Cytokines derived from cultured skeletal muscle cells after mechanical strain promote neutrophil chemotaxis in vitro

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Peterson JM, Pizza FX. Cytokines derived from cultured skeletal muscle cells after mechanical strain promote neutrophil chemotaxis in vitro. J Appl Physiol 106: 130–137, 2009. First published October 30, 2008; doi:10.1152/japplphysiol.90584.2008.—We tested the hypothesis that cytokines derived from differentiated skeletal muscle cells in culture induce neutrophil chemotaxis after mechanical strain. Flexible-bottom plates with cultured human muscle cells attached were exposed to mechanical strain regimens (ST) of 0, 10, 30, 50, or 70 kPa of negative pressure. Conditioned media were tested for the ability to induce chemotaxis of human blood neutrophils in vitro and for a marker of muscle cell injury (lactate dehydrogenase). Conditioned media promoted neutrophil chemotaxis in a manner that was related both to the degree of strain and to the magnitude of muscle cell injury (ST 70 > ST 50 > ST 30). Protein profiling using a multiplex cytokine assay revealed that mechanical strain increased the presence of IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor, macrophage inflammatory protein (MCP)-1, and IL-6 in conditioned media. We also detected 14 other cytokines in conditioned media from control cultures that did not respond to mechanical strain. Neutralization of IL-8 and GM-CSF completely inhibited the chemotactic response for ST 30 and ST 50 and reduced the chemotactic response for ST 70 by 40% and 47%, respectively. Neutralization of MCP-1 or IL-6 did not reduce chemotaxis after ST 70. This study enhances our understanding of the immunobiology of skeletal muscle by revealing that skeletal muscle cell-derived IL-8 and GM-CSF promote neutrophil chemotaxis after injurious mechanical strain.

IN SKELETAL MUSCLE, the events following eccentric contractions (6, 15, 19, 28, 38), mechanical loading of atrophic muscle (10, 36), and trauma (26, 27) all share a common theme of injury accompanied by neutrophil accumulation. The identity and cellular source of molecules recruiting neutrophils to injured skeletal muscle, however, are unknown. Because neutrophils have been demonstrated to injure skeletal muscle in vitro (20, 29) and in vivo (6, 30), identification of the molecule(s) that promotes their migration into and within skeletal muscle may reveal potential avenues for alleviating neutrophil-mediated muscle injury.

Multiple cell types residing in skeletal muscle, such as skeletal muscle cells, endothelial cells, fibroblasts, and resident macrophages, are capable of producing molecules that attract neutrophils (13). Our laboratory has developed a cell culture model to focus on the interplay between differentiated human skeletal muscle cells after mechanical strain and neutrophil responses (39). With this model, we demonstrated that mechanical strain of differentiated human skeletal muscle cells results in the release of one or more soluble molecules that elicit neutrophil chemotaxis (39). These data allow for further exploration into the identity of muscle-derived chemoattractants after mechanical strain.

Neutrophil migration to and within injured skeletal muscle is thought to occur via chemotaxis, which is dependent on a soluble concentration gradient of one or more chemoattractants. Skeletal muscle cells are capable of producing and releasing a variety of molecules, including cytokines and chemokines (CXC and CC) (3, 8, 23, 31). Some members of these families are known to promote neutrophil chemotaxis (12, 25), whereas the majority of cytokines/chemokines produced by skeletal muscle cells have yet to be tested for their potential to induce neutrophil chemotaxis. Granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, IL-6, IL-8, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and TNF-α have the potential to directly or indirectly influence neutrophil chemotaxis after mechanical loading of skeletal muscle because they are known to influence various aspects of the inflammatory response (7, 41). Little is known, however, about how mechanical loading of skeletal muscle cells influences the production and/or release of these and other cytokines.

The overall goal of this project was to identify skeletal muscle cell-derived neutrophil chemoattractants after mechanical strain. To achieve this goal, several foundational experiments were conducted. First, we tested whether neutrophil chemotaxis induced by factors derived from cultured differentiated skeletal muscle cells after mechanical strain (conditioned medium) was responsive to the degree of muscle cell injury. Second, we boiled conditioned media to provide information on whether the induced chemotaxis was attributable to soluble proteins or lipids. Results from these experiments revealed that neutrophil chemotaxis was responsive to the degree of strain-induced injury and that one or more chemoattractants for neutrophils were likely to be proteins. We then hypothesized that cytokines derived from skeletal muscle cells induce neutrophil chemotaxis after mechanical strain. We performed protein profiling, using a quantitative multiplex approach to determine whether the appearance of certain cytokines in conditioned media paralleled the response of neutrophil chemotaxis after varying degrees of mechanical strain. Candidate cytokines were then tested for their ability to promote neutrophil chemotaxis.

METHODS

Muscle cell culture. Human primary myoblasts obtained from a female donor were negative for mycoplasma, hepatitis B virus, hep-
atitis C virus, and human immunodeficiency virus (Cambrex, Charles City, IA) and were used at passage 4. Myoblasts (10,000 cells/cm²) suspended in skeletal muscle growth medium (Cambrex) were seeded on collagen I triple-coated flexible-bottom microwell plates (Flexcell International, Hillsborough, NC) in a 37°C, 5% CO₂ humidified environment. Differentiation of myoblasts was induced by replacing growth medium with DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 2% heat-inactivated FBS (GIBCO Invtrogen, Carlsbad, CA), 2 mg/ml human EGF (Cambrex), 0.3 mg/ml gentamicin (Sigma), and 0.015 µg/ml amphotericin B (Sigma) when ~90% confluence was reached. On the fifth day of differentiation, differentiated skeletal muscle cells were experimentally treated.

**Treatment of skeletal muscle cells.** Differentiated skeletal muscle cells were washed with HBSS, bathed in 3 ml of basal medium (DMEM), and then exposed to either a control or strain protocol in a 37°C, 5% CO₂ humidified environment. DMEM rather than differentiation medium was used for experiments to control for the influence that serum factors may have on neutrophil chemotaxis. Cells were mechanically strained for 30 min with a vacuum-based system (FX-4000 Flexercell Strain Unit; Flexcell), which creates nonuniform multiaxial (radial and circumferential) strain of the membrane of the tissue culture plate. Negative pressure was applied to individual wells of the tissue culture plates at a frequency of 0.25 Hz. Negative pressures of 10 [strain regimen (ST) 10], 30 (ST 30), 50 (ST 50), or 70 (ST 70) kPa drew the membranes to which the skeletal muscle cells were attached downward for 2 s, followed by a 2-s release (4-s cycles). The manufacturer’s loading posts and base plates were not used, to avoid compression of the membrane of tissue culture plates. Our ST 10, ST 30, ST 50, and ST 70 protocols correspond to manufacturer-defined percentages of 1.5%, 4.9%, 9.5%, and 15% when the loading posts and base plate are used. The manufacturer does not provide percent strains for conditions when the loading posts and base plate are not used. Rubber stoppers were applied to control wells to prevent mechanical strain. Three hours after exposure to mechanical strain, medium was collected for a total experimental time of 3.5 h. This time point was selected because neutrophil accumulation in muscle is evident within 2–6 h after muscle injury (28). Medium was then centrifuged, and supernatants were stored at −80°C (conditioned media). Conditioned control medium was collected from cultures that were bathed in DMEM for 3.5 h but did not undergo strain (ST 0).

**Skeletal muscle cell injury.** Lactate dehydrogenase (LDH) in conditioned media was measured according to manufacturer’s instructions (CytoTox 96; Promega, Madison, WI) and used as an indicator of muscle cell injury. Our prior work (39) supports the use of LDH release as an indicator of muscle cell injury by demonstrating that LDH release after mechanical strain occurred when cultured muscle cells showed ultrastructural signs of membrane rupture. Maximal LDH release (positive control) was determined by lysing muscle cells (0.9% Triton X-100; 10 min), whereas basal LDH release (ST 0 medium) was used as the negative control. An injury index was calculated for each mechanical strain condition with the following equation: [(experimental value − ST 0 value)/(maximal release value − ST 0 value)] × 100.

**Neutrophil isolation.** Heparinized venous human blood was obtained from healthy volunteers after written informed consent was obtained. The University of Toledo Human Subjects Review Board approved procedures involving human subjects.

Polymorphonuclear cells (PMNs) were isolated from other cells in the blood by density-gradient centrifugation as previously described with slight modifications (39). Whole blood was layered onto 1-Step Polymorphs cell separation medium (Accurate Chemical, Westbury, NY) and centrifuged. The gradient layer containing PMNs was aspirated, washed with calcium- and magnesium-free HBSS, and centrifuged. Red blood cells were removed by hypotonic lysis with distilled H₂O. Cells were then washed, centrifuged, and suspended in basal medium at a concentration of 3.2 × 10⁶ cells/ml for chemotaxis experiments. In some chemotaxis experiments, neutrophils were suspended in conditioned media to test for specificity of chemotaxis. Cytospin-prepared slides of the final cell suspension revealed that >95% of the isolated cells were neutrophils.

**Neutrophil chemotaxis.** Neutrophil chemotaxis was measured in a 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) as previously described (39). All media for chemotaxis were supplemented with 1% BSA. Lower wells of the chamber were loaded with basal medium (negative control), 10⁻⁷ M N-formylmethionyl-leucyl-phenylalanine (FMLP, positive control; Sigma), or conditioned media. A 3-μm-pore polycyprydolone-free polycarbonate filter (Neuro Probe) was placed over the filled wells of the lower chamber. The upper chamber wells were filled with 1.6 × 10⁶ neutrophils suspended in basal medium. The chamber was then incubated at 37°C for 30 min. Nonmigrated cells were removed from the top surface of the filter by washing with PBS and then scraping against a rubber edge. Migrated cells were fixed in absolute methanol and stained with Wright-Giemsa (Fisher Scientific, Pittsburgh, PA). The total numbers of migrated neutrophils in 20 high-powered fields (×1,000) per well were counted in duplicate and averaged. Neutrophil chemotaxis was expressed as an index with the following equation: [(experimental value − basal medium value)/(FMLP value − basal medium value)] × 10⁴ (14).

To test whether IL-8 alone, at concentrations observed in conditioned media, could elicit chemotaxis, recombinant human IL-8 (rHIL-8; BD Biosciences, San Jose, CA) was placed in lower wells of the chemotaxis chamber and assayed for chemotaxis. Neutralizing antibodies to IL-8 and MCP-1 and an isotopic control antibody (mouse IgG1) were purchased from BD Biosciences, whereas those for GM-CSF and IL-6 were purchased from R&D Systems (Minneapolis, MN). Neutralizing antibodies were incubated at room temperature with conditioned media for 45 min before neutrophil exposure. In boiling experiments, conditioned medium was boiled for 5 min and cooled to room temperature before being loaded into the lower wells of the chamber.

To reduce the likelihood that the observed neutrophil migration was due to random movement (chemokinesis) rather than directional migration (positive chemotaxis), the following experiment was performed. Upper wells of the chemotaxis chamber were loaded with human neutrophils suspended in basal medium, undiluted ST 70 conditioned medium, or ST 70 conditioned medium diluted in half with basal medium. Neutrophils were allowed to migrate for 30 min toward undiluted ST 70 conditioned medium, half-diluted ST 70 conditioned medium, or basal medium, and neutrophil chemotaxis was determined as described above.

**Multiplex cytokine assay.** A multiplex suspension bead array immunoassay run on a Luminex® analyzer (Luminex, Austin, TX) was used to screen for the presence of cytokines in conditioned media. The premixed human 22-plex cytokine kit (Linco Research, St. Charles, MO) was prepared according to the manufacturer’s instructions. Conditioned media from individual wells were analyzed in triplicate. Briefly, wells of the assay plate were blocked, and then assay buffer, appropriate samples, and the antibody-immobilized bead solution were added to wells. The plate was incubated in the dark for 2 h and washed, and the detection antibody cocktail was added. After a 30-min incubation in the dark with agitation, streptavidin-phycocerythrin was added to wells and the plate was incubated in the dark for 30 min with agitation. Wells were thoroughly washed, sheath fluid was applied, and the plate was analyzed. Cytokine concentrations were calculated based on standard curve data with a weighted five-parameter logistic fit analysis (MasterPlex QT software; MiraiBio, Alameda, CA).

**ELISA kits.** Colorimetric ELISA kits were used to confirm the presence of cytokines identified as responsive to strain with the multiplex assay. The concentrations of IL-8, MCP-1 (BD Biosciences), and GM-CSF (BioLegend, San Diego, CA) in conditioned media and IL-8 and MCP-1 in skeletal muscle cells were determined according to manufacturer’s instructions. Cells were washed with
HBSS, lysed with ice-cold extraction buffer (0.1% IGEPAL CA-630 in PBS; Sigma) supplemented with protease inhibitors [1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 5 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin; Sigma], and incubated on ice for 10 min (32). Total protein content in cell lysates was determined (22). Protein in cell lysates was measured so that cytokines measured in skeletal muscle cells could be expressed relative to total protein (pg/μg of protein). Conditioned medium samples were applied undiluted, whereas lysates of skeletal muscle cells were diluted in ELISA diluent (1:5). Optical densities of the standards and samples were measured at a wavelength of 450 nm (SpectroMax 190; Molecular Devices, Sunnyvale, CA).

Statistical analysis. Data sets were tested for normality and equal variance. Separate one-way ANOVAs were utilized to evaluate the influence of mechanical strain on injury index and to determine IL-8 and MCP-1 ELISA medium concentrations (SigmaStat; Sigma). The ability of rhIL-8 to elicit neutrophil chemotaxis was analyzed with a one-way repeated-measures (RM) ANOVA. The effects of boiling and antibody neutralization on neutrophil chemotaxis were determined with a two-way RM ANOVA. A Kruskal-Wallis one-way ANOVA on ranks was used to analyze ELISA GM-CSF medium concentrations and ELISA IL-8 and MCP-1 muscle cell concentrations, whereas the influence of mechanical strain on neutrophil chemotaxis was analyzed with a Friedman one-way RM ANOVA on ranks. The Student-Newman-Keuls post hoc test was used to locate the differences between means when the observed F ratio was statistically significant (P ≤ 0.05). Data are reported as means ± SE.

RESULTS

Skeletal muscle cell injury. The injury index, measured by the amount of LDH in conditioned media, revealed that regimens of ST 30, 50, and 70 were injurious compared with ST 10 (Fig. 1A). Additionally, increasing degrees of muscle cell injury were detected with each successive mechanical strain regimen (Fig. 1A).

Neutrophil chemotaxis. Characterization of mechanical strains that elicited varying degrees of injury enabled us to determine whether neutrophil chemotaxis induced by strained myotubes was associated with the degree of cell injury. Significant neutrophil chemotaxis was not observed after the lowest strain regimen, ST 10 (Fig. 1B). Neutrophil chemotaxis however, progressively increased for ST 30, ST 50, and ST 70 (Fig. 1B). The chemotactic response for ST 30, ST 50, and ST 70 paralleled the magnitude of strain-induced injury.

Because conditioned media could cause random neutrophil movement (chemokinesis) rather than directional migration (chemotaxis), an analysis was performed to reduce the likelihood that our results were nonspecific. Dilution of ST 70 conditioned medium by half decreased the chemotaxis index by approximately half, while addition of ST 70 conditioned medium to the upper wells of the chemotaxis chamber did not result in an increase in the chemotaxis index (data not reported). These data indicate that the observed neutrophil chemotaxis was dose dependent and likely attributable to chemotaxis rather than increased chemokinesis.

Conditioned media from ST 0, ST 30, and ST 70 were boiled as a rudimentary means for determining whether the neutrophil chemoattractants in our media were proteins. Boiling of conditioned media after mechanical strain resulted in a blunted chemotactic response (Fig. 1C). These data indicated that the neutrophil chemoattractants in our conditioned media were likely proteins.

Cytokines. In an effort to determine the complexity of the environment created after mechanical strain and to reveal the identity of some chemoattractants, a multiplex cytokine assay was utilized. The majority of cytokines measured in the multiplex assay (18 of 22) were detected in conditioned control medium, although some cytokines were detected at
Cytokines insensitive to strain or not detected in conditioned media (Table 1 and Fig. 2). Concentrations of IL-8 and MCP-1 increased with each of the injurious strain regimens (Fig. 2). GM-CSF concentrations increased marginally after ST 30 and ST 50 and were highest after ST 70 (Fig. 2). Granulocyte colony-stimulating factor (G-CSF) concentrations were below the lowest standard until ST 70, while IL-6 concentrations were below detectable limits until ST 70 (Fig. 2).

Quantitative ELISA kits were used to confirm the presence of IL-8, MCP-1, and GM-CSF in conditioned media after mechanical strain and to determine the concentrations of IL-8 and MCP-1 in skeletal muscle cells. In all cases ELISAs confirmed the presence of these cytokines in conditioned media. The concentrations of IL-8 and MCP-1 from ELISAs, however, were higher than those found by the multiplex assay. ELISA concentrations of GM-CSF, on the other hand, were similar to those found in the multiplex assay.

Media concentrations of IL-8 via ELISA were significantly elevated after ST 30, ST 50, and ST 70 compared with ST 0 (Fig. 3A). However, no concentration differences between ST 30, 50, and 70 were detected, indicating that IL-8 release was not responsive to the degree of muscle cell injury (Fig. 3A). Skeletal muscle cell IL-8 concentrations were elevated after ST 30, ST 50, and ST 70 relative to ST 0 (Fig. 3B). Elevated concentrations of IL-8 were detected in muscle cells after ST 70 compared with ST 30 and ST 50 (Fig. 3B). MCP-1 ELISA concentrations in conditioned media were significantly elevated above ST 0 after ST 30 and ST 70 (Fig. 3C). Additionally, ST 70 concentrations were also elevated above ST 10 and ST 50 concentrations (Fig. 3C). Skeletal muscle cell MCP-1 concentrations of both ST 30 and ST 70 were elevated above all other conditions (Fig. 3D). ELISA for GM-CSF revealed concentrations in the range of 2–4 pg/ml for control and conditioned media.

**Table 1. Cytokines insensitive to strain or not detected in multiplex kit**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Concentration, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detected but insensitive to strain</strong></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>7.16±2.08</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5.31±0.14</td>
</tr>
<tr>
<td>IL-1α</td>
<td>6.88±0.35</td>
</tr>
<tr>
<td>IL-2</td>
<td>3.04±0.19</td>
</tr>
<tr>
<td>IL-5</td>
<td>4.13±0.02</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.68±0.14</td>
</tr>
<tr>
<td>IL-12</td>
<td>6.52±0.05</td>
</tr>
<tr>
<td>IL-13</td>
<td>6.62±0.21</td>
</tr>
<tr>
<td>IL-15</td>
<td>2.27±0.32</td>
</tr>
<tr>
<td>IL-17</td>
<td>4.13±0.02</td>
</tr>
<tr>
<td>IP-10</td>
<td>2.93±0.65</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>7.95±0.57</td>
</tr>
<tr>
<td>RANTES</td>
<td>6.80±0.05</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5.26±0.02</td>
</tr>
<tr>
<td><strong>Below detectable limits</strong></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-4</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-7</td>
<td>N/A</td>
</tr>
</tbody>
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Values are means ± SE; n = 7. Concentrations are those detected in conditioned control medium (ST 0). IP-10, interferon-inducible protein-10; MIP, macrophage inflammatory protein; N/A, not applicable.

**Fig. 2. Cytokines measured in the multiplex assay that increased after 1 or more strain protocols. n = 8 except ST 0 (n = 7).** MCP, monocyte chemotactic protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor.

**Contribution of IL-8 and GM-CSF to neutrophil chemotaxis.**

To determine whether IL-8 was contributing to neutrophil chemotaxis after mechanical strain, conditioned medium was supplemented with a neutralizing antibody for IL-8 and assayed for chemotaxis. Recombinant IL-8 at a concentration known to be chemotactic for neutrophils (100 ng/ml) was used to determine the optimal concentration of neutralizing IL-8 antibody. These experiments revealed that 100 μg/ml of an anti-IL-8 antibody completely blocked neutrophil chemotaxis to rhIL-8 (100 ng/ml), a finding that is consistent with prior work (34).

Treatment of conditioned media with 100 μg/ml of anti-IL-8 significantly reduced chemotaxis for ST 30, 50, and 70 compared with normal medium and medium supplemented with an isotypic control antibody (Fig. 4A). The chemotactic response for ST 30 and ST 50 was completely blocked by IL-8 neutralization, while the response to ST 70 was reduced by 40% (Fig. 4A). However, rhIL-8 alone, at concentrations detected in conditioned media after mechanical strain (20–70 pg/ml), was not sufficient to elicit neutrophil chemotaxis (data not reported).

Because GM-CSF was modestly elevated in conditioned media after mechanical strain and can act synergistically with IL-8 to potentiate the chemotactic response of neutrophils (1, 34), we neutralized GM-CSF in conditioned media and assayed for neutrophil chemotaxis. The concentration of neutralizing antibody used (30 μg/ml) was at a greater antibody-to-cytokine ratio than that previously reported to block neutrophil chemotaxis (1, 34). Antibody neutralization of GM-CSF completely blocked chemotaxis induced by conditioned media from ST 30 and ST 50 and reduced the chemotactic response to ST 70 by 47% (Fig. 4B).

To further evaluate the chemotactic response to ST 70, we performed double neutralization experiments. Treatment of ST 70 conditioned medium with neutralizing antibodies for IL-8 (100 μg/ml) and GM-CSF (30 μg/ml) reduced the chemotactic response to ST 70 by 47% (n = 3). These findings confirm that...
IL-8 and GM-CSF cause neutrophil chemotaxis after our most injurious strain protocol.

**Contribution of MCP-1 and IL-6 to neutrophil chemotaxis.**

The failure of IL-8 and GM-CSF to completely explain the chemotactic response elicited by our most injurious strain regimen indicated that other chemoattractants were present in ST 70 conditioned medium. Because the multiplex assay indicated that MCP-1 and IL-6 concentrations were increased after ST 70, we performed neutralization experiments for both MCP-1 and IL-6. The concentrations of neutralizing antibodies (cytokine-to-antibody ratio) for MCP-1 (90 ng/ml) and IL-6 (1.2 μg/ml) used in these experiments had previously been reported to block monocyte chemotaxis (21, 33) and tissue invasion of tumor cell lines (24, 37), respectively. Because MCP-1 and IL-6 are not directly chemotactic for neutrophils, we were unable to determine the optimal concentration of neutralizing antibodies to inhibit neutrophil chemotaxis to recombinant forms of MCP-1 and IL-6. Neutralization of MCP-1 or IL-6 did not reduce neutrophil chemotaxis to conditioned medium from ST 70 compared with normal ST 70 medium or ST 70 medium supplemented with an isotypic control antibody (Table 2). These data indicate that neither MCP-1 nor IL-6 promoted chemotaxis after our most injurious strain regimen. The lack of an effect of MCP-1 and IL-6 neutralization, however, could be the result of a suboptimal neutralizing antibody concentration. Neutralization experiments for MCP-1 and IL-6 were not performed for ST 30 and ST 50 because neutralization of either IL-8 or GM-CSF completely blocked neutrophil chemotaxis to conditioned media from these strain protocols.

**DISCUSSION**

The major finding of this study was that IL-8 and GM-CSF released from differentiated skeletal muscle cells after mechanical strain promote neutrophil chemotaxis. Neutralization of MCP-1 or IL-6 did not reduce neutrophil chemotaxis to conditioned media from ST 70 compared with normal ST 70 medium or ST 70 medium supplemented with an isotypic control antibody (Table 2). These data indicate that neither MCP-1 nor IL-6 promoted chemotaxis after our most injurious strain regimen. The lack of an effect of MCP-1 and IL-6 neutralization, however, could be the result of a suboptimal neutralizing antibody concentration. Neutralization experiments for MCP-1 and IL-6 were not performed for ST 30 and ST 50 because neutralization of either IL-8 or GM-CSF completely blocked neutrophil chemotaxis to conditioned media from these strain protocols.
strain. IL-8, a well-characterized neutrophil chemoattractant, did not reach sufficient concentrations in conditioned media to induce chemotaxis alone. However, neutralization of IL-8 completely prevented chemotaxis from ST 30 and ST 50 conditioned media and reduced the chemotactic response from ST 70 conditioned medium by 40%. Neutralization of GM-CSF resulted in a loss of the chemotactic response similar to that achieved by neutralizing IL-8, indicating that IL-8 and GM-CSF were likely functioning synergistically to promote neutrophil chemotaxis. Neutralization experiments for MCP-1 and IL-6 revealed that neither cytokine directly or indirectly (e.g., synergy) contributes to the chemotaxis elicited by conditioned medium from ST 70. These data advance our understanding of the immunobiology of skeletal muscle by revealing that human skeletal muscle cells, after injurious mechanical strain, promote neutrophil chemotaxis by releasing IL-8 and GM-CSF into their local environment.

IL-8 is a member of the ELR⁺ CXC chemokine family, which are potent chemotactic factors for neutrophils (17, 25). IL-8 was originally described as a monocyte-derived product that elicits neutrophil chemotaxis (43), and subsequent studies have revealed that many cell types are capable of producing IL-8 (17), including skeletal muscle cells (2, 39). Maximal IL-8-induced chemotaxis from human neutrophils in vitro occurs at concentrations ranging from 80 to 150 ng/ml, with marginal chemotaxis observed at much lower concentrations (0.8 – 5 ng/ml) (9, 11, 14, 43). In this study, the ELISA for IL-8 revealed concentrations ranging from 51 to 63 pg/ml in conditioned media after mechanical strain. These concentrations proved to be chemotactic, because neutralization of IL-8 blunted the chemotactic response. However, recombinant IL-8 alone at the concentrations observed in conditioned media does not induce neutrophil chemotaxis (present study; Ref. 14). We interpret these findings to indicate that other soluble factors were synergizing with IL-8 to promote chemotaxis after injurious mechanical strain.

GM-CSF, a hematopoietic growth factor, has been reported to influence various aspects of neutrophil function. Wolach et al. (41) reported that prior exposure to GM-CSF primes neutrophils, resulting in enhanced polarization, chemotaxis, and reactive oxygen species production in response to subsequent fMLP stimulation. GM-CSF has also been reported as a direct chemoattractant for neutrophils, albeit at a higher concentration (125 pg/ml) than that found in our conditioned media (~5 pg/ml) (34). Finally, neutrophil chemotaxis in the presence of suboptimal or optimal IL-8 concentrations is potentiated by the presence of GM-CSF (1, 34). In the present study, neutralization of GM-CSF completely blocked the chemotactic response elicited by conditioned media from ST 30 and ST 50 and impaired neutrophil chemotaxis to ST 70 by 47%. Interestingly, the magnitude of reductions seen in neutrophil chemotaxis after neutralizing GM-CSF was similar to those found after neutralization of IL-8. These data, in conjunction with the finding that IL-8 at the concentrations observed in conditioned media is not chemotactic for neutrophils, may indicate that IL-8 and GM-CSF are functioning cooperatively to induce neutrophil chemotaxis after injurious strain of skeletal muscle cells. G-CSF concentrations were also detected in conditioned media; however, Shen et al. (34) previously revealed that there was no additional enhancement of neutrophil chemotaxis when a cocktail of IL-8, G-CSF, and GM-CSF was tested compared with the response observed when only IL-8 and GM-CSF were combined. It is therefore unlikely that G-CSF and GM-CSF are working together to promote IL-8-induced chemotaxis. It remains to be determined whether any other cytokines are synergizing with IL-8 and GM-CSF to induce neutrophil chemotaxis after mechanical strain.

Partial chemotaxis from ST 70 conditioned medium after neutralization of IL-8 and GM-CSF indicated that either a single chemoattractant or multiple chemoattractants working in synergy promoted chemotaxis after our most injurious strain protocol. MCP-1 concentrations in ST 70 conditioned medium were elevated by mechanical strain, indicating that it was a potential candidate for contributing to neutrophil chemotaxis after our most injurious strain protocol. Neutralization of MCP-1, however, did not influence the chemotactic response to ST 70 conditioned medium. In contrast to our findings, Gouwy et al. (11) reported MCP-1 as a neutrophil chemoattractant when in the presence of IL-8. The concentrations of IL-8 and MCP-1 observed in conditioned media in the present study may not have been high enough to elicit synergy between these two chemokines. IL-8 and MCP-1 concentrations in ST 70 conditioned medium were detected at 63 and 45 pg/ml, respectively, whereas Gouwy and colleagues (11) revealed synergy between IL-8 and MCP-1 at much higher concentrations (5 and 100 ng/ml, respectively).

Of the cytokines we measured that responded to mechanical strain, IL-6 was the last potential candidate to explain chemotaxis induced by ST 70 because it was the only cytokine that revealed increased concentrations solely after the most injurious strain protocol. Borish et al. (5) previously revealed that IL-6 is not directly chemotactic for neutrophils, but to our knowledge the ability of IL-6 to synergize with other cytokines to promote chemotaxis has not been tested. Neutralization of IL-6 did not, however, reduce neutrophil chemotaxis in ST 70 conditioned medium, revealing that the presence of IL-6 does not influence neutrophil chemotaxis after injurious mechanical strain.

The mechanism by which mechanical strain caused cytokine concentrations to increase in conditioned media is unknown. Because cytokines can be synthesized by skeletal muscle cells and localized to their extracellular matrix, the accumulation of selected cytokines in conditioned media after mechanical strain may have occurred because of alterations to the extracellular matrix and/or as a result of membrane ruptures (39). In the absence of mechanical strain, agonists for cytokine production during an inflammatory response are generally thought to be other cytokines. However, we observed the release of IL-8 and MCP-1 without concurrent elevations in cytokines that are potent regulators of IL-8 and MCP-1 (e.g., IL-1 and TNF-α) (4, 18, 35). Little is known about how mechanical strain signals the production of cytokines in skeletal muscle cells, but these
data may indicate that the release of IL-8 and MCP-1 is likely regulated by signaling events associated with the mechanical strain itself. Previous investigators have revealed that mechanical strain induces the production of IL-8 and MCP-1 in smooth muscle, endothelial, and epithelial cells (16, 40, 42). Future studies are needed to reveal the mechanisms of mechanical strain-induced cytokine production from skeletal muscle cells.

Although our prior work did not test the contribution of IL-8 to neutrophil chemotaxis, we did report that IL-8 concentrations in conditioned media decreased after mechanical strain (39). In the present study we found that strain increased IL-8 release from skeletal muscle cells. These differences may be attributable to differences in the mechanical strain protocol. Our prior work utilized less strenuous strain protocols but for a longer duration (2 h) (39), whereas the present study used more rigorous strain protocols for a shorter period of time (30 min). Additional work is needed to determine how duration and magnitude of mechanical strain influence signaling pathways involved in the production and release of cytokines from skeletal muscle cells.

In addition to the cytokines tested, we found 14 cytokines in conditioned media that were not responsive to mechanical strain. Whether one or more of these cytokines contributed to the observed neutrophil chemotaxis after mechanical strain either by potentiating the IL-8/GM-CSF response or by syner-
gizing with other unknown factors remains to be determined. Another novel aspect of the present study is that we detected, via a multiplex approach, numerous cytokines in media obtained from control cultures of differentiated skeletal muscle cells. Little is known about the profile of cytokines released from cultures containing myotubes because the majority of studies reporting cytokine release have studied proliferating from cultures containing myotubes because the majority of cells. Little is known about the profile of cytokines released from cultures containing myotubes because the majority of studies reporting cytokine release have studied proliferating from cultures containing myotubes. However, the majority of studies have used these myoblasts contributed to the observed cytokine production. Additional work is needed to confirm the constitutive release of these cytokines from cultures containing myotubes.

The present study revealed that neutrophil chemotaxis observed after mechanical strain of skeletal muscle cells is responsive to the degree of muscle cell injury. In an attempt to identify the chemoattractants triggering neutrophil chemotaxis, we found that IL-8 and GM-CSF promoted the chemotactic response after injurious mechanical strain. The presence of IL-8 at concentrations that do not elicit neutrophil chemotaxis alone and the similar pattern of reduction when IL-8 and GM-CSF were neutralized may indicate that IL-8 and GM-CSF are functioning in synergy to promote neutrophil chemotaxis. Furthermore, the inability of IL-8 and GM-CSF to explain all the chemotactic response after the highest strain regimen may indicate that additional chemoattractants are operating after our most rigorous strain protocol. Future studies aimed at revealing the identity of additional skeletal muscle cell-derived neutrophil chemoattractants will further our understanding of the immunobiology of skeletal muscle after exercise-induced injury. This knowledge may reveal pharmacological opportunities for reducing the number of neutrophils in skeletal muscle injured by mechanical loading in an effort to alleviate neutrophil-mediated muscle injury (6, 20, 29, 30).

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