perceptibly. Histological analysis revealed structures that appeared to be small hemangiomas that colocalized with β-gal-labeled regions of the leg (Figure 3). However, the VEGF-expressing myofibers, detectable by their expression of β-gal, were sparsely distributed around the implantation site and were interspersed with regions of mononuclear cells as described above and by blood pools typically associated with hemangiomas (Cotran et al., 1994).
Figure 2. Recruitment of Endothelial Cells by Day 11 of VEGF Production in Muscle
(A) Low magnification showing entire leg cross-section, stained with X-gal. Box denotes region shown at higher magnification in (B). Infiltrating cells (arrows) are not blue and are therefore not derived from implanted myoblasts. (C) Identical neighboring section stained with hematoxylin/eosin (H/E); white arrows correspond to the arrows in (B). Box denotes region enlarged in (D) to show the absence of neutrophils, shown in inset for comparison. (E) Double immunofluorescent labeling of a similar region of mononuclear cells in a single section, showing staining for PECAM-1 (green; top) in many cells and von Willebrand factor (red; bottom) in very few cells. (F) Double labeling with antibodies against PECAM-1 (green; top) and VE-cadherin (red; middle). Yellow color in the double exposure (bottom) indicates overlap of red and green caused by the presence of both antigens on the same cells. (G) Same as in (F), but red is immunostaining for CD34. (H) Same as in (F), but red is F4/80 macrophage staining. Separate red and green color in the double-exposure panel indicates the presence of the antigens on two distinct cell populations. Bars, (A) 1 mm; (B and C) 100 μm; (D) 20 μm; (E) 100 μm; and (F) 50 μm.

By day 44-47, 100% of the legs injected with VEGF myoblasts had become dark in color and very large, encompassing more than twice the diameter of control legs (Figure 4). All contralateral uninjected legs and control legs injected with LacZ myoblasts were normal in size. At this point, the mice were still ambulatory, but the experiments were terminated and the mice were euthanized. Dissection of the large legs revealed that this growth effect was limited to the muscles of the lower leg, into which the myoblasts had been implanted. Histologically, the legs injected with VEGF myoblasts (Figures 4A and 4B) now consisted primarily of hemangioma and pools of blood, with very few surviving β-gal-labeled myofibers. In contrast, the uninjected contralateral legs and control legs injected with LacZ myoblasts (as shown in Figures 4C-4E at the same magnification as the large legs) appeared normal and showed no signs of hemangioma or mononuclear cell infiltration. One mouse was kept alive until day 50 and was analyzed by nuclear magnetic resonance imaging (MRI) using T2-weighted imaging to visualize fluid. No pooled fluid was observed in the contralateral leg by MRI, whereas the legs injected with VEGF myoblasts possessed what appeared to be a lattice of fluid-filled compartments (Figure 4F), consistent with the identification of the VEGF-induced structures as hemangiomas.

Higher magnification observations revealed that the hemangiomas in the legs injected with VEGF myoblasts consisted of cellularized structures that appeared as tendrils in cross-section, presumably corresponding to the borders of the “channels” visible by MRI (Figure 4A). Immunofluorescence revealed that the exterior but not the interior of these tendrils expressed PECAM-1 (Figure 5A). Many individual macrophages were observed in the interior of the tendrils (Figure 5C). Immunostaining of these structures with multiple antibodies showed that
the outer layer of PECAM-1-expressing endothelial cells generally was not associated with vWF, although a minority of the channels were bounded by cells that expressed both vWF and PECAM-1 (Figure 5D). vWF immunostaining was observed consistently in blood vessels, platelets, blood clots, and megakaryocytes in the bone marrow (data not shown), but the staining for vWF in the vascular channels was inconsistent, as previously reported for hemangiomas (Booth and Sundberg, 1995). The layer of cells directly below the endothelial layer reacted strongly with an antibody against smooth muscle actin (Skalli et al., 1986), with a pattern similar to that of a normal blood vessel, whereas a less intense filamentous staining pattern was observed throughout the interior of the tendrils (Figures 5E and 5F). Uncharacteristically large vessels and even larger channels were observed that possessed an inner lining that sometimes contained vWF (Figures 5G and 5H). None of these effects were observed in contralateral uninjected legs (data not shown). Control myoblasts not expressing VEGF fused into muscle fibers and produced β-gal as expected, with no evidence of mononuclear cells or disruption of tissue architecture (Figures 5I and 5J). Of particular interest was the observation that, as at the earlier time points, an increase in the number of visible vessels was not observed in the muscle tissue adjacent to the hemangiomas, despite the intense hypervascular activity at the site of implantation.

Effects on Capillary Density

In previous studies as discussed above, delivery of VEGF has resulted in an increase in the capillary/myofiber ratio in ischemic muscle. In order to ascertain whether the density of capillaries had been affected either locally or at sites distant from the site of myoblast implantation, five legs representative of different conditions and controls were sectioned and immunofluorescently stained with the PECAM-1 antibody to label capillaries (Figures 6A and 6B). For each leg, 32 randomly chosen microscope fields taken from four different planes throughout the muscle were photographed and the capillaries were counted in each field. Double labeling with anti-PECAM antibodies and Hoechst dye (data not shown) confirmed that the red dots visualized by PECAM-1 staining were not nuclei. In the legs injected with VEGF cells, capillaries and fibers were counted in regions of muscle adjacent to hemangioma tissue. No significant difference in capillary/myofiber ratios was detected in the non-ischemic muscles analyzed here (Figure 6C).
Figure 5. Late-Stage Formation of Vascular Structures by Day 44–47, High Magnification

For orientation, representative lumens of vascular channels or blood vessels are denoted with asterisks. (A) Part of a hemangioma caused by injection of VEGF-expressing cells, such as that in Figure 4B, showing tendrils and blood channels visualized by H/E staining. (B) Similar sample double labeled with antibody to the endothelial cell marker PECAM-1 (red) and nuclear stain Hoechst 33258 (blue), illustrating that the tendrils only possess endothelial cells on the outside. (C) Similar sample visualized by double immunofluorescent staining for PECAM-1 (red) and the macrophage antigen F4/80 (green), showing that the interior of the tendril structures are abundant in macrophages. (D) Similar sample but double labeled with antibodies to PECAM-1 (red) and von Willebrand factor (green); yellow color results from colocalization of red and green staining. Only a minority of the channels stain both red and green (hence yellow); most only stain red. Platelets, which also express von Willebrand factor, are seen as small green specs. (E) Normal blood vessels double immunostained with antibodies against PECAM-1 (red) and smooth muscle actin (green; overlapping regions are yellow) illustrating the usual presence of an endothelial intima layer directly over a smooth muscle media layer. (F) Hemangioma tissue tendrils similarly double labeled, showing an endothelial layer covering a strongly staining smooth muscle layer and a smooth muscle-like stroma. (G and H) Lower magnification of neighboring sections containing a very large vascular pool stained with H/E and von Willebrand factor antibody. (I and J) Neighboring sections from a mouse injected with control myoblasts stained with X-gal and H/E. Arrows point to corresponding locations. Bars, 100 μm in all panels.

Serum Levels of VEGF

VEGF was not detectable by ELISA in serum from day 21 by retro-orbital bleeds of four control mice and four mice implanted with VEGF myoblasts (limit of detection <8 pg/ml). However, when blood was collected in the same manner on day 43, VEGF was detected at an average level of 40 ± 21 pg/ml in the four mice that received VEGF myoblasts, and was undetectable in the four control mice. On the following day, when two VEGF myoblast-injected animals were sacrificed, extensive bleeding occurred upon dissection of the enlarged legs. Blood samples were collected from the bleeding region next to the leg and the sera from these samples were assayed and determined to be 230 and 159 pg/ml, respectively; therefore, the local concentration of VEGF was significantly higher than that measured in the systemic circulation the day before.

Discussion

We present here the novel findings that exogenously delivered VEGF can exert a physiological effect in normal, non-ischemic muscle, and that too much VEGF expression can have deleterious effects. In addition, our data demonstrate that a single growth factor can lead to the recruitment and organization of multiple cell types into complex vascular structures. It is notable that the physiological response to VEGF delivery occurred in
A model is shown in which lower VEGF levels or shorter durations of exposure to VEGF induces angiogenesis in ischemic muscle, whereas higher levels or longer durations induces vasculogenesis in non-ischemic muscle.

Figure 6. Capillary Densities Are Unaltered by VEGF Delivery to Normal Muscle
(A) Cross section of muscle immunostained for PECAM-1, showing capillaries between fibers. Bar, 100 \( \mu \)m. (B) Graph of capillary/myo-fiber ratios, showing the mean ± standard deviation for the following several legs: leg injected with VEGF cells, day 23; two legs injected with VEGF cells, day 44; contralateral uninjected leg from an animal injected with VEGF cells, day 44; and leg injected with control cells not expressing VEGF, day 44. In the legs injected with VEGF cells, the capillary counts were derived from regions of muscle adjacent to the hemangiomas.

non-ischemic muscle, whereas previous reports of angiogenesis induced by VEGF delivery have involved either naturally occurring or induced muscle ischemia associated with an inadequate supply of blood and oxygen (Banai et al., 1994a; Takeshita et al., 1994a, 1994b, 1996; Harada et al., 1996; Isner et al., 1996; Tsurumi et al., 1996; Mack et al., 1998). Indeed, when VEGF protein was administered intravenously to animals with one ischemic leg, new vessels were formed but only in the ischemic limb (Bauters et al., 1995). A relationship between ischemia and VEGF is well documented, as hypoxia induces upregulation of expression of both VEGF and its receptors (Claffey et al., 1992, 1998; Banai et al., 1994b; Brogi et al., 1996; Detmar et al., 1997). These previous findings have led to the prevalent theory that ischemia is necessary for VEGF to exert its effects in muscle. The data presented here show that this is not the case.

One possible explanation as to why the effects in non-ischemic muscle reported here have not previously been observed is that the effects of VEGF may be dose-dependent. High serum VEGF levels (200 pg/ml) were detected at the implantation site, where we observed a network of channels so extensive as to form a hemangiomatoma. This effect was seen in adult muscle that was not ischemic. By contrast, the lower serum levels of VEGF in the systemic circulation (40 pg/ml) had no effect on vessel number or size in adjacent or contralateral muscles, which were non-ischemic as well. Notably, this lower level is comparable to that observed by others in the serum (35 pg/ml) after transfection of a plasmid encoding VEGF into the arterial wall, which sufficed to induce angiogenesis but only in ischemic muscle (Takeshita et al., 1996). We propose that this differential effect may be explained by a multiple threshold model, in which a specific threshold level of VEGF is required to affect ischemic adult muscle and a higher level of VEGF is required to affect non-ischemic adult muscle.

The data presented here raise the possibility that the network of channels observed in normal adult muscle at high VEGF levels was due to vasculogenesis, a process of new vessel formation previously thought only to occur in embryogenesis. In support of this hypothesis, there was no evidence of augmented branching of blood vessels leading into the VEGF-induced hemangiomas, which would be characteristic of angiogenic vessel sprouting (Wilting et al., 1992). Instead, the hemangiomas appeared to be essentially self-contained and not integrated with the surrounding tissue. During embryonic development, a network of channels comprising the vasculature arises by vasculogenesis, the fusion of primordial blood islands consisting of hematopoietic precursor cells surrounded by endothelial cell precursors known as angioblasts (Pardanaud et al., 1989; Risau and Flamme, 1995). We postulate that if this is the case, then the multiple threshold model proposed above may be modified whereby a specific level of VEGF is required for angiogenesis in ischemic muscle, and a higher level can stimulate vasculogenesis in non-ischemic muscle (Figure 7).

How might these vascular structures arise? We postulate that localized constant production of high levels of VEGF can lead to the recruitment of circulating endothelial precursors, or angioblasts, and induce them to undergo differentiation in non-ischemic adult muscle. Several lines of evidence suggest this intriguing possibility. We show here that the initial response to VEGF expression in the muscle is an accumulation of isolated cells that express PECAM-1 but not vWF and do not appear to be connected to neighboring blood vessels. Angioblasts in the embryo and in cultured embryoid bodies that form from dissociated embryonic cells express PECAM-1, VE-cadherin, and CD34, but not vWF (Wang et al., 1992; Baldwin et al., 1994; Nishikawa et al., 1998),
which are properties shared by the cell masses that were recruited by VEGF in our study. Bone-marrow-derived endothelial precursor cells, thought to be angioblasts or to be derived from them, have been reported to persist in the adult circulation and to potentially play a role in the endothelialization of vascular grafts (Asahara et al., 1997; Shi et al., 1998). Microinjection of VEGF protein into quail embryos early in development is known to cause vasculogenesis in normally avascular regions and the appearance of “massive vascular sacs” (Drake and Little, 1995), which appear to be similar to the channels that we observed. Taken together, these observations are consistent with the hypothesis that the implantation of VEGF-expressing myoblasts may have caused the chemoattraction of distant precursor cells and triggered post-embryonic vasculogenesis or a similar process. This hypothesis may also provide an explanations for previous observations that the implantation of VEGF-expressing fibroblasts resulted in small clusters of one to eight endothelial cells not connected to blood vessels (Mesri et al., 1995). In addition, when C6 glioblastomas were engineered to express VEGF in vivo, “vascular sacs” similar to those described here were observed, which may have also arisen through the recruitment of precursor cells. Clearly, the identification and characterization of this putative precursor is of major interest.

That exogenous delivery of a single factor that primarily affects endothelial cells could result in such marked effects on multiple cell types leading to the formation of a complex tissue was quite unexpected. The structure of the tissue surrounding the vascular channels is similar to that of clinical hemangiomas, consisting of smooth muscle and macrophage-like cells lined with endothelial cells (Williams, 1980; Kojimahara, 1986). Furthermore, the structure is reminiscent of that of the intima and media of normal blood vessels. Although macrophages, which are also known to be chemoattracted to VEGF (Shen et al., 1993), can produce angiogenic factors themselves and could play a synergistic role (Moore and Sholley, 1985), the macrophages recruited along with the endothelial cells in this report are not likely to suffice to cause the observed response. Indeed, in our previous studies, hemangiomas have not resulted from myoblast-mediated gene delivery of colony-stimulating factor-1 (CSF-1), although a profound effect on macrophage accumulation was observed (Dhawan et al., 1996). Thus, VEGF itself appears to be the critical factor that triggers this effect, regardless of the possible role of macrophages as mediators. Although, in theory, edema due to the vascular permeability enhancing properties of VEGF could have contributed to the observed increase in leg size, this possibility seems unlikely. Both the histological and MRI analyses shown in Figures 4 and 5 demonstrate that the growth of the leg is due primarily to the growth of hemangiomas. Therefore, VEGF alone, delivered from a nontransformed, non-endothelial source, is able to directly induce the differentiation of the multiple cell types required for blood vessel formation.

Our results make abundantly clear that VEGF delivered at excessive levels or durations can have deleterious results and that previous speculations on this matter were well founded. Isner et al. (1996) noted that small hemangiomas did indeed form in the ischemic leg of a human patient treated by VEGF plasmid transfection of the arterial wall; however, these hemangiomas apparently resolved themselves as the temporary expression of VEGF ceased. Indeed, the inherently transient gene expression resulting from plasmid transfection and adenoviral gene delivery, which are the two VEGF gene delivery techniques currently in clinical trials, may be desirable in this case. An advantage of retrovirally transduced myoblast implantation is that it allows localized delivery of VEGF or other growth factors at sustained physiological levels. Furthermore, delivery of these genes under the control of a regulatable promoter has the potential for precise control of the time or level of delivery. This possibility can now be tested using novel regulatable vector systems in which gene expression can be activated, levels modulated, and extinguished at will (Hofmann et al., 1996; Kringlestein et al., in press; Rossi et al., unpublished data).

Experimental Procedures

Construction of MFG-VEGF Retrovirus and Genetic Engineering of Primary Mouse Myoblasts

The murine VEGF cDNA clone was a generous gift of K. Claffey (Beth Israel Deaconess Medical Center, Boston, MA). PCR was used to engineer BspHI and BamHI sites at the 5′ and 3′ ends, respectively. The BspHI-BamHI fragment was excised and ligated into the standard NcoI (compatible with BspHI) and BamHI sites in the MFG retroviral plasmid (provided by P. Robbins, University of Pittsburgh, PA). The ligation product was sequenced across the junctions and through the open reading frame to confirm the absence of PCR-related mutations. Both the PCR product and the original clone were confirmed to have the sequence GGA GAG (Gly Glu) at positions 91 and 92 instead of GAG AGA (Glu Arg) in the published murine VEGF sequence; however, the published sequences for VEGF of four other species all show Gly Glu at these positions. The resulting plasmid, pMFG-VEGF, was transfected into Phoenix packaging cells (Pear et al., 1993; Achacoso and Nolan, unpublished data) and retroviral supernatants were collected, frozen on dry ice. Primary myoblasts already expressing LacZ from a retroviral promoter (Rando and Blau, 1994) were transduced at high efficiency (Springer and Blau, 1997) with four successive exposures to MFG-VEGF virus.

Immunosays of Cells and Culture Media

Immunoblots were probed with a goat anti-mouse VEGF polyclonal antibody (R&D Systems, Minneapolis, MN) at 0.2 μg/ml for 2–3 hr and then probed with HRP-conjugated rabbit anti-goat secondary antibody (Calbiochem, La Jolla, CA) at 1:10,000 dilution for 1 hr. VEGF bands were visualized by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL). For immunofluorescence, cells were grown in tissue culture dishes and secretion was blocked for 3 hr with the drug monensin to allow secreted proteins to accumulate in the Golgi apparatus (Tassin et al., 1985). The cells were then fixed in 1.5% formaldehyde in PBS for 15 min at room temperature, followed by permeabilization and blocking in 0.1% saponin and 0.5% BSA in PBS (SBPBS) for 30 min at room temperature. The cells were then incubated with the primary antibody described above at 1:100 dilution in SBPBS for 2 hr, rinsed several times with SBPBS, and incubated with a Texas red-conjugated rabbit anti-goat secondary antibody and Hoechst 33258 dye in SBPBS for 1 hr.

ELISA analyses of cell culture supernatants and of mouse serum were carried out using a commercially available anti-mouse VEGF ELISA kit (R&D Systems) according to the manufacturer’s instructions. To quantitate the amount of VEGF secreted by the myoblasts in culture, medium was harvested from both VEGF cells and control cells after 4 hr of incubation and was then removed, filtered, and stored at -80°C. The cells were then counted in a hemacytometer. Thawed culture supernatants were analyzed in duplicate by ELISA,
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