Macrophages, not neutrophils, infiltrate skeletal muscle in mice deficient in P/E selectins after mechanical reloading

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Macrophages, not neutrophils, infiltrate skeletal muscle in mice deficient in P/E selectins after mechanical reloading. Am J Physiol Regul Integr Comp Physiol 285: R727–R732, 2003. First published June 26, 2003; 10.1152/ajpregu.00175.2003.—Our objective was to test the hypothesis that endothelial selectins, P and E selectins, are necessary for leukocyte migration after muscle injury from unloading/reloading. Mice hindlimbs were suspended for 10 days followed by reloading periods of 6 or 24 h after which the soleus muscle was dissected. Light microscopic observations showed that macrophages, but not neutrophils, were able to invade soleus muscles in mice deficient in P/E selectins (P/E−/−) during reloading periods. The recruitment efficiency of neutrophils after 6 and 24 h of reloading was minimal in P/E−/− mice relative to unloaded animals. The recruitment of macrophages in the soleus muscle was preserved in P/E−/− mice. The concentration of macrophages increased by 8.1-fold compared with unloaded muscles in double-mutant mice after 24 h of reloading. The accumulation of macrophages in reloaded muscles did not lead to fiber necrosis. Together, these findings indicate that macrophages can invade skeletal muscle through cellular mechanisms that do not involve P/E selectins during skeletal muscle reloading.

MODIFICATIONS IN SKELETAL muscle use and muscle injury caused by direct trauma, thermal or mechanical stresses, or muscle reperfusion after periods of ischemia can induce an increase in the concentration of inflammatory cells in muscle (10, 24, 38). Several investigators have speculated that these inflammatory cells can increase muscle fiber injury after modified muscle use (7, 39), which has been supported by experimental evidence showing that neutrophil depletion before muscle reperfusion results in less muscle damage (6, 18). By contrast, macrophages are expected to play a beneficial role in muscle repair. Macrophages can induce apoptosis of neutrophils (30), phagocyte tissue debris (28), and release factors that can regulate cell proliferation and myogenesis (28, 35). Thus the diametrically opposed roles of these leukocyte subsets give credence to the notion that inflammatory cells are a double-edged sword in that they are necessary for efficient tissue healing but responsible for at least part of the secondary damage.

The recruitment of leukocytes from the vasculature into inflammatory sites is a multistep process initiated by rolling of leukocytes along the endothelium, followed by firm adhesion and diapedesis (4). Members of the selectin family, including L/P and E selectins, are thought to be largely responsible for the early rolling of leukocytes in postcapillary venules during the acute phase of inflammation (22). The availability of mice genetically deficient for either L/P or E selectin, as well as mice deficient in two or all selectins, has opened new avenues for dissecting the specific roles of selectins in the recruitment of leukocytes into inflammatory sites. For example, mice deficient in P selectin presented a delay of 2–4 h in the recruitment of leukocytes into inflamed peritoneum, but ultimately the number of inflammatory cells reached near normal levels (29). Mice lacking E selectin displayed a mild phenotype comprising increased leukocyte rolling velocities and reduced leukocyte stable arrest on cytokine-activated endothelium (27, 31). However, the phenotype of animals deficient in both endothelial selectins was much greater than either single knockouts. Indeed, P/E−/− mice exhibited extreme leukocytosis, defects in leukocyte rolling on activated endothelium, and severe reduction in neutrophil accumulation in peritoneum after injection with proinflammatory agents (3, 13). Similar alterations were observed in mice lacking all three selectins (20, 36). Together, these results clearly demonstrate that both endothelial selectins cooperate to recruit neutrophils into inflammatory sites.

Because little is known about the mechanisms through which inflammatory cells invade skeletal muscle and that blocking inflammatory cell invasion may significantly improve the outcome of several inflammatory pathologies, we tested the hypothesis that P/E selectins are necessary for inflammatory cell recruitment in skeletal muscle by modifying mechanical load-
ing to produce muscle dysfunction (12, 40). In this model, weight bearing is removed from the mouse hindlimb muscles followed by a period of normal, weight-bearing ambulation, during which inflammatory cells invade shortened and weakened soleus muscles. This muscle unloading/reloading procedure was applied to wild-type and P/E−/− mice, after which the concentration of invading populations of neutrophils and macrophages and the percentage of injured fibers were assessed.

MATERIALS AND METHODS

Experimental protocol. P/E−/− mice were generated by gene targeting (13) and were backcrossed seven generations into the C57BL/6 background, and wild-type C57BL/6 (purchased from Charles River, Quebec, Canada) were used for mechanical unloading and reloading studies. All female mice were aged between 8 and 12 wk and housed at Laval University. All female mice were subjected to hindlimb unloading for 10 days using an apparatus similar to that described by Morey-Holton and Globus (32). Mice were removed from the suspension apparatus at the end of the unloading period and either immediately killed or allowed to reload their hindlimbs during normal cage activity for periods of 6 or 24 h. Mice were anesthetized with pentobarbital sodium (10 mg/kg), and soleus muscles were excised with the intact tendons, frozen and sectioned as described by St.-Pierre and Tidball (39). All studies and procedures were approved by the Animal Research Committee at Laval University.

Immunohistochemistry. Sections were processed for immunohistochemistry with the following antibodies: 1) F4/80 (rat anti-mouse IgG; diluted 1:100; Bioproducts for Science), which recognizes a plasma membrane component on mature macrophages, and 2) Ly-6G (rat anti-mouse IgG; diluted 1:300; Pharmingen), which binds specifically to peripheral neutrophils. The sections labeled for macrophages or neutrophils were then washed in PBS and incubated with biotinylated anti-rat IgG (diluted 1:200; Vector Laboratories) for 1 h. After rinsing in PBS for 30 min, the sections were incubated with horseradish peroxidase-avidin (1:1,000; Vector Laboratories) and the endothelial cell marker platelet endothelial cell adhesion molecule-1 (PECAM-1; rat anti-mouse CD31; diluted 1:100; Pharmingen) to confirm macrophage invasion. Macrophages were labeled with F4/80 as described above, followed by incubation with an alkaline phosphatase-avidin (1:1,000; Vector Laboratories) instead of horseradish peroxidase-conjugated avidin. Sections were then incubated at 37°C and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium for 30 min. The primary antibody was omitted from control sections. The concentration of inflammatory cells labeled with each antibody was measured in duplicate in two mid-belly sections separated by 1 mm from left and right solei. Therefore, eight sections per mouse were examined by light microscopy using Nomarski optics. The number of labeled cells in each section was counted, and the total area of the section was determined manually by moving methodically the quadrant in each area to cover the whole section. Intravascular leukocytes, which represented <2% of counted leukocytes, were excluded during neutrophil and macrophage counting, and the data were normalized relative to unloaded mouse groups.

Blood collection. Blood was collected by cardiac puncture and placed in polypropylene tubes containing EDTA. These samples were analyzed in the Department of Hematology at Centre Hospitalier de l’Université Laval. Complete blood counts were determined using an automic cell counter (Beckman Coulter) and differential counts on Wright-stained smears.

Statistical analysis. All values are reported as means ± SE. The effect of hindlimb suspension on the number of neutrophils and macrophages as well as the peripheral blood counts was assessed by one-way ANOVA to test whether the variation among experimental groups was significant at \( P < 0.05 \). When significant \( F \) ratio was obtained, post hoc multiple comparison testing was done with a Fisher’s protected least-significant differences test to determine where specific differences had occurred.

RESULTS

To evaluate the role of endothelial selectins in the inflammatory response after muscle reloading, we subjected mice to a 10-day period of hypogravity followed by a 6- or 24-h reloading challenge. The soleus muscle was dissected, and mid-belly sections were cut and immunolabeled for neutrophil (Gr-1)- or macrophage (F4/80)-specific antigens. Reloading under these conditions leads to the recruitment of inflammatory cells in the challenged skeletal muscles, which can readily be determined by immunohistochemistry (Fig. 1).

The number of neutrophils in the soleus muscle of wild-type mice was increased by 2.7- and 2.4-fold after 6 and 24 h of reloading, respectively, compared with unloaded animals. In contrast, the number of neutrophils migrated in the soleus muscle of double-mutant mice after 6 and 24 h of reloading was similar to those of unloaded animals (Fig. 2). These results suggest that the recruitment of neutrophils in skeletal muscle challenged with reloading is dependent on endothelial selectins.

To evaluate the recruitment of macrophages, sections were stained with the F4/80 antibody. We found that the concentration of macrophages in the soleus muscle of wild-type and double-mutant mice decreased during the unloading period and increased by 4.8- and 8.1-fold, respectively, after 24 h of reloading (Fig. 3). The increases of macrophage concentrations in soleus muscles at 24 h of reloading are consistent with the observation that the concentration of monocytes in circulation is more important in P/E−/− mice than wild type. In contrast to the results obtained with neutrophils, the recruitment efficiency of macrophages was not reduced in P/E−/− mice. The concentration of macrophages was in fact significantly higher in P/E−/− mice compared with wild-type after 24 h of reloading, indicating that the mechanisms mediating macrophage recruitment were preserved and efficient in soleus muscles lacking both endothelial selectins.

To ascertain that changes in the concentrations of neutrophils and macrophages in soleus muscle were not caused by alterations in the number of circulating leukocytes, blood cell counts were determined at different periods of unloading and reloading in wild-type and double-mutant mice. These results corroborate previ-
ous observations showing that double-mutant mice exhibit severe leukocytosis (13). Importantly, the period of reloading had no impact on leukocyte counts in either wild-type or P/E

To evaluate the severity of muscle necrosis in wild-type and double-mutant mice, we counted the number of macrophages that invaded muscle fibers under different experimental conditions. We found that the proportion of muscle fibers that were invaded by F4/80 macrophages did not exceed 0.36% of the total number of fibers (Fig. 4). Modifications in mechanical loading did not significantly change the number of necrotic fibers in either strains.

**DISCUSSION**

The sequence and time course of inflammatory cell invasion are believed to be similar and well preserved in all damaged tissues. However, recent results have shown that leukocyte recruitment in skeletal muscle varies depending on type of insult. For example, eccentric contractions (lengthening contractions) induce damage and loss in muscle force (9), but immunohistochemical and myeloperoxidase data indicate that monocyte recruitment occurs without extravasation of neutrophils (25). In contrast, ischemia and reperfusion

**Fig. 1.** Micrograph of cross sections of soleus muscles from mice deficient in P/E selectins. A: negative controls (no primary antibody) show no staining. B: double-immunolabeling of macrophages (black) and endothelial cells. Soleus muscles were sectioned and labeled with antibodies specific for macrophages and the endothelial cell marker platelet endothelial cell adhesion molecule-1. Macrophages accumulated in the interstitial space between myofibers (arrows). Capillaries were observed around myofibers (arrowheads). Bars, 50 μm.

**Fig. 2.** Percent change for neutrophil accumulation in wild-type (WT) and double-mutant [knockout (KO)] mice from ambulatory controls (AMB) and animals reloaded (R) for 6 or 24 h. Results were expressed relative to unloaded animals (UNL); n = 6 for all WT groups, and n = 7 for all KO groups except for 24-h reloaded (n = 5). Values are means ± SE. *Significantly different from unloaded control, P < 0.05. #Significantly different from its WT matched group, P < 0.05.

**Fig. 3.** Percent change for macrophage accumulation in WT and KO mice from ambulatory controls and animals reloaded for 6 or 24 h. Results were expressed relative to unloaded animals; n = 6 for all WT groups, and n = 7 for all KO groups except for 24-h reloaded (n = 5). Values are means ± SE. *Significantly different from unloaded control, P < 0.05. #Significantly different from its WT matched group, P < 0.05.
by injured skeletal muscle that can chemotactically attract neutrophils and macrophages (5). Macrophage inflammatory protein-1 represents another potential candidate because it is a powerful chemoattractant molecule for inflammatory cells (26), and its concentration increases in circulation after strenuous exercise (34). However, we cannot exclude the possibility that nonmuscle cells such as endothelial cells, adipocytes, or endogenous macrophages may also be a source of leukocyte chemoattractants during muscle reloading.

In the present study, we tested the hypothesis that P/E selectins are necessary for leukocyte invasion in a model of muscle dysfunction caused by hindlimb suspension followed by reloading periods. Here we show that P/E selectins are not required for macrophage invasion in skeletal muscle, indicating that other adhesion molecules participate in the recruitment of this leukocyte subset. The most plausible candidate for bypassing P/E selectin function is the \( \alpha_4 \)-integrin/vascular cell adhesion molecule-1 pathway, because \( \alpha_4 \)-integrin is highly expressed on eosinophils and monocytes (7, 16) and can mediate the rolling and adhesion of these leukocytes under in vitro and in vivo conditions (1, 19). Interestingly, intravital microscopy observations in a model of IL-4-induced inflammation of the cremaster muscle revealed that \( \alpha_4 \)-integrin can initiate leukocyte-endothelial cell interactions in the absence of selectins in vivo and that the recruitment of eosinophils and mononuclear cells is preserved (17). This is also consistent with the recent observation by Jung and Ley (21) indicating that mice lacking all three selectins presented dramatic reductions in leukocyte rolling and neutrophil recruitment, but monocyte recruitment on the vessel wall was almost unaffected. Together, these findings provide strong evidence that neutrophil and monocyte recruitment in muscle and nonmuscle tissues appears to operate through distinct mechanisms.

Experimental studies on cardiac and skeletal muscles indicate that neutrophil infiltration plays a significant role in some tissue injury (15, 24, 42, 43). For example, an injurious role for inflammatory cells in skeletal muscle has been demonstrated after ischemia and reperfusion where the extent of muscle injury during reperfusion is diminished in animals depleted of circulating neutrophils (6, 18). In addition, the administration of free radical scavengers prevents muscle damage, suggesting that free radicals generated by inflammatory cells may cause injury during reperfusion (37). The present findings indicate that inflammatory cell invasion does not exacerbate muscle damage where only 0.36% of the fibers were necrotic after 24 h of reloading. Previous results have shown that the increase in inflammatory cell concentration was not associated with any detectable reduction in muscle force and that the inability to activate the contractile machinery was the primary mechanism for the loss in force production during the reloading periods (12). The incubation of soleus muscles in physiological solution containing caffeine, which acts directly on \( \text{Ca}^{2+} \) release channels of the sarcoplasmic reticulum, revealed that at least 40% of the reduction in maximal force production originated from a failure in the excitation-contraction coupling process at a step preceding the opening of the sarcoplasmic reticulum \( \text{Ca}^{2+} \) release channel (12).

Our results also suggest that endogenous macrophage survival is influenced by mechanical stress. The absence of mechanical loading leads to significant reductions of resident macrophage concentration in both wild-type and P/E selectin-deficient mice. A role for...
mechanical tension in regulating cell survival has been demonstrated in other cell types, such as endothelial cells (8) and fibroblasts (14). For example, fibroblasts in mechanically loaded collagen matrices showed little or no apoptosis, but the release of mechanical conditions led to apoptosis (14). Whether macrophages also undergo apoptosis after an unloading period remains to be defined, but recent observations showed that macrophages can respond to mechanical deformation with selective augmentation of matrix metalloproteinases and induction of immediate early genes (41). Because a phenotypic continuum exists between fibroblasts and macrophages (2), the possibility that muscular unloading induces macrophage apoptosis should be the subject of future studies.

In summary, our results clearly show that P/E selectins were essential for neutrophils but not required for monocytes to infiltrate reloaded soleus muscle. These results raise interesting questions regarding the design of therapies for inflammatory myopathies in which neutrophils can cause secondary damage. Anti-endothelial selectin therapy may therefore serve a dual purpose as it blocks neutrophil invasion but permits the recruitment of monocytes/macrocyphages, which are likely positive regulators of cell proliferation and myogenesis.

DISCLOSURES

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REFERENCES


