Satellite cell-mediated angiogenesis in vitro coincides with a functional hypoxia-inducible factor pathway

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Satellite cells are muscle precursor cells that occupy the “satellite” cell position in relation to skeletal muscle fibers (20). Normally quiescent in adult skeletal muscle, satellite cells activate upon muscle injury and become the primary effectors of muscle repair (25, 39). In an effort to understand the stimulus for revascularization of injured muscle, Phillips and Knighton (21) demonstrated that damaged muscle releases factor(s) capable of stimulating angiogenesis. We and others have shown that satellite cells produce a number of growth factors, including hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), some of which are known to be angiogenic (1, 9, 16, 22). Current evidence suggests that one factor is VEGF, a key regulator of the vasculature that enhances vessel permeability, endothelial cell survival, and migration (8, 11, 27). During skeletal muscle ischemia, strong VEGF gene expression and protein abundance are found in regenerating muscle fibers but not undamaged fibers (23), and exogenous intramuscular administration significantly improves muscle capillary density in this setting (37).

Hypoxia-inducible factor (HIF), a transcription factor expressed in response to hypoxia, plays a central role in the restoration of cellular oxygen homeostasis, which may occur in cases of tissue growth or repair (26). As such, HIF drives the transcription of >100 genes involved in metabolism, erythropoiesis, and angiogenesis, including VEGF (17). Microarray analysis demonstrates the upregulation of the hypoxia-inducible angiogenic pathway of HIF and VEGF in ischemic muscle (36). In addition, the strongest HIF and VEGF signals correspond to the location of presumptive satellite cells (12, 23, 36).

Taken together, these observations indicate that a hypoxia-inducible angiogenic pathway may be viable in skeletal muscle and further suggest that promotion of skeletal muscle angiogenesis may reside in satellite cells.

As a first step toward studying the possibility that satellite cells may promote angiogenesis, we developed a coculture model consisting of a three-dimensional microvascular fragment (MVF) construct and primary satellite cells. In addition, we examine what role the HIF-VEGF pathway may play in satellite cell-driven angiogenesis.

MATERIALS AND METHODS

Materials. All animal care and use was conducted according to National Research Council guidelines and approved and supervised by the University of Arizona Institutional Animal Care and Use Committee. In all experiments, 9- to 12-mo-old male Sprague-Dawley rats were used. Cell culture medium and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT).
Satellite cell isolation and culture. Primary satellite cells were isolated from male Sprague-Dawley rats according to Allen et al. (2). Briefly, muscle groups from the hind limb and back were excised, trimmed of fat and connective tissue, hand minced with sterile scissors, and digested for 1 h at 37°C with 1.25 mg/ml pronase. Cells were separated from muscle fiber fragments and tissue debris by differential centrifugation and plated in tissue culture dishes coated with 20 μg/cm² poly-lysine (0.1 mg/ml in distilled water) and a 10 μg/ml solution of fibronectin in sterile PBS.

In vitro MVF culture model. MVF were isolated from male Sprague-Dawley rats as previously described (28, 38), with slight modifications. Briefly, after rats were killed, epididymal fat pads were collected, minced, and digested in a collagenase solution (2 mg/ml in PBS containing 0.1% BSA) for 8–10 min. MVF were pelleted by centrifugation, washed, and resuspended in PBS containing 0.1% BSA. Isolation of MVF from tissue debris and single cells was achieved by differential selection through a 500- /H11011 and 30- /H9262 m screen, respectively. MVF were suspended in ice-cold, pH-neutralized rat tail type I collagen (3 mg/ml final concentration using 2.5X DMEM) at ~15,000 MVF/ml for plating (0.25 ml/well) in 48-well culture plates. Following collagen polymerization, gels were immersed in an equal volume of media.

Angiogenic growth of MVF in collagen gels is characterized by the formation of smooth vascular sprouts that are morphologically distinct from the rough, smooth muscle-associated appearance of the parent vessel (15, 28, 38). The sprouting assay has been determined by measurement of a line traced along each sprout were captured using a digital camera. Vessel sprout length was averaged from replicate experiments within each treatment group and /H11001 included a minimum of 30 sprouts per treatment.

Coculture model. An in vitro coculture model composed of satellite cells and MVF or muscle-derived fibroblasts and MVF was developed. Isolated MVF are pH-neutralized rat tail type I collagen (3 mg/ml final concentration using 2.5X DMEM) at ~15,000 MVF/ml for plating (0.25 ml/well) in 48-well culture plates over a satellite cell or fibroblast monolayer (at 50% confluency) and cultured in DMEM with 2% FBS. Muscle-derived fibroblasts were prepared as described by Allen et al. (1). Briefly, fibroblasts were clonally derived from cultures that had been passaged three to six times. Fibroblast cultures were stained for the presence of desmin to ensure that myogenic cells were not present.

Collection of satellite cell conditioned medium. Conditioned medium (CM) was collected from rat satellite cells (144 h after harvest, maintained in DMEM + 2% horse serum + 1% penicillin-streptomycin + 0.05% gentamicin for 24 h immediately before collection) and muscle-derived fibroblasts (80% confluency). Briefly, plates were rinsed twice with pre-CM (DMEM + 0.1% penicillin-streptomycin + 1X TTS; Cambrex Bio Science, Walkersville, MD) to remove residual serum. Following rinses, cells were cultured in pre-CM for 16 h and the medium was removed and centrifuged for 5 min at 1,500 g to remove any cellular debris. CM was concentrated 10-fold (10X) using conical filter units (10,000 molecular weight cutoff; Amicon Ultra Centrifugal Filter Devices, Millipore, Bedford, MA). Pre-CM that was not used on cells was also placed in conical filter units for control conditions. The medium was then filtered using Steriflip Filter Units (0.22 μm, Millipore), and FBS was added to each type of medium to a final concentration of 2%. To inhibit satellite cell-derived VEGF, soluble VEGF receptors were added to satellite cell CM using concentrations described previously (37). Briefly, 100 ng/ml recombinant mouse VEGF soluble receptor 1 (sFlt-1)/Fc Chimera (R&D Systems, Minneapolis, MN) + 30 ng/ml recombinant mouse VEGF soluble receptor 2 (sFlk-1)/Fc Chimera (R&D Systems) were added to satellite cell CM (containing 2% FBS) or DMEM (containing 2% FBS). The medium was then added to MVF or stored at 4°C for <2 wk.

Measurement of HIF transcriptional activity. Primary rat satellite cells (100,000 cells/well in 6-well dishes) were transiently transfected with the hypoxia response element (HRE) luciferase reporter plasmid (35; 0.25 μg/well) and LacZ plasmid (0.75 μg/well) using FuGENE 6 transfection reagent (Roche, Indianapolis, IN). Transfection medium containing plasmids was removed after ~5 h and replaced with F-10 + 2% FBS + 1% penicillin-streptomycin + 0.05% gentamicin for treatments. Wells were then treated in triplicate with one of the following treatments: normoxia (21% O2), hypoxia (1% O2), or cobalt chloride (CoCl2; 150 μM) for 24 h. Treatment was discontinued after 24 h, and wells were rinsed twice with PBS (pH 7.4). After the PBS rinses, the cells were lysed with 200 μl of Reporter Lysis Buffer (Promega, Madison, WI), and plates were placed in ~80°C for later analysis.

Luciferase activity in cell lysates was measured using Luciferase Assay Reagent (LAR; Promega) by luminometer (Bio-Tek Clarity Luminescence Microplate Reader, Bioteck Instruments, Winooski, VT) per manufacturer’s instructions. The β-galactosidase was assayed in a 96-well plate using a β-galactosidase Enzyme Assay System (Promega) and read at absorbance 420 nm (Molecular Devices Spectra-Max M2, Molecular Devices, Sunnyvale, CA).

Vascular endothelial growth factor ELISA for conditioned medium. Rat satellite cells were seeded at 25,000 cells/well on 24-well plates, cultured in F-10 + 2% FBS + antibiotics and subjected to one of the following treatments for 48 h: normoxia (21% O2), 150 μM CoCl2, or hypoxia (1% O2). Empty wells (no cells) kept at 21% O2 with F-10 + 2% FBS + antibiotics served as a baseline control for the amount of VEGF present in the medium alone. Each treatment was performed in triplicate. Following treatment, medium was collected from each well, centrifuged at 5,000 g for 5 min to remove any cellular debris, and stored at ~80°C until the assay was performed. The VEGF analysis was performed using the Quantikine Rat VEGF Immunoassay kit (R&D Systems). To normalize VEGF protein on a cell number basis, the DNA content for each well was measured using the CyQUANT Cell Proliferation Assay Kit (Invitrogen, Eugene, OR) as described in the manufacturer’s protocol.

Analysis of gene expression. Total RNA was isolated from cells using the QIAGEN RNaseqy Mini Kit (QIAGEN, Germantown, MD) with on-column DNase digestion to remove genomic DNA contamination. Quantity and quality of RNA were assessed by absorbance at 260 nm and agarose gel electrophoresis. Total RNA (500 ng) was reverse transcribed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR was performed with rat-specific primers for VEGF (forward primer: 5’-AGGCTG-CACCCACGACAGAAG-3’; reverse primer: 5’-TCACCCTGGG-GCTTGTCA-3’). Empty wells (no cells) kept at 21% O2 with F-10 + 2% FBS + antibiotics served as a baseline control for the amount of VEGF present in the medium alone. Each treatment was performed in triplicate. Following treatment, medium was collected from each well, centrifuged at 5,000 g for 5 min to remove any cellular debris, and stored at ~80°C until the assay was performed. The VEGF analysis was performed using the Quantikine Rat VEGF Immunoassay kit (R&D Systems). To normalize VEGF protein on a cell number basis, the DNA content for each well was measured using the CyQUANT Cell Proliferation Assay Kit (Invitrogen, Eugene, OR) as described in the manufacturer’s protocol.

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cDNA prepared and pooled from experimental samples. Each assay plate contained negative controls and a standard curve (five serial dilutions of a pooled cDNA sample) to determine amplification efficiency of the respective primer pair. Unknown sample expression was then determined from the standard curve, adjusted for HPRT expression, and expressed as a fold of normoxia expression.

Immunohistology. Skeletal muscle tissue (Vastus) from an in vivo stretch model (34) and control rats were embedded in OCT and snap frozen in liquid N₂-cooled isopentane. Six-micron-thick slides were cut and fixed in 2% paraformaldehyde in PBS for 20 min and were then permeabilized with 10% FBS and 1% Triton X-100 in PBS for 1 h at room temperature. The slides were incubated in HIF-1α or VEGF goat polyclonal IgG (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After four 10-min washes in PBS, the slides were stained with Alexa Fluor 594 rabbit anti-goat IgG (1:250 in PBS; Invitrogen) for 1 h at room temperature in the dark. Following PBS washes, the slides were blocked with Doublestain Block (DAKO, Carpinteria, CA) for 1 h at room temperature and then incubated in mouse anti-Pax-7 supernatant (1:6 in PBS; Developmental Studies Hybridoma Bank, Iowa City, IA) or mouse monoclonal HIF-1α antibody (1:100 in PBS for VEGF/HIF-1α double staining; Novus Biologicals, Littleton, CO) at 4°C overnight. Alexa Fluor 488 rabbit anti-mouse IgG (1:250 in PBS; Invitrogen) was then used as secondary antibody, and the slides were applied with DAPI for nuclear staining. For control slides, the secondary antibodies were applied but had no access to primary antibodies. Images were taken using a Leica DM 5500 B microscope equipped with Image-Pro software.

Statistical analysis. General analysis of variance procedures were employed to analyze experimental results using general linear model procedures from the statistical software Minitab (State College, PA). Data are represented as least-squares means with standard errors, and statistically significant differences at P < 0.05 are denoted by differing letters.

RESULTS

To characterize the potential communication between myogenic and angiogenic cell types, we developed an in vitro coculture model composed of rat satellite cells and MVF. In this system, MVF are suspended in a three-dimensional collagen matrix and layered over a rat satellite cell monolayer propagated for 48 h in culture. Low-magnification images (Fig. 1) indicate a dramatic stimulation of angiogenesis in cocultured MVF compared with MVF cultured alone. Upon proper stimulation, MVF cultures exhibit characteristics of angiogenesis (endothelial cell sprouting, tubule formation, and extensive branching), providing a useful angiogenic bioassay. To quantitatively assess the angiogenic capacity of satellite cells, cocultures of satellite cells and MVF were grown for 6 days and then analyzed for sprout number and length. Because satellite cell preparations are sometimes contaminated with low levels (<5%) of fibroblasts, we cocultured MVF with muscle-derived fibroblasts as a control. MVF cultured in the presence of satellite cells exhibited more than fivefold greater sprout numbers formed compared with fibroblast coculture (P < 0.01), and the average sprout length was ~178% greater in satellite cell versus fibroblast coculture (P < 0.01; Fig. 2).

To determine whether the greater degree of angiogenesis we observed in the coculture setting was due to soluble factors produced by satellite cells or direct participation of cells from the satellite cell preparation, we cultured MVF in the presence

![Fig. 1. Promotion of angiogenesis by satellite cells. A and B: image of rat microvascular fragment (MVF) cultured alone. C: image of rat satellite cells (RSC) cocultured with MVF. MVF and myotubes are indicated by red and black arrows, respectively. In each image, a bar equivalent to 20 µm in length is provided.](http://ajpcell.physiology.org/)

![Fig. 2. Quantification of satellite cell- or muscle-derived fibroblast stimulation of in vitro angiogenesis. MVF embedded in collagen were layered over monolayer satellite cell- (48 h after isolation) or skeletal muscle-derived fibroblast cultures. After 5 days of coculture, pictures of MVF were taken and angiogenesis was quantified. Bars with different letters differ at P < 0.01.](http://ajpcell.physiology.org/)
of satellite cell CM. A positive dose-dependent effect of satellite cell CM on MVF growth was observed (data not shown and Fig. 3A). As shown in Fig. 3B, 10×-concentrated CM derived from satellite cells was approximately 3.5- and 2.5-fold more potent than 10×-concentrated fibroblast-derived CM in stimulating sprout formation and length, respectively \((P < 0.01)\). This result suggested that satellite cells secrete soluble-acting angiogenic growth factor(s); therefore we investigated the potential angiogenic role of satellite cell-derived VEGF. To do this, soluble VEGF receptors (sFlt and sFlk) were added to satellite cell CM to specifically block VEGF action (Fig. 4). The addition of sFlt and sFlk to satellite cell CM eliminated positive satellite cell effects on MVF sprout length and number (Fig. 4), and this reduction in angiogenic activity could be rescued by the addition of excess VEGF to the CM containing soluble VEGF receptors (data not shown).

In related experiments focusing on satellite cell activation, we conducted an initial microarray analysis of gene expression in cultured satellite cells undergoing activation in vitro and observed significant changes in the gene expression of HIF-1α, a key regulatory transcription factor involved in the regulation of VEGF and angiogenesis (X. Liu and R. E. Allen, unpublished observations). This observation, along with data presented in Figs. 1 and 2, prompted the notion that an angiogenesis-promoting program may be initiated in satellite cells during muscle regeneration. We examined expression of HIF-1α in greater detail using
RT-PCR during all phases of satellite cell culture. HIF-1α gene expression was first identified at 24 h in culture, when cells are undergoing activation, and continued to be detected during proliferation and differentiation phases (Fig. 5A). After confirming that satellite cells produce HIF-1α mRNA in culture, we sought to localize HIF-1α protein in satellite cells in vitro. Figure 5B shows HIF-1α localization in cultured satellite cells at 48 h in culture. Next, we treated proliferating satellite cells with positive regulators of HIF-1 in an attempt to manipulate satellite cell HIF activity, quantified using a

Fig. 5. A: expression of hypoxia-inducible factor-1α (HIF-1α) in cultured RSC. RT-PCR was used to detect the presence of HIF-1α message in cultured satellite cells from 24 h through 144 h. The β-actin message served as a control for equal amounts of starting RNA among samples. Two representative samples are shown at each time point. B: immunolocalization of HIF-1α in activated (48 h) RSC in culture. Cells were counterstained with DAPI to identify nuclei.

Fig. 6. Modulation of HIF-1 and VEGF gene expression and protein in primary RSC. For each experiment, subconfluent RSC were cultured in six-well plates (100,000 cells/well) containing DMEM with 20% FBS before experimental treatment. A: RSC were cultured in DMEM with 20% FBS and subjected to normoxic (20% O2), hypoxic (1% O2), or cobalt chloride (CoCl2; 150 μM) conditions for 24 h before RNA isolation and real-time PCR analysis of HIF-1α gene expression. B: RSC were transfected with 0.25 μg hypoxia response element (HRE) luciferase reporter plasmid (pHRE) before normoxic, hypoxic, or CoCl2 exposure. After 24 h, cell lysates were harvested and assayed for luciferase activity. C: RSC were treated as described in A before real-time PCR analysis of VEGF gene expression. D: RSC were cultured in DMEM with 2% FBS and subjected to normoxic, hypoxic, or CoCl2 conditions. At the end of 48 h, conditioned media were collected and VEGF protein concentration was determined by ELISA and normalized to DNA content per well. Bars with different letters differ at P < 0.05.
luciferase reporter gene controlled by a promoter containing a HRE (35). Hypoxia (1% O2) and CoCl2 reduced HIF-1α gene expression by ~20% and 35% (P < 0.05), respectively (Fig. 6A). Despite the reduction in HIF-1α gene expression, hypoxia and CoCl2 treatment increased satellite cell HIF activity by approximately 2- and 10-fold (P < 0.05), respectively (Fig. 6B). Increased HIF activity during hypoxic and CoCl2 conditions was positively associated with satellite cell VEGF gene expression and VEGF protein content in satellite cell CM (Fig. 6, C and D).

Finally, we sought to identify the extent of HIF-1α expression in satellite cells in vivo following mild muscle trauma. Therefore, we applied an in vivo model of muscle stretch to the adult rat hind limb which results in the activation of normally quiescent satellite cells with modest tissue damage (34). Localization of HIF-1α, VEGF, and Pax7 (a satellite cell marker) in longitudinal sections of rat muscle 48 h poststretch is shown in Fig. 7. Figure 7, A–C, shows a section stained with HIF-1α (red) and Pax7 (green). Arrows point to two pairs of HIF-1α-positive nuclei in addition to four single nuclei. The single nucleus in the bottom right portion of each image and one member of each pair are also positive for Pax7. Although not apparent in this reduced figure, we often find that the other cell in the pair is weakly positive for Pax7. Although not apparent in this reduced figure, we often find that the other cell in the pair is weakly positive for Pax7. In Fig. 7, E–G, VEGF and Pax7 are shown; three Pax7 cells are seen, and VEGF is located in the near vicinity. VEGF would be expected to be secreted and bound to the extracellular matrix near active satellite cells. The colocalization of HIF-1α and VEGF (Fig. 7, I–K) show VEGF staining along the membrane of fibers with the most intense staining near HIF-1α-positive nuclei. We see very little HIF-1α (Fig. 7D) or VEGF (Fig. 7H) staining in control unstretched muscles; arrows identify Pax7-positive nuclei, but little evidence of HIF-1α or VEGF is seen.

Fig. 7. Immunolocalization of VEGF (D and H), HIF-1α (A and G), and Pax7 (B and E) in rat muscle 48 h after stretch, a process that activates quiescent satellite cells. C, F, and I: composite immunolocalization images plus DAPI staining to indicate nuclear position. Arrows indicate colocalization of proteins within putative satellite cells. J: HIF-1α and VEGF colocalize in mononucleated cells in frozen sections. I and K: images correspond to secondary antibody use alone. In each image, a bar equivalent to 20 μm in length is provided for size reference.
DISCUSSION

A striking observation in the present study is the ability of satellite cells to promote vascular growth from preexisting blood vessel fragments, a process defined as angiogenesis. This finding is critical in the context of muscle regeneration where angiogenesis is an essential process during the restoration of skeletal muscle to a fully functional state. In this respect, our coculture is particularly relevant because of the three-dimensional structure and multicellular (consisting of endothelial, pericyte, and smooth muscle cells) composition of the MVF used in the model.

A second key observation is the ability of the satellite cell to stimulate angiogenesis whether in close proximity to (coculture conditions) or physically removed (through the use of CM) from the MVF. We did not discern whether satellite cells stimulated angiogenesis via direct contact with the MVF in the coculture setting. However, recent data by Christov et al. (4) indicate that satellite and endothelial cells are tightly juxtaposed in the muscle niche, suggesting that direct contact may be an important means of cellular communication. We clearly demonstrate that satellite cells secrete soluble-acting factors to promote angiogenesis in agreement with those observed by Christov et al. (4) where CM from human myogenic precursor cells was found to be proangiogenic for human umbilical vascular endothelial cells (HUVECs) in culture. Our results also provide strong evidence that VEGF is an important factor involved in satellite cell-mediated angiogenesis.

HIF, a transcription factor expressed in response to hypoxia, plays a central role in the restoration of cellular oxygen homeostasis, which may occur in cases of tissue growth or repair (26). HIF-1α is a heterodimeric complex composed of an inducible α-subunit (HIF-1α) and a constitutive β-subunit (HIF-1β/ARNT). Under normoxic conditions, a cascade of events targets HIF-1α for degradation in the 26S proteasome. Such posttranslational modifications include hydroxylation of HIF-1α, interaction with von Hippel-Lindau tumor suppressor proteins (pVHLs), and subsequent ubiquitination and degradation. Under hypoxic conditions, the absence of HIF-1α hydroxylation prevents binding by pVHLs, and HIF-1α escapes ubiquitination and degradation (26). This permits HIF-1α migration to the nucleus, dimerization with HIF-1β, and activation of gene transcription. HIF-1α protein is usually not present at high levels in tissues or cells in a normoxic environment. However, we have observed via immunohistochemistry that HIF-1α protein is present in satellite cells under normoxic conditions (data not shown), and we were able to measure the activity of HIF-1 through the HRE luciferase reporter plasmid in this study. We detected HIF-1α activity under normoxia, albeit at lower levels than under hypoxia or CoCl2. The HIF-1α protein has also been detected in skeletal muscle under normoxic conditions (18, 30). The presence of HIF-1α during normoxia suggests that alternate mechanisms of regulation exist in satellite cells. For example, HIF-1α has been shown to be modulated in other cell types by, among other factors, nitric oxide (NO) and the IGFs (10, 24, 40). HIF-1 activity may also be stimulated at the transcriptional level by mitogen action through receptor tyrosine kinases, even in the absence of hypoxia (reviewed in Ref. 7). NO is another especially attractive component to examine for the regulation of HIF-1α in satellite cells under normoxic conditions because NO has been shown to mediate the activation of satellite cells (3). It is tempting to speculate that, because NO is able to regulate both the activation of satellite cells through HGF release and HIF-1α under normoxic conditions in other cell types, NO may be a regulator of HIF-1α in satellite cells under normoxia as well. This may be a plausible theory because we hypothesized that satellite cells are the vehicle for muscle-regulated angiogenesis, and satellite cells would need to be activated for muscle repair and subsequent angiogenesis. These lines of evidence support an intriguing possibility that HGF may initiate a signaling cascade involving HIF that culminates in a putative hypoxia-inducible angiogenic pathway and VEGF production during satellite cell activation.

In a mild muscle trauma model, we observed the appearance of HIF-1α protein in satellite cells coincident with their activation from quiescence, prompting the notion that HIF-1 activity may be a necessary component of satellite cell activation. Pivotal factors to the satellite cell activation process, such as NO and HGF (1, 3, 33, 34), also appear to regulate HIF-1 activity in other cell types (24, 29, 31, 32). We and others have demonstrated that HIF-1 activity in primary satellite cells and myogenic cell lines (C2C12 and L6) can be modulated by hypoxia and CoCl2 (5, 6, 18). Collectively, these initial observations suggest that a heretofore unexplored aspect of satellite cell biology is the initiation of a proangiogenic program, which warrants further investigation.

In summary, experiments reported herein provide evidence that active satellite cells possess a potent proangiogenic program that may participate in revascularization of damaged muscle. Coordination of myogenesis and angiogenesis may therefore be accomplished through the secretion of soluble factors, such as VEGF, HGF, IGFs, and FGFs, by satellite cells participating in skeletal muscle regeneration.

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