Skeletal muscle satellite cell activation following cutaneous burn in rats

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1. Introduction

Severe burns are estimated to occur in over 1 million persons in the United States annually and constitute about 5–10% of all warfare military casualties [1, 2]. A persistent hypercatabolic state and concomitant skeletal muscle atrophy is initiated after acute burn, remains elevated for several months following injury, and is considered a major complication that limits patient recovery [3–6].

Dramatic changes in physiology affect organs distal to the burn site, including skeletal muscle where significant decreases in mass and function are observed [7–10]. To date, the majority of studies directed toward understanding burn-induced skeletal muscle atrophy have focused on anabolic and catabolic processes at the tissue level with less attention being given to specific cell types that are involved. Satellite cells, adult stem cells located between the basal lamina and the plasmalemma of muscle fibers [11], play a key role in regulating skeletal muscle mass and are responsive to local and systemic changes due to a
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variety of muscle diseases and injuries (for review see Ref. [12]). Since their importance in skeletal muscle repair, regeneration, and disease is well established, they are a logical target for gaining a better understanding of cellular changes that occur in muscle following burn.

The majority of satellite cells in adult skeletal muscle reside in a quiescent state where they are primed to participate in skeletal muscle regeneration. Their activation, or exit from quiescence, following local injury enables the restoration and regeneration of skeletal muscle and has been well described in a wide variety of injury and hypertrophy models [13–16]. Given their anatomical location, it is intuitive that they are affected by focal skeletal muscle injuries, including muscle directly below burned skin [17]; however, their role in the regeneration of tissue distant from injury is less well characterized. Since their activation is substantially supported by a wide variety of studies highlighting their importance in a number of repair and hypertrophy models, a logical hypothesis is that their activation is diminished when atrophic conditions predominate. Conversely, several lines of evidence support increases in satellite cell activity in response to a number of cytokines that are elevated following acute burn [18–23].

In light of the potential role of satellite cells in regulating muscle mass following burn, it is interesting to consider the preferential atrophy and strength loss of fast-twitch muscles that occurs over slow-twitch muscles [24,25], despite a higher frequency of satellite cells in slow-twitch fiber types than fast-twitch [26,27]. These concepts present the possibility that fiber-type specific changes following burn may have a satellite cell component. Since skeletal muscle atrophy occurs following burn, the hypotheses of the current study were that satellite cell activation would be decreased in skeletal muscle following cutaneous burn, and that decreases in satellite cell activation would be greater in fast-twitch than slow-twitch skeletal muscle. Therefore, the purpose of this study was twofold: to characterize the activation of satellite cells in response to acute burn and to determine whether there are differences in satellite cell activation between slow- and fast-twitch muscles.

2. Methods

This study has been conducted in compliance with the Animal Welfare Act and the implementing Animal Welfare Regulations and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals and was conducted in the animal facility at the US Army Institute of Surgical Research. Rats were housed individually in a temperature-controlled environment with a 12-h light/dark cycle.

2.1. Burn

Male Lewis Inbred rats (approximately 350 g, Harlan®, Houston, TX) in both the sham and burn groups were anesthetized with 1.5–3% isoflurane (Forane®, Baxter Healthcare Corp., IL) in 100% oxygen using a nose cone, and then the dorsal and ventral surface of the trunks were shaved. Animals in the burn group received a 40% total burn surface area (TBSA) full thickness of burn by immersing the dorsum in 100 °C heated water for 10 s and ventral surface for 2 s according to the modified Walker–Mason burn model [28,29]. Burned rats were resuscitated with 20 ml of intraperitoneal Ringer’s lactate solution immediately following the burn. Both sham and burn animals received buprenorphine (0.05 mg/kg, q 12 h subcutaneously) for 24 h after injury.

2.2. Satellite cell isolation and culture

Satellite cells were isolated similar as described by Allen et al. [30]. Following euthanasia, muscles were minced and digested for 1 h at 37 °C with 1.25 mg/ml of protease type XIV (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) (pH 7.2). Digested material was centrifuged at 1500 × g for 5 min and the supernatant was discarded. The pellet was triturated, resuspended in PBS, and centrifuged at 500 × g for 8 min after which the supernatant was retained. Trituration of the pellet, resuspension in PBS, centrifugation, and supernatant retention were repeated using centrifugations at 500 × g for 5 and 1 min. The supernatants were pooled, centrifuged at 1500 × g for 5 min, and pre-plated on a tissue culture–treated dish for 2 h in a pre-plate medium [Dulbecco’s modified eagle medium (DMEM) containing 10% horse serum, 1% antibiotic–antimycotic mixture, and 0.5% gentamicin, pH 7.2)] (Invitrogen, Grand Island, NY) to increase myogenic purity. Non-adherent cells were seeded on chamber slides as described below and cultured in growth medium (F-10 medium containing 20% fetal bovine serum, 1% antibiotic-antimycotic mixture, and 0.5% gentamicin, pH 7.2) in a humidified incubator with 5% CO2 at 37 °C. This methodology has been capable of achieving high levels of myogenic purity [30,31]. To confirm the purity of satellite cell cultures, a subset of cells from sham and burn animals was seeded on chamber slides and the percentage of cells positive for MyoD and Pax7 was determined 48 h after plating using a mouse monoclonal anti-MyoD antibody (BD Pharmingen, San Diego, CA) or mouse monoclonal anti-Pax7 (Developmental Studies Hybridoma Bank, University of Iowa) a fluorescence conjugated anti-mouse antibody (1:200, NL-557 anti-mouse IgG, NorthernLights™, R&D Systems®), Minneapolis, MN), and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) labeling to identify nuclei.

2.3. Satellite cell activation in vivo

The determination of satellite cell activation in vivo was performed similar to that previously described [18,31,32]. Thirty-two hours after burn, both burn and sham rats were injected with bromodeoxyuridine (BrdU, 50 mg/kg; Sigma, St. Louis, MO) intraperitoneally to label activated satellite cells in vivo. Sixteen hours later, predominantly fast-twitch muscles [tibialis anterior (TA), extensor digitorum longus (EDL), plantaris (PL), and gastrocnemius (GJ)] (n = 3) or a slow-twitch muscle (soleus, SOL) (n = 3) were weighed and satellite cells isolated from them as described above. One-tenth of the final volume of cell suspension from fast (500 μl) and slow (100 μl) muscles was plated in two wells of Matrigel™-coated (0.1 mg/ml, BD Bioscience, San Jose, CA) two-well chamber slides in GM (n = 6 wells per condition). The entire well was counted to determine satellite cell yield and expressed per gram of tissue. Cells were fixed 24 h later for BrdU detection as described below.
2.4. **Determination of MyoD+ and Pax7+ satellite cell number in muscle fibers**

Two days after burn, EDL and soleus muscles from sham (n = 3) or burn rats (n = 3) were dissected, prepared in OTC medium, and snap-frozen in liquid nitrogen following immersion in cold methylbutane. Cross sections (8 μm) from the mid-belly of muscles were cut using a cryostat (−20°C) for immunohistochemical detection of Pax7 and MyoD. Cryosections were fixed in 4% paraformaldehyde for 10 min, incubated in 0.3% Triton X-100/PBS for 15 min, and blocked (5% donkey serum in PBS) for 1 h at room temperature. After three washes in PBS, sections were incubated overnight at 4°C with mouse monoclonal anti-MyoD (1:50 dilution, BD Pharmingen, San Diego, CA) or mouse monoclonal anti-Pax7 (1:20 dilution, Developmental Studies Hybridoma Bank, University of Iowa) together with rabbit polyclonal anti-laminin (1:100 dilution, Abcam, Cambridge, MA) for double staining. Following three washes in PBS, the sections were incubated with fluorescence conjugated secondary antibody (NL-493 anti-rabbit IgG and NL-557 anti-mouse IgG at 1:200 dilution, NorthernLights™, R&D Systems®, Minneapolis, MN). After three PBS washes sections were mounted with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, Vector Shield Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA). The cells labeled for MyoD or Pax7 and nuclei were quantified in three sections for each muscle. The total number of MyoD or Pax7 positive cells or nuclei within the laminin positive membrane was counted and is expressed per 100 muscle fibers.

2.5. **In vitro activation assay**

The effect of circulating factors on satellite cell activity was determined by evaluating BrdU incorporation in response to serum treatment. The whole blood of either burn or sham rats was collected in serum collection tubes (BD Bioscience, San Jose, CA), and the serum was separated by centrifugation at 2500 × g for 20 min at 4°C and collected and stored at −70°C. The satellite cells isolated from quadriceps, gastrocnemius, plantaris, and biceps femoris muscles were plated at Matrigel™-coated eight-well chamber slides in growth medium with 20% serum from burn or sham burn rats. Satellite cells were pulsed with 20 nM BrdU (20 mM) for the final 2 h (46–48 h) and BrdU immunocytochemistry performed as described below.

2.6. **Immunofluorescence staining and microscopy**

For BrdU staining, the cells were fixed with 4% paraformaldehyde for 20 min, denatured with 2N hydrochloride in PBS for 30 min at 37°C, and blocked (5% donkey serum in PBS) for 1 h at room temperature. Fixed cells were incubated with monoclonal anti-BrdU antibodies (1:50 dilution, Novus Biologicals, Littleton, CA) at 4°C overnight. Following three washes in PBS, the cells were incubated with secondary fluorescence conjugated anti-mouse antibody (1:200, NL-557 anti-mouse IgG, NorthernLights™, R&D Systems®, Minneapolis, MN). The total number of cells and BrdU positive cells from both fast and slow muscles were then counted and expressed as a percentage of the total number of cells as determined with light and fluorescence microscopy.

2.7. **Statistics**

All data are expressed by mean ± standard error of mean. Two-way analysis of variance was used to analyze differences between groups. Student’s t-test was used to analyze differences between groups at single time points where appropriate. Significance was established at p < 0.05.

3. **Results**

3.1. **Muscle weight**

Similar to our previous observations indicating a atrophy of fast-twitch muscles following burn [7], the absolute muscle weights of individual fast-twitch muscles (EDL, G + PL) and the total weight of the EDL, TA, and G + PL (from both sides), were significantly lower in burned rats compared to those of sham rats at day 2 after burn (p < 0.05; Table 1). The absolute muscle

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<th>Table 1 – Muscle weights 2 days after burn.</th>
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<td><strong>Group</strong></td>
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<td><strong>Muscle weight (g)</strong></td>
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<td><strong>Burn</strong></td>
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<td><strong>Muscle weight/body weight (g/kg)</strong></td>
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The absolute muscle weights of the extensor digitorum longus (EDL), gastrocnemius and plantaris (G + PL), and combined EDL, tibialis anterior (TA), and G + PL (from both sides) isolated from burn animals 2 days after injury were significantly less than those isolated from sham (p < 0.05). The absolute weights of the slow-twitch soleus muscles and TA muscles isolated from burn animals were not different from those isolated from sham animals. The normalized muscle weights (absolute muscle weight/body weight) of the EDL, TA, G + PL, and combined EDL, TA, and G + PL (from both sides) isolated from burn animals 2 days after injury were significantly less than those isolated from sham animals (p < 0.05). The normalized muscle weights of the slow-twitch soleus muscles isolated from burn animals and were not different than soleus muscles isolated from sham animals. All values are mean ± standard error of the mean.

*Statistical difference between burn and sham for fast muscles.
weights of the SOL and TA were not different between burn and sham rats (Table 1). The normalized muscle weights of the individual fast-twitch muscles (EDL, G + PL) and the total weight of the EDL, TA, and G + PL (from both sides), were significantly lower in burned rats compared to those of sham rats at day 2 after burn (p < 0.05; Table 1). The normalized muscle weights of the SOL were not different between burn and sham rats (Table 1).

3.2. Satellite cell yield

The myogenic purity of the cell preparation was greater than 90% as determined by MyoD and Pax7 immunostaining in companion cultures, which is in agreement with a number of studies using this methodology [30,31,33] (Fig. 1). The satellite cell yield per gram of muscle tissue was significantly greater in fast muscles in burn than sham (p < 0.05; Fig. 1). Conversely,
there were no significant differences in satellite cell yield from the slow muscle between burn and sham rats. The average satellite cell yield per gram of tissue for fast muscles was 1226 ± 128 and 2948 ± 318 cells for sham and burn, respectively. The average satellite cell yield per gram of tissue for slow muscles was 12,493 ± 909 and 12,733 ± 678 for sham and burn, respectively. These findings caused us to speculate that satellite cell activation may be increased in vivo in fast-twitch muscles with burn treatment.

3.3. Satellite cell activation in vivo

In agreement with the increase in satellite cell yield that we observed, satellite cell activation as determined by BrdU incorporation during in vivo exposure was 29% higher in fast muscles in burn than in sham, and there was no difference in slow muscles between burn and sham rats (p < 0.05; Fig. 2). Importantly, the satellite cell activation in vivo using BrdU incorporation in sham cells is similar to that reported in control muscle by others using a similar experimental design [18,32]. To determine whether satellite cell number was increased in vivo, presumably a result of prior activation, cross sections were stained for Pax7, which is a well-known marker for quiescent, activated, and proliferating satellite cells but not for myonuclei [34,35]. A higher incidence of satellite cells in the soleus muscle compared to the EDL as determined with Pax7 in the current study is consistent with an increased frequency of satellite cells in slow versus fast skeletal muscles [26,27]. In agreement with increased cell yield and in vivo BrdU incorporation, we observed a 3.2-fold increase in the number of cells per fiber that express Pax7 in the fast muscles from burn animals as compared to sham; however, there was no difference in the percentage of Pax7-positive cells in the slow muscle between burn and sham (p < 0.05; Fig. 3). As a final verification of satellite cell activation, the percentage of cells expressing the muscle regulatory factor MyoD was determined as an indication of activation [36,37]. In agreement with our other findings, there was a 2.7-fold increase in the number of cells per fiber that express MyoD in the fast muscles from burn animals as compared to sham; however, there was no difference in the percentage of MyoD-positive cells in the slow muscle between burn and sham (p < 0.05; Fig. 4). The low sample size could be considered a limitation to these findings, however, the data presented here for sham muscles is consistent with previous measurements of MyoD and Pax7 positive cells per fiber under control conditions in other studies [38,39]. Collectively, satellite cell activation was increased in fast, but not in slow, muscles of burned but not sham rats using several accepted methods for determining satellite cell activation.

3.4. Effects of burn serum on satellite cell activation

Within each serum treatment, there was no difference between satellite cells isolated from sham or burn animals; however, for this experiment, no attempt was made to separate muscle types and various muscle types were pooled. Interestingly, the treatment of satellite cells with burn serum significantly increased satellite cell activation as compared to treatment with sham serum, regardless of whether satellite cells were isolated from sham or burn animals (p < 0.05; Fig. 5).

4. Discussion

Severe burn causes sustained loss of lean body mass and skeletal muscle atrophy, which, in addition to the burn wound and hypertrophic scarring, is one of the major complications that hampers recovery from the injury. The impact of burn on skeletal muscle mass and persistence of muscle atrophy has been well characterized, and the influence of satellite cells in regulating muscle size has been under investigation for decades. Accordingly, the purpose of this study was to characterize the effect of burn on the first step in the progression of satellite cells for muscle repair; namely, satellite cell activation.

Since satellite cell activation accompanies hypertrophy and repair following injury, a logical hypothesis is that in the context of burn satellite cell activation is diminished since skeletal muscle atrophy is present. However, in the current study, four separate observations support the notion that satellite cell activation in distal limb muscles is increased following burn to the trunk area, despite rapid onset of skeletal muscle atrophy. The first observation that caused us to speculate that differences in satellite cell activation may exist was the increased cell yield from burn animals (Fig. 1). The association between increased satellite cell activity and increased yields have been previously reported [40]. This initial observation was followed up with an additional
Fig. 3 – Burn increases the expression of MyoD extensor digitorum longus (EDL), but not soleus muscles. Soleus (A–F) and EDL (G–L) muscles from sham (A–C, G–I) or burn (D–F, J–L) were isolated 2 days after burn to quantify MyoD expression (M). All nuclei appear blue due to labeling by DAPI, the basal laminae appears green due to labeling by anti-laminin, and satellite cells expressing MyoD are labeled in red due to labeling by anti-MyoD. The mean number of MyoD+ cells per 100 fibers is increased with burn for the EDL but not the soleus muscles (M). For all figures, arrowheads denote activated satellite cells (MyoD+/DAPI+) within the basal lamina. Boxed insets show magnified views of the area indicated by the white box. Bars represent mean ± standard error of the mean for culture wells per group. Bar = 50 μM. *Statistical difference between burn and sham for fast muscles.

The hypermetabolic response causing sustained muscle protein catabolism and increase in myonuclear apoptosis following burn implies that a deterioration of skeletal muscle cell homeostasis may contribute post-burn muscle atrophy [41–43]. Evidence that the response is specific to a change in the muscle microenvironment can be found in the observation that all of the indices of increased activation occurred in the fast muscles, where the majority of atrophy occurs. Skeletal muscle fibers contain post-mitotic nuclei and rely on satellite cells as the major myogenic source to replace lost myonuclei following muscle injury and disease. It then follows that increased satellite cell activation may be an acute response to prepare satellite cells to replace deteriorating myonuclei. Regardless, this expansion of myogenic cells is insufficient, at least initially, to inhibit the rapid onset of atrophy in fast-twitch fibers. Of the few other conditions where increased satellite cell activation occurs during atrophic conditions, the sequelae of events following nerve transaction is the best characterized. Nerve transaction results in an initial increase in the activation of satellite cells; however, myonuclear apoptosis contributes to an eventual decrease in myonuclear number and overall depletion of the myogenic machinery required to sustain muscle size [44]. Although it was beyond the scope of the current study to investigate the long-term changes that result from an increased activation, a logical hypothesis is that alterations of satellite cell functionality subsequent to the initial activation occur. For example, a plausible hypothesis is that satellite cell proliferation and differentiation may be decreased and inhibit the ability of satellite cells to effectively contribute to muscle mass maintenance. The observation that the proliferative activity of myoblasts is diminished in the TA muscle supports this idea [45].

It is noteworthy to consider the anatomical distinction between the location of the burn and our current observations. Previous morphological and genetic changes in skeletal muscles distal to the afflicted area have been reported [10]. The systemic changes subsequent to burn affect a myriad of other tissues and organs, including skeletal muscle. For example, increased cell proliferation has been reported in the liver and small bowel concomitant with acute damage and
atrophy of these organs [46,47]. A possibility is that satellite cell activation is the result of a general response to trauma that causes the elevation of a vast array of cytokines involved in multi-organ adaptations. For example, changes in the levels of hepatocyte growth factor (HGF), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and insulin-like growth factor 1 (IGF-1) occur after burn [19,20], all of which play different roles for satellite cell activation, proliferation, differentiation, and

Fig. 4 – Burn increases the expression of Pax7 in the extensor digitorum longus (EDL), but not soleus muscles. Soleus (A–F) and EDL (G–L) muscles from sham (A–C, G–I) or burn (D–F, J–L) were isolated 2 days after burn to quantify Pax7 expression (M). All nuclei appear blue due to labeling by DAPI, the basal laminae appears green due to labeling by anti-laminin, and satellite cells expressing Pax7 are labeled in red due to labeling by anti-Pax7. The mean number of Pax7+ cells per 100 fibers is increased with burn for the EDL but not the Soleus muscles (M). For all figures arrowheads denote satellite cells (Pax7+/DAPI+) within the basal lamina. Boxed insets show magnified views of the area indicated by the white box. Bars represent mean ± standard error of the mean for culture wells per group. Bar = 20 μM. *Statistical difference between burn and sham for fast-twitch muscles.

Fig. 5 – Burn serum increases satellite cell activation in vitro. Satellite cells isolated from quadriceps, gastrocnemius, plantaris, and biceps femoris of sham (A, B) or burn rats (C, D) were treated with media containing either 20% serum from sham (A, C) or burn (B, D) animals. Brdu was pulse-labeled between 46 and 48 h after seeding. Immunofluorescent detection of Brdu was accomplished with anti-BrdU (red; A–D), quantified, and expressed as a percentage of all cells as determined with light microscopy. Burn serum significantly increased the activation of satellite cells derived from both sham and burn animals. Forty-six to 48 h after seeding and express was used to estimate satellite cell activation. Bar = 100 μM. *Statistical difference between burn and sham for fast muscles.
apoptosis [18,21–23]. Thus, it is likely that a balance of these factors ultimately controls the temporal cascade of satellite cells in skeletal muscle following burn. Although specific cytokines were not investigated, the net outcome of these factors was an increase in activation as determined by the application of burn serum to satellite cell cultures (Fig. 5). Since the effects of serum on satellite cell activation were in vitro observations, they may not be representative of changes in the activation of satellite cells residing in their niche. Therefore, the influence of other factors within the cellular microenvironment may be overlooked. Nonetheless, a logical speculation is that HGF modulates satellite cell activation following burn. This possibility is based on previous observations of HGF increases following burn and the well-characterized role for HGF in modulating satellite cell activation [18,20,48]. As stated above, since this study investigated only the initial cellular response in the acute phase following burn, the long-term outcomes remain to be determined. Whether this balance is modified at later time points to influence the progression of satellite cells is not known. Satellite cells in the skeletal muscles, as well as probably all the progenitor cells in different organs, may be capable of contributing to regenerative process acutely after injury but may fail if the intensity and duration of injury are enhanced.

Currently, there is a significant void in the understanding of the myogenic cell response within skeletal muscle following burn. It is well accepted that the coordinated regulation of skeletal muscle regeneration includes an interaction among several cell and tissue types. Although the focus of the current study was specifically directed toward understanding the role of the satellite cell, the potential for other cell types to play a role in atrophy following burn should not be disregarded.

In conclusion, we demonstrate that severe burn induces an increase in satellite cell activation in hindlimb fast-twitch muscles where there is atrophy, but not in slow-twitch muscles. Increased activation with burn serum suggests that systemic factors are responsible for changes in myogenic cells distant from the burn area. A better understanding of the impact of burn on satellite cell functionality will allow us to identify the cellular mechanisms of long-term muscle atrophy after burn and potentially therapeutic targets.

Conflict of interest

The authors declare no conflict of interest.

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