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Myogenic and angiogenic pathways are sequentially activated during postnatal muscle regeneration

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ABSTRACT

Recently, it has been demonstrated that functional muscle regeneration occurs with combined delivery of angiogenesis and myogenesis factors. Although this new approach simultaneously targets distinct aspects of the regenerative process, the temporal relationship between myogenesis and angiogenesis during muscle regeneration is not fully characterized. Thus, we examined the expression of several important molecules that regulate myogenesis and angiogenesis following chemically-induced muscle injury. MRFs (MyoD and myogenin) exhibited strong increase in day 5 and returned to the control level by day 14. HIF-1α protein increased to a peak at day 5 and then VEGF protein increased to a peak at day 7. VEGF receptors (Flt-1, KDR/Flk-1, neuropilin-1) protein increased to a peak between 7 and 14 days. Moreover, we examined the effects of inhibition of VEGF signaling with selective VEGF receptor inhibitors, SU1498, on muscle regeneration. The skeletal muscles forced to regenerate in the presence of SU1498 were of poor repair with small regenerating myofibers and increased connective tissues. These results suggest that myogenic and angiogenic pathways are sequentially activated during postnatal muscle regeneration and angiogenesis plays an important role in the success of muscle regeneration.

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KEYWORDS: angiogenesis, myogenesis, muscle regeneration

ABBREVIATIONS

EBD, evans-blue dye; HIF-1, hypoxia-inducible factor 1; Flt-1, fms-like tyrosine kinase-1; KDR/Flk-1, kinase insert domain-containing receptor/fetal liver kinase-1; MRFs, myogenic regulatory factors; PECAM-1/CD31, platelet/ endothelial cell adhesion molecule-1/cluster of differentiation 31; VEGF, vascular endothelial growth factor

INTRODUCTION

Skeletal muscle exhibits a high capacity to regenerate/repair damaged tissue following injury. During muscle regeneration, various cellular responses are coordinately activated [1]. Upon injury, damaged tissue is infiltrated by fibroblasts, neutrophils, and monocytes/macrophages. Myogenic cells are activated to proliferate, differentiate, and fuse with the existing myofibers or fuse together to form new myofibers [1]. Myogenesis is orchestrated through a series of transcriptional controls governed by the myogenic regulatory factors (MRFs). It has been well established that activated satellite cells are characterized by expression of MyoD and Myf5, whereas myoblast terminal differentiation is characterized by expression of myogenin and MRF4 [2, 3].

Revascularization also occurs during muscle regeneration. There is close relationship between

maintaining a sufficient blood supply and muscle regeneration [4], indicating that revascularization contribute to the success of muscle regeneration [5]. The process of revascularization during muscle regeneration has been well documented. The endothelial cells and pericytes of capillaries undergo degeneration and subsequently new capillaries begin to develop along the existing capillary basement membrane [6]. The new capillaries sprout out from surviving capillaries toward the center of the injured area [7]. Angiogenesis is a biological process by which new capillaries are formed from pre-existing vessels [8]. It is essential in many physiological conditions such as embryogenesis, ovulation and wound repair [8]. It is tightly regulated by angiogenesis-regulated factors. Hypoxia-inducible factor 1 (HIF-1) plays a critical role in angiogenesis by activating transcription of genes encoding angiogenic growth factors such as vascular endothelial growth factor (VEGF) [9], which is a key regulator of physiological angiogenesis [10]. VEGF exerts its biological effects through two tyrosine kinase receptors, the fms-like tyrosine kinase (Flt-1) and the kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/Flk-1), which are expressed primarily on endothelial cells [10]. Neuropilin-1 also acts as coreceptors to modulate VEGF signaling [11]. Recently, it has been reported that functional muscle regeneration occurs with combined delivery of angiogenic and myogenic factors [12]. Understanding how myogenic and angiogenic pathways respond to muscle injury will provide a valuable insight into strategy for therapeutic intervention for muscle disease. However, the temporal relationship between myogenesis and angiogenesis during muscle regeneration is not fully characterized [13].

In the present study, we examined the expression of several important molecules that regulate myogenesis and angiogenesis following chemically-induced muscle injury. Moreover, we examined the effects of inhibition of VEGF signaling with selective VEGF receptor inhibitors, SU1498, on muscle regeneration. Our results suggest that myogenic and angiogenic pathways are sequentially activated during postnatal muscle regeneration and angiogenesis plays an important role in the success of muscle regeneration.

MATERIALS AND METHODS

Animal care

Male 4-week-old C57BL/6J mice (Clea Japan, Meguro, Tokyo) were used and housed in the animal care facility under a 12-h light/12-h dark cycle at room temperature (23 ± 2_C) and 55 ± 5% humidity. The mice were maintained on a diet of CE-2 rodent chow (Clea Japan) and given water ad libitum. The mice were procured after approval for the present study from The University of Tokyo Animal Ethics Committee.

Induction of muscle degeneration/regeneration

Degeneration of skeletal muscle was induced by intramuscular injection of 50% glycerol [14]. After anesthesia by intraperitoneal injection of pentobarbital (50 mg/kg), the mouse's lateral and posterior surfaces of the lower-left hindlimb were shaved and prepared. The gastrocnemius muscles were exposed by making a 1.5-cm-long incision through the skin overlying the muscle belly. The belly region of the gastrocnemius was determined by verifying by palpation that this site was the bulkiest portion of the gastrocnemius when the right ankle joint fixed at 90° and was marked with a permanent marker. Following the injury, the skin incision was closed using a 6-0 silk suture and treated with hydrogen peroxide. We made a mark on the wound site with a permanent marker and checked it so that the marker should not disappear every day. Preliminary experiments were performed to verify to what extent muscle injury occurs by intramuscular injection of glycerol. Before inducing muscle injury, Evans-blue dye (EBD) (0.5 mg/0.05 ml PBS) was injected into the tail vein of the mice without anesthesia to visualize the degenerating myofibers [15]. After 1 h, gastrocnemius muscles were removed from mice and transversely sectioned in the usual manner. EBD injected intravenously stained only degenerating myofibers which were visible as blue fibers macroscopically and could also be seen as red fluorescent myofibers microscopically (Figure 1). We counted the number of EBD-positive degenerating myofibers in the entire section by fluorescent microscopy. Mean percentage of damaged-myofibers in relation to the whole skeletal muscle section was approximately 60-70%. The gastrocnemius muscles were isolated at

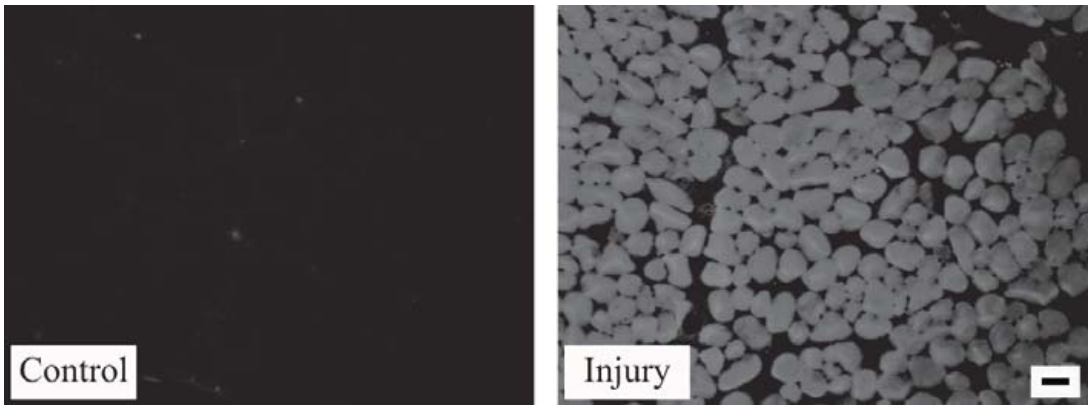

Figure 1. Histochemical evaluation of Evans blue staining after intravenous dye injection into mice. Tissues were isolated, frozen, cryosectioned, and then extensively washed with acetone. Sections were observed under a fluorescence microscope. Control mice showed no dye uptake into their myofibers. There were very few myofibers with a positive dye staining on sections of control animals, which were otherwise EBD negative. In injured muscle, the intracellular staining was diffusely distributed across the myofiber cytoplasm. Most of the EBD-positive fibers showed intense staining, whereas in some others, the signal was moderate. *Scale bar* = 100μm.

various time points (0.5, 1, 3, 5, 7, 10, 14, or 28 days) after muscle injury. Similarly, the gastrocnemius muscles were isolated from age-matched control mice without surgery at various time points. All procedures in the animal experiments were performed in accordance with the guidelines presented in the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, published by the Physiological Society of Japan.

Treatment of (ε)-3-(3,5-diisoproply-4-hydroxyphenyl)-2-[3-phenyl-n-propyl) amino-carbonyl] acrylonitrile (SU1498)

To inhibit VEGF-KDR/Flk-1 signaling, 50 μl of SU1498 (10 μM) or DMSO was introduced into the muscle surrounding the injury site by direct intramuscular injection at day 1, 3, 5, and 7 after the initial injury. The gastrocnemius muscles were isolated at day 10 for histochemical analysis.

Immunoblot analysis

Total cellular protein extracts were prepared from whole gastrocnemius muscle with PBS, and then scraping the cells directly into sample buffer. Protein concentration was determined by BCA assay. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Inc., Lincoln, NE) and incubated in primary antibody overnight at 4°C. Membranes were then incubated with appropriate secondary antibody for 1 hour at room temperature and analyzed with an Odyssey Infrared Imaging System (LI-COR). The antibodies are listed in Table 1. The intensity of bands was quantified by using ImageJ software (Ver. 1.42, http://rsb.info.nih.gov/ij/). To verify equal loading, membranes were probed with anti-β-Tubulin antibody or stained with IRDye Blue Protein Stain (LI-COR).

Histochemical analysis

The tissues were transversely sectioned at 8 μm using a cryostat at -20°C and thawed on 3-amino propylethoxysilane-coated slides. The sections were fixed with 3.7% paraformaldehyde for 10 min, and then stained with hematoxylin and eosin for evaluation of general muscle architecture. For morphometrical analysis, randomly selected fields were photographed (original magnification, ×100; six fields per sample). Fiber-cross sectional area (FCSA) was determined using ImageJ software.

RESULTS

Expression of myogenic regulatory factors during muscle regeneration

We examined the expression levels of MyoD and myogenin proteins during muscle regeneration.

Table 1. List of antibodies used in this study.

Antibody	Clone	Ig type	Epitope	Supplier	Dilution
Primary antibodies					
MyoD (C-20)	Polyclonal	Rabbit IgG	C-terminus (mouse)	Santa Cruz Biotechnology, Inc.	1:5000
Myogenin (m-225)	Polyclonal	Rabbit IgG	Amino acids 1-225 full length (rat)	Santa Cruz Biotechnology, Inc.	1:5000
Myosin (sarcomere) (MF 20)	Monoclonal	Mouse IgG2b	(chicken)	DSHB	1:500
HIF-1α	Polyclonal	Rabbit IgG	Amino acids 530-825 (mouse)	Novus Biologicals, Inc.	1:1500
VEGF (A-20)	Polyclonal	Rabbit IgG	N-terminus (human)	Santa Cruz Biotechnology, Inc.	1:3000
Flt-1 (C-17)	Polyclonal	Rabbit IgG	C-terminus (human)	Santa Cruz Biotechnology, Inc.	1:3000
KDR/Flk-1 (C-20)	Polyclonal	Rabbit IgG	C-terminus (mouse)	Santa Cruz Biotechnology, Inc.	1:2500
Neuropilin-1	Polyclonal	Rabbit IgG	C-terminus (rat)	Oncogene Research Products, Inc.	1:2000
PECAM-1 (CD31) (M-20)	Polyclonal	Goat IgG	C-terminus (mouse)	Santa Cruz Biotechnology, Inc.	1:2000
β-Tubulin (EP1331Y)	Monoclonal	Rabbit IgG	C-terminus (human)	Epitomics, Inc.	1:5000
Secondary antibodies			Conjugate		
Rabbit IgG	-	Goat	Alexa Fluor 680	Molecular Probes Inc.	1:4000
Mouse IgG	-	Rabbit	IRDye800	Rockland Immunochemicals, Inc.	1:4000
Goat IgG	-	Donkey	Alexa Fluor 680	Molecular Probes Inc.	1:4000

DSHB, developmental studies hybridoma bank.

Muscle regeneration in gastrocnemius muscle was induced following injection of glycerol, which causes extensive and reproducible muscle necrotic injury. The myogenic differentiation is initiated within 2 days followed by extensive regeneration within 7-14 days after initiation of muscle injury [14]. Figure 2 shows the temporal changes in the amount of MyoD and myogenin proteins during muscle regeneration. The amount of MyoD protein increased to a peak at day 5, followed by a progressive decrease by day 7, and returned to the control level by day 14. The pattern of temporal changes in myogenin protein levels was similar to that observed in the MyoD, exhibiting the same strong increase in day 5. We also examined the expression of sMyHC, terminal differentiation marker, during muscle regeneration. The amount of sMyHC protein rapidly decreased at day 0.5, followed by a progressive decrease by day 3, and increased by 2-fold at day 28, compared to control level.

Expression of angiogenesis-regulated factors during muscle regeneration

Figure 3 shows the temporal changes in the amount of angiogenesis-regulated factors during muscle regeneration. The amount of HIF-1α protein increased to a peak at day 5, followed by a progressive decrease by day 7, and returned to the control level by day 14. VEGF protein was detected to a small extent in control muscle. After muscle injury, the amount of VEGF protein increased to a peak at day 7 and remained above basal levels observed in control animals up to day 28. The amount of Flt-1 protein rapidly decreased at day 0.5, followed by a progressive decrease by

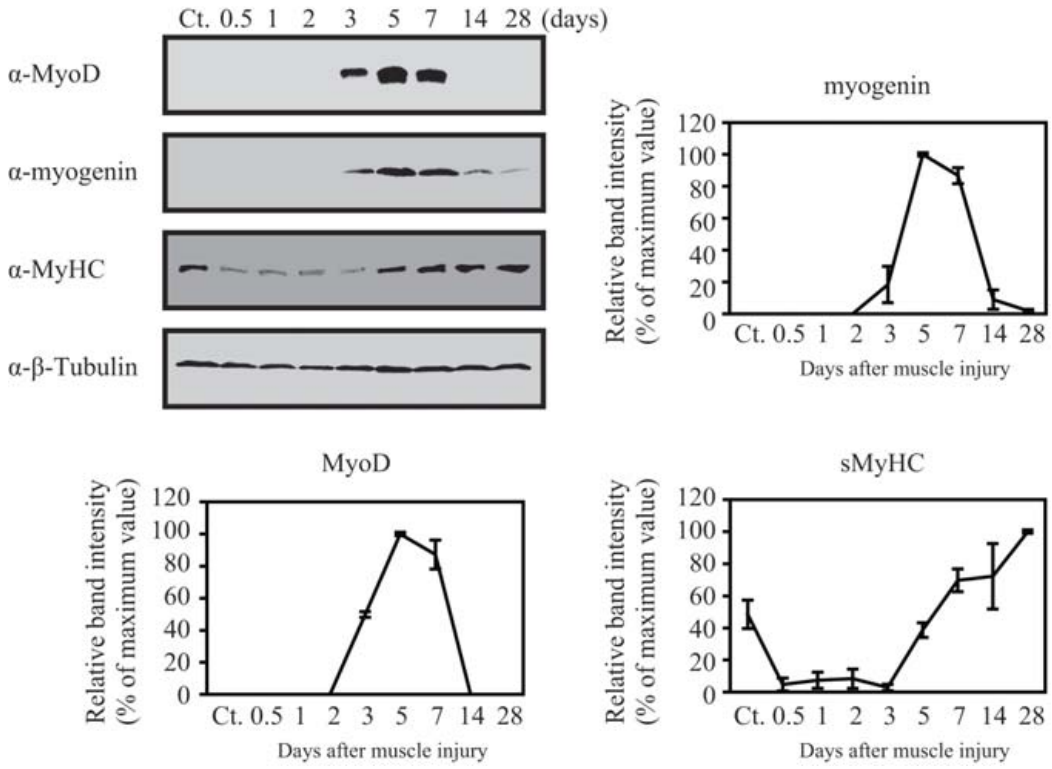

Figure 2. Time course of changes in amounts of MyoD, myogenin, and sarcomeric myosin heavy chain (sMyHC) during muscle degeneration/regeneration. Tissues were isolated at various time points. Samples were subjected to SDS-PAGE and immunoblotted using the indicated antibodies. Proteins were visualized to correspond to their expected size: MyoD (45-kDa), myogenin (36-kDa), sMyHC (220-kDa), and β-Tubulin (55-kDa). Quantification of immunoblot analysis of total extracts from muscle tissues. The intensities of bands were measured and normalized to the maximum value observed during muscle degeneration/regeneration. β-Tubulin was used as a loading control. Because of the extensive myofiber necrosis at early time points of muscle degeneration/regeneration, the amount of β-Tubulin was not equal across all the time points, as expected. The data are the means ± SD of at least three independent experiments.

day 3, increased to a peak at day 14, and returned to the control level by day 28. Skeletal muscle tissue from control mice expressed the mature 230-kDa forms (KDR/Flk-1), whereas that from injured mice expressed both unglycosylated 150-kDa and mature 230-kDa forms. The amount of KDR/Flk-1 (230-kDa) protein rapidly decreased at day 0.5, followed by a progressive decrease by day 3, increased to a peak at day 14, and returned to the control level by day 28. Unglycosylated 150-kDa form was firstly detected at day 3, increased to a peak at day 7, and then not detected at day 28. The amount of neuropilin-1 protein increased to a peak at day 7 and returned to the control level by day 14. The amount of PECAM-1/CD31 protein remained unchanged from day 0.5 to day 7 and increased by 3-4-folds at day 28, compared to control level.

Effects of SU1498 on muscle regeneration

The biological effects of VEGF are mediated by at least two tyrosine kinase receptors, Flt-1 and KDR/Flk-1 which bind VEGF with high affinity [10]. There are significant differences with respect to biological activity between KDR/Flk-1 and Flt-1. KDR/Flk-1 but not Flt-1 can mediate the mitogenic and chemotactic effects of VEGF and activate signaling pathways [10]. SU1498, an inhibitor of KDR/Flk-1, has been used successfully to study the physiological manifestations of receptor functions [16]. To elucidate the possible role of angiogenesis during muscle regeneration, we examine the effect

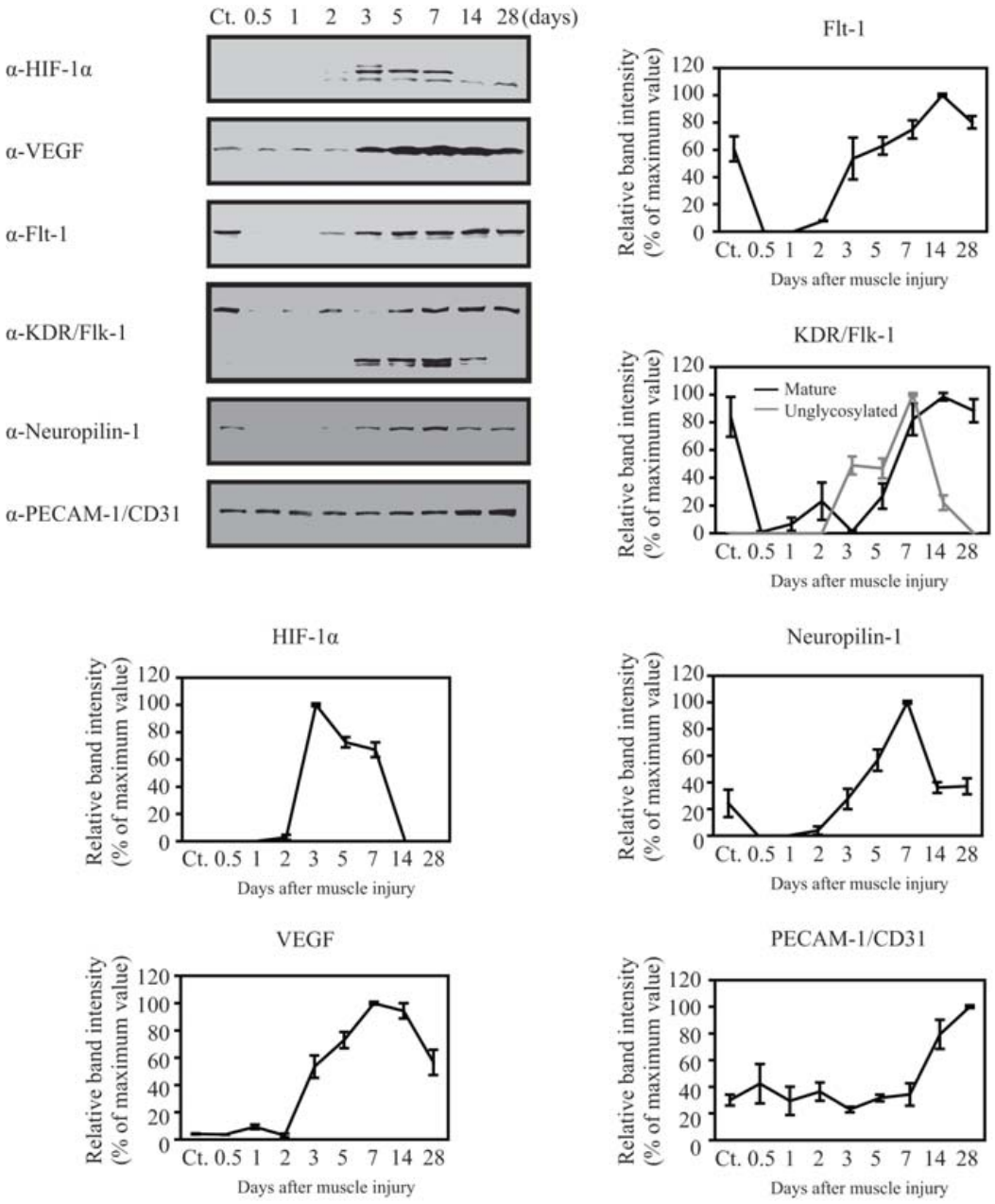

Figure 3. Time course of changes in amounts of HIF-1α, VEGF, Flt-1, KDR/Flk-1, Neuropilin-1, and PECAM-1/CD31 during muscle degeneration/regeneration. Proteins were visualized to correspond to their expected size: HIF-1α (120-kDa), VEGF (23-kDa), Flt-1 (180-kDa), KDR/Flk-1 (150-and 230-kDa), Neuropilin-1 (130-kDa), and PECAM-1/CD31 (130-kDa). β-Tubulin was used as a loading control as shown in Figure. 2. The data are the means ± SD of at least three independent experiments.

of SU1498 on muscle regeneration. Glycerol was injected into gastrocnemius muscles of control and SU1498-treated mice. Ten days after initial injury, the muscles were removed, cryosectioned, and stained with hematoxylin and eosin. The control muscles contained numerous nascent myofibers with centrally located nuclei, whereas the skeletal muscles forced to regenerate in the presence of SU1498 were of poor repair with small myofibers and increased connective

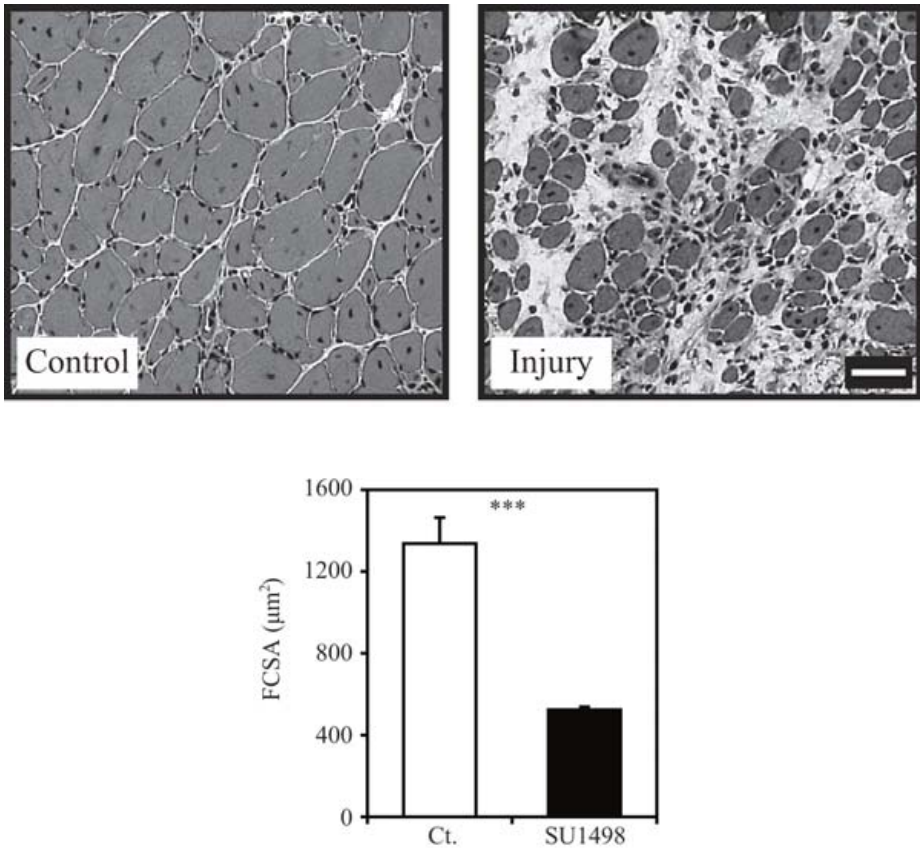

Figure 4. Effects of SU1498 treatment on muscle regeneration. Tissue were isolated for histochemical analysis 10 days after initiation of muscle injury, frozen, cryosectioned, fixed, and then stained with hematoxylin and eosin. *Scale bar* = 100μm. The data are the means ± SD of at least six independent experiments. There are statistically significant differences compared to the control: ***P<0.001.

tissues (Figure 4). A quantitative analysis showed FCSA of regenerating myofibers was smaller than that of control myofibers.

DISCUSSION

In the present study, we examined the expression of several important molecules that regulate myogenesis and angiogenesis following chemically-induced muscle injury. Moreover, we examined the effects of inhibition of VEGF signaling with selective VEGF receptor inhibitors, SU1498 on muscle regeneration. The results of this study show that (1) myogenic and angiogenic pathways are sequentially activated during postnatal muscle regeneration; (2) angiogenesis plays an important role in the success of muscle regeneration.

We showed simultaneous upregulation of MRFs (MyoD and myogenin) during muscle regeneration. Overall, they are expressed simultaneously and relatively late in the process of myogenesis [2, 3]. Grounds *et al.* showed that increased MyoD and myogenin mRNA transcripts in mononuclear cells were detected as early as 6 h after injury, peaked between 24 and 48 h, and thereafter declined to pre-injury levels at 8 days using *in situ* hybridization technique [17]. We previously reported that MyoD and myogenin mRNA transcripts were simultaneously upregulated at day 3 after freeze muscle injury using real-time PCR analysis [18]. The expression pattern of their proteins exhibited the same strong increase at day 5 using immunoblot analysis [19]. Füchtbauer *et al.* demonstrated that both MyoD and myogenin proteins were detected in the regenerating myofibers using immunohistochemical analysis [20], suggesting that they may be coexpressed in the regenerating myofibers. MyoD induces terminal cell cycle arrest

during myogenic differentiation by increasing the expression of cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$ [21]. The MRFs activate transcription of muscle-specific genes by binding, upon heterodimerization with ubiquitous E proteins [22], the E-box consensus sequence in skeletal muscle gene promoters and enhancers [23]. Considering their role in myogenesis, elevated expression levels of MyoD and myogenin in regenerating myofibers may reflect an increased demand for its basal functions during cell cycle growth arrest and/or the progressive expression of the muscle phenotype.

The time course and relationship of inflammation, angiogenesis and myogenesis following femoral artery occlusion have been already reported [13]. They showed that mitotic activity began in endothelial and muscle satellite cells 3 days after occlusion, resulting in new formation of capillaries and myofibers. They concluded that myogenesis and angiogenesis occur concomitantly in regenerating skeletal muscle because of ischemia-induced cell death and inflammation. However, our results indicate that activation of angiogenetic pathway occurs after that of myogenic pathway. The temporal difference of activation between myogenesis and angiogenesis may be, at least in part, supported by the observation that many newly formed regenerating myofibers with poorly organized capillary distribution were observed until day 5-7 after freeze muscle injury [24]. Considering their protein expression patterns observed in this study, there is temporal difference between myogenesis and angiogenesis during muscle regeneration. However, we cannot exclude the possibility that this discrepancy may be attributed to experimentation differences between the laboratories. Indeed, it is pointed out that the degree of muscle healing is dependent on the severity and age of the injury, the animal model used, and the outcome used to follow the healing process [25].

The biological activity of HIF-1 is determined by the expression and activity of the HIF-1α subunit [26]. HIF-1α dimerizes with HIF-1β and binds DNA to activate transcription of a number of target genes involved in the regulation of angiogenesis such as VEGF [27]. In the present study, increased expression of HIF-1α preceded expression of VEGF, suggesting that its expression may be dependent upon HIF-1 transcriptional activity. In support of this, cobalt-mediated HIF-1α stabilization increased VEGF expression in C2C12 cells [18, 28]. When HIF-1 activity is enhanced by suppression of prolyl and asparaginyl hydroxylase activity by dimethyloxalylglycine, VEGF production increased in skeletal muscle [29]. Adeno-associated virus-mediated transduction of a stabilized form of HIF-1α markedly increased capillary sprouting and proliferation in skeletal muscle [30]. Taken together, HIF-1 plays an important role in the regulation of VEGF expression. However, HIF-1 independent regulation of VEGF has been also reported. Arany *et al.* showed that transcriptional coactivator PGC-1α (peroxisome-proliferator-activated receptor-γ coactivator-1α) was induced by a lack of nutrients and oxygen, and PGC-1α powerfully regulated VEGF expression in cultured muscle cells and skeletal muscle *in vivo* [31]. The induction of VEGF by PGC-1α did not involve the canonical hypoxia response pathway and HIF-1 [31]. Instead, PGC-1α coactivated the orphan nuclear receptor ERRα (oestrogen-related receptor α) on conserved binding sites found in the promoter and in a cluster within the first intron of the VEGF gene [31]. Similarly, PGC-1β also induced the expression of VEGF *in vitro* and *in vivo*. The induction of VEGF by PGC-1β required coactivation of ERRα and is independent of the HIF pathway [32]. We previously reported that PGC-1β not PGC-1α was induced during muscle regeneration [33] suggesting that, PGC-1β also may stimulate the expression of VEGF during muscle regeneration.

It has been reported that the Flt-1 receptor gene is also regulated by HIF-1. Flt-1 promoter includes a sequence matching the HIF-1 consensus binding site previously found in other hypoxia-inducible genes such as the VEGF gene and erythropoietin gene [34]. An adenoviral vector encoding a constitutively stable hybrid form of HIF-1α increased Flt-1 expression in endothelial cells [35]. Besides HIF-1-dependent regulation, it is likely that VEGF affects Flt-1 expression. Sato *et al.* reported that VEGF stimulated Flt-1 expression and the combination of anti-Flt-1 and anti-KDR monoclonal antibodies almost completely inhibited VEGF-mediated induction Flt-1 in endothelial cells [36]. Considering that upregulation of VEGF

was followed by increased Flt-1 expression in the process of muscle regeneration, Flt-1 expression may be regulated by HIF-1 transcriptional activity and VEGF.

In the present study, increased expression of VEGF occurred before expression of mature form and unglycosylated form of KDR/Flk-1 peaked during muscle regeneration. This may show that VEGF directly regulates expression of KDR/Flk-1. This possibility is supported by the previous study demonstrating that (1) VEGF treatment induced a time-dependent increase in KDR/Flk-1 protein; (2) Blockade of VEGF with a neutralizing anti-VEGF monoclonal antibody abolished VEGF-induced KDR/Flk-1 upregulation; (3) VEGF treatment resulted in an increase in KDR/Flk-1 receptor present on the endothelial cell surface; (4) VEGF treatment increased KDR/Flk-1 promoter activity and mRNA expression [37]. Taken together, upregulation of KDR/Flk-1 induced by VEGF may represent an important positive feedback mechanism for VEGF action in muscle injury-induced angiogenesis.

It has been proposed that Flt-1 may not be primarily a receptor transmitting a mitogenic signal, but rather a 'decoy' receptor, able to regulate in a negative fashion the activity of VEGF on the vascular endothelium, by preventing VEGF binding to KDR/Flk-1 [38]. In contrast, KDR/Flk-1 is the major mediator of the mitogenic, survival, angiogenic, and permeability-enhancing effects of VEGF [10]. In the present study, both Flt-1 and KDR/Flk-1 was simultaneously upregulated during muscle regeneration. This suggests that they may be coordinately expressed to control the effects of VEGF on endothelial cells during muscle regeneration.

Neuropilin-1 is originally characterized as a neuronal receptor for certain secreted members of the collapsin/semaphorin family [39, 40], and also plays a role in vasculogenesis and angiogenesis. Neuropilin-1 mutant mice exhibited cardiovascular defects [41], while mice treated with anti-neuropilin-1 antibody exhibited impaired wound angiogenesis [42]. Neuropilin-1 functions as a coreceptor for $VEGF_{165}$, enhancing its binding to KDR/Flk-1 and its bioactivity [11]. This study is, to our knowledge, the first to deal with the potential regulation of neuropilin-1 expression during muscle regeneration. We found neuropilin-1 increased in injured muscle, suggesting that this receptor may be associated with angiogenesis during muscle regeneration. Interestingly, both KDR/Flk-1 and neuropilin-1 proteins simultaneously increased in injured muscle, suggesting that they may be coordinately expressed to function as a coreceptor for VEGF.

We used PECAM-1/CD31, vascular endothelial marker, to evaluate the extent of capillarization during muscle regeneration. PECAM-1/CD31 protein increased approximately 3-fold at day 28, compared to control level, suggesting that excessive growth of new capillaries may occur in the process of muscle regeneration. Winkel *et al.* reported morphological adaptation of the process of neovascularization when the gracilis anterior muscles were devascularized and transplanted into the femoral region [43]. They observed that the loose connective tissue between the slender regenerating myofibers was filled by a closely meshed network of capillaries and sinusoids after surgery. They suggest that this excessive capillarization may be typical of endothelial cell proliferation as well as a reaction to the need for an increased oxygen supply to the regenerating myofibers. This adaptive response may indicate that the capillary supply adjusts to the maximum aerobic capacity as well as to the mitochondrial content of the myofibers [44].

Pharmacologic inhibition of VEGF-KDR/Flk-1 signaling attenuated muscle regeneration, suggesting that it induces an insufficient capillary network, resulting in impairing a cooperative relationship between myofibers and capillaries. However, we cannot exclude the possibility that SU1498 has a direct effect on myogenic cells because KDR/Flk-1 was expressed in myoblasts, myotubes, and regenerating myofibers [45-50]. Several evidences indicate that VEGF-KDR/Flk-1 signaling plays an important role in cell survival and differentiation. Germani *et al.* reported that VEGF administration to differentiating C2C12 myoblasts prevented apoptosis, while inhibition of VEGF signaling with SU1498 or a neutralizing Flk-1 antibody, enhanced cell death [46]. Arsic *et al.* also reported that treatment with SU1498 abolished the protective effect of VEGF on camptothecin-induced cell apoptosis in cultured

myoblasts and C2C12 cells [47]. Messina *et al.* reported that VEGF treated *mdx* mice, a model of Duchenne muscular dystrophy, showed reduced necrotic fibers area and increased regenerating myofibers area with an augmented number of myogenin-positive satellite cells and of developmental myosin heavy chain-positive myofibers [50]. We observed that treatment with SU1498 can block the differentiation of myoblasts into myotubes (our unpublished data). Therefore, VEGF-KDR/Flk-1 signaling may have an impact on both myogenic and angiogenic pathways.

In conclusion, we demonstrated that myogenic and angiogenic pathways were sequentially activated during muscle regeneration and pharmacologic inhibition of VEGF-KDR/Flk-1 signaling resulted in attenuating muscle regeneration. Understanding how myogenic and angiogenic pathways respond to muscle injury will provide a valuable insight into strategy for therapeutic intervention for muscle disease. Recently, Borselli *et al.* reported that functional muscle regeneration occurs with combined delivery of angiogenic and myogenic factors [12]. The dual delivery of VEGF/insulin-like growth factor-1 (IGF-1) from an injectable biodegradable hydrogel leads to a complete functional recovery of ischemic injured skeletal muscle compared to the delivery of VEGF or IGF-1 alone. Our data may contribute to the strategy of simultaneously targeting distinct aspects of the regenerative process.

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