Intramuscular VEGF repairs the failing heart: role of host-derived growth factors and mobilization of progenitor cells

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Zisa D, Shabbir A, Mastri M, Suzuki G, Lee T. Intramuscular VEGF repairs the failing heart: role of host-derived growth factors and mobilization of progenitor cells. Am J Physiol Regul Integr Comp Physiol 297: R1503–R1515, 2009. First published September 16, 2009; doi:10.1152/ajpregu.00227.2009.—Skeletal muscle produces a myriad of mitogenic factors possessing cardiovascular regulatory effects that can be explored for cardiac repair. Given the reported findings that VEGF may modulate muscle regeneration, we investigated the therapeutic effects of chronic injections of low doses of human recombinant VEGF-A165 (0.1–1 μg/kg) into the dystrophic hamster muscle in a hereditary hamster model of heart failure and muscular dystrophy. In vitro, VEGF stimulated proliferation, migration, and growth factor production of cultured C2C12 skeletal myocytes. VEGF also induced production of HGF, IGF2, and VEGF by skeletal muscle. Analysis of skeletal muscle revealed an increase in myocyte nuclei [531 ± 12 VEGF 1 μg/kg vs. 364 ± 19 for saline (number/mm²) saline] and capillary [591 ± 80 VEGF 1 μg/kg vs. 342 ± 21 for saline (number/mm²)] densities. Skeletal muscle analysis revealed an increase in Ki67+/H11001 nuclei in the VEGF 1 μg/kg group compared with saline. In addition, VEGF mobilized c-kit+/CD31+/CXCR4+ progenitor cells. Mobilization of progenitor cells was consistent with higher SDF-1 concentrations found in hamstring, plasma, and heart in the VEGF group. Echocardiogram analysis demonstrated improvement in left ventricular ejection fraction (0.60 ± 0.02 VEGF 1 μg/kg vs. 0.45 ± 0.01 mm for saline) and an attenuation in ventricular dilation (5.59 ± 0.12 VEGF 1 μg/kg vs. 6.03 ± 0.03 mm for saline (mm)) 5 wk after initiating therapy. Hearts exhibited higher cardiomyocyte nuclear [845 ± 22 VEGF 1 μg/kg vs. 519 ± 40 for saline (number/mm²)] and capillary [2,159 ± 119 VEGF 1 μg/kg vs. 1,590 ± 66 for saline (number/mm²)] densities. Myocardial analysis revealed ~2.5-fold increase in Ki67+ cells and ~2.8-fold increase in c-kit+ cells in the VEGF group, which provides evidence for cardiomyocyte regeneration and progenitor cell expansion. This study provides novel evidence of a salutary effect of VEGF in the cardiomyopathic hamster via induction of myogenic growth factor production by skeletal muscle and mobilization of progenitor cells, which resulted in attenuation of cardiomyopathy and repair of the heart.

vascular endothelial growth factor; β-sarcoglycan; intramuscular injection

VASCULAR ENDOTHELIAL GROWTH factor (VEGF) has come into the clinical realm for treatment of cardiovascular and peripheral vascular ischemia (58, 65). Clinical trials aimed at therapeutic angiogenesis have been conducted using VEGF protein, plasmid DNA, or viral vectors, showing that administration of VEGF is generally safe. However, controlled VEGF trials for heart disease completed so far have not demonstrated convincing clinical efficacy, presumably due to rapid degradation rate of VEGF and inadequate delivery regimens (12, 49, 58), thus necessitating development of alternative strategies for efficacious cardiovascular growth factor therapy.

Skeletal muscle provides a unique tissue environment for delivery of therapeutic growth factors since it is highly vascularized, easily accessible, and comprises a large portion of the body mass, allowing for repeated therapeutic injections if necessary. Direct injection of VEGF viral vectors into skeletal muscle, for example, has been shown to promote skeletal muscle regeneration in ischemic and dystrophic muscles (4, 32). This therapeutic effect of VEGF on skeletal muscle is perhaps not unexpected given that skeletal myocytes are known to express the VEGF receptors VEGF-R1 and VEGF-R2 (4, 43), and VEGF can modulate skeletal myocyte function and promote vascular sympathetic innervation (16, 31).

The ability of skeletal muscle to function as a trophic factor-producing organ has increasingly been recognized (40, 62). Cytokines and growth factors produced and released by skeletal muscle, collectively designated as myokines, can potentially exert numerous trophic actions, including autocrine, paracrine, and endocrine effects on other organs. For instance, skeletal muscle produces several well-known cardioprotective growth factors, such as FGF, hepatocyte growth factor (HGF), and VEGF (22, 43, 56), which have all been used in preclinical or clinical studies for cardiovascular therapy (34, 52, 58). These growth factors are thought to mediate cardiac repair through their cytoprotective, myogenic, angiogenic, anti-fibrotic, and stem cell mobilizing properties.

A prominent feature of the growth factor network is a cross-talk mechanism that enables induction or amplification of more than one growth factor by another (7, 50, 53). This activation of the growth factor production capacity of skeletal muscle can potentially be an attractive therapeutic strategy for cardiac repair. Taking advantage of this unique feature of skeletal muscle and its ability to respond to VEGF, we investigated whether direct intramuscular VEGF injections into skeletal muscle could be used for heart therapy. Using a hereditary TO2 hamster model of heart failure and muscular dystrophy, we show here that intramuscular injections of recombinant human VEGF-A165 stimulate skeletal muscle regeneration, production of growth factors, and mobilization of progenitor cells, culminating in attenuation of disease progression and robust repair of the failing hamster heart.

MATERIALS AND METHODS

Cell culture. Mouse C2C12 myocytes (64) were cultured in DMEM with nutrient mixture F-12 (DMEM/F-12) supplemented with 10% FBS, 2 mM glutamine, 50 μg/ml gentamycin, and 0.125 μg/ml Fungizone, and incubated in a humidified 5% CO₂ atmosphere. Cells

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were washed with HBSS, trypsinized, and plated as described in the figure captions.

**Cell proliferation and transwell migration assays.** Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (61). In brief, C2C12 cells were plated onto 24-well plates (1,000 cells/well). After overnight attachment, cells were washed three times with HBSS, and VEGF was added at concentrations of 10 and 100 ng/ml to the wells in a serum-free medium. Assays were performed at day 0 prior to VEGF addition and both 2 and 4 days after VEGF addition. The trans-well migration assay was performed with C2C12 cells in a 24-well plate using 8-μM pore size inserts (BD Falcon) and were plated at a density of 10⁵ cells/insert in serum-free MEM. The next day, the lower compartment of the chamber was filled with 0.7 ml of medium or with or without VEGF. After 24 h of incubation, inserts were taken off the plate, and the cells on the upper side of the insert were removed by wiping 3 times with a cotton swab. The cells on the lower side of the insert were fixed with 4% paraformaldehyde, washed, and stained by the DAPI. Nuclei were counted from five random fields at ×100 magnification using Image J Software (National Institutes of Health, Bethesda, MD). Migration index was calculated by dividing the number of migrated cells in the presence of VEGF by the number of migrated cells in the absence of VEGF.

**Animals.** Bio-F1B (normal) and Bio-TO2 (cardiomyopathic) male hamsters were obtained from Bio Breeders (Watertown, MA). All procedures and protocols conformed to institutional guidelines for the care and use of animals in research.

**Intramuscular VEGF injection.** Four-month-old TO2 hamsters were injected twice per week, over the 5-wk course of the study, in both left and right hamstrings with either saline, or recombinant human VEGF-A165 (R&D Systems, Madison, WI). Each injection site was 0.5 ml of saline or VEGF protein solution (10 or 100 ng/ml). The lower-dose VEGF group received 0.1 µg VEGF protein per kg body wt on each injection day, and the higher-dose VEGF group received 1 µg/kg. No tissue inflammation, edema, or tumor growth was noted throughout the course of the study. Blood samples were collected via retroorbital eye bleed or cardiac puncture (for terminal stage only). Tissues were harvested and processed by one of three methods: fixed in freshly prepared 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound, or snap frozen in liquid nitrogen for RNA and protein preparations.

**Quantitative RT-PCR.** RNA extraction and purification from C2C12 skeletal myocytes and hamster tissues were performed using Qiagen RNA isolation kits, and qRT-PCR protocols were performed as described previously (29). β2-microglobulin was used as the reference gene for calculations. Data were analyzed by the 2^ΔΔCT method. Oligonucleotides were synthesized by Midland (Midland, TX). Primer sequences are listed in Table 1.

**ELISA analysis.** Concentrations of HGF, IGF-2, NGF, SDF-1, and VEGF in cell culture media, animal plasma, and homogenized tissues were measured using ELISA kits from R&D Systems: mouse HGF DuoSet (#DY2207), mouse IGF-2 DuoSet (#DY792), mouse NGF DuoSet (#DY556), mouse stromal cell-derived factor 1 (SDF-1) (#DY460), rat VEGF DuoSet (#DY564), and human VEGF DuoSet (#DY293B) following manufacturer’s protocol. Snap-frozen hamstring tissues were homogenized in an ice-cold lysis solution containing 0.1% TX-100 and 2 mM EDTA. Tissue homogenates were clarified, diluted to 1 mg proteins/ml, and used for ELISA. Protein concentrations were determined using the bicinchoninic acid assay kit (Thermo Scientific, Waltham, MA). Circulating cardiac troponin-I (cTnI) was assayed with a rat cTnI ELISA kit (Life Diagnostics, West Chester, PA) using plasma samples collected 5 wk after VEGF administration.

**Capillary and nuclear density quantification.** Freshly excised tissues were immersed in OCT, frozen in liquid nitrogen, and stored at −80°C until use. Ventricular and injected hamstring cross sections 5 μm thick were obtained using a cryostat and fixed in acetone: ethanol mixture (3:1 ratio) for 5 min. Sections were blocked with normal saline supplemented with 0.025% Tween-20 and 2% nonfat milk powder for 45 min. Fluorescein-labeled Griffonia Simplicifolia Lectin I Isotip B4 (GSL-IB4; #FL-1201; Vector Laboratories, Burlingame, CA) diluted 1:100 was incubated with the tissue sections overnight at 4°C. Cardiomyocytes were stained with a mouse cardiac Troponin T (cTnT) antibody (#MS-295; Thermo Scientific), and skeletal myocytes were stained with a rabbit troponin I (TnI) antibody (#sc15368; Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h. Sections were then incubated with Alexa 555 conjugated anti-mouse or anti-rabbit secondary antibody for 1 h and then mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were taken in 10–20 random fields using Zeiss’s AxioImager fluorescence microscope at ×200 magnification. The number of capillaries (FITC channel) and total nuclei count (DAPI channel) were quantified by ImageJ software using the analyze particle feature. Noncardiomyocyte nuclei quantified from the merged images by their lack of cTnT staining were subtracted from total nuclei count to determine cardiomyocyte nuclear density. Skeletal myocyte nuclear density was determined similarly by subtracting out nonmyocyte nuclei identified by the absence of TnI staining. Black areas from

### Table 1. Primer sequences

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images were subtracted out using Photoshop-aided quantification of black pixels to calculate total tissue area. Capillary and myocyte nuclear density were normalized to total tissue area in mm².

Ki67 & c-kit staining. Actively proliferating cells in the injected hamstring and heart were identified by nuclear staining with a Ki67 antibody on paraffin sections. Antigen retrieval was performed using pH 6.0 citrate buffer at 95°C for 20 min. Sections were permeabilized with 0.1% Triton X-100, blocked with normal saline supplemented with 0.025% Tween-20 and 2% nonfat milk powder for 30 min and double stained with the Ki67 antibody (#RM-9106; Thermo Scientific) and either the cTnT antibody for cardiac muscle or the myosin heavy chain antibody (25) for skeletal muscle. C-kit+ progenitor cells were identified and quantified in the heart using a c-kit antibody (DAKO, Glostrup, Denmark) along with the cTnT antibody for cardiac muscle. C-kit and Ki67 antibody staining along with cTnT was also performed to identify progenitor cells that are actively proliferating. Stained tissue sections were imaged with the Zeiss Axioimager Z1 epifluorescence microscope. A minimum of 10 images were taken from random fields on at least two sections from each tissue. Total nuclei and Ki67⁺ nuclei in each image were counted using Image J software. Cardiomyocyte nuclei count was determined by counting and excluding the noncardiomyocyte nuclei. C-kit⁺ cells in the heart were quantified using Image J software.

Flow cytometry. Flow cytometry was carried out, as described previously (48). In brief, peripheral blood mononuclear cells were isolated 5 wk after initiation of VEGF administration (0.1 or 1 μg/kg). After blocking with an FcR receptor blocker for 20 min, cells were labeled with phycoerythrin (PE) conjugated c-kit (#12-1171; eBioscience, San Diego, CA), PE-conjugated CD31 (#12-0311; eBioscience), and PE-conjugated CXCR4 (#12-9991; eBioscience) antibodies. Dead cells were excluded by 7-amino-actinomycin D (7-AAD) counterstaining. Flow cytometry was performed on ~50,000 cells, and data were analyzed using FCS Express (De Novo Software, Los Angeles, CA).

Echocardiography. Echocardiograph measurements were performed by an operator who was blinded to treatment groups, as described in our recent work (33).

Quantification of apoptosis. Analysis of apoptosis was performed using frozen sections prepared as described above using the ApopTag kit (Millipore) per the manufacturer’s instructions. cTnT antibody was used to identify apoptotic myocytes, and analysis was performed similarly as described above. All apoptotic nuclei in each section were counted and normalized to total myocytes and nonmyocytes.

Fibrosis and cardiomyocyte diameter quantification. Masson-Trichrome sections were used for fibrosis analysis and cardiomyocyte diameters. Fibrosis was performed by Photoshop-aided quantification of image pixels. The blue color range was selected to represent fibrotic areas. At least 15 random fields at ×200 magnification were assessed for each slide. Artifactual spaces (white clear areas) from images were subtracted using Photoshop-aided quantification of white pixels to calculate total tissue area. The ratio of fibrotic area-to-total tissue area was calculated as % fibrotic area. For quantification of cardiomyocyte diameters, at least 200 random cardiomyocytes were measured from each animal using the measurement tool in AxioVision LE software (Carl Zeiss, Oberkochen, Germany).

Statistical analysis. Statistical analysis was performed using Sigma Stat ver. 3.0. All results are expressed as means ± SE. Statistical significance was evaluated using the unpaired Student t-test for comparisons between two means. Statistical significance for multiple groups was evaluated by one-way or two-way ANOVA analysis followed by the Holm-Sidak method for multiple comparisons, as appropriate. Significance was set at P < 0.05.
RESULTS

Effects of recombinant human VEGF-A165 on skeletal muscle were evaluated using both in vitro and in vivo assays, and cardiac therapeutic efficacy was determined using the TO2 hamster heart failure model.

**VEGF induces growth factor production by C2C12 skeletal myocytes in vitro.** The mouse C2C12 skeletal myocyte culture was first used to characterize the response of skeletal myocytes to VEGF. We have previously demonstrated that VEGF-transduced bone marrow mesenchymal stem cells produce increased levels of growth factors and mediate functional recovery in pressure-overloaded swine hearts (29, 63). We, therefore, assessed whether VEGF might stimulate growth factor production in cultured C2C12 skeletal myocytes, which are known to express VEGF receptor 1 and 2 (16). qRT-PCR analysis demonstrated that VEGF stimulation of C2C12 skeletal myocytes induced expression of a broad spectrum of growth factor genes, most notably angiopoietin-1 (Angpt), FGF2, HGF, and VEGF (Fig. 1A). Increased expression of growth factors was further confirmed at the protein level by ELISA analysis of C2C12 conditioned media for HGF, IGF2, NGF, and VEGF (Fig. 1B), showing that there was statistically significant increases in secreted HGF (~83%), NGF (~21%), and VEGF (~135%) after VEGF treatment. These data indicated that cultured C2C12 skeletal myocytes produced therapeutically important growth factors, and this capacity can be further boosted by VEGF.

**VEGF stimulates proliferation and migration of C2C12 skeletal myocytes in vitro.** VEGF is well known for its ability to mediate proliferation and migration of endothelial cells (9). We have demonstrated that VEGF also stimulates proliferation of mesenchymal stem cells (29). However, the effect of VEGF on myocytes is not well characterized. Using the C2C12 cell system and MTT cell proliferation assay, we demonstrated that VEGF at 100 ng/ml, but not 10 ng/ml, stimulated C2C12 skeletal myocyte proliferation (Fig. 2A). We then determined whether VEGF could induce migration of C2C12 cells using a transwell migration system. We observed a VEGF dose-dependent increase in C2C12 migration (Fig. 2B). These results together indicate that VEGF has both proliferative and chemotactic effects on skeletal myocytes in vitro.

**VEGF induces growth factor production by skeletal muscle.** Having demonstrated the multiple stimulatory effects of VEGF on skeletal myocytes in vitro, we next determined whether VEGF might exert similar effects on skeletal muscle in vivo. Skeletal muscle is easily accessible and capable of producing growth factors that are of importance to cardiac repair (22, 37). Therefore, activation of skeletal muscle by VEGF can be a logistically appealing strategy. To distinguish between the administered (exogenous) and host-derived (endogenous) VEGF, we used human VEGF-A165 for the injection. VEGF was administered at two low dosages compared with documented therapeutic trials (8, 42). TO2 hamsters received chronic VEGF protein injections (0.1 or 1 μg/kg) into both left and right hamstring muscles. Injections were initiated when the TO2 hamster started to exhibit signs of heart failure, at 4 mo of age (33) and were continued for 5 wk. We found that the levels of the injected human VEGF at 3 days, 2 wk, and 5 wk were too low to be detected by human VEGF-specific ELISA using both plasma and muscle tissue samples. This finding was consistent with documented rapid degradation rates of VEGF in vitro and in vivo (12, 49).

Skeletal muscle expression and production of growth factors in response to 1 μg/kg VEGF were further quantified using both qRT-PCR and ELISA. Injected hamstring muscle from treated and saline control animals after 3 days and 5 wk of VEGF injections were further quantified by ELISA analysis. qRT-PCR showed that there was significant induction of multiple growth factor genes both 3 days and 5 wk after treatment (Fig. 3A). Specifically, there was an increase of angiopoietin-1, FGF1, IGF1, NGF, and VEGF at both 3 days and 5 wk. Significant increases of FGF2, HGF, and IGF2 were also detectable after 5 wk. ELISA assays were performed for HGF, VEGF, IGF2, and NGF to corroborate the RNA data. Fig. 3B shows that increased production of HGF, IGF2, and VEGF could be detected at 5 wk. Our data clearly indicated that skeletal muscle actively produced growth factors, and this ability was further boosted in response to VEGF in vitro and in vivo.

The effect of VEGF on myogenic and angiogenic regeneration. Since increased production of skeletal muscle trophic factors is expected to promote muscle growth, we next...
sought to determine whether VEGF injections might promote skeletal muscle regeneration. The TO2 hamster harbors a defective $\beta$-sarcoglycan gene, causing muscular dystrophy and dilated cardiomyopathy (33, 47). Fig. 4A shows representative images for saline and VEGF hamstring muscle 5 wk after initiation of injections showing nuclei (blue), capillaries (green), and myocytes (red). Upon analyzing hamstring tissue after 5 wk of 1 $\mu$g/kg VEGF injections, we observed an increase in myofiber nuclear density compared with the saline group (531 number/mm$^2$ VEGF vs. 364 number/mm$^2$ saline, Fig. 4C). Because VEGF is a potent inducer of angiogenesis, we also determined whether VEGF induced angiogenesis in the dystrophic muscle. Fig. 4D demonstrated that there was also a significant increase in capillary density (591 number/mm$^2$ VEGF 1 $\mu$g/kg vs. 364 number/mm$^2$ saline, Fig. 4C). Because VEGF is a potent inducer of angiogenesis, we also determined whether VEGF induced angiogenesis in the dystrophic muscle. Fig. 4D demonstrated that there was also a significant increase in capillary density (591 number/mm$^2$ VEGF 1 $\mu$g/kg vs. 364 number/mm$^2$ saline, Fig. 4C).

VEGF-mediated mobilization of bone marrow progenitor cells. Mobilization of bone marrow progenitor cells plays an important role in tissue repair, and this ability of VEGF is known to contribute to postnatal neovascularization (5, 26). Peripheral blood cells expressing c-kit, CD31, and CXCR4 have been shown to originate from the bone marrow compartment and contribute to tissue repair (21). We, therefore, investigated whether bone marrow progenitor cells might be mobilized in response to intramuscular VEGF injection. Flow cytometric analysis of peripheral blood mononuclear cells showed that VEGF, in a dose-dependent manner, increased circulating progenitor cells expressing c-kit surface marker (Fig. 5A, left). VEGF also increased peripheral blood circulating CD31$^+$ cells at both 0.1 $\mu$g/kg and 1 $\mu$g/kg dosages (Fig. 5A, center). The number of CXCR4$^+$ cells in the peripheral blood was significantly increased by the 1 $\mu$g/kg VEGF dosage (Fig. 5A, right).

VEGF modulates SDF-1 levels and recruits CXCR4 cells to the peripheral blood. It has been reported that VEGF modulates SDF-1 levels in vivo and may be involved in the mechanism of VEGF-induced progenitor cell mobilization via SDF-1 mediated recruitment through its receptor CXCR4 (19).
Our results indicate that VEGF mobilizes CXCR4+ cells into the peripheral blood at the 1 μg/kg dosage. Therefore, we investigated whether the increased percentage of CXCR4+ cells in the peripheral blood was associated with changes in SDF-1 levels in hamstring, plasma, or heart tissue. VEGF injection of hamstring muscle induced a statistically significant increase in SDF-1 protein at both 3 days and 5 wk (Fig. 5B). Plasma analysis revealed there was also an increase in SDF-1 at 3 days and 5 wk in VEGF-treated hamsters (Fig. 5C). Analysis of the heart also revealed a statistically significant increase in SDF-1 concentration at 5 wk but not at 3 days (Fig. 5D). These data indicate that VEGF modulates SDF-1 levels in the hamstring, plasma, and heart, which is consistent with increased peripheral blood mobilization of CXCR4+ cells in VEGF-treated animals.

Intramuscular VEGF injections improve ventricular function. Having demonstrated mitogenic effects on skeletal muscle by VEGF, we then determined whether intramuscular VEGF injections could improve cardiac function in the TO2 hamster. Four-month-old cardiomyopathic hamsters were again injected with human VEGF-A165 into the hamstring muscle at 0.1 or 1 μg/kg dosages for 5 wk. Echocardiography was performed prior to injection, and 2 and 5 wk postinitiation of injections. Interestingly, VEGF at both dosages significantly improved cardiac function, as shown by increased left ventricular ejection fraction (LVEF) at 2 wk and 5 wk compared with
control (Fig. 6A). VEGF administered at 0.1 μg/kg improved LVEF by 20% at 2 wk and 25% at 5 wk. VEGF at 1 μg/kg increased LVEF by 30% at 2 and 5 wk, showing a more potent therapeutic benefit than the 0.1 μg/kg dose. Although both VEGF dosages decreased left ventricular diastolic diameter (LVDd) at 2 wk (~5% decrease for 0.1 μg/kg and ~10% decrease for 1 μg/kg), only the 1 μg/kg dosage decreased LVDd at 5 wk (Fig. 6B). Notably, additional injection experiments revealed that repeated VEGF injections were necessary for maintaining the therapeutic effect since discontinuing the VEGF injections resulted in a steady decline in ventricular function (data not shown).

Intramuscular VEGF injections attenuate myocardial injury and fibrosis. Ventricular dysfunction in the failing hamster heart has been associated with increased myocardial cell death and fibrosis (44, 45). We, therefore, assessed whether functional improvement after VEGF injections was associated with attenuated myocardial tissue injury and fibrosis. Circulating cardiac troponin-I levels were measured at 5 wk, comparing the F1B, TO2 saline control, TO2 VEGF 0.1 μg/kg, and TO2 VEGF 1 μg/kg dosage groups. Fig. 7A shows that there was a significant decrease in apoptosis of both cardiomyocytes and noncardiomyocytes (Fig. 7B). There was not a significant difference in cardiomyocyte or noncardiomyocyte apoptosis between the two VEGF dosages. Trichrome staining of heart tissue sections revealed clear differences in the extent of fibrosis (Fig. 7C). Quantitative computer-assisted image analysis revealed significant reductions in myocardial fibrosis in both VEGF injection groups compared with TO2 saline control (Fig. 7D). There was not a significant difference in fibrosis between the two VEGF dosages.

Intramuscular VEGF injections promote myocardial regeneration. Morphometric studies were performed to determine whether VEGF injections could induce myocardial myogenesis and angiogenesis. The myocardium of hamsters given 1 μg/kg VEGF had a higher cardiomyocyte nuclear density compared with TO2 saline (845 ± 22 number/mm² TO2 VEGF 1 μg/kg vs. 519 ± 40 number/mm² TO2 saline, Fig. 8A). Myocardial capillary density was also improved by both 0.1 and 1 μg/kg VEGF doses compared with TO2 saline (2,159 ± 119 number/mm² TO2 VEGF 1 μg/kg, 2,030 ± 53 number/mm² TO2 VEGF 0.1 μg/kg vs. 1,590 ± 66 number/mm² TO2 saline, Fig. 8B). Because these findings suggested active myocardial regeneration after VEGF injections, we further quantified cell cycle activity by staining heart sections with a Ki67 antibody. Both cardiomyocyte and noncardiomyocyte nuclei stained positive for the Ki67 antigen (Fig. 8C). Compared with the normal F1B hamster heart, the failing TO2 heart exhibits higher levels

Fig. 5. VEGF injection mobilizes bone marrow progenitor cells and modulates SDF-1 levels in tissue and plasma. VEGF denotes 1 μg/kg dosage. A: Circulating c-kit+, CD31+, or CXCR4+ cells were quantified by flow cytometry 5 wk after VEGF injection. Cell numbers per million peripheral blood mononuclear cells were presented (n = 6 per group). **P < 0.001 vs. saline. *P < 0.05 vs. saline. †P < 0.05 vs. VEGF 0.1 μg/kg. B: ELISA assay of SDF-1 using injected hamstring tissue homogenates from 3 days and 5 wk. *P < 0.05 vs. saline. C: ELISA assay of SDF-1 levels in plasma from 3 days and 5 wk. *P < 0.005 (n = 4 per group). D: ELISA assay of SDF-1 using heart tissue homogenates from 3 days and 5 wk. **P < 0.001 vs. saline. †P < 0.001 vs. 3 days (n = 3–6 per group).
cardiomyocyte regeneration of cell cycle and stem cell markers such as cyclins, VEGF-mediated cardiac repair. Proliferation of cardiac progenitor cells may contribute to heart tissue revealed that c-kit augmented in the VEGF-treated hearts. Antibody staining of preformed double staining with c-kit and Ki67 antibodies. We determine whether the c-kit, as documented previously (13). To F1B animals (Fig. 8A). Active proliferation of cardiac progenitor cells compared with TO2 control (Fig. 8F). Because of the preexisting injury in the heart, TO2 animals have a higher percentage of c-kit+ cell than normal F1B animals (Fig. 8F), as documented previously (13). To determine whether the c-kit+ cells were also proliferating, we preformed double staining with c-kit and Ki67 antibodies. We found that a significant proportion of the c-kit+ cells also stained with Ki67, indicating the c-kit+ progenitor cells were actively proliferating in the stem cell niche (Fig. 8G). Active proliferation of cardiac progenitor cells may contribute to VEGF-mediated cardiac repair.

qRT-PCR analysis shown in Fig. 9A demonstrates increased expression of cell cycle and stem cell markers such as cyclins, c-kit, and CD31 in the VEGF group. Cardiomyocyte regeneration was revealed by analysis of the average cross-sectional fiber size, which inversely correlated with the myocardial nuclear densities (Fig. 9B) and reflected the smaller fiber sizes of the newly regenerated cardiomyocytes (14.5±0.47 μm for VEGF vs. 18.2±0.5 μm for saline). Fiber-size distribution profiles illustrated the abundance of smaller cardiomyocytes in the VEGF group and larger cardiomyocytes in the saline control group (Fig. 9C). These data combined suggested that repeated skeletal muscle injections of VEGF-A165 induced active regeneration in the failing hamster heart, and this was marked by increased cardiomyogenesis and angiogenesis, as well as attenuated myocardial apoptosis and fibrosis, culminating in the improved ventricular function.

DISCUSSION

The present study demonstrated for the first time that chronic intramuscular injections of low-dose VEGF-A165 (0.1–1 μg/kg) can be used for heart failure treatment. Activation of skeletal muscle was evidenced by improved local tissue regeneration and increased growth factor output after VEGF injections. Activation and amplification of the skeletal muscle growth factor network provide a novel trophic factor repair mechanism for the failing hamster heart, indicated by the improved ventricular function, attenuated myocardial injury and fibrosis, and the increased cardiomyogenesis and angiogenesis.

Targeting skeletal muscle for cardiac repair. VEGF has recently been recognized to have a novel nonangiogenic role important for the cardiovascular system (66). VEGF exerts regulatory effects on many different cell types, including but not limited to cardiomyocytes, skeletal myocytes, smooth muscle cells, epithelial cells, osteoblasts, neurons, and stem cells. With respect to skeletal muscle, direct intramuscular injections of VEGF viral vectors have been shown to promote skeletal muscle regeneration in ischemic and dystrophic muscles (4, 16, 32). This potent regenerative effect of VEGF on injured skeletal muscle is confirmed by the current study. Further, we demonstrate that VEGF enhances proliferation, migration, and growth factor production of cultured skeletal myocytes, suggesting that the muscle-activating function of VEGF observed in the TO2 hamster can be achieved independent of the angiogenic role of VEGF. This conclusion is consistent with the findings that skeletal myocytes express functional VEGF receptors (VEGF-R1 and VEGF-R2) (4, 16, 43).

A practical consideration is that skeletal muscle is highly vascularized and easily accessible, allowing for repeated therapeutic injections if necessary. Indeed, we have found that repeated intramuscular VEGF injections are necessary for maintaining the cardiac therapeutic effect in the TO2 hamster heart failure model. Despite the frequent biweekly VEGF injections, we did not observe angiomata formation, tissue edema, or inflammation on gross inspection, which have been documented in several therapeutic studies using intramyocardial VEGF injection (27, 46). The lack of these side effects in our VEGF therapeutic trial may be related in part to the use of low VEGF protein dosages (0.1–1 μg/kg) in contrast to the high-protein dosages, exceeding 10 μg/kg used in other therapeutic angiogenesis (42).

Skeletal muscle as a major trophic factor organ. In the adult, VEGF-A is expressed in all vascularized tissues, such as
skeletal muscle, myocardium, and large blood vessels, and the low levels of VEGF-A are deemed essential for the maintenance of the vascular system under normal physiological conditions (30). Skeletal muscle is a dynamic tissue with an impressive ability to continuously respond to environmental stimuli (2). Given its adaptive ability to regenerate after injury or ischemic insult, skeletal muscle can be a major source of VEGF-A due to the large body mass of the tissue. Tissue-specific VEGF gene deletion studies by Tang et al. (54) and Giordano et al. (17) suggest that myocytes may indeed be a critical paracrine source of VEGF for regulating vascular and contractile function. In addition to VEGF, the ability of skeletal muscle to function as a trophic factor-producing organ has increasingly been recognized (40, 62). Several cytokines and growth factors, including EGF, FGF, HGF, IGF, and NGF have all been shown to be produced by skeletal myocytes (3, 15, 22, 56, 60). However, the capacity for trophic factor production can be aberrantly downregulated, as seen in human dilated cardiomyopathy for VEGF (1) and human heart failure for NGF (24). Significantly, we demonstrate here that the trophic factor production capacity of skeletal myocytes can be stimulated by VEGF in vitro and in vivo, thus providing a strong rationale for the proposed intramuscular VEGF injection regimen for heart failure therapy. Further, the use of the VEGF-A165 isoform, which contains a heparin binding domain involved in extracellular matrix interaction (35), can provide a trapping mechanism for the injected VEGF, potentially allowing for more efficacious stimulation of skeletal muscle.

It should be noted that controlled VEGF trials for heart disease completed so far have not demonstrated convincing clinical efficacy, presumably due to rapid degradation rate of VEGF and inadequate delivery regimens. With a VEGF half-life of 4-6 min in plasma, VEGF is rapidly degraded by endothelial cell proteases, and suggests that the drug delivery system be optimized to enhance bioavailability. In addition, the VEGF-A165 isoform, which contains a heparin binding domain involved in extracellular matrix interaction (35), can provide a trapping mechanism for the injected VEGF, potentially allowing for more efficacious stimulation of skeletal muscle.

Fig. 7. Intramuscular VEGF injections attenuate myocardial tissue injury and fibrosis. A: plasma levels of cardiac troponin-I 5 wk after VEGF treatment (1 μg/kg). Cardiac troponin-I was not detected (ND) in F1B plasma. B: quantification of cardiomyocyte and noncardiomyocyte apoptosis using the ApopTag kit. Apoptotic cells were not detected (ND) in F1B hearts. C: representative images of trichrome-stained heart sections showing fibrosis (blue), ×200. D: computer-assisted quantification of fibrotic areas using Trichrome-stained sections. *P < 0.05 vs. TO2 saline control. **P < 0.001 vs. TO2 saline control. #P < 0.05 vs. F1B (n = 3 for each panel).
life of 3 min in the circulation (14), it is not surprising that we were unable to detect the injected human VEGF protein 3 days after intramuscular administration. On the other hand, VEGF of host origin could be readily detected in the hamster muscle homogenate samples, and the levels of host-derived VEGF could be further enhanced by VEGF injections. Our study, which implicates the importance of skeletal muscle-derived VEGF, is in agreement with a study published by Tateno et al. (55). It was discovered that injection of peripheral blood mononuclear cells did not produce sufficient angiogenic factors to induce the extent of neovascularization observed in a hind limb ischemia model but instead stimulated endogenous skeletal myocytes to produce angiogenic factors. Along this line, we have also found that intramuscular VEGF injection improved ventricular function in the porcine hibernating myocardium (data not shown). This preliminary finding suggests that the simple intramuscular VEGF injection approach can be equally applicable to a large animal model or even the clinical setting.

Mechanisms of attenuation of heart failure progression and cardiac repair. Although early preclinical studies of cardiac stem cell therapy suggested therapeutic mechanisms mediated by stem cell transdifferentiation or fusion (38, 57), it has become apparent that these cellular mechanisms do not contribute significantly to the observed functional improvement after stem cell administration. Additionally, trophic factors derived from the implanted stem cells, including VEGF, have been found to mediate the cardiovascular therapeutic effect.

Fig. 8. Intramuscular VEGF injections promote myocardial regeneration. A: cardiomyocyte nuclear densities expressed as numbers of nuclei per mm². B: myocardial capillary densities expressed as GSL-IB4 lectin-stained capillaries per mm². C: representative image showing Ki-67⁻ (pink nuclei) cardiomyocytes and noncardiomyocytes. Cardiomyocytes were stained green with a cardiac troponin T antibody, and nuclei were stained blue with DAPI, ×630. D: total Ki67⁺ nuclei and Ki67⁻ cardiomyocytes. E: representative image showing c-kit staining of interstitial cells (pink cytoplasmic) in the heart. Cardiomyocytes were stained green with a cardiac troponin T antibody, and nuclei were stained blue with DAPI, ×630. F: quantification of % c-kit⁻ cells in the heart. Nuclei were stained blue with DAPI, ×630. *P < 0.01 vs. TO2 saline control. **P < 0.05 vs. TO2 saline control. #P < 0.05 vs. F1B. ##P < 0.01 vs. F1B. †P < 0.01 vs. TO2 0.1 μg/kg VEGF (n = 3 for each panel).
Our results indicate that a significant systemic effect of recurring VEGF injections can be attributed to mobilization of bone marrow progenitor cells and to stimulation of host muscle tissue growth factor production. VEGF has been well known for its ability to mobilize bone marrow progenitor cells (5), and in the current study, we demonstrated that VEGF mobilizes populations of progenitor cells, expressing c-kit, CD31, or CXCR4 surface markers. The VEGF-mobilized progenitor cells can actively repopulate the skeletal muscle and myocardium, as shown here by increased expression of cell cycle markers (Ki67 and cyclins) and stem cell markers (c-kit, and CD31) in both tissues, and participate in endogenous repair mechanisms. These recruited progenitor cells can further participate in cardiomyogenesis and angiogenesis directly or indirectly, as indicated by increased capillary and cardiomyocyte nuclear densities and abundance of smaller cardiomyocytes.

Mobilization of progenitor cells from the bone marrow may also occur indirectly through VEGF-induced SDF-1 expression and contribute to attenuation of heart failure in addition to endogenous cardiac repair. Plasma elevation of SDF-1 is involved in bone marrow stem cell mobilization (10). VEGF-injected skeletal muscle increased production of SDF-1, which was associated with a rise in plasma SDF-1 levels. The increase of SDF-1 in the plasma may contribute to the mobilization of CXCR4+ cells from the bone marrow and to increased progenitor cells being recruited to the injured myocardium.

Intramuscular VEGF injections amplified the production, by skeletal myocytes, of many therapeutically relevant and functionally synergistic growth factors, including but not limited to angiopoietin-1, FGF1, FGF2, HGF, IGF1, IGF2, NGF, and VEGF. These growth factors are capable of mediating cardiac repair through their stem cell-mobilizing, cytoprotective, myogenic, angiogenic, and antifibrotic properties. VEGF-A165 can boost the function of mesenchymal stem cells and endothelial progenitor cells (23, 63). The well-known vascular permeability effect of VEGF on the capillary endothelium can facilitate the release of these growth factors into circulation (11). In addition, VEGF has been shown to promote differentiation of stem cells into cardiomyocytes and endothelial cells (39, 51).

Myogenesis typically requires synergistic interactions among multiple trophic factors (41). Similarly, blood vessel maturation and stability requires angiopoietins in addition to VEGFs and other angiogenic factors such as FGFs and HGF (6). Induction of NGF by VEGF is consistent with a cross talk between VEGF signaling and neural stem cells and may be
involved in postganglionic innervation of blood vessels (28, 31). Regeneration of the failing heart, as well as attenuation of the disease process, can be accomplished in part by the coordinated actions of the myriad of trophic factors derived from the VEGF-stimulated host muscle as supported by our present findings. This noninvasive approach may be beneficial in patients afflicted with muscular dystrophy who also suffer from cardiomyopathy.

**Perspectives and Significance**

Exercise and physical activity are known to prevent the onset of coronary artery disease and reduce cardiovascular symptoms (59). Although the precise mechanism by which exercise therapy reduces mortality in heart patients remains poorly defined, it could be partly mediated by increased production of protective trophic factors from contracting skeletal muscle that mediate progenitor cell mobilization into peripheral blood (40, 62). The intramuscular VEGF injection regimen for heart failure treatment, as demonstrated here, draws significant comparison to the relationship between active skeletal muscle and low cardiovascular risk and highlights the critical link between the skeletal muscle and cardiovascular systems. As this therapeutic module hinges upon host tissue response to low doses of VEGF, which is transient and short-lived, chronic intramuscular VEGF injections are needed for efficacious and long-term cardiac repair. This noninvasive growth factor therapeutic regimen contrasts vividly with the traditional intramuscular VEGF injections are needed for efficacious and low doses of VEGF, which is transient and short-lived, chronic cardiomyopathy.

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**GRANTS**

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