Recombinant Vascular Endothelial Growth Factor Secreted From Tissue-Engineered Bioartificial Muscles Promotes Localized Angiogenesis

Yongxin Lu, MD; Janet Shansky, MSc; Michael Del Tattoo, BS; Paulette Ferland; Xiaoyun Wang, MD; Herman Vandenburgh, PhD

Background—Therapeutic angiogenesis by the administration of recombinant vascular endothelial growth factor (rVEGF) is a novel strategy for the treatment of ischemic disorders. rVEGF has been delivered as a protein, by plasmid DNA, and by genetically engineered cells with different pharmacokinetic and physiological properties. In the present study, we examined a new method for delivery of rVEGF using implantable bioartificial muscle (BAM) tissues made from genetically modified primary skeletal myoblasts. Our goal was to determine whether the rVEGF delivered by this technique promoted controlled angiogenesis in nonischemic and/or ischemic adult mouse tissue.

Methods and Results—Primary adult mouse myoblasts were retrovirally transduced to secrete human or mouse rVEGF and tissue-engineered into implantable 1×10 to 15-mm BAMs containing parallel arrays of postmitotic myofibers. In vitro, they secreted 290 to 511 ng of bioactive mouse or human VEGF/BAM per day. rVEGF BAMs implanted subcutaneously into syngeneic mice caused a 30-fold increase in the number of CD31-positive capillary cells within the BAM by 1 week compared with control BAMs. Implantation of rVEGF-secreting BAMs into ischemic hindlimbs resulted in a 2- to 3-fold increase in capillary density of neighboring host muscle by 1 week and out to 4 weeks with no evidence of hemangioma formation.

Conclusions—Local delivery of rVEGF from BAMs rapidly increases capillary density both within the BAM itself and in adjacent ischemic muscle tissue. Genetically engineered muscle tissue provides a method for therapeutic protein delivery in a dose-regulated fashion. (Circulation. 2001;104:594-599.)

Key Words: angiogenesis ■ muscles ■ ischemia ■ gene therapy
cloned into the BAM H1 site of pLgXSN (gift of Dr Dusty Miller, Fred Hutchinson Cancer Center, Seattle, Wash.). pMFG-mVEGF, an MFG retroviral construct containing the cDNA encoding recombinant murine VEGF<sub>164</sub>, was a gift of Dr Helen M. Blau (Stanford University, Palo Alto, Calif.). Recombinant human growth hormone (rhGH) cDNA was used as a soluble, secretable marker of gene activity. It was excised from the MFG-6G revertial construct (gift of Dr Jeffrey Morgan, Shriners Burn Institute, Cambridge, Mass.) and subcloned into pLgXSN as described above for rhVEGF<sub>165</sub>.

**Generation of Replication-Deficient Retroviral Producer Cell Lines**

Retroviral producer cell lines were generated for LghVEGF<sub>165</sub>SN, LghGHSN, and LgXSN after a 2-step transfection/transduction protocol optimized for primary adult mouse myoblasts by use of E86 ectropic and PT67 amphotropic packaging cells. Virus-containing medium was collected from high-titer PT67 clones and stored at −80°C. pMFG-mVEGF was transfected into Phoenix packaging cell (gift of Dr Garry Nolan, Stanford University) to generate virus-containing medium containing mVEGF retrovirus, and β-galactosidase retroviral medium was collected from a stably transduced packaging cell line (CRE BAG 2; CRL-1858, ATCC).

**Primary Mouse Myoblast Culture, Transduction, and Tissue Engineering Into BAMs**

Primary mouse myoblasts were isolated from the hind limbs of 4- to 6-week-old male C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, Me) and maintained in culture according to standard procedures.<sup>13,14</sup> Isolated myoblasts were transduced with polybrene-supplemented virus-containing medium according to a centrifugation protocol.<sup>15</sup> BAMs for subcutaneous implants were formed from 2×10<sup>6</sup> transduced myoblasts and were 1×15 mm,<sup>16</sup> whereas those implanted into ischemic hind limbs were 10 mm long and were formed from 1.5×10<sup>6</sup> myoblasts. BAMs were treated with cytochrome arabinoside (1 µg/mL) for 3 to 6 days before implantation to eliminate proliferating cells as previously described.<sup>10</sup>

**Growth Factor Analyses**

mVEGF and rVEGF protein levels in culture medium from BAMs and mouse serum were measured with ELISA kits (R&amp;D Systems). The minimum detectable dose with these kits is 3 to 5.0 pg/mL. To measure tissue levels of extracellular matrix-bound mVEGF or hVEGF, BAMs were homogenized in protein lysis buffer.<sup>17</sup> Total protein was measured by the BCA protein assay (Pierce). hGH levels in culture medium and serum were assayed by a radioimmunoassay technique that does not cross-react with mouse GH. For Western blots, aliquots of conditioned culture medium containing 6 ng of hVEGF<sub>164</sub>, subjected to electrophoresis on 12% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, probed with anti-hVEGF (sc152, Santa Cruz Biotechnology), and developed with ECL detection reagent (Amersham).

Stimulation of human umbilical vein endothelial cell (HUVEC, Clonetics) proliferation by conditioned medium from BAMs was used as a measure of VEGF bioactivity.<sup>18</sup> Anti-hVEGF monoclonal antibody (R&amp;D) was added to some culture wells, and rhVEGF<sub>165</sub> (R&amp;D, 10 ng/mL) was used as a positive control.

**Surgical Procedures: Implantation of BAMs and Ischemic Model**

All experimental animal procedures were approved by the Institutional Animal Care and Use Committee and conformed to the guiding principles of the American Physiological Society. After 14 days in vitro, BAMs were implanted into 4- to 6-week-old male C3HeB/FeJ mice. Mice receiving rhVEGF or rhGH BAM implants were immunosuppressed with cyclosporine (60 mg/kg daily) because of the potential for formation of antibodies to the human protein. Subcutaneous BAM implants were as previously described<sup>11</sup> with either 1 BAM (rVEGF implants) or 2 BAMs (rhGH implants) implanted into the back of each animal. For the ischemic model, the femoral and saphenous arteries were ligated in 1 hind limb of each mouse, and side branches were removed.<sup>19</sup> One BAM was implanted in the ischemic hindlimb between the tibialis anterior muscle fascia and overlying skin and secured in place on the fascia by fibrin sealant (Tisseel VH; Baxter Hyland) to maintain myofiber tension.

**Tissue Histochemistry and Quantification of Capillary Density in BAMs**

BAM and host muscle explants were either frozen in isopentane or fixed with 0.25% glutaraldehyde for cryostat sectioning. Capillary density was examined by quantification of endothelial cells in cryostat sections stained with anti-mouse CD31 (Pharmingen), an antibody specific for mouse endothelial cells, following standard immunoperoxidase procedures and development with DAB. The primary antibody was omitted from negative controls. Five nonoverlapping microscopic fields were analyzed from each explanted BAM by use of the Zeiss KS 300 Version 3.0 Image Analysis System, and the area that stained positive for CD31 was quantified and expressed as a percentage of the total area analyzed.

For β-gal staining, glutaraldehyde-fixed BAMs were cryosectioned and stained with an X-gal Substrate Set (Kirkegaard & Perry Laboratories).

**Statistical Analyses**

Results are expressed as mean±SEM, and comparisons were by unpaired t tests, with P<0.05 taken as a statistically significant difference.

**Results**

**Tissue-Engineered Primary Mouse Myoblast BAMs Express Biologically Active VEGF In Vitro**

Transduced primary mouse skeletal myoblasts were tissue-engineered into BAMs by suspending the cells in a collagen-Matrigel extracellular matrix solution and casting the suspension into silicone rubber molds with artificial end attachment points.<sup>16</sup> Internal longitudinal tensions develop within the cell-gel mixture as it dehydrates, causing the formation of a cylindrical structure 1 mm in diameter and containing parallel arrays of multinucleated postmitotic myofibers. Hematoxylin-eosin staining of BAM cross sections revealed no morphological difference between rVEGF and control BAMs (data not shown).

Western blotting of culture medium from rhVEGF BAMs under reducing conditions showed 2 bands with molecular weights of 28 and 23 kDa, with the majority of the secreted protein in the 28-kDa band; rhVEGF standards showed a major band at 26 kDa and a minor band at 23 kDa (data not shown). The BAMs in vitro secreted consistent levels of hVEGF<sub>165</sub>, were subjected to electrophoresis on 12% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, probed with anti-hVEGF (sc152, Santa Cruz Biotechnology), and developed with ECL detection reagent (Amersham).

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The biological activity of secreted rhVEGF was determined by its ability to increase endothelial cell proliferation. HUVECs were incubated with conditioned medium from either rhVEGF BAMs (rhVEGF concentration of ≈10
ng/mL) or control BAMs. The mitogenic activity on HUVECs increased 50±6% with conditioned medium from rhVEGF BAMs, compared with an increase of 5±3% with medium from control BAMs relative to unsupplemented medium (Figure 1). The growth response elicited by rhVEGF-conditioned medium was only partially neutralized by an antibody specific for hVEGF, probably because of the synergistic stimulation of HUVEC proliferation by other growth factors present in the conditioned medium (eg, insulin-like growth factor-1).

BAMs Implanted Subcutaneously Into Syngeneic Mice Can Survive for at Least 5 Weeks In Vivo

rmVEGF-BAG BAMs and BAG BAMs were implanted subcutaneously into syngeneic mice. Explanted BAMs showed areas of healthy myofibers that stained β-gal–positive after 1 to 5 weeks in vivo, with no β-gal staining outside the area of the implant (Figure 2), indicating that the transduced cells in the BAMs have not migrated from the implant site.

mVEGF Levels Are Greater in Implanted rmVEGF BAMs Than in Control BAMs

One and 2 weeks after implantation, the mVEGF content of rmVEGF BAMs was 2.5- to 3.0-fold higher than in control BAMs (Figure 3A). In BAMs explanted after 3 and 5 weeks, the level of mVEGF in rmVEGF BAMs was similar to that of control BAMs and comparable to levels in normal mouse tibialis anterior muscle (Figure 3A). This decrease after 2 weeks in vivo is not due to cell death or promoter inactivation, because rhGH BAMs engineered with the same LgXSN construct and from the same primary myoblasts expressed soluble hGH for >10 weeks (Figure 3B).

Vascularization Is Accelerated Within VEGF-Secreting BAMs

Capillary ingrowth into subcutaneously implanted BAMs showed a significantly higher density of CD31-positive cells in mVEGF BAMs than in control BAMs at all time points (Figure 4). After 1 week, 23.8±2.5% of the total cross-sectional area stained positive for CD31 in rmVEGF BAMs, compared with only 0.8±0.2% in nonsecreting BAMs (Figure 5). This increase was sustained out to 6 weeks (28.9±1.7% versus 10.1±1.8% in rmVEGF-secreting and control BAMs, respectively). Similar results were observed in short-term studies of immunosuppressed mice implanted with rhVEGF-secreting BAMs, but longer-term implant studies were not performed with rhVEGF BAM implants because of the high level of immunosuppressant required. In no instance did hemangiomas form in or around the rVEGF BAM implants.

Angiogenesis of Ischemic Muscle Is Accelerated by rmVEGF BAMs

Capillary ingrowth in the ischemic tibialis anterior muscle was significantly increased as early as 1 week in mice receiving rmVEGF-secreting implants compared with control BAMs or no implants and continued to increase for up to 4 weeks (Figure 6). Capillary density in the tibialis anterior muscle was greatest near the implant at 1 week, but by 4 weeks there was no difference along the length of the muscle (data not shown).

Systemic Levels of mVEGF Are Not Increased by rmVEGF BAM Implants

Serum levels of mVEGF were assayed from mice implanted with rmVEGF BAMs, control BAMs, and mice with no implants. There were no significant differences between any of the groups in mice receiving either subcutaneous or ischemic hindlimb implants for up to 6 weeks (38.6 to 56.1 pg/mL for both control and rmVEGF implants), demonstrating that rmVEGF BAMs act locally rather than systemically to stimulate angiogenesis.
Vascularization of Implanted BAMs Can Be Predicted From Preimplant mVEGF Secretion Levels

One advantage to using BAMs as a delivery platform for a foreign gene product is the ability to monitor protein secretion levels before implantation. To determine whether it is possible to accurately predict the in vivo biological effect of mVEGF from in vitro mVEGF secretion levels, we compared the secretion level from preimplant rmVEGF BAMs (156 to 336 ng mVEGF · BAM⁻¹ · d⁻¹) to their capillary density after implantation for 3 weeks (Figure 7). A linear relationship exists between the area staining positive for CD31 and preimplantation secretion levels of the BAMs (r² = 0.83), indicating that stimulation of capillary growth by rmVEGF BAMs in vivo can be predicted from preimplantation in vitro mVEGF secretion levels.

Discussion

In this study, we show that primary adult mouse myoblasts can be genetically engineered to secrete rhVEGF or rmVEGF and tissue-engineered into bioartificial muscles (BAMs). rhVEGF BAMs secreted hVEGF₁₆₅ with molecular weights of 28 and 23 kDa. Similar results have been reported in other studies and may represent glycosylation variants of rhVEGF₁₆₅. Bioactivity of rhVEGF secreted from BAMs was demonstrated by its ability to stimulate the growth of human umbilical vein endothelial cells in vitro. Subcutaneous implantation of rhVEGF- or rmVEGF-secreting BAMs into syngeneic mice resulted in significantly increased vascularization of rVEGF-secreting BAMs compared with nonsecreting BAMs, confirming the bioactivity of the secreted rmVEGF and rhVEGF in vivo. In addition, implantation of rmVEGF-secreting BAMs into an ischemic hindlimb stimulated localized angiogenesis of neighboring host muscle tissue. These results suggest that tissue-engineered skeletal muscle may be a practical platform to secrete biologically active rVEGF in order to stimulate angiogenesis in neighboring ischemic tissue.
rVEGF gene therapy has been shown to promote therapeutic angiogenesis in preclinical models of tissue ischemia and in human clinical trials. Therapeutic angiogenesis is not risk-free, however. Some possible negative side effects using various methods of rVEGF delivery are the production of nonfunctional leaky vessels and enhancement of vascular permeability, development of hemangiomas, and the stimulation of angiogenesis in tumors. It is therefore important to determine a means of optimally inducing localized angiogenesis with minimal effects systemically and to find an appropriate dose of rVEGF that minimizes the potential deleterious effects on nearby tissue.

Delivery of rVEGF from BAMS is shown in the present study to target a local area with no elevation in serum levels, no harmful effects on neighboring tissue, and no hemangioma formation for up to 6 weeks in vivo. In another study, implantation of mVEGF-engineered proliferating myoblasts into nonischemic mouse leg muscles or the heart led to capillary growth into the subcutaneous implants in mice and increases capillary growth into endothelial cell growth into the subcutaneous implants in mice and increases capillary growth into nearby host muscle in an ischemic animal.

In summary, cell-based delivery of rVEGF from a “living protein delivery platform” composed of fused, postmitotic muscle cells results in the stimulation of endothelial cell growth into the subcutaneous implants in mice and increases capillary growth into nearby host muscle in an ischemic condition. The use of retroviral vectors in our studies resulted in the stable integration of the rVEGF gene into the host cell genome and long-term expression when implanted in vivo. Adenoviral vectors are characterized by a progressive loss of gene expression, because they are not integrated into the host genome. mVEGF levels in explanted BAMSs were significantly elevated after 1 and 2 weeks in vivo but decreased to that of normal mouse skeletal muscle by 3 to 4 weeks (Figure 3A). In contrast, hGH secretion from BAMSs genetically engineered with the same retroviral construct persists for months (Figure 3B). It is not known why the BAM mVEGF levels decrease. Myofibers survive adequately in the BAM for ≥5 weeks on the basis of β-gal staining (Figure 2), so decreased secretion due to myofiber death is unlikely. Possibly a feedback mechanism exists, such that once the blood supply is increased into the “ischemic” BAM, the myofibers no longer synthesize rmVEGF. The LTR promoter may be shutting off, an explanation that seems unlikely, because β-gal gene expression, also driven by the LTR promoter, persists in the implants for >5 weeks. It seems most likely that once the BAMSs are well vascularized, rmVEGF is still expressed but is rapidly delivered to localized host tissue by the newly formed blood vessels and no longer accumulates in the BAM itself.

One advantage of implanting genetically engineered postmitotic myofibers is that secretion levels of growth factors can be monitored in vitro, before implant surgery. In a previous study, we showed that in vivo systemic levels of rhGH from implanted BAMSs could be predicted from preimplantation secretion levels. We demonstrate here that biological activity of mVEGF secreted from BAMSs can also be predicted from in vitro secretion levels (Figure 7), because higher mVEGF secretion levels resulted in a higher density of capillary growth. Protein delivery by injected myoblasts or by intramuscular plasmid DNA injection is limited by the variability in the number of postmitotic muscle fibers that take up and express the foreign gene, making secretion levels difficult to predict. With BAM technology, the desired in vivo biological effect can be regulated by engineering BAMSs with varying numbers of growth factor–secreting myofibers or by implanting varying numbers of BAMSs into each animal.
hindlimb model. Extending rVEGF BAM technology to human skeletal muscle offers great potential for the treatment of ischemic disease. We showed in a previous study that human adult skeletal muscle cells isolated from elderly congestive heart failure patients and genetically engineered to secrete rhGH can be formed into rhGH-secreting BAMs.29 The subsequent implantation of human BAMs for gene therapy would offer the advantage of a predictable delivery platform having a high protein synthesis capacity and long-term survival (decades for skeletal myofibers). Several issues remain to be addressed in both small and large animal models before effective clinical trials are possible, including the regulation of secretion levels, effectiveness of implants at different target sites, and the potential existence of unfused myoblasts in the BAMs that could migrate to distant sites. With the resolution of these issues, rhVEGF BAM gene therapy could provide a new option for the future clinical treatment of ischemic disease as well as other medical conditions in which angiogenesis is beneficial.

Acknowledgments
This work was supported by grants from the National Institutes of Health (R01-HL-60502, R01-AG-15415) and NASA (NAG2-1205). We thank Marcy Silver at St Elizabeth’s Medical Center for training in the ischemic hindlimb model surgery and Dr Robert Valentini for critical reading of the manuscript.

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_Circulation_. 2001;104:594-599
doi: 10.1161/hc3101.092215

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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