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Original article

Time-dependent gene expression analysis after mouse skeletal muscle contusion

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Abstract

Background: Though the mechanisms of skeletal muscle regeneration are deeply understood, those involved in muscle contusion, one of the most common muscle injuries in sports medicine clinics, are not. The objective of this study is to explore the mechanisms involved in muscle regeneration after contusion injury.

Methods: In this study, a total of 72 mice were used. Eight of them were randomly chosen for the control group, while the rest were subjected to muscle contusion. Subsequently, their gastrocnemius muscles were harvested at different time points. The changes in muscle morphology were assessed by hematoxylin and eosin (HE) stain. In addition, the gene expression was analyzed by real-time polymerase chain reaction.

Results: The data showed that the expression of many genes, i.e., specific markers of immune cells and satellite cells, regulatory factors for muscle regeneration, cytokines, and chemokines, increased in the early stages of recovery, especially in the first 3 days. Furthermore, there were strict rules in the expression of these genes. However, almost all the genes returned to normal at 14 days post-injury.

Conclusion: The sequence of immune cells invaded after muscle contusion was neutrophils, M1 macrophages and M2 macrophages. Some *CC* (*CCL2*, *CCL3*, and *CCL4*) and *CXC* (*CXCL10*) chemokines may be involved in the chemotaxis of these immune cells. *HGF* may be the primary factor to activate the satellite cells after muscle contusion. Moreover, 2 weeks are needed to recover when acute contusion happens as used in this study.

Keywords: Chemokines; Contusion; Cytokines; Gene; Macrophages; Satellite cells; Skeletal muscle

1. Introduction

Muscle injuries are common musculoskeletal problems. The mechanisms of skeletal muscle recovery are becoming clearer recently. The healing process of injured skeletal muscle consists of three distinct phases: degeneration and inflammation, regeneration, and fibrosis.[1–3] The first phase is characterized by local swelling at the injury site, formation of hematoma, necrosis of muscle tissue, degeneration, and inflammatory response. The second phase includes phagocytosis of the damaged tissue and regeneration of the injured muscle. And the final phase is characterized by scar tissue formation if the injury is serious.

The first phase usually consists of the infiltration of activated macrophages and neutrophils into the injured tissue. Many chemokines may play important roles in the chemotaxis of these immune cells.[4,5] In the regeneration phase, many growth factors can regulate the activation, proliferation, and differentiation of satellite cells, which are necessary for the regeneration of injured skeletal muscles.[6]

As we mentioned above, there is an appreciable understanding of the mechanisms of skeletal muscle regeneration among researchers. However, our understanding in this domain has been limited to the injury models of toxicant injection, freeze-induced injury, burn, disuse muscle atrophy, mdx mice, *etc*.[7–10] As a result, the mechanisms involved in muscle contusion, one of the most common muscle injuries in sports medicine clinics, are still not fully understood. Therefore, in the present study, we construct a model of skeletal muscle contusion with the objective of exploring the mechanisms involved in muscle regeneration. At different time points post-injury, we studied many genes expression

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such as the specific markers of neutrophils (*MPO*),[11] M1 macrophages (*CD68*) and M2 macrophages (*CD163*),[3,12] proliferation (*MyoD*), and differentiation (*myogenin*)[12–14] of satellite cells. Moreover, we examined the mRNA levels of inflammatory cytokines (i.e., *TNF-α*, *IL-1β*, *IL-6*, and *IL-10*) and chemokines (i.e., *CCL2*, *CCL3*, *CCL4*, and *CCL8*; *CXCL9*, *CXCL10*, and *CXCL12*).[3,5] We also analyzed the transcript levels of some regulatory factors which play important roles in satellite cell activation and muscle regeneration (i.e., *HGF*, *uPA*, *IGF-I*, *MGF*, and *myostatin*).[15–19]

2. Methods

2.1. Mice

Seventy-two C57BL/6 male mice (weighing 18.2–22.9 g, purchased from Shanghai Lab. Animal Research Center, Shanghai, China) were provided food and water ad libitum and maintained on a 12 h:12 h light–dark cycle. Eight mice were randomly chosen for the uninjured control group ($n = 8$), while the rest were subjected to muscle contusion ($n = 64$). In preparing the mice for muscle injury induction, they were anesthetized with 400 mg/kg chloral hydrate administered intraperitoneally. The study was approved by the Ethics Review Committee for Animal Experimentation of Shanghai University of Sport.

2.2. Contusion model

A simple and reproducible muscle contusion model in mice was used.[20,21] The animals' hind limbs were positioned on a board, dorsiflexing the ankle to 90°. A 16.8 g (diameter 15.9 mm) stainless steel ball was dropped from the height of 100 cm through a tube (interior diameter of tube:16 mm) onto an impactor[20] resting with a surface of 28.26 mm^2 on the middle of the gastrocnemius muscle of the mouse. The instantaneous force delivered by a falling object with these characteristics was calculated to equal 0.58 $N{\cdot}m/cm^2$, where 1 N·m is equal to the force of an object weighing 100 g falling over a distance of 1 m.[22] The muscle contusion created by this method was a high-energy blunt injury that created a large hematoma and was followed by massive muscle regeneration,[20,23] healing processes that are very similar to those seen in humans.[24] The mice that had bone fracture (fracture rate of 2.7%) were foreclosed. The injured mice in this study had signs of unrelieved pain such as piloerection of fur, reluctance to ambulate, overgrooming of the injured limb, and abnormal gait or posture.[25] At different time points (6 h, 12 h, 1, 3, 5, 7, 14, and 21 days) post-injury, the mice were killed by cervical dislocation while under anesthesia and then gastrocnemius muscles were harvested.

2.3. Histology

At the time points of 1, 3, 7, 14, and 21 days post-injury, the right gastrocnemius muscles were collected and embedded in paraffin. Cross sections were cut 8 μm from the midbelly of each gastrocnemius muscle and were stained with hematoxylin and eosin (HE) for morphological analysis. Using a 40 lens objective, images were captured for each muscle section (Labphot-2; Nikon, New York, NY, USA).

2.4. RNA extraction and cDNA synthesis

Approximately 60 mg of tissue (from the middle of the left gastrocnemius muscle) was homogenized using an Ultra-Turrax homogenizer (IKA, Staufen, Germany) in a solution of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated using a modified guanidiniumisothiocyanate-CsCl method,[26,27] and the concentration and purity were determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer (NanoDrop 2000; Thermo Scientific, Wilmington, MA, USA). Total RNA was reverse transcribed into cDNA using the Revertaid™ First Strand cDNA Synthesis Kit from Fermentas (Fermentas, Vilnius, Lithuania). cDNA was synthesized using 2 μg of total RNA, 0.2 μg of random primers, 20 mmol/L dNTP mix, 5 × reaction buffer (Fermentas), 20U RiboLock™ RNase Inhibitor and 200 U of Revertaid™ M-MuLVreverse transcriptase in a total volume of 20 μL. The reaction was carried out at 25°C for 5 min followed by another 60 min at 42°C and was terminated by the deactivation of the enzyme at 70°C for 5 min. Control reactions lacking either reverse transcriptase or template were included to assess carry-over of genomic DNA and non-specific contamination.[28,29]

2.5. Real-time polymerase chain reaction (PCR)

Quantitative PCR was carried out in triplicate in reactions consisting of 12.5 μL 2 × Maxima SYBR Green/ROX qPCR Master mix (Fermentas), 1 μL cDNA, nuclease-free water and 300 nmol/L of each primer. Primer specifications are listed in Table 1. Amplifications were performed on a Rotor-Gene 3000 thermal cycler (Corbett, Sydney, Australia) with the following parameters: activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. The threshold cycle (CT, the number of cycles to reach threshold of detection) was determined for each reaction, and the levels of the target mRNAs were quantified relatively to the level of the housekeeping gene *GAPDH* using $2^{-\triangle\triangle CT}$ method.[30]

2.6. Statistical analysis

All values are expressed as mean ± SD, and statistical significance was set at $p < 0.05$. Mean values were compared between groups by one-way ANOVA with the Bonferroni method as a *post hoc* test, or non-parametric Kruskal–Wallis test. Data were analyzed using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Muscle morphology

HE stain was used to assess changes in muscle morphology after injury. On Day 1 post-injury, cross sections of gastrocnemius muscles showed substantial fiber damage and edema, and a large number of inflammatory cell infiltration. On Day 3 post-injury, a small quantity of centronucleated myofibers were observed. On Days 7 and 14 post-injury, central nucleation phenomenon became more pronounced. On Day 21 post-injury, central nucleation almost disappeared. Since centrally nucleated myofibers are a sign of regeneration in injured muscle,[19] it means