Delayed angiogenesis and VEGF production in CCR2−/− mice during impaired skeletal muscle regeneration

Oscar Ochoa,1 Sun D, Reyes-Reyna SM, Waite LL, Michalek JE, McManus LM, Shireman PK. Delayed angiogenesis and VEGF production in CCR2−/− mice during impaired skeletal muscle regeneration. Am J Physiol Regul Integr Comp Physiol 293: R651–R661, 2007. First published May 23, 2007; doi:10.1152/ajpregu.00069.2007.—The regulation of vascular endothelial growth factor (VEGF) levels and angiogenic events during skeletal muscle regeneration remains largely unknown. This study examined angiogenesis, VEGF levels, and muscle regeneration after cardiotoxin (CT)-induced injury in mice lacking the CC chemokine receptor 2 (CCR2). Muscle regeneration was significantly decreased in CCR2−/− mice as was the early accumulation of macrophages after injury. In both mouse strains, tissue VEGF was similar at baseline (no injections) and significantly decreased at day 3 post-CT. Tissue VEGF in wild-type (WT) mice was restored within 7 days postinjury but remained significantly reduced in CCR2−/− mice until day 21. Capillary density (capillaries/mm2) within regenerating muscle was maximal in WT mice at day 7 and double that of baseline muscle. In comparison, maximal capillary density in CCR2−/− mice occurred at 21 days postinjury. Maximal capillary density developed concurrent with the restoration of tissue VEGF in both strains. A highly significant, inverse relationship existed between the size of regenerated muscle fibers and capillaries per square millimeter. Although this relationship was comparable in WT and CCR2−/− animals, there was a significant decrease in the magnitude of this response in the absence of CCR2, reflecting the observation that regenerated muscle fiber size in CCR2−/− mice was only 50% of baseline at 42 days postinjury, whereas WT mice had attained baseline fiber size by day 21. Thus CCR2-dependent events in injured skeletal muscle, including impaired macrophage recruitment, contribute to restoration of tissue VEGF levels and the dynamic processes of capillary formation and muscle regeneration.

adipogenesis; angiogenesis; CCL2; muscle regeneration; monocyte/macrophage

Tissue responses following skeletal muscle injury are highly complex and coordinated processes involving the interactions of many different cell populations that promote angiogenesis and muscle regeneration. A better understanding of the interactions of these complex events may promote therapies that will improve tissue regeneration. For instance, macrophage depletion results in impaired muscle regeneration (37, 42); these cells may be directly involved in skeletal muscle regeneration through the removal of necrotic debris (12), the production of growth factors (27, 41), or the modulation of angiogenesis (25, 28, 29, 38). Thus, diminished monocyte/macrophage recruitment following injury could have profound effects on the muscle reparative process via impairments in angiogenesis.

Monocyte chemotactic protein-1 (MCP-1, also known as CCL2) and its CC chemokine receptor 2 (CCR2) are important in monocyte/macrophage recruitment to sites of injury (7). Previously, we have documented impaired macrophage recruitment in ischemia-injured muscle in CCR2−/− mice in conjunction with delayed muscle regeneration despite normal restoration of perfusion by scanning laser Doppler imaging (8). These observations are consistent with extensive reports of impaired macrophage recruitment in CCR2−/− mice (3, 14, 19, 22, 23, 26, 30, 46). Nevertheless, the role of the MCP-1/CCR2 axis on angiogenesis in relation to skeletal muscle regeneration following myofiber injury has been poorly defined. Given the importance of angiogenesis in muscle regeneration (4, 5), we hypothesize that impairments in muscle regeneration in CCR2−/− mice result from decreased angiogenesis.

The aim of the current study was to comprehensively examine the consequences of CCR2 deficiency on the relationship between muscle regeneration, tissue vascular endothelial growth factor (VEGF) levels, and capillary density following cardiotoxin (CT) injury of mouse hindlimb muscles. CT is derived from snake venom and contains many different lytic factors. The toxins work primarily through forming pores that cause depolarization and degradation of the muscle plasma membrane [reviewed in Harris (13)]. CT injection results in muscle necrosis with a subsequent inflammatory and regenerative response similar to the injury-repair response observed in muscle after ischemic injury (8). Because impairments in collateral artery formation (arteriogenesis) have been variable after the induction of hindlimb ischemia in CCR2−/− mice (8, 14, 39), a CT injury model was used to preferentially investigate capillary formation (angiogenesis) while limiting the influence resulting from possible differences in arteriogenesis between mouse strains.

MATERIALS AND METHODS

Experimental animals. CCR2−/− mice on a C57Bl/6J background were derived as previously described (19) and backcrossed to C57Bl/6J mice from Jackson Laboratories (Bar Harbor, ME) for six generations. Original CCR2−/− breeders were a kind gift from William A. Kuziel (Protein Design Laboratories, Fremont, CA).

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CCR2−/− mice on the C57Bl/6d background were bred at the Audie Murphy Veterans Hospital, and C57Bl/6d wild-type (WT) control mice were purchased from Jackson Laboratories. Male mice 4–6 months were used in this study. All procedures complied with the National Institutes of Health Animal Use and Care Guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and at the South Texas Veterans Health Care System.

Mouse CT model. Both WT and CCR2−/− mice received intramuscular CT (Calbiochem, San Diego, CA) injections in the right hindlimb muscles below the knee to induce myonecrosis. Two 50-μl CT (2.5 μM) injections were delivered uniformly in the muscles of the anterior compartment (AC). Similarly, the posterior compartment received four 50-μl CT injections. The left leg was injected with identical volumes of normal saline (NS) and served as a control. With the use of this method, 50–80% injury was consistently induced in the tibialis anterior (TA) muscle, the largest muscle of the AC. The anterior and posterior compartments were removed en bloc, weighed, and the entire compartment was either snap-frozen in liquid nitrogen, placed in 10% neutral buffered formalin (NBF), processed for frozen sections, immediately used to prepare muscle lysates or processed for fluorescent-activated cell sorting (FACS) analysis (see below). Baseline specimens were obtained from mice without injections of NS or CT. The AC muscles consisting of the TA, extensor digitorum longus, extensor digitorum lateralis, long peroneal, and flexor hallucis longus were predominately used in the current study, except for FACS analysis, in which both the AC and posterior compartment muscles were used.

Tissue weights and lysate preparation. Mice were killed at baseline (no injections) and at various time points following CT and NS injections (days 1, 3, 7, 14, and 21, n = 4/time point), and AC muscles of the hindlimb were removed en bloc, weighed, and immediately used to prepare tissue lysates as previously described (34).

Measurement of protein, lactate dehydrogenase activity, MCP-1, and VEGF. Protein in tissue lysates was determined by the Pierce BCA protein assay (Pierce Biotechnology, Rockford, IL), using a microtiter plate format; BSA from ICN Biomedicals (Costa Mesa, CA) in lysate buffer was used as the standard, as previously described (34). Absorption in all microtiter plate assays was monitored in a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA), at 562 nm. Results were analyzed with SOFTmax PRO software. Reaction rates for lactate dehydrogenase (LDH) were determined as previously described (34). LDH activity was normalized to milligram of protein measured in each lysates and expressed as units per milligram protein.

Murine MCP-1 (BioSource, Camarillo, CA) and VEGF (R&D Systems, Minneapolis, MN) tissue levels were assessed by an ELISA by as per the manufacturer’s protocol with slight modification; the VEGF assay measured murine VEGF164 and VEGF120. Standards and unknowns were diluted in lysate buffer and adjusted for the amount of protein in the tissue lysates, and results are expressed as picogram per milligram protein, as previously described (34). The dynamic range was 78–1,000 and 7.8–50 pg/ml for MCP-1 and VEGF, respectively.

Determination of percent total body fat. Determination of percent total body fat on baseline WT and CCR2−/− mice was performed using the PIXImus Mouse Densitometer (General Electric, Waukesha, WI). Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg; Abbott Laboratories, Chicago, IL) before scanning.

Tissue macrophage quantification. Tissue macrophages at day 3 post-CT injection were measured using FACS as previously described (8). Briefly, all muscles between the knee and ankle were removed from WT and CCR2−/− mice 3 days post-CT. Cells were enzymatically dissociated by incubation in a solution containing 1% collagenase type II and 2.4 U/ml dispase (both from Invitrogen, Carlsbad, CA) in PBS supplemented with calcium chloride to a final concentration of 2.5 mM at 37°C for 90 min. The suspension was passed through a 40-μm cell strainer (BD Biosciences, San Jose, CA). The filtrate was centrifuged at 350g to harvest the dissociated cells, which were further quantitatively analyzed for macrophages by FACS. Macrophages were defined as CD11b+/Gr1− cells. For this assay only, CT was injected in the anterior and posterior compartment muscles of bilateral legs, and all four compartments were harvested and combined to create a single cell suspension for each mouse for FACS analysis (n = 5 mice/strain). The absolute number of macrophages was determined in the single cell suspension and divided by the weight of the harvested tissue.

Histology and histochemistry. Mice were killed at baseline and at 1, 3, 7, 14, 21, 28, or 42 days following CT and NS injections (right and left leg, respectively, n = 6–11 mice/time point/strain). Hindlimb tissues were collected en bloc from the AC and placed in 10% NBF for routine paraffin embedding or snap-frozen in liquid nitrogen-cooled isopentane; OCT Embedding Media (Tissue-Tek, Torrance, CA) were used to attach the muscle to cork during freezing. Routine, indirect immunohistochemical procedures were used for the localization of monocytes/macrophages (F4/80; Serotec, Raleigh, NC), as previously described (34).

Capillary density, muscle fiber cross-sectional area, and percent fat. The biotinylated lectin, Griffonia (Bandeiraea) simplicifolia lectin I (Vector Laboratories, Burlingame, CA), at 1:50 dilution was used to identify endothelial cells on paraffin-embedded cross sections. Endogenous peroxidase activity was blocked by incubation in 3% H2O2, and nonspecific binding was blocked by treatment of sections with 1.67% horse serum in PBS. After incubation with lectin, sections were sequentially incubated with HRP-streptavidin followed by diaminobenzidine tetrahydrochloride and counterstained with hematoxylin and eosin (H&E). Capillary density could only be reliably performed in tissues without extensive necrosis; areas of tissue necrosis resulted in high background staining. Capillary counts and morphometric analyses were performed on images captured using an Eclipse TE2000-U microscope equipped with a high-resolution DXM 1200F digital camera (Nikon, Melville, NY) interfaced with a personal computer equipped with Metamorph (Nikon) software.

For morphometric analyses, 2- to 3-μm cross sections were obtained through the midportion of the AC specimen and stained with H&E. Additional sections were processed for capillary density. The TA is the largest muscle in the AC and was easily identifiable in the cross sections. Within each section, four nonoverlapping areas of the TA muscle were digitally captured (×20 magnification) using phase contrast microscopy to enhance the identification of cell borders and lectin staining; areas containing large blood vessels or fibrous tissue bands between muscle bundles were excluded, and care was taken to avoid TA specimens with tangential or longitudinal presentation of myofibers. For each histomorphometric parameter, results from all images derived from a given section were averaged to obtain a single value for each animal.

In each of the digitally captured images, intermuscular adipocytes (i.e., between and among individual muscle fibers in a given muscle bundle) were manually outlined and divided by the total area of the image (0.278 μm2) to calculate the percent fat.

The average cross-sectional area (μm2) of individual muscle fibers for a given animal was determined after manually outlining individual muscle fibers in each of the digitally captured images for a given TA; fibers that were only partially present within the images were excluded from fiber size analysis. Only regenerated fibers, identified by the presence of centrally located nuclei (6), were measured in the post-CT specimen, whereas, in the baseline specimen, only fibers with peripherally located nuclei (i.e., mature, nonregenerated fibers) were measured for both WT and CCR2−/− mice.

Capillary counts were performed on TA specimens using the four fields described above. The number of capillaries and muscle fibers in each image were counted using NIS-Elements Software (Nikon). Only capillaries associated with muscle fibers were counted and expressed as capillaries per muscle fiber (C/μF). In addition, areas of fat, fibrosis, AJP-Regul Integr Comp Physiol • VOL 293 • AUGUST 2007 • www.ajpregu.org
and residual necrosis were manually outlined and subtracted from the total area, and capillary density was expressed as capillaries per square millimeter. Extensive muscle necrosis was present in TA specimen shortly following CT injection, precluding quantification of capillary density until 7 and 14 days postinjury in WT and CCR2−/− mice, respectively.

Data analysis. SAS software (SAS, Cary, NC) was used for all statistical analyses. Results from corresponding time points of each group were averaged and used to calculate descriptive statistics (mean ± SE). All statistical testing was two sided with a significance level of 5%.

Protein/weight, weight, LDH activity, fiber cross-sectional area, capillary density VEGF, and MCP-1 data were analyzed by a Dunnett’s corrected multiple-comparison procedure using a two-way ANOVA of least-square means to determine whether significant differences existed at different time points (1, 3, 7, 14, 21, 28, and 42 days after injection) compared with baseline values. Bonferroni-corrected P values were used to determine significant differences between WT and CCR2−/− mice at individual time points. For lysates samples with MCP-1 below the level of detection in the ELISA (<78 pg/ml), a value of 78 pg/ml was assigned to these samples (16), and this value was corrected for the protein in each specimen.

Tissue macrophages and percent total body fat were analyzed by an unpaired Student’s t-test. Percent intermuscular fat data was analyzed by the Wilcoxon Signed-Rank test using a Bonferroni correction. An additional analysis investigated the relationship between capillaries per square millimeter and muscle fiber cross-sectional area for each strain using a linear regression model of capillaries per square millimeter on muscle fiber cross-sectional area, strain, and an interaction effect of cross-sectional area and strain. To evaluate the effect of time, separate linear regression models for each strain were fit for capillaries per square millimeter in terms of cross-sectional area, day, and the interaction.

RESULTS

CT-induced changes in muscle weight and lysate protein content. Tissue weight of the AC muscles was measured following CT injection as an indication of injury and edema. In the NS-injected AC muscles, there were no significant differences in weight compared with baseline values in either mouse strain (data not shown). In both WT and CCR2−/− mice (Fig. 1A), AC tissue weights increased acutely after injury at day 1 compared with baseline (P ≤ 0.01) and returned to values similar to baseline by day 3. Although AC tissue weights in WT mice were below baseline at day 7 (P = 0.03), weights increased after this point, returning to baseline values by day 14 and above baseline (P = 0.03) by day 21, paralleling an ongoing increase in tissue protein production (see below). In contrast, although not statistically significant, tissue weights in CCR2−/− mice trended in a decreasing manner at days 14 and 21 compared with baseline. At 21 days post-CT injection, however, CCR2−/− mice muscle weights were significantly decreased (P = 0.04) compared with WT.

Muscle lysate protein content was normalized to the weight of the tissue and expressed as a ratio of milligrams protein per gram of tissue. In the NS-injected AC muscles, there were no significant differences in protein/weight compared with baseline values in either mouse strain at any time point (data not shown). Both strains were similar at baseline and experienced a significant (P ≤ 0.001) decrease in protein/weight at 1 day following injury. After day 1, protein/weight ratios gradually increased in WT mice, reaching values similar to baseline by day 14 post-CT. In comparison, protein/weight ratios in CCR2−/− mice remained depressed and were significantly decreased compared with both baseline (P = 0.001) and WT values (P = 0.008) at day 14 post-CT. Finally, by 21 days post-CT, CCR2−/− mice protein/weight ratios returned to values similar to baseline. These results suggested impaired protein production in regenerating muscle following injury in CCR2−/− mice.

Histologic evaluation of muscle injury, inflammation, and regeneration post-CT injection. In the control, NS-injected muscle of both mouse strains, there were rare foci of skeletal muscle regeneration, a pattern that corresponded to muscle damage incurred by the needle. Furthermore, the NS-injected specimen exhibited a similar histologic appearance as the baseline specimen. In WT mice, at day 1 after CT injection, skeletal muscle injury was widespread, and an inflammatory infiltrate consisting mainly of polymorphonuclear neutrophilic leukocytes (neutrophils) was present in conjunction with edema (Fig. 2A). Irreversibly injured myofibers were easily identified as intensely eosinophilic cells lacking nuclei. At 3 days, a robust, predominately mononuclear, inflammatory infiltrate was evident within injured muscle in WT animals (Fig. 2B).
Fig. 2. Impaired macrophage recruitment and muscle regeneration with increased intermuscular fat accumulation in CCR2−/− mice post-CT. Mouse strain and day (d) post-CT injection (in parentheses) are indicated on each image. Sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemical localization of the macrophage marker F4/80, followed by counterstaining with hematoxylin. *Necrotic muscle fiber; n, normal muscle fiber.
fibers progressively increased thereafter and returned to values similar to baseline by day 21. In contrast, the cross-sectional area of regenerated fibers of CCR2/−/− mice was significantly (P < 0.001) decreased through day 42 post-CT injection. In fact, the fiber cross-sectional area in CCR2/−/− mice was approximately one-half of baseline values 42 days after injury. Furthermore, the cross-sectional area of regenerated myofibers in CCR2/−/− mice was significantly (P < 0.001) decreased at all post-CT time points compared with WT mice.

Percent total body fat and intermuscular fat accumulation. No significant differences were observed in the PIXImus-measured percent total body fat of baseline WT or CCR2/−/− mice (15.7 ± 0.6 and 17.5 ± 0.9%, respectively, n = 10–11 mice/strain).

Intermuscular fat in regenerated muscle was estimated by histomorphometry. At baseline, no intermuscular fat was identified in either mouse strain. CCR2/−/− mice had significantly (P ≤ 0.02) increased intermuscular fat at days 14, 21, and 28 compared with WT mice (Fig. 4).
Capillary density was measured in baseline and post-CT in TA muscles of CCR2−/− mice, and C/F was significantly (P = 0.03) increased in WT mice compared with baseline at post-CT days 3 (931 ± 150 pg/mg) and 7 (410 ± 114 pg/mg) with maximal MCP-1 levels occurring at day 3. Furthermore, MCP-1 levels were significantly (P = 0.04) increased in CCR2−/− mice at post-CT day 3 compared with WT mice. Thus CCR2−/− mice were characterized by an increased and sustained level of tissue MCP-1 compared with WT mice.

VEGF levels were also measured in the AC tissue lysates. At baseline, similar levels of VEGF were observed in both mouse groups. At baseline and post-CT in TA muscles of CCR2−/− mice, capillaries per square millimeter were significantly (P = 0.006) increased above baseline at post-CT days 14, 21, and 28. Thus the dynamic process of capillary formation with return to baseline density was substantially altered in CCR2−/− mice.

Inverse relationship between muscle fiber cross-sectional area and capillary density during skeletal muscle regeneration. The relationship between capillary density and regenerating muscle fiber size in WT and CCR2−/− mice was

Fig. 5. Monocyte chemotactic protein (MCP)-1 and vascular endothelial growth factor (VEGF) tissue levels following CT-induced injury. MCP-1 (A) and VEGF (B) levels were measured in tissue lysates prepared from the anterior compartment muscles of WT and CCR2−/− mice. Baseline results were derived from samples collected without injections. Samples with values below the level of detection were defined as ND; n = 4 mice/time point/strain; *significant difference (P ≤ 0.001) compared with baseline; #significant difference (P ≤ 0.001) between CCR2−/− and WT groups at corresponding time points. Data are presented as means ± SE.

Fig. 4. Intermuscular fat in the tibialis anterior muscle following CT-induced muscle injury. Fat area (%) in regenerated tibialis anterior muscle following CT injection. Data are presented as means ± SE; n = 6–8 mice/time point/strain; *significant difference (P = 0.03) compared with baseline; #significant difference (P = 0.02) between CCR2−/− and WT groups at corresponding the time points. Baseline specimens for both WT and CCR2−/− mice did not contain any intermuscular fat and were defined as not detectable (ND).

Intercellular fibrosis development in TA muscles of CCR2−/− mice. Representative H&E stained cross-sections of TA muscles. A, WT; B, CCR2−/−. As a measure of the extent of fibrosis, the percentage of the cross-sectional area occupied by fibrosis was quantified. Data are presented as means ± SE; n = 6–8 mice/group; *significant difference (P = 0.03) compared with baseline; #significant difference (P = 0.02) between CCR2−/− and WT groups at corresponding the time points. Data are presented as means ± SE.

A different pattern emerged when capillary density was expressed per area (Fig. 6B). In WT mice, capillaries per square millimeter were significantly (P < 0.001) increased and maximal at post-CT day 7 (2,291 ± 116); this capillary density was ~220% of baseline values. Subsequently, capillary density progressively decreased but remained significantly (P ≤ 0.02) elevated through day 21 post-CT (Fig. 7A) before returning to near baseline levels by day 28. In contrast, in CCR2−/− mice, capillaries per square millimeter progressively increased to a maximum at day 21 (1,821 ± 78, Fig. 7C); this capillary density was ~190% of baseline values. In CCR2−/− mice, capillaries per square millimeter were significantly (P ≤ 0.006) increased above baseline at post-CT days 14, 21, and 28. Thus the dynamic process of capillary formation with return to baseline density was substantially altered in CCR2−/− mice.

Tissue levels of MCP-1 and VEGF post-CT injection. Levels of the CCR2 ligand, MCP-1, were measured in the post-CT tissue lysates derived from the AC muscles. At baseline, MCP-1 was not detectable in the AC muscles of either WT or CCR2−/− mice but increased in both strains following CT injections (Fig. 5A). In WT mice, MCP-1 levels were significantly (P ≤ 0.01) increased over baseline at post-CT days 1 (255 ± 32 pg/mg) and 3 (116 ± 27 pg/mg) with maximal MCP-1 levels occurring at day 1. In CCR2−/− mice, MCP-1 levels were significantly (P ≤ 0.03) increased over baseline at post-CT days 3 (931 ± 150 pg/mg) and 7 (410 ± 114 pg/mg) with maximal MCP-1 levels occurring at day 3. Furthermore, MCP-1 levels were significantly (P = 0.04) increased in CCR2−/− mice at post-CT day 3 compared with WT mice. Thus CCR2−/− mice were characterized by an increased and sustained level of tissue MCP-1 compared with WT mice.

VEGF levels were also measured in the AC tissue lysates. At baseline, similar levels of VEGF were observed in both mouse strains (80 ± 2 vs. 77 ± 4 pg/mg; Fig. 5B). Subsequently, tissue VEGF in WT mice decreased below baseline (P < 0.001) only at day 3 post-CT before returning to baseline at day 7. In contrast, tissue VEGF was significantly (P ≤ 0.002) decreased in CCR2−/− mice at post-CT days 1, 3, 7, and 14 before returning to baseline at day 21. Thus CCR2−/− mice had a more rapid and sustained decrease in VEGF tissue levels, and the levels were significantly (P = 0.002) decreased in CCR2−/− mice at 7 days post-CT compared with WT mice.

Capillary density post-CT injury. Capillary density was measured in baseline and post-CT in TA muscles of CCR2−/− and WT mice and expressed as either C/F or capillaries per square millimeter. In CT-injured muscles, only areas of regeneration (identified by centrally located nuclei in the muscle fibers) were used. Widespread muscle necrosis was evident in both strains acutely following CT injection such that capillary density could not be reliably quantified at the early time points of 1 and 3 days post-CT. Not until post-CT day 7 in WT and day 14 in CCR2−/− mice was quantification of capillary density possible.

At baseline, there were no significant differences in C/F (2.12 ± 0.05 vs. 1.83 ± 0.12; Fig. 6A) or capillaries per square millimeter (974 ± 34 vs. 1,052 ± 69; Fig. 6B) in the TA of CCR2−/− and WT mice, respectively. However, in the post-CT TA, C/F was significantly (P = 0.003) increased in WT mice at day 21 compared with baseline (Fig. 6A). In contrast, C/F was significantly (P ≤ 0.004) decreased in CCR2−/− mice compared with both baseline and WT mice (Fig. 6A) at days 14, 21, 28, and 42.

Fig. 6. Inverse relationship between muscle fiber cross-sectional area and capillary density during skeletal muscle regeneration. A, inverse relationship between muscle fiber cross-sectional area (A) and capillary density (B) measured in TA muscles of CCR2−/− mice. Data are presented as means ± SE; n = 6–8 mice/group; *significant difference (P ≤ 0.001) compared with baseline; #significant difference (P ≤ 0.001) between CCR2−/− and WT groups at corresponding the time points. Data are presented as means ± SE.

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examine the effects of MCP-1 and CCR2 on tissue levels of VEGF in relation to the processes of angiogenesis and skeletal muscle regeneration. Although tissue VEGF was similarly decreased in both WT and CCR2-/- mice after injury, VEGF returned to baseline at day 7 in WT mice but remained decreased until day 21 in CCR2-/- mice. Interestingly, maximal capillary density (capillaries/mm²) postinjury developed concomitant with the restoration of tissue VEGF to baseline levels in both mouse strains. Furthermore, muscle regeneration was severely impaired in CCR2-/- mice, attaining only 50% of baseline fiber size at 42 days postinjury, whereas WT mice were similar to baseline by 21 days. Taken altogether, these findings indicate that CCR2-dependent events not only promote angiogenesis and subsequent muscle regeneration but also modulate tissue VEGF levels in the inflammatory microenvironment to alter the balance of capillary formation and regression following muscle injury.

Initially identified as important for monocyte/macrophage recruitment (7, 19), the MCP-1/CCR2 axis may be involved in angiogenesis through direct signaling activities in vascular cells. First, MCP-1 and CCR2 are expressed on endothelial cells, and MCP-1 production is increased after injury in vitro (44). Second, MCP-1 directly induces vascular smooth muscle cell proliferation (31) and migration of both vascular smooth muscle (36) and endothelial cells (44). Moreover, a recent study suggested that VEGF alone is insufficient to elicit an angiogenic response in mild ischemic conditions in the absence of tissue necrosis and a subsequent inflammatory cell response (24). Finally, the contributing effects of the MCP-1/CCR2 axis on angiogenesis may not be limited to local endothelial cell effects but may also involve recruitment of bone marrow-derived endothelial progenitor cells (35). Given the above, it is conceivable that, in addition to macrophage recruitment, there may be other essential CCR2-dependent cellular events that significantly impact angiogenesis in the current study.

Further enhancement of angiogenesis may occur via potential synergistic effects of MCP-1 and VEGF. MCP-1 activation of CCR2 on endothelial cells induces hypoxia-inducible factor-1α, which increases VEGF production (15). In turn, VEGF induces MCP-1 production via the activated protein-1 binding site of the MCP-1 promoter region (45). Thus MCP-1 and VEGF may synergistically enhance angiogenesis through multiple, complimentary interactions involving multiple cell types. Importantly, despite large increases in tissue MCP-1 in CCR2-/- mice in the present study, VEGF remained depressed for an extended interval following injury. Thus, in CCR2-/- animals, disruption of MCP-1 signaling via CCR2 may have contributed to delays in VEGF restoration.

Although VEGF is important in angiogenesis, it also influences muscle regeneration. VEGF receptor-1 and -2 are present on satellite cells and regenerating muscle fibers (2, 11). VEGF also enhances myoblast migration in vitro and prevents apoptosis of myoblasts in cell culture and in skeletal muscle following ischemia (11). In addition, overexpression of VEGF-A improved muscle fiber reconstitution in a dose-dependent fashion following ischemic and toxic injury (2). Thus VEGF may contribute to the reparative process by enhancing both angiogenesis and muscle regeneration. Indeed, in the present investigation, major increases in muscle fiber size.

Fig. 6. Muscle capillary density in baseline and regenerated tibialis anterior muscle. Capillaries/muscle fiber (C/F; A) and capillaries/mm² (B) are shown. Capillary density could not be quantified at day 7 post-CT in CCR2-/- mice because of extensive residual necrosis; n = 6–8 mice/time point/strain; *significant difference (P ≤ 0.02) compared with baseline; #significant difference (P ≤ 0.05) between WT and CCR2-/- groups at the corresponding time points. Data are presented as means ± SE.
occurred in both mouse strains only after restoration of tissue VEGF to baseline.

In this study, VEGF was constitutively present in skeletal muscle before injury. Following CT injection with extensive muscle necrosis and inflammation, tissue VEGF decreased acutely in both mouse strains followed by a subsequent differential restoration to baseline values. In contrast to the current observations, previous studies have documented an increase in muscle VEGF protein (9, 21) and mRNA (24) following ischemic injury. These seemingly conflicting findings may be explained by the severity of injury in each model and was elegantly demonstrated by Tang et al. (40) where the effects of chronic vs. acute hindlimb ischemia were compared. With chronic ischemia, in the absence of necrosis and inflammation, tissue VEGF increased above baseline. However, severe, acute ischemia with prominent necrosis and inflammation resulted in decreased tissue VEGF compared with baseline. Thus VEGF levels in tissue are dependent not only on hypoxia but also on the amount of necrosis present within the tissue and time after injury. Furthermore, tissue necrosis in hindlimb ischemia models can be variable and is dependent on the extent of arterial disruption and location of the sampled muscle (reviewed in Ref. 32). Decreased tissue VEGF after CT injection in the current study occurred in conjunction with significant necrosis in both strains of mice and exhibited a pattern similar to acute ischemia, described above (40). With the loss of cellular elements that contribute to tissue VEGF, such as endothelial cells, VEGF levels decreased. In conjunction with muscle regeneration and restoration of capillary networks, VEGF levels returned to baseline. Macrophages were probably not a major source of VEGF, since the nadir of tissue VEGF in WT mice occurred at day 3, a time when a robust macrophage infiltrate was present. Rather, the current results suggest that other CCR2-dependent events modulate the restoration of tissue VEGF levels.

To determine the effects of CCR2 deficiency on angiogenesis, capillary density was analyzed. Parameters commonly used to objectively define capillary density often include capillaries per square millimeter and C/F; however, C/F may be more representative of muscle fiber size when significant differences in muscle fiber size are present. Thus capillary density expressed as capillaries per square millimeter may be a better measurement of angiogenesis during skeletal muscle regeneration given the progressive increases in muscle fiber size to baseline over time.

Capillary density (capillaries/mm²) was maximal and double that of baseline muscle within 7 days after injury in WT mice; this time point corresponded to restoration of tissue VEGF to baseline and regeneration of muscle fibers. Interestingly, the subsequent reduction of capillary density to baseline paralleled an increase in regenerated muscle fiber size in WT mice. It is conceivable that this vascular remodeling results from the production of angiostatic factors within the regenerating tissue (20). Similar patterns of capillary formation and regression observed in WT mice were also exhibited in the CCR2−/− animals, albeit in a delayed fashion. As tissue VEGF levels were restored to baseline at 21 days in CCR2−/− mice, capillary density was maximal in CCR2−/− animals. Previously, we reported similar capillary density in WT and CCR2−/− mice after ischemic injury (8). However, a single, 21-day time point was examined, and it is conceivable that significant differences in capillary density between WT and CCR2−/− mice existed before and after the 21-day time point. The current study represents a more comprehensive approach to estimation of capillary density than our previous report after ischemic injury (8). These combined results support the hypothesis that a temporally sequenced integration of angiogenic and angiostatic events is crucial in the normal progression of skeletal muscle fiber regeneration following injury.

The present study also demonstrated an interesting inverse relationship between regenerated muscle fiber size and capillary density (capillaries/mm²) in both mouse strains (Fig. 8B) and suggests that establishment of a capillary network is essential for regenerating muscle fiber growth. Growing, re-
generating muscle fibers probably have an increased need for nutrient delivery, which can be accomplished by increased capillary density. As the fiber attains baseline size, the metabolic demands may decrease, leading to the expression of angiostatic factors (20) and the regression of capillaries. Conceivably, the establishment of capillary networks may be significantly affected by macrophage infiltration, since restoration of VEGF and maximal capillaries per square millimeter in WT mice occurred only after robust macrophage accumulation at day 5, whereas, in CCR2−/− mice, macrophage accumulation was severely reduced, VEGF restoration delayed, maximal capillary density postponed, and muscle regeneration impaired. Despite the altered events in CCR2−/− mice, it is interesting to note that the inverse relationship between fiber size and capillary density in regenerating muscle was maintained in both mouse strains.

This study also documented significant increases in intermuscular adipocyte accumulation in the regenerating muscle of CCR2-deficient mice following CT injury; similar results have also been observed in ischemic (8) and freeze-injury (43) models. Although the cellular origin of intermuscular adipocytes is unknown, possibilities include transdifferentiation of myogenic progenitor cells (18) or bone marrow-derived progenitor cells (17) due to impaired CCR2-mediated signaling as well as migration of adipocytes from surrounding areas (1). Irrespective of the cellular etiology of intermuscular adipose tissue, it is important to note that this histologic finding was observed in conjunction with alterations in both angiogenesis and muscle regeneration. Additional studies are required to determine the mechanistic and/or interactive basis for these abnormal processes.

Interestingly, the histologic appearance of injured muscle was similar after ischemic (8) and CT-induced injury. Furthermore, recovery from freeze-induced injury (43) was also similar in CCR2−/− mice, i.e., impaired muscle regeneration with increased intermuscular fat accumulation. Thus, regardless of the method used for muscle injury, the inflammatory and regenerative responses were remarkably similar in all three injury models. Moreover, although CT injury may not directly mimic human disease, the subsequent inflammatory and repair process induced after CT injury is likely similar to that which occurs in humans following necrotic muscle injury.

With the combined observations from our current and previous studies (8, 33, 34), a working model has been developed to describe the temporal sequence of cellular and mediator events of inflammation, angiogenesis, and skeletal muscle regeneration (Fig. 9). In brief, following acute injury, widespread muscle necrosis and injury recruits inflammatory cells, initially neutrophils, to the injured tissue (day 1). At the same time, endothelial cell production of MCP-1 promotes recruitment and activation of macrophages. As neutrophils are...
placed by macrophages, phagocytosis of the extracellular matrix and angiogenesis are initiated and followed by myogenic progenitor cell proliferation. During the removal of necrotic tissue, tissue LDH and VEGF levels decrease (day 3). VEGF restoration subsequently occurs to facilitate an increase in endothelial cells (i.e., maximal capillary density at day 7), macrophage infiltration regressions, and muscle progenitor cells continue to proliferate and differentiate into myoblasts. Established capillary networks allow for effective muscle regeneration with restoration of tissue LDH activity as myoblasts continue to undergo differentiation and fusion (day 14). As muscle fiber size increases, the metabolic demand of the growing muscle fiber decreases, and capillary density regresses as regenerated fibers attain a normal size. Thus muscle fiber size and capillary density demonstrate an inverse relationship and may ultimately influence each other to maintain homeostasis at baseline levels (day 28).

In summary, the results of the present study document that CCR2-dependent impairments in macrophage accumulation and reductions in tissue VEGF were juxtaposed and followed by delayed angiogenesis and impaired skeletal muscle regeneration. Most notably, capillaries per square millimeter in both mouse strains were maximal when tissue VEGF was restored to baseline, and capillaries per square millimeter returned to baseline in WT mice concordantly with the growth of muscle fibers to a normal cross-sectional area. In the absence of CCR2, alterations in this sequence of events leads to the conclusion that CCR2-dependent events in regenerating skeletal muscle contribute to the restoration of tissue VEGF levels and to the subsequent dynamic process of capillary formation and muscle regeneration. Further studies are required to more precisely define the cellular mechanisms that regulate angiogenesis during skeletal muscle regeneration.

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