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Sequential Expression of Vascular Endothelial Growth Factor, Flt-1, and KDR/Flk-1 in Regenerating Mouse Skeletal Muscle

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Summary

We investigated the expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and KDR/Flk-1) during muscle regeneration by immunohistochemistry and real-time RT-PCR. On days 5 and 7 after the induction of injury, VEGF and Flt-1 were detected in the cytoplasm and KDR/Flk-1 in the cytoplasm and on cell membranes of the same regenerating muscle fibers. The levels of these proteins in the regenerating muscle fibers gradually decreased until day 20. In contrast, these proteins were not detected in the fibers of normal muscle. This suggests that regenerating muscle fibers express VEGF and its receptors in response to injury. In addition, we found that the VEGF mRNA transcript transiently increased after 12 h of muscle injury and then returned to the basal levels observed in normal muscles on day 1. The expression of Flt-1 and KDR/Flk-1 mRNA transcripts also peaked on day 3 and then returned to the basal levels observed in normal muscles on day 10. These findings suggest that regenerating muscle fibers are an important source of VEGF and that VEGF signaling through Flt-1 and KDR/Flk-1 may be involved in the process of muscle regeneration *in vivo*.

Key words
Muscle regeneration • EGF • Flt-1 • KDR/Flk-1

Introduction

Skeletal muscles exhibit a high capacity to repair and regenerate after injury. This capacity for tissue regeneration is bestowed by satellite cells, which are located between the basal lamina and the sarcolemma of mature muscle fibers (Mauro 1961). Upon injury, satellite cells become activated, proliferate, and fuse with the existing muscle fiber or fuse together to form a new muscle fiber (Hawke and Garry 2001). The activity of satellite cells is affected by various factors such as fibroblast growth factor, hepatocyte growth factor, insulin-like growth factor-I, -II, transforming growth factor-β, leukemia inhibitory factor, and interleukin-6 (Charge and Rudnicki 2004).

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor that plays an important role in vascular development as well as in physiological and pathological angiogenesis (Cross *et al.* 2003). The biological activity of VEGF is mediated by two tyrosine kinase receptors, fms-like tyrosine kinase (Flt-1) and kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/Flk-1) (Ferrara 2001), which are expressed almost exclusively on endothelial cells (Petrova *et al.* 1999). However, several studies indicate that the effects of VEGF might extend to a variety of

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other cell types such as neuronal cells (Jin *et al.* 2000, Ogunshola *et al.* 2002), osteoblasts (Midy and Plouet 1994), cardiac myocytes (Seko *et al.* 1998) and myogenic cells (Germani *et al.* 2003, Arsic *et al.* 2004).

VEGF may regulate muscle fiber formation through a direct effect on satellite cells (Germani *et al.* 2003, Arsic *et al.* 2004). Exogenous administration of VEGF stimulates myoblast migration (Germani *et al.* 2003), protects myogenic cells from apoptosis (Germani *et al.* 2003, Arsic *et al.* 2004), and promotes myogenic cell growth (Arsic *et al.* 2004). VEGF (Rissanen *et al.* 2002, Tuomisto *et al.* 2004), Flt-1 (Germani *et al.* 2003, Arsic *et al.* 2004), and KDR/Flk-1 (Rissanen *et al.* 2002, Germani *et al.* 2003, Arsic *et al.* 2004, Tuomisto *et al.* 2004) are expressed in the regenerating muscle fibers after ischemia- or chemically-induced muscle damage, suggesting that muscle fibers also produce VEGF and its receptors in response to injury. Unfortunately, previous immunohistochemical studies have not been conducted on the same muscle fibers. To further elucidate the role of these proteins in muscle regeneration, more information is needed about the spatiotemporal expression patterns of VEGF, Flt-1, and KDR/Flk-1 proteins. Additionally, to our knowledge, the chronological expression patterns of these mRNAs in the early process of muscle regeneration remain to be elucidated.

In the present study, we investigated in detail the expression patterns of VEGF, Flt-1, and KDR/Flk-1 proteins and mRNA transcripts in regenerating muscle by immunohistochemistry and real-time reverse transcription-polymerase chain reaction (RT-PCR). We studied the effects of freeze injury, a model system frequently used to investigate muscle regeneration (Creuzet *et al.* 1998, Pavlath *et al.* 1998, Warren *et al.* 2002). Similar to other models, including treatment with bupivacaine (Benoit and Belt 1970) or cardiotoxin (Couteaux *et al.* 1988), freeze injury was highly effective at eliciting muscle degeneration.

Methods

Animal care

Male, 10-week-old CD1 mice were purchased from CLEA (Tokyo, Japan). The mice were housed in the animal 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) and given water *ad libitum*. 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 and based on the Helsinki Declaration of 1964. This study was also approved by the Animal Committee of the National Institute of Fitness and Sports, Japan.

Induction of muscle regeneration

Regeneration of skeletal muscle was induced by a single freeze injury of the tibialis anterior (TA) muscle by standard procedures as previously described (Warren *et al.* 2002). Under anesthesia, the mouse's lateral and anterior surfaces of the lower-left hindlimb were shaved and prepared. The TA muscles were exposed by making a 1.5-cm-long incision through the skin overlying the muscle belly. Injury was induced by applying a steel probe cooled in liquid nitrogen to the TA muscle belly for 5 s. The probe consisted of a 6-mm diameter rod with a sharp tip. Following the injury, the skin incision was closed using a 6-0 silk suture and treated with hydrogen peroxide. The TA muscles were isolated at various time points (12 hours, 1, 3, 5, 7, 10, or 20 days) after freeze injury and weighed using an electronic balance.

Histology and immunohistochemistry

The TA muscles were isolated at various time points (3, 5, 7, 10, or 20 days) after the freeze injury. The TA muscles from untreated mice were used as a control. Serial cross-sections (8-10 μm thickness) were cut using a cryostat at –20 °C and thawed on 3-amino propyl-ethoxysilane-coated slides. The sections were stained with hematoxylin and eosin (H&E) for evaluation of general muscle architecture. For immunohistochemical analysis, the sections were fixed with cold acetone (–20 °C) or with 4 % paraformaldehyde for 10 min and then washed with 0.1 M phosphate-buffered saline (PBS). To quench endogenous peroxidase activity, the sections were incubated with a solution of methanol containing 1 % H_2O_2 for 60 min. The sections were washed with 0.1 M PBS, blocked with 0.1 M PBS containing 1 % bovine serum albumin and 0.1 % Triton X-100, and then incubated overnight at 4 °C with rabbit anti-VEGF antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Flt-1 antibody (1:800; Santa Cruz Biotechnology) or rabbit anti-Flk-1 antibody (1:800; Santa Cruz Biotechnology) in 0.1 M PBS containing 1 % bovine serum albumin and 0.1 % Triton X-100. The sections were incubated with horseradish peroxidase-conjugated secondary antibody (1:500; Molecular Probes,

Table 1. Oligonucleotide primer sequences and amplification conditions

Gene	Primer sequences (5' - 3')	Forward and Reverse	Annealing temperature (°C)	Amplicon size (bp)
VEGF[a]	GGAGATCCTTCGAGGAGCACTT	GGCGATTTAGCAGCAGATATAAGAA	55	129
Flt-1[a]	GAGGAGGATGAGGGTGTCTATAGGT	GTGATCAGCTCCAGGTTTGACTT	60	115
KDR/Flk-1[a]	GCCCTGCTGTGGTCTCACTAC	CAAAGCATTGCCCATTCGAT	60	115
GAPDH[b]	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	60	54

[a] Shih *et al*. (2002), and [b] RTPrimerDB (Real Time PCR Primer and Probe Database)

OR, USA) in 0.1 M PBS containing 1 % bovine serum albumin and 0.1 % Triton X-100 for 30 min at room temperature. To detect immunoreactive protein, the sections were incubated with 0.1 M PBS containing 0.05 % 3-diaminobenzidine and 0.01 % H_2O_2 for 10 min at room temperature.

RNA extraction and cDNA synthesis

The TA muscles were isolated at various time points (12 hours, 1, 3, 5, 7, 10 or 20 days) after freeze injury. The TA muscles from untreated mice were used as a control. Tissues were then transferred to glass homogenizers on ice and 1 ml TRI reagent (Molecular Reserch Center, OH, USA) was added per 50 mg tissue. RNA integrity was confirmed by denaturing agarose gel electrophoresis and the concentration was quantified by measuring the optical density (OD) at 260 nm. All samples had an optical density ratio (OD_{260}/OD_{280}) of at least 1.9. The DNase-treated total RNA (1 μg) was then converted to cDNA using a First-strand cDNA synthesis system for quantitative RT-PCR (Marligen Biosciences, MD, USA). The cDNA samples were aliquoted and stored at −80 °C.

Real-time polymerase chain reaction (PCR) analysis

Real-time PCR was performed using an OPTICON™ DNA Engine (MJ Research, MA, USA) according to the manufacturer's instructions. Amplification was carried out using SYBR Premix Ex Taq™ (TAKARA BIO, Shiga, Japan). All primers used in this study (Table 1) were obtained from ESPEC OLIGO SERVICE (Ibaraki, Japan). For each set of primers, PCR thermal cycle conditions were optimized to achieve a single ethidium bromide-stained band following electrophoresis on a 2 % agarose gel. Differences in gene expression were calculated relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using the standard curve method. GAPDH was confirmed as an appropriate normalizer by comparing the differences in raw threshold cycle values (the number of amplification cycles at which the signal is detected above the background and is in the exponential phase). A standard curve was constructed from 10-fold serially diluted cDNA from TA muscle. Each sample was normalized by its GAPDH content. The final results were expressed as relative fold changes compared to sedentary mice.

Statistical analysis

Data are expressed as means ± S.E.M. Differences in muscle weights were compared using Student's t-test. The real-time RT-PCR data were compared using one-way analysis of variance (ANOVA) with Bonferroni's post-test. A 0.05 level of probability was used as the criterion for statistical significance.

Results

Morphological changes during muscle regeneration

After injury, the muscle-to-body weight ratio transiently increased at hour 12 and day 1, decreased during days 3 to 10, and returned to nearly normal levels after 20 days (Fig. 1). Figure 2 shows the morphological changes during muscle regeneration revealed by H&E staining. Control muscle fibers were characterized by regular outlines and peripherally located nuclei, and they showed no signs of necrosis. At day 20 post-injury, muscle regeneration had progressed appreciably, although muscle fibers centrally located nuclei were still present. In three independent experiments at various time points, similar morphological changes were repeatedly observed.

Localization of VEGF, Flt-1 and KDR/Flk-1 during muscle regeneration

To determine the cellular localization of VEGF and its receptors during muscle regeneration, we

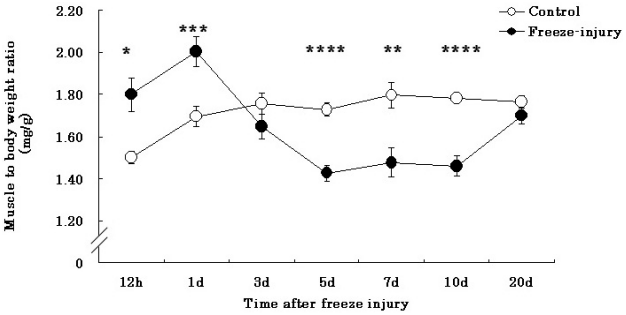

Fig. 1. Muscle-to-body weight ratio at post-injury hour 12, days 1, 3, 5, 7, 10, and 20. The ratio was calculated by dividing the muscle weight (mg) by the body weight (g). The data are expressed as means ± S.EM. (n=9-18 per time point). The TA muscle was carefully dissected, removed, and immediately weighed. Significantly different from control: ** $p<0.01$, *** $p<0.002$, **** $p<0.001$.

performed immunohistochemical analysis in normal muscles as well as at various time points after freeze injury (Fig. 2). In normal muscle, VEGF and Flt-1 were detected in vascular structures but not in muscle fibers, whereas KDR/Flk-1 was scarcely detected in muscle tissue. When muscle regeneration was initiated by freeze injury, VEGF and its receptors were readily observed in the same regenerating muscle fibers. At day 5 post-injury, VEGF and Flt-1 were detected in the cytoplasm of regenerating muscle fibers. KDR/Flk-1 was also located in the cytoplasm and on the cell membranes of the regenerating muscle fibers. Approximately 90 % of the regenerating muscle fibers simultaneously expressed VEGF, Flt-1, and KDR/Flk-1 proteins. On day 7 post-injury, similar to the observations at day 5, VEGF, Flt-1, and KDR/Flk-1 were expressed in the regenerating muscle fibers. On day 10 post-injury, staining for VEGF and KDR/Flk-1 was present on the cell membranes of regenerating muscle fibers, whereas the staining for Flt-1 returned to the basal level observed in normal muscles. At day 20 post-injury, the staining for VEGF and KDR/Flk-1 was markedly decreased in the regenerating muscle fibers.

Expression of VEGF, Flt-1 and KDR/Flk-1 mRNA in regenerating muscle

To investigate the gene expression for VEGF and its receptors during muscle regeneration, we performed real-time RT-PCR analysis in normal muscle as well as at various time points after freeze injury. VEGF, Flt-1, and KDR/Flk-1 mRNA transcripts were readily detected in normal and regenerating muscle. Figure 3 shows the changes in gene expression levels after freeze injury. The VEGF mRNA transcripts were increased 1.9-fold after 12 hours of muscle injury and then returned to the basal levels observed in control muscle on day 1. The expression of Flt-1 (4.6-fold) and KDR/Flk-1 (7.1-fold) mRNA transcripts peaked on day 3 and then returned to the basal levels observed in control muscles on day 10.

Discussion

The present study yielded two major findings. First, we found that VEGF and it receptors (Flt-1 and KDR/Flk-1) are expressed in the same regenerating muscle fibers. Second, we found that their gene expression appears to be coordinated with muscle regeneration. The present study further shows that regenerating muscle fibers potentially express VEGF and its receptors, suggesting that VEGF signaling through Flt-1 and KDR/Flk-1 may be associated with the process of muscle regeneration *in vivo*.

Immunohistochemical analyses by Germani *et al*. (2003) have established the presence of immuno-reactive VEGF, Flt-1, and KDR/Flk-1 in quiescent satellite cells. The cells expressing Flt-1 and KDR/Flk-1 at the edges of normal muscle fibers are stained with an antibody to anti-M cadherin, a cell adhesion molecule expressed in quiescent and activated satellite cells. Furthermore, they showed that VEGF protein is also detected in satellite cells. In contrast to this study, VEGF (Rissanen *et al.* 2002) and its receptors (Rissanen *et al.* 2002, Arsic *et al.* 2004) are not detectable in quiescent satellite cells. We also observed similar characteristics of VEGF, Flt-1, and KDR/Flk-1 staining in normal skeletal muscle. Although we did not attempt to localize satellite cells in this study, we did not find VEGF, Flt-1, and KDR/Flk-1 proteins in normal skeletal muscles even though we tried to stain the muscles with higher concentrations of primary antibodies (data not shown). The levels of VEGF, Flt-1, and KDR/Flk-1 in normal muscle appear to be very low (Germani *et al.* 2003). Thus this difference may be at least partially explained by variations in the immunohistochemical techniques.

Previous studies (Cherwek *et al.* 2000, Milkiewicz *et al.* 2003, 2004) have shown that the expression of VEGF and its receptors is up-regulated in response to ischemia-induced muscle injury, but its role has been thought to be mainly restricted to angiogenesis. However, some evidence suggests that VEGF and its receptors participate in muscle regeneration. After the

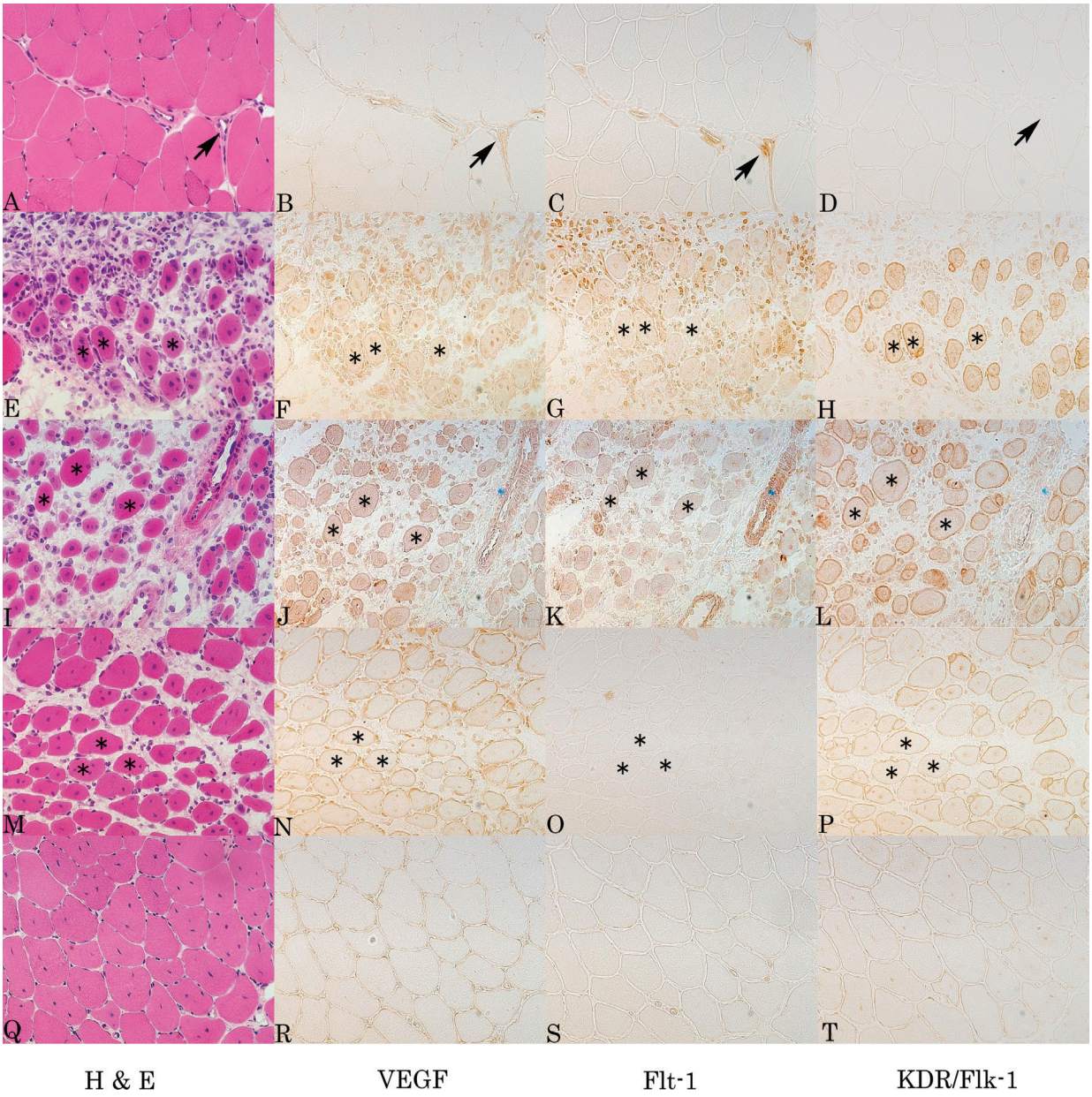

Fig. 2. VEGF, Flt-1, and KDR/Flk-1 expression in skeletal muscle tissue from normal (A-D) and injured mice on day 5 (E-H), 7 (I-L), 10 (M-P), and 20 (Q-T). Serial cross-sections were immunostained for VEGF, Flt-1, and KDR/Flk-1. H&E staining indicates staged muscle regeneration after injury. In normal muscle, VEGF (B) and Flt-1 (C) but not KDR-Flk-1 (D) were detected in vascular structures (indicated by arrows) but not in muscle fibers. VEGF and its receptors were expressed in regenerating muscle fibers on days 5 (F-H) and 7 (J-L). Asterisks indicate the same muscle fibers. On day 10, VEGF and KDR/Flk-1 but not Flt-1 were still detectable in regenerating muscle fibers (N-P). The expression of VEGF and KDR/Flk-1 was markedly decreased on day 20 when the regenerative process progressed considerably (R-T). Magnification, ×20.

induction of freeze injury, VEGF and its receptors were identified in muscle fibers with centrally located nuclei. This finding is consistent with previous studies showing VEGF (Rissanen *et al.* 2002, Germani *et al.* 2003), Flt-1 (Germani *et al.* 2003, Arsic *et al.* 2004), and KDR/Flk-1 (Rissanen *et al.* 2002, Germani *et al.* 2003, Arsic *et al.* 2004) expression in regenerating muscle fibers. Thus, we speculate that VEGF may have a direct autocrine or paracrine action *via* KDR/Flk-1 on muscle regeneration. In support of this hypothesis, Arsic *et al.* (2004) demonstrated that transfer of the VEGF gene markedly improved muscle fiber reconstitution after ischemia-, glycerol- or cardiotoxin-induced damage. Their results suggest that the effect of VEGF gene transfer is mediated by KDR/Flk-1 rather than Flt-1 because transfer of the gene for placental growth factor, a VEGF family member that interacts with Flt-1, is less effective at promoting muscle regeneration. Furthermore, they observed that the regenerative effects of VEGF gene transfer are greater when the treatment starts 5 days after injury. This is

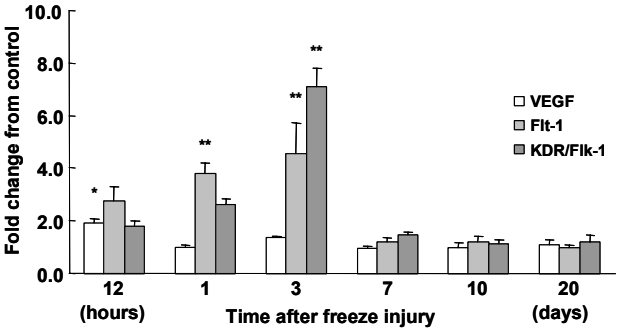

Fig. 3. Time course for VEGF, Flt-1 and KDR/Flk-1 expression in skeletal muscle tissue after freeze injury. The TA muscle was obtained from control and injured mice at the times indicated. The muscle tissues were analyzed for VEGF, Flt-1, and KDR/Flk-1 mRNA transcripts by real-time PCR. The expression of VEGF, Flt-1 and KDR/Flk-1 was normalized by GAPDH mRNA levels from corresponding samples and is presented as the fold change *vs.* control. The values represent the means ± SEM from 6 animals per time point. Significantly different from control: * $p<0.05$, ** $p<0.01$.

consistent with our demonstration of high levels of KDR/Flk-1 on the cell membrane of regenerating muscle fibers at the same time point. Collectively, these findings suggest that KDR/Flk-1 may be the main mediator of VEGF effect on myogenic cells during muscle regeneration.

In the present study, VEGF appeared to be colocalized with Flt-1 in the regenerating muscle fibers until 7 days after injury. Because Flt-1 has a very high affinity for VEGF but weak mitogenic activity in endothelial cells, it is assumed to act as a decoy receptor (Shibuya 2001). Therefore, we hypothesize that Flt-1 in myogenic cells primarily functions not as a signaling receptor but rather as a negative regulator of VEGF activity by making it less available to KDR/Flk-1. However, there is no direct evidence to support this hypothesis, so further studies are needed to elucidate the role of Flt-1 in the process of muscle regeneration.

We analyzed the gene expression using whole muscle tissue. Therefore, care should be taken not to overestimate the expression of VEGF and its receptors mRNA transcripts in the regenerating muscle because VEGF is expressed in smooth muscle cells (Ferrara *et al.* 1991) and macrophages (Xiong *et al.* 1998), whereas its receptors are expressed in endothelial cells (Petrova *et al.* 1999). It has been reported that VEGF mRNA and protein do not increase in ischemic rabbit quadriceps muscle during the first week following femoral artery ligation (Deindl *et al.* 2001). However, most studies have shown that VEGF mRNA and protein expression are up-regulated in ischemic skeletal muscle (Rissanen *et al.* 2002, Milkiewicz *et al.* 2003, Tuomisto *et al.* 2004). For instance, VEGF mRNA is up-regulated in muscle following 24-48 h of ischemia due to an embolism or a major thrombus in a native artery or prosthesis (Tuomisto *et al.* 2004). VEGF protein is also increased in ischemic skeletal muscle 11 days after femoral artery ligation (Milkiewicz *et al.* 2004). In the present study, we demonstrated by real-time PCR that freeze-induced muscle injury results in an early increase in VEGF expression characterized by a single peak that reaches a maximum 12 h after injury. The early response of VEGF mRNA may be due to a decrease in the local oxygen level, which is known to be a major regulator of VEGF mRNA expression (Shweiki *et al.* 1992). Specifically, hypoxia increases VEGF mRNA stability (Shima *et al.* 1995) and the rate of VEGF mRNA transcription (Levy *et al.* 1995).

Few studies have addressed the expression of Flt-1 and KDR/Flk-1 mRNA in the early stage of muscle regeneration. Both Flt-1 and KDR/Flk-1 increased in the process of muscle regeneration. However, the up-regulation of Flt-1 and KDR/Flk-1 mRNA transcripts was delayed relative to the increase in the VEGF mRNA transcript. The expression of Flt-1 and KDR/Flk-1 has been reported to be affected by hypoxia, although to a lesser extent than that of VEGF (Neufeld *et al.* 1999). Hypoxia regulates Flt-1 expression at the level of transcription (Gerber *et al.* 1997) and KDR/Flk-1 expression at post-transcriptional steps (Waltenberger *et al.* 1996). This hypoxia-induced change in the expression of Flt-1 and KDR/Flk-1 may be indirect because VEGF potentiates the expression of both receptor types (Wilting *et al.* 1996, Barleon *et al.* 1997). This is consistent with the early increase in the expression of VEGF mRNA in regenerating skeletal muscle. The delay in up-regulation of both receptor mRNA transcripts relative to the increase in VEGF may suggest that the expression of these genes is coordinated with the process of muscle regeneration.

These findings suggest that VEGF signaling through Flt-1 and KDR/Flk-1 may participate in the process of muscle regeneration *in vivo* although the significance of VEGF, Flt-1, and KDR/Flk-1 expression in regenerating muscle fibers remains to be determined.

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