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Increased MAPK signaling during in vitro muscle wounding

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Abstract

Regeneration of skeletal muscle upon injury is a complex process, involving activation of satellite cells, followed by migration, fusion, and regeneration of damaged myofibers. Previous work concerning the role of the mitogen activated protein (MAP) kinase signaling pathways in muscle injury comes primarily from studies using chemically induced wounding. The purpose of this study was to test the hypothesis that physical injury to skeletal muscle cells in vitro activates the MAP kinase signaling pathways. We demonstrate that extracellular signal regulated kinases (ERKs) 1, 2, and p38 are rapidly and transiently activated in response to injury in C2C12 cells, and are primarily localized to cells adjacent to the wound bed. Culture medium from wounded cells is able to stimulate activation of p38 but not ERK in unwounded cells. These results suggest that both ERK and p38 are involved in the response of muscle cells to physical injury in culture, and reflect what is seen in whole tissues in vivo.

Keywords: C2C12; Muscle; Wounding; Regeneration; Signaling; p38; ERK; MAP kinase

Skeletal muscle has the remarkable ability to adapt to physiological demands such as growth, exercise, and injury [1]. The ability of skeletal muscle to regenerate after injury reflects the plasticity of muscle tissue, due primarily to the regenerative capacity of satellite cells. Quiescent satellite cells are located beneath the basal lamina surrounding each muscle fiber, and constitute 2–10% of the cell nuclei associated with each myofiber [1]. Satellite cells can be activated by a variety of factors released from injured muscle, such as scatter factor (SF; otherwise known as hepatocyte growth factor [HGF]) [2], leukemia inhibitory factor (LIF) [3], and transforming growth factor β (TGFβ) [4]. Upon injury, satellite cells enter the cell cycle, proliferate, migrate, and undergo myogenesis to replace the lost fibers in the injured area [1].

Investigating the changes in the signaling events that occur during skeletal muscle regeneration is one means of identifying which genes participate in the processes of muscle cell activation, migration, and differentiation [5]. The mitogen activated protein (MAP) kinase pathways are the major signaling modules through which cells translate extracellular stimuli into changes in gene expression [6]. The MAP kinases regulate the activity of several transcription factors, which in turn initiate the expression of various immediate and delayed response genes [7]. Currently, little is known regarding the role of the MAP kinase signaling pathways involved in regulating the satellite cell population during muscle wounding and regeneration. Therefore, the use of reproducible experimental injury protocols will be important for defining the regulation of the satellite cell population. This information will be essential to our understanding of anomalies in the regeneration process, such as in atrophic muscle, senescence, or in myopathic conditions [1,8].

Our present study focuses on the signaling cascades activated in response to muscle wounding in vitro. We use the murine C2C12 myoblastic cell line, derived from mouse muscle satellite cells and considered to be an excellent model for myogenesis [9,10]. We show that there is a rapid activation of the extracellular signal regulated kinases (ERKs) 1, 2, and p38 upon physical wounding of a confluent monolayer of muscle cells, and

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that this activation is largely confined to the cells adjacent to the wound edge. The increase in ERK activation is similar to that seen when muscle cells are treated with interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα). In addition, culture medium from wounded cells is able to provoke an increase in phosphorylated p38 levels in unwounded cells, suggesting the release of injury-induced factor(s). Our findings are the first to report the upregulation of these signaling pathways in muscle cells in vitro, using a novel system to study physical injury on cultured cells.

Materials and methods

Reagents. All tissue culture reagents were obtained from Gibco BRL, unless otherwise stated. Anisomycin was purchased from Calbiochem (La Jolla, CA, USA). TNFα and IL-1 were obtained from Peprotech (Tebu, Le Perray-en-Yvelines). Antibodies against phosphorylated p38 MAP kinase (clone P38-TY) and ERK (clone MAPK-YT) were from Sigma (Saint Quentin Fallavier, France), as were the anti-p38 total (M0800) and -ERK total (M5670) antibodies. Fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibodies were obtained from Dako (Trappes, France).

Cell culture. Mouse C2C12 myoblasts (ATCC no. CRL-1772) were cultured at 37 °C in an atmosphere of 5% CO_2 in growth medium (GM) consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% vol/vol penicillin/streptomycin.

Cell wounding. Confluent C2C12 cells were wounded as previously described, using a device specially constructed for this purpose [11]. Briefly, this apparatus enables the generation of a continuous, curvilinear wound throughout the entire surface area of a tissue culture dish. When performed on a 100 mm diameter culture dish, the wound length reached 15–20 m [11]. After wounding, the plate was returned to the incubator (37 °C, 5% CO_2) for varying lengths of time (as stated in the figure legends) before the cells were lysed and protein extracts were harvested.

Culture media "swap" experiments. Confluent C2C12 cells were wounded, and left in the incubator for 15 min. Culture medium from the plates of injured cells was collected in 50 ml conical tubes (Falcon), and added to separate cultures of intact confluent C2C12 cells. After 10 min, the cells were lysed and protein extracts were prepared as described below.

Immunoblotting. Cells were treated as indicated in the figures, rinsed, and solubilized in ice cold lysis buffer [50 mM Hepes, pH 7.4, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, supplemented with protease inhibitors: aprotinin (2 μg/ml), leupeptin (10 μM), and AEBSF (1 mM)]. Equal amounts of protein were separated by SDS–PAGE on a 10% acrylamide gel. Proteins were transferred to Hybond-C extra membrane (Amersham), stained with ponceau red to verify an even transfer, and then the blots were incubated in blocking buffer (TBS 1X, 0.1% Tween20, and 5% nonfat powdered milk) for 1 h at room temperature. The membranes were washed in washing buffer (TBS 1X, 0.1% Tween20) three times, for 5 min per wash, probed with the primary antibody and then with the secondary antibody for 1 h in TBS 1X, 0.1% Tween20, and 1% milk. Each of the primary antibodies used in this study was diluted 1:6000 for Western blots. After each incubation, membranes were washed three times, 10 min per wash, in washing buffer. Then proteins were visualized using the Amersham ECL system. Band intensity was quantified using PCBas.

Immunofluorescence. C2C12 cells were grown to confluence on 22 mm glass coverslips. Several scratches were performed on the confluent cell monolayer using a sterile scalpel blade, and the cells were then placed back in the incubator for 5–10 min. All the following steps were performed at room temperature. Coverslips were rinsed once in sterile PBS, and the cells were permeabilized in 0.5% Triton X-100 in PBS, for 15 min, followed by washing in three separate changes of PBS, 10 min each. The coverslips were then fixed in 5% paraformaldehyde in PBS, for 10 min, and washed in three 10-min changes of PBS. Blocking was performed for 1 h using 3% BSA in PBS. The cells were incubated in primary antibody (either anti-phospho p38 or anti-phospho ERK, at a 1:100 dilution in PBS with 1% BSA), for 1 h. The coverslips were washed in three changes of PBS (10 min each), and incubated in FITC-conjugated goat anti-mouse secondary antibody (1:100 dilution) in the dark. The coverslips were then washed in at least five changes of PBS, in the dark, over a 45-min period. The coverslips were mounted in Dako mounting medium supplemented with DAPI (1 μg/ml), and visualized on an Olympus BH2 epifluorescence microscope. Photomicrographs were taken with a Nikon Coolpix digital camera.

Results

The wounding system used in this study, which has recently been described [11], uses a scalpel blade mounted on a rotating platform, enabling the generation of evenly spaced wounds across a large surface area on confluent monolayers of cultured cells. This technique augments the number of cells that participate in the wounding–regeneration response, when compared to classical wounding techniques such as laceration [11]. In this manner, we aimed to establish the cellular signaling response to physical wounding in cultured C2C12 muscle cells. The C2C12 cell line, derived from murine myoblasts, provides an effective and convenient model system in which to develop an in vitro model for studying muscle cell wounding–regeneration.

The results depicted in Fig. 1 show that the mechanical wounding of a confluent monolayer of C2C12 cells resulted in an approximately 50 μm wide wound bed, devoid of cells. At 2 h post-wounding, cells from the adjacent regions began to protrude into the cell-free area. Greater numbers of cells were seen in this region at 4 h post-wounding, and by 6 h post-wounding, the wound bed was completely covered with cells. The speed at which the wound bed is covered suggests that cell migration, rather than proliferation, is involved. Indeed, cell counts at 6 h post-wounding do not indicate an increase in the number of cells in wounded plates as compared to control plates (unpublished observations).

The MAP kinase pathways are known to be activated in response to a wide array of stimuli [12]. Similarly, activation of the stress kinases is seen following exposure to stressors such as osmotic shock, thermal shock, UV, and anisomycin treatment [12]. To study the initial signaling response of C2C12 cells to injury, we examined the activation of the p38 and ERK signaling pathways in C2C12 cells upon physical wounding. Protein extracts were harvested from the cells at various time points after wounding, and analyzed by Western blotting using phospho-specific p38 and ERK 1, 2 antibodies. The levels of activated p38 increased at 3 min post-wound-

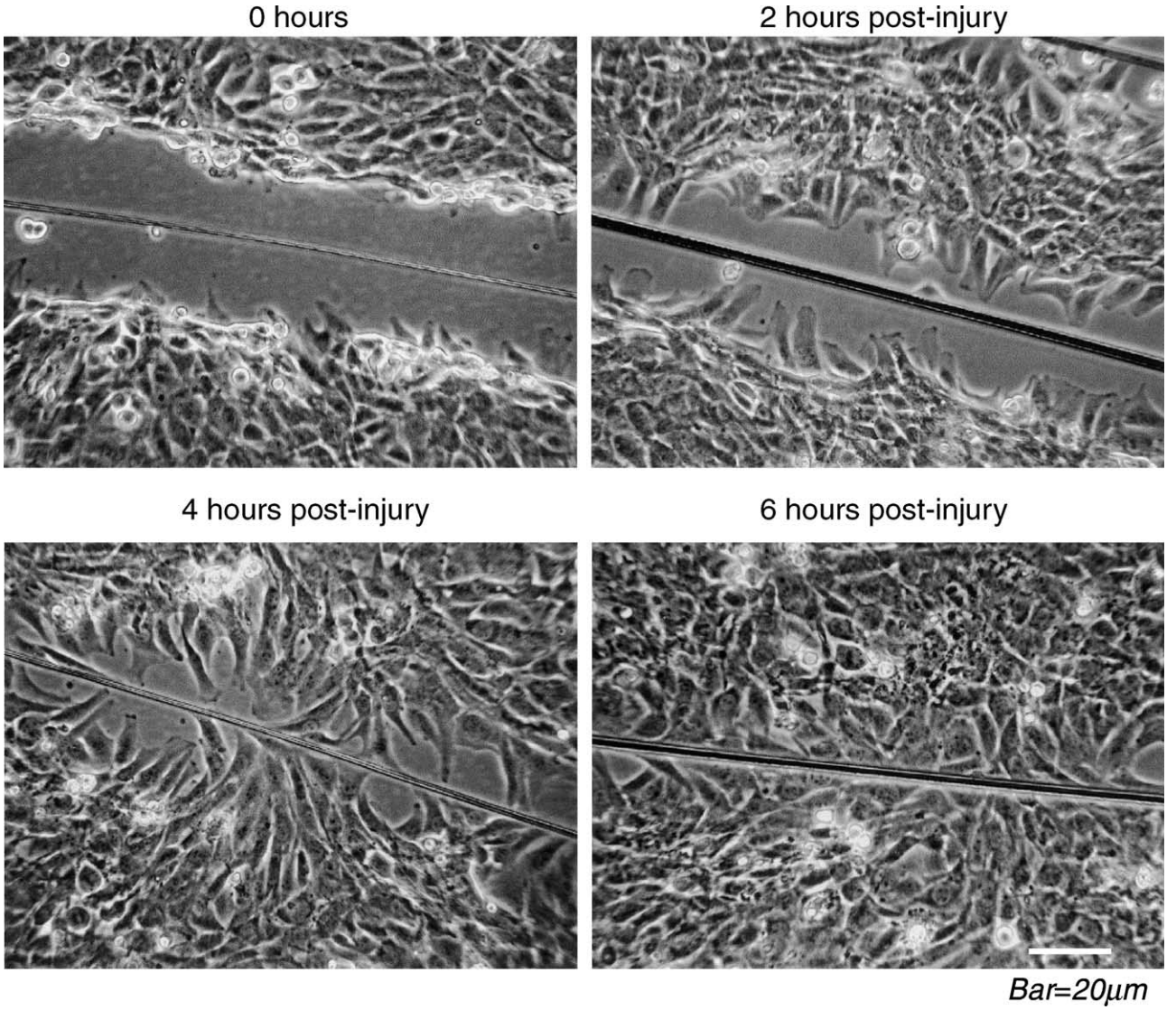

Fig. 1. Migration of C2C12 cells after wounding. Use of the wounding device described in [11] results in a series of parallel, circular, and cell-free areas in between regions of unwounded cells. Photographs were taken at different times (0, 2, 4, and 6 h) after wounding.

ing, and peaked at 10 min post-wounding (Fig. 2). At 30 min, the active p38 levels began to decline, reaching basal levels at 1 h post-wounding. An anisomycin-treated sample (protein extracts from C2C12 cells treated with 50 ng/ml anisomycin) was included for comparison. Control unwounded cells expressed lower, basal levels of phosphorylated p38. We did not observe any apparent increases in the levels of phosphorylated Jun, although total Jun levels remained equal at all time points (results not shown).

Phosphorylated ERK 1 and 2 levels increased dramatically at 3 min post-wounding, and peaked at 10 min. After this point, the activated ERK levels declined, reaching baseline levels by 1 h post-wounding. Unwounded control cells exhibited negligible amounts of these proteins. The patterns of activation of both p38 and ERK seem to be similar. Western blotting at later time points after wounding (2, 4, 8, and 24 h) showed no further increase in the levels of activated p38 and ERK in the cells (not shown). Probing of the same samples with a pan-p38-specific antibody and a pan-ERK-specific antibody (Fig. 2) indicated that there were no changes in the total protein levels of these signaling molecules.

We next focused on the localization of the phosphorylated p38 and ERK signals in the wounded cells. Immunofluorescence was performed on cells grown to confluence on coverslips, and scratched using a sterile scalpel blade. Staining of the cells using both the anti-phosphorylated p38 and ERK antibodies revealed that the most intense level of staining occurred in the wound margins, i.e., in the cells directly adjacent to the wound bed. In the case of phosphorylated p38, the apparently more intense staining at the wound edges was partially

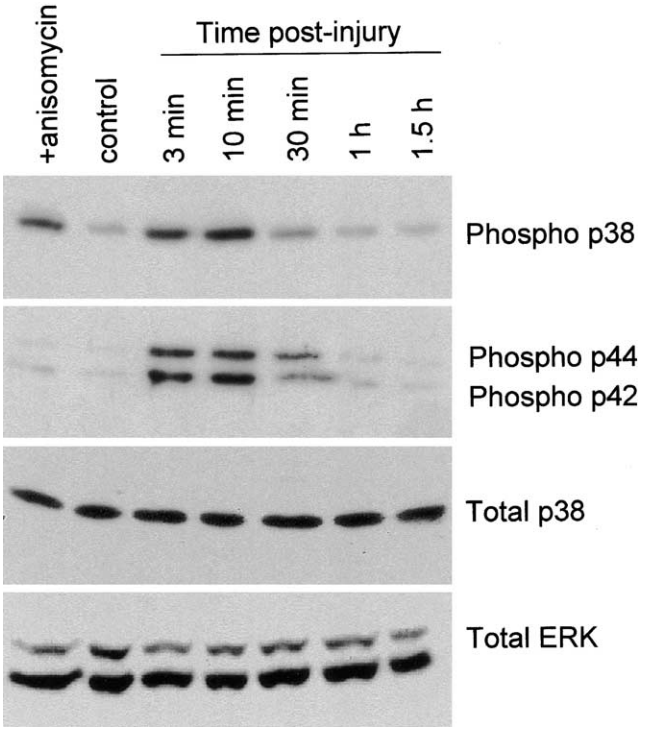

Fig. 2. Phosphorylation of p38 and ERK after wounding. Cell extracts were harvested at different time points after injury, and proteins were separated by SDS–PAGE. Western blots were performed using phospho-specific p38 and ERK antibodies, as well as anti-total p38 and total ERK antibodies. Protein extracts from unwounded cells, as well as cells treated with 50 ng/ml anisomycin, were included as controls.

due to cells that had retracted slightly from the wounding procedure, resulting in more densely packed cells. Phosphorylated p38 staining was also present in regions of unwounded cells further away from the wound bed (Fig. 3A). Higher magnification showed that the phosphorylated p38 staining occurred in a somewhat mottled pattern in the cell nucleus (Fig. 3A, DAPI staining). Control cells from coverslips not subjected to injury showed negligible levels of staining with the phospo-p38 antibody (Fig. 3A, Control). Phosphorylated ERK levels were more pronounced in cells at the edges of the wound bed (Fig. 3B). The staining occurred in a diffuse fashion throughout the cytoplasm. Levels of staining decreased in cells further away from the wound bed, with the most dramatic fluorescence occurring in the cells directly adjacent to the wound. Control, intact cells from coverslips not subjected to injury showed barely detectable levels of phosphorylated ERK (Fig. 3B, Control).

It is known that injured muscle cells are able to secrete various growth factors and cytokines, which can act at great distances to affect satellite cells [1]. Along these lines, we wished to determine whether injured C2C12 cells could affect unwounded, intact cells. We harvested culture medium from plates of confluent, injured cells, and used this medium to treat confluent, unwounded cells. Fig. 4 depicts a Western blot of protein extracts from unwounded C2C12 cells treated with culture medium (CM) from wounded cells (“+ wounded CM”), unwounded cells (“+ control CM”), or the untreated cells (“Control”). Levels of phosphorylated p38 were similar in the control lanes (untreated cells and cells treated with control culture medium). However, protein extracts from cells treated with culture medium from injured cells showed higher levels of phosphorylated p38. Levels of total p38 were equal in all lanes. We did not observe any changes in the levels of phosphorylated ERK (Fig. 4).

To compare the injury-induced response with responses to other stresses, we treated C2C12 cells with UV, as well as the cytokines TNFα and IL-1. Fig. 5 depicts the resulting increases in activated p38 and ERK seen using these treatments. Treatment with TNFα and IL-1 resulted in an increase in the levels of phosphorylated p38 and ERK in confluent C2C12 cells. In contrast, UV treatment resulted only in a minor increase in phosphorylated ERK levels, as compared to cytokine treatment. Thus, the response of C2C12 cells to wound injury more closely reflects the response seen with cytokine, rather than UV, treatment. The blots were probed with an anti-total ERK antibody to verify equal protein loading. Immunoblots from wounded cells were included for comparison (Fig. 5, right panels).

Discussion

The muscle response to trauma is characterized by the onset of a complex series of events, involving satellite cell activation, proliferation, migration, and differentiation to regenerate the damaged myofibers [1]. The characterization of the molecular signaling pathways that are involved in regulating these events will be important for understanding how muscle trauma translates to changes in gene expression. This will be valuable for understanding the molecular events deregulated in conditions such as cachexia and myopathies. Several commonly used models for studying muscle regeneration include localized crush [3], freeze [13], chemical- [14], or cardiotoxin-induced [15,16] injury. However, one of the disadvantages of using the whole tissue is that it is impossible to ascertain whether responses are due to skeletal muscle tissue or due to other cell types present in the sample, such as blood and connective tissue [17,18]. To circumvent some of these problems, we have used a novel method for injuring cells in vitro [11], to study the responses of the C2C12 myoblastic cell line to physical injury.

Our results show that the p38 and ERK pathways are activated in response to physical injury in C2C12 cells in vitro. It is well established that the stress kinases are activated by a variety of cellular stresses, including heat

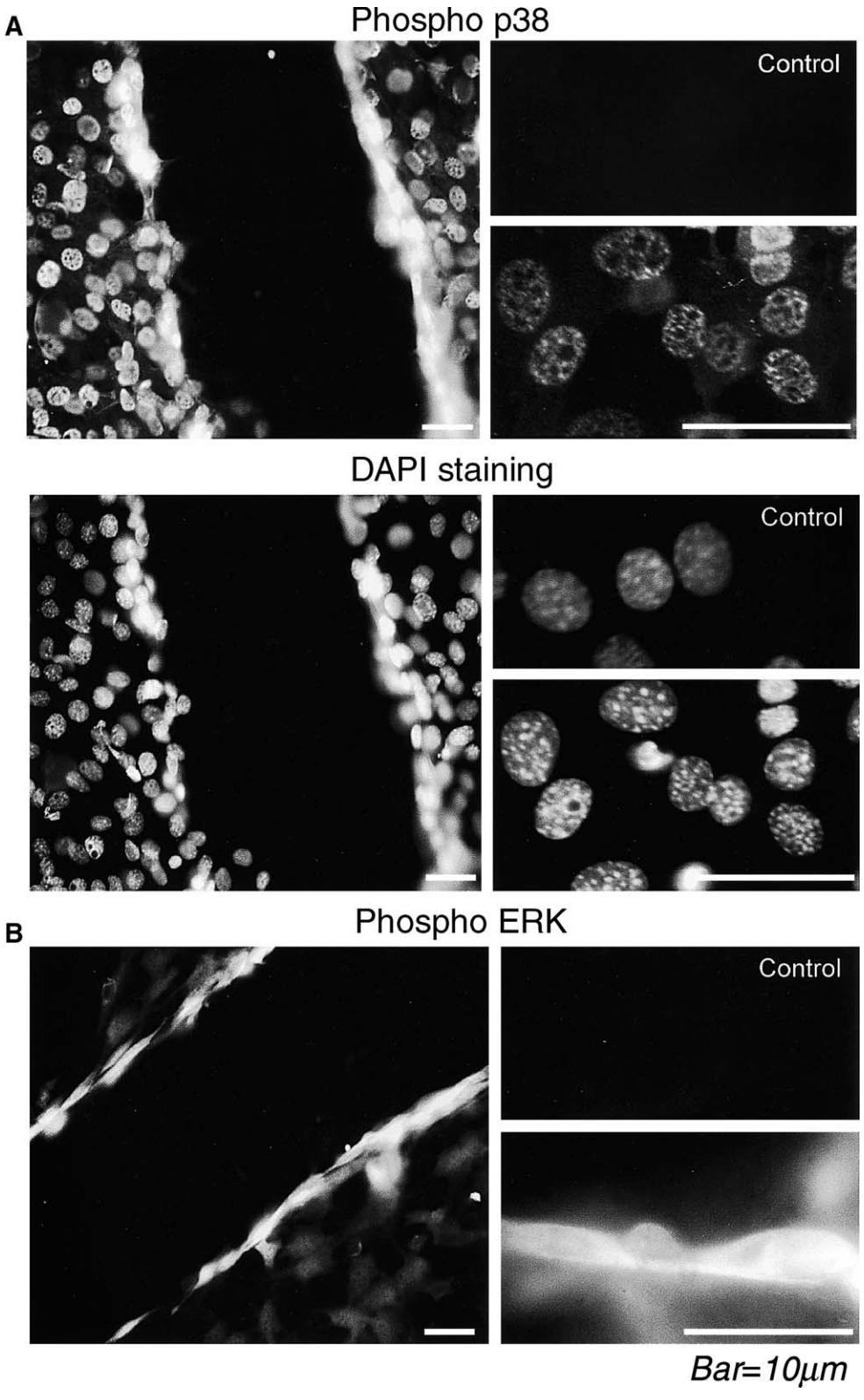

Fig. 3. Immunolocalization of phosphorylated p38 and ERK in wounded cells. C2C12 cells were grown to confluence on coverslips, and scratch wounds were performed with a sterile scalpel blade. The cells were stained using anti-phosphorylated p38 antibody and DAPI (A) and anti-phosphorylated ERK antibody (B). The phosphorylated p38 and DAPI images shown in A depict the exact same fields of cells, demonstrating that the phosphorylated p38 staining is nuclear. "Control" images were taken from cells on separate coverslips, which were not subjected to any physical wounds.

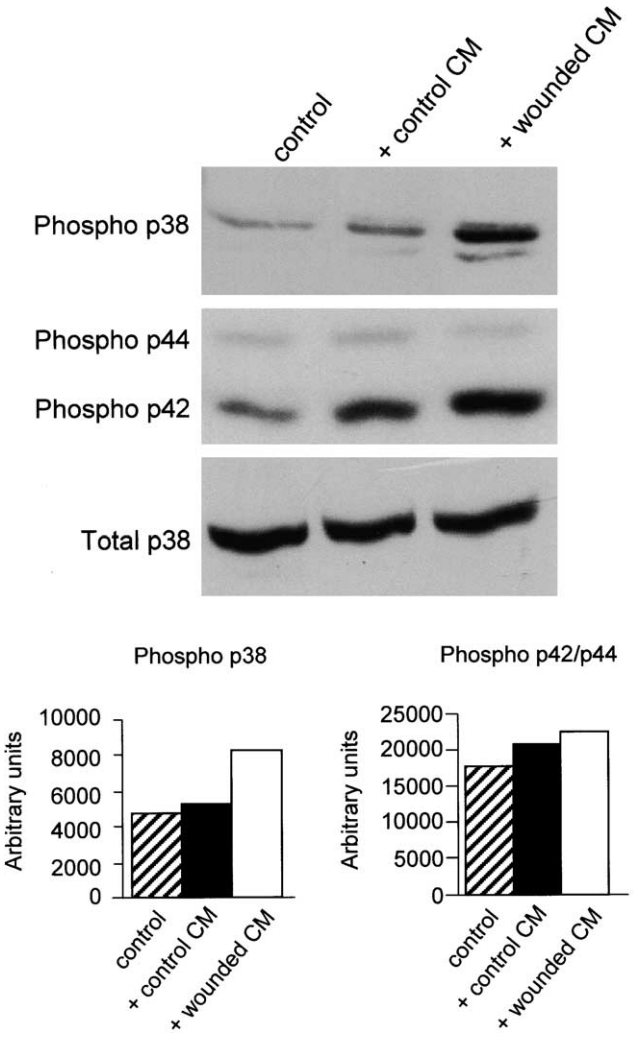

Fig. 4. Culture medium from wounded cells stimulates p38 phosphorylation in intact, unwounded C2C12 cells. Culture medium was collected from confluent cells 15 min post-wounding, and this medium was added to intact, confluent C2C12 cells. After 10 min, the unwounded cells were lysed and protein extracts were prepared for immunoblotting using anti-phosphorylated p38, anti-phosphorylated ERK, and total p38 antibodies. The phosphorylated p38 and ERK bands were quantified using PCBas, and are depicted in the histograms.

shock, UV irradiation, and pro-inflammatory cytokines [12]. We show that p38 is rapidly and transiently activated upon physical injury in C2C12 cells. This reflects the previously published work on the activation of these signaling pathways in response to various types of muscle traumas in vivo, including ischemia/reperfusion injury [19], needle biopsy [20], and hydrogen peroxide treatment [21]. The ERK pathway is activated in muscle cells in response to physical exercise [22–24], where activation of ERK increases proportionately with the intensity of exercise [25]. Although the exact role of the ERK pathway in muscle injury has yet to be defined, it is possible that it may play a protective role, such as that which occurs during recovery from simulated ischemia

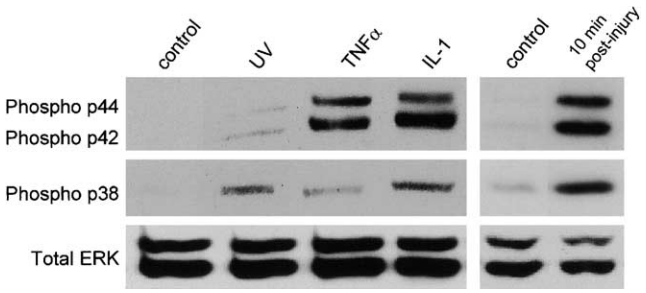

Fig. 5. Signaling response of C2C12 cells to other stresses. Confluent cells were treated with either 1000 U/ml TNFα or IL-1, or 2 min under a UV lamp (UVC; 40 J/m^2). The cells were lysed after 15 min and protein extracts were prepared. Immunoblotting was performed using anti-phosphorylated p38 and ERK antibodies, as well as with an antibody to total ERK as a loading control. Immunoblots from wounded cells at 10 min post-injury (showing upregulation of phosphorylated p38 and ERK) are included for comparison.

[19,26]. However, ERK could also mediate the cell migratory response [27]. Further work needs to be done to define the roles of these signaling pathways in response to muscle cell injury.

In comparison to other stress stimuli (such as UV and cytokine treatment), the response of C2C12 cells to physical wounding more closely reflects that seen with cytokine treatment. Treatment of the cells with either TNFα or IL-1 resulted in activation of ERK and p38 after 15 min. In contrast, UV treatment resulted only in a minor increase in phophorylated ERK levels. UV light is known to stimulate ERK activity in certain cell types through a membrane-mediated mechanism [28], which may not be present in C2C12 cells. On the other hand, muscle cells can both produce and respond to cytokines [2,8,29]; thus it is not surprising that the C2C12 cell response to wounding is similar to that seen with cytokine treatment.

In this study, the activation of ERK was localized to the cells adjacent to the wound bed. These results reflect what has been shown in needle biopsies of vastus lateralis muscle tissue, where activation of the MAP kinase signaling molecules was restricted to the area of injured muscle [20]. On the other hand, we show that p38 activation was seen in cells adjacent to the wound bed as well as in unwounded cells further away. In exercised subjects, the increase in ERK phosphorylation was limited to the exercised muscle whereas the increase in p38 was also found in non-exercised, control muscle [30]. This suggests that the ERK response is mediated by local, injury-induced factors, whereas the p38 response is affected systemically [25,30]. Our results corroborate this hypothesis: conditioned medium from wounded cells is able to increase phosphorylated p38 levels but not ERK in unwounded cells. In addition, the immunofluorescence data show phospho-p38 staining in regions of unwounded cells further away from wounded areas, whereas coverslips with only intact cells showed

no staining. Phospho-ERK staining occurred primarily in the cells adjacent to the wound bed, but not in regions further away. This suggests that injured C2C12 cells release certain factor(s) which can act on unwounded cells to trigger p38 (but not ERK) phosphorylation. Injury to muscle tissue is known to trigger the release of various factors, including HGF [31], TNFα [32], LIF [3], and IL-6 [33], as well as to induce the upregulation of genes such as the thrombin receptor PAR-1 [34]. These factors are thought to regulate the progression of cell activation, migration, and differentiation that are part of the tissue regeneration process [1]. Further work needs to be done to characterize the identities and roles of the factors released by C2C12 cells upon physical injury.

To our knowledge, these findings are the first report of the upregulation of phosphorylated p38 and ERK after physical injury in muscle cells in vitro. The use of an in vitro system such as ours facilitates the isolation of responses specific to muscle cells (without contamination by surrounding tissue), as well as it enables the study of the effects of activators and inhibitors of specific signaling pathways in a controlled and reproducible manner. It would also be informative to perform these experiments using muscle cell lines derived from disease models, such as the mdx mouse. Characterization of the exact roles played by the MAPK signaling pathways in satellite cell migration, proliferation, and differentiation will be essential for understanding the regulation of the wound healing process in muscle. This may ultimately lead to the discovery of novel treatments for disorders of skeletal muscle regeneration.

Acknowledgments

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