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Macrophage depletion by clodronate liposome attenuates muscle injury and inflammation following exhaustive exercise

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ABSTRACT

Exhaustive exercise promotes muscle injury, including myofiber lesions; however, its exact mechanism has not yet been elucidated. In this study, we tested the hypothesis that macrophage depletion by pretreatment with clodronate liposomes alters muscle injury and inflammation following exhaustive exercise. Male C57BL/6J mice were divided into four groups: rest plus control liposome ($n=8$), rest plus clodronate liposome ($n=8$), exhaustive exercise plus control liposome ($n=8$), and exhaustive exercise plus clodronate liposome ($n=8$). Mice were treated with clodronate liposome or control liposome for 48 h before undergoing exhaustive exercise on a treadmill. Twenty-four hours after exhaustive exercise, the gastrocnemius muscles were removed for histological and PCR analyses. Exhaustive exercise increased the number of macrophages in the muscle; however, clodronate liposome treatment reduced this infiltration. Although exhaustive exercise resulted in an increase in injured myofibers, clodronate liposome treatment following exhaustive exercise reduced the injured myofibers. Clodronate liposome treatment also decreased the mRNA expression levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the skeletal muscle after exhaustive exercise. These results suggest that macrophages play a critical role in increasing muscle injury by regulating inflammation.

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1. Introduction

Skeletal muscle injury is caused by execution of prolonged exercise, such as running a marathon. Muscle injury not only induces muscle soreness, but also reduces exercise performance as a result of muscle fatigue [1]. Several studies in humans indicate that prolonged exercise causes skeletal muscle injury, as evidenced by an increase in the serum levels of intracellular cytosolic enzymes such as creatine kinase (CK) and lactate dehydrogenase (LDH) [2–4]. As described by McNeil et al. [5], histological analysis demonstrated that eccentric exercise, such as downhill running, caused myofiber lesions, including membrane damage [5]. Similarly, Malaguti et al. [6] reported that exhaustive exercise not only increased CK and LDH levels, but also induced myofiber structure lesions in rats [6]. These findings show that exhaustive exercise can promote muscle injury, including myofiber structure lesions. However, the mechanism of exhaustive exercise-induced muscle injury has not yet been elucidated. Our previous studies showed that pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and monocyte chemoattractant protein (MCP)-1, were secreted after prolonged exercise in humans [7,8]. Similarly, several studies showed that exhaustive exercise increased mRNA level and protein concentration of tumor necrosis factor (TNF)-α in rat skeletal muscle [9,10]. Interestingly, Su et al. [11] reported that non-steroidal anti-inflammatory drugs were effective at reducing serum CK level following downhill running [11], suggesting that inflammation may be of central importance in the induction of exercise-induced muscle injury.

Macrophages secrete pro-inflammatory cytokines and contribute to induction of muscle inflammation [12], and macrophage-mediated inflammation plays a major role in muscle injury. However, following injection of clodronate-encapsulated liposome, macrophage-depleted mice showed significantly less skeletal muscle injury and inflammation caused by freeze treatment [13]. Moreover, Wehling et al. [14] reported that depletion of macrophages by anti-F4/80 antibody injections decreased myofiber injury, including membrane lesions of dystrophin-deficient mice [14]. As observed with several muscle injury models, including cardiotoxin injection, macrophage infiltration occurs in

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injured muscle after eccentric exercise, such as downhill running [15,16]. Similarly, Malaguti et al. [17] reported that exhaustive exercise increased the number of mononuclear cells, including macrophages, in rat skeletal muscle [17]. Therefore, the exhaustive exercise-induced infiltration of macrophages is an important factor in the development of muscle injury; however, it has not been demonstrated whether macrophages can promote muscle injury following exhaustive exercise. Here, we tested the hypothesis that macrophage depletion by injection of clodronate liposome affects muscle injury and inflammation following exhaustive exercise, and demonstrated that macrophages are involved in exercise-induced muscle damage.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice ($n=32$) were purchased from Kiwa Laboratory Animals (Wakayama, Japan) at 9 weeks of age, and four mice were housed together in one cage in a controlled environment under a light–dark cycle (lights on at 9:00 and off at 21:00). The experimental procedures followed the Guiding Principles for the Care and Use of Animals in the Waseda University Institutional Animal Care and Use Committee, and were approved (2013-A110). All mice were randomly divided into four groups: rest plus control liposome (R, $n=8$), rest plus clodronate liposome (RC, $n=8$), exhaustive exercise plus control liposome (E, $n=8$), and exhaustive exercise plus clodronate liposome (EC, $n=8$). Mice in all groups were allowed food *ad libitum*.

2.2. Injection of clodronate-encapsulated liposome

Clodronate liposome and control liposome were purchased from FormuMax Scientific (Sunnyvale, CA, USA). Clodronate liposome or control liposome (150 μL) was intraperitoneally injected 48 h before exercise.

2.3. Exercise protocol

Before acute exhaustive exercise, all mice were initially acclimated to running on a motorized treadmill (Natsume, Kyoto, Japan) at 20 m/min, 0% grade, for 20 min/day for 1 week. On the day of the experiment, mice were placed on a treadmill at 7% slope and the speed was increased to 24 m/min. The speeds used for determination of exhaustion time were 10 m/min for 15 min, followed by 15 m/min and 20 m/min for 15 min each, and then 24 m/min until exhaustion. Exhaustion was defined as the point at which a mouse refused to run despite being given the shock grid five times. Mice were removed from the belt upon exhaustion as judged by their inability to remain on the belt. The mean running time to exhaustion for the E and the EC groups was 161.0 ± 14.2 and 181.7 ± 13.8 min, respectively. No significant difference in running time to exhaustion was observed between the E and EC groups.

Mice were sacrificed under light anesthesia with inhaled isoflurane (Abbott, Tokyo, Japan) 24 h after the exhaustive exercise. The gastrocnemius was promptly removed, frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.4. Histological analysis

A portion of the gastrocnemius was orientated on pieces of cork and secured with gum tragacanth, and then snap frozen by immersing the samples in pre-cooled isopentane at −80 °C. Immunofluorescence staining was applied to frozen gastrocnemius sections in order to examine the expression of F4/80. The 6-μm serial sections were incubated in 4% paraformaldehyde. F4/80 (Abcam, Cambridge, MA, USA) and Dystrophin (Abcam) primary antibodies were added to 1% BSA solution and then transferred to the sections being incubated. Secondary Alexa Fluor 555 Goat Anti-Rat IgG (Life Technologies, Carlsbad, CA, USA) and Alexa Fluor 488 Goat Anti-Rabbit IgG (Life Technologies) were dissolved in phosphate-buffered saline (PBS) and added to the sections being incubated. Antibodies were diluted to concentrations each below: F4/80 (20 μl/ml), Dystrophin (20 μl/ml), Alexa Fluor 555 Goat Anti-Rat IgG (10 μl/ml), and Alexa Fluor 488 Goat Anti-Rabbit IgG (10 μl/ml). PBS contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. The stained section was visualized by fluorescence microscopy (KEYENCE, Osaka, Japan), F4/80-positive cells were counted on four random high-power fields (200) per slide using BZ-2 software (KEYENCE), and the average value for each section was calculated. F4/80 positive cells were detected by visual judgement of the observer.

IgG staining was applied to frozen gastrocnemius sections to examine muscle-fiber membrane lesions using previously described methods, with some modifications [18]. Presence of IgG in the muscle-fiber cytosol indicates the presence of muscle-membrane lesions, including induction of cell-membrane permeability. The 6-μm serial sections were incubated in 1% BSA solution with IgG fluorescein isothiocyanate (FITC)-conjugated Mouse Anti-IgG (Vector, Burlingame, CA, USA) and Anti-Dystrophin primary antibody (Abcam). Secondary Alexa Fluor 488 Goat Anti-Rabbit IgG (Life Technologies) was dissolved in PBS buffer and added to the sections being incubated. Antibodies were diluted to concentrations each below: IgG FITC-conjugated Mouse Anti-IgG (15 μl/ml), Dystrophin (20 μl/ml), and Alexa Fluor 488 Goat Anti-Rabbit IgG (10 μl/ml). The stained section was visualized by fluorescence microscopy (KEYENCE), and the number of injured fibers showing muscle-fiber cytosolic fluorescence and total number of fibers were counted on four random high power fields (200) per slide using BZ-2 software (KEYENCE). IgG positive muscle fibers were detected by visual judgement of the observer.

2.5. Real-time quantitative PCR

Total RNA was extracted from the gastrocnemius homogenate using the RNeasy Fibrosis Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The purity of total RNA was assessed using the NanoDrop system (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was reverse transcribed to cDNA using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA), according to the manufacturer's instructions. PCR was performed with the Fast 7500 real-time PCR system (Applied Biosystems) using Fast SYBR Green PCR Master Mix (Applied Biosystems). The thermal profiles consisted of denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 3 s, and annealing at 60 °C for 15 s. The 18S ribosomal RNA was used as the housekeeping gene and all data were represented relative to its expression as fold change based on the values of the other genes plus the control liposome group. The specific PCR primer pairs for each gene are shown in Table 1.

2.6. Statistical analyses

All statistical analyses were performed using SPSS version 19.0 (IBM, Chicago, IL, USA). The statistical significance of differences between groups in the number of IgG- and F4/80-positive cells and mRNA expression was determined using two-way analysis of variance. If significant interactions were observed, comparisons were performed using Tukey's honestly significant difference post hoc test. The level of significance was set at $p < 0.05$.

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