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

Noriaki Kawanishi [a,b], Tsubasa Mizokami [c], Hiroyuki Niihara [c], Koichi Yada [c], Katsuhiko Suzuki [d,*]

[a] Institute for Nanoscience & Nanotechnology, Waseda University, Tokyo, Japan
[b] Research Fellow of the Japan Society for the Promotion of Sciences, Tokyo, Japan
[c] Graduate School of Sport Sciences, Waseda University, Tokorozawa, Saitama, Japan
[d] Faculty of Sport Sciences, Waseda University, 2-579-15 Mikajima, Tokorozawa, Saitama 359-1192, Japan

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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.

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

* Corresponding author.
E-mail addresses: kawanishinoriaki@akane.waseda.jp (N. Kawanishi), o-hisyo.w@akane.waseda.jp (T. Mizokami), h.niihara@akane.waseda.jp (H. Niihara), k.yada@aoni.waseda.jp (K. Yada), katsu.suzu@waseda.jp (K. Suzuki).

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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 $p < 0.05$.

Table 1
Primer sequences for real-time RT-PCR analysis.

Genes	Forward	Reverse
18S ribosomal RNA	CGGCTACCACATCCAAGGA	AGCTGGAATTACCGCGGC
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
MCP-1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
TNF-α	CCTCCCTCTCATCAGTTCTA	ACTTGGTGGTTTGCTACGAC
IL-1β	GGGCCTCAAAGGAAAGAATC	TTGGTTGGGATCCACACTCT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC

MCP; monocyte chemoattractant protein, TNF; tumor necrosis factor, IL; interleukin.

3. Results

3.1. Effects of macrophage depletion and exhaustive exercise on macrophage infiltration into the skeletal muscle

Macrophage infiltration, which is thought to play a critical role in muscle injury, was investigated by assessing the content of macrophages in the gastrocnemius muscle by F4/80 immunofluorescence staining. While exhaustive exercise increased the number of F4/80-positive cells (macrophages) in the gastrocnemius muscle, injection of clodronate liposome reduced this infiltration (Fig. 1A). The number of F4/80-positive cells was significantly higher in the E group as compared to the C group ($p < 0.01$). However, the F4/80-positive cells in the EC group were significantly lower than those observed in the E group ($p < 0.01$; Fig. 1B). Similarly, we found that the mRNA levels of F4/80 and MCP-1 were significantly increased in the E group as compared to the C group ($p < 0.01$); however, F4/80 mRNA levels were significantly lower in the EC group relative to the E group ($p < 0.01$; Fig. 1C). No significant difference in MCP-1 mRNA levels in the muscle was observed between macrophage depletion and non-depletion at 24 h post exercise (Fig. 1C).

3.2. Effects of macrophage depletion on exhaustive exercise-induced muscle injury

Sections of gastrocnemius muscle were immunolabeled with FITC-conjugated anti-mouse IgG to examine the presence of extracellular protein in the muscle-fiber cytoplasm. Presence of extracellular protein in the muscle-fiber cytoplasm indicates the

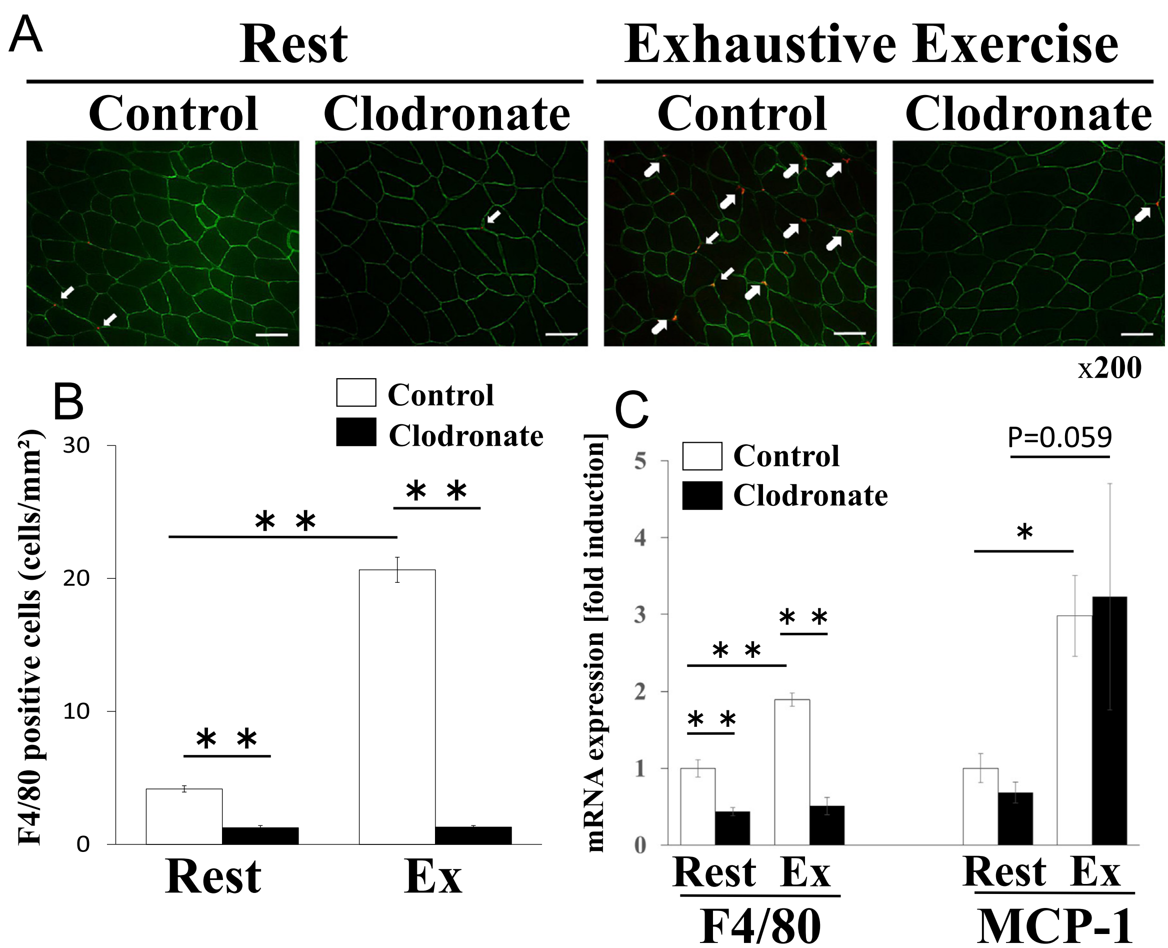

Fig. 1. Effects of exhaustive exercise and clodronate liposome treatment on macrophage infiltration in the skeletal muscle. (A) F4/80 immunofluorescence staining [red, F4/80-positive cells (arrows); green, dystrophin] of gastrocnemius muscle sections. Scale bar is 50 µm. (B) Number of F4/80-positive cells and (C) mRNA levels of F4/80 and MCP-1 in the gastrocnemius muscle. Values represent means ± SEM. * $p < 0.05$; ** $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

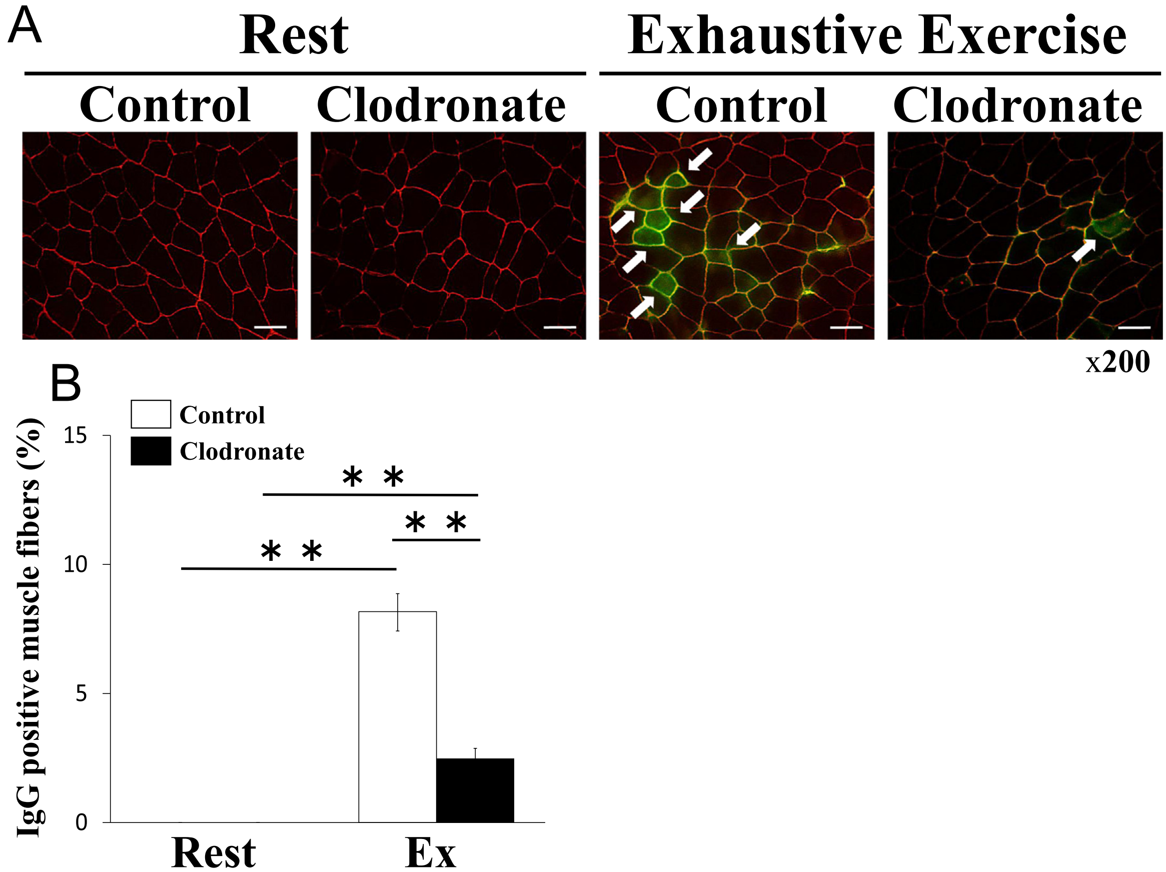

Fig. 2. Effects of exhaustive exercise and clodronate liposome treatment on muscle damage. (A) IgG immunofluorescence staining [green, IgG positive muscle fibers (arrows); red, dystrophin] of gastrocnemius muscle sections. Scale bar is 50 μm. (B) Number of IgG-positive cells. Values represent means ± SEM. $^{*}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

presence of muscle-fiber injury, including fiber-membrane lesions. The muscle fibers containing cytosolic IgG were not present in the R group (Fig. 2A), while the E group showed 8.2% IgG-positive muscle fibers (Fig. 2B). However, the percentage of IgG-positive muscle fibers in the EC group was significant lower than that observed in the E group ($p < 0.01$, Fig. 2B).

3.3. *Effects of macrophage depletion on muscle inflammation following exhaustive exercise*

TNF-α, IL-1β, and IL-6 are markers of muscle inflammation. The changes in these cytokine mRNA expression levels are shown in Fig. 3. The mRNA levels of TNF-α and IL-6 were significantly higher in the E group relative to those in the C group (TNF-α, $p < 0.05$; IL-6, $p < 0.01$). However, the mRNA levels of TNF-α, IL-1β, and IL-6 decreased significantly following injection of clodronate liposome (IL-6, $p < 0.05$; TNF-α and IL-1β, $p < 0.01$).

4. Discussion

Exhaustive exercise causes skeletal muscle injury and inflammation; however, the mechanism underlying exhaustive exercise-induced skeletal muscle injury and inflammation remains

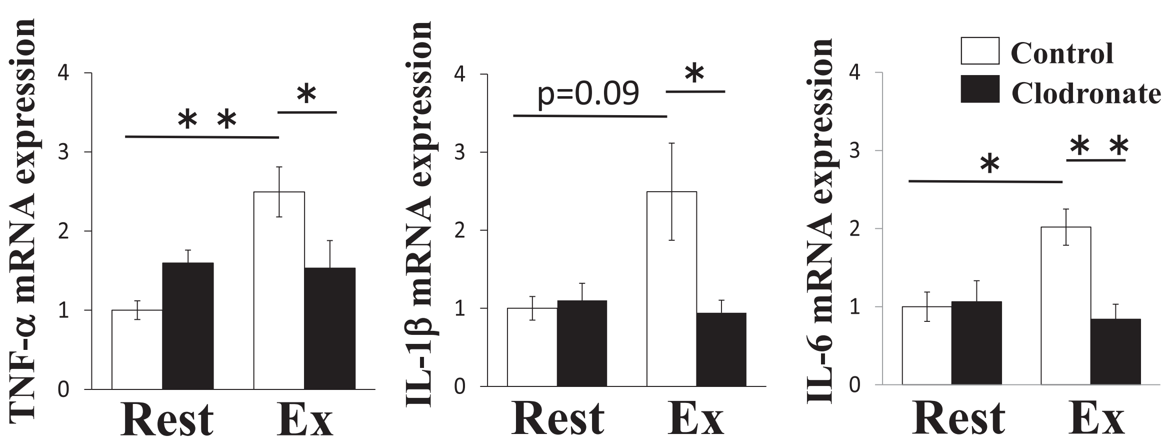

Fig. 3. Effects of exhaustive exercise and clodronate liposome treatment on pro-inflammatory cytokine response in skeletal muscle. The mRNA levels of TNF-α, IL-1β, and IL-6 in the gastrocnemius muscle. Values represent means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$.

unclear. We examined whether attenuated macrophage infiltration by clodronate liposome injection influenced muscle-fiber injury and inflammation that occurs during the first 24 h after exercise.

Macrophage infiltration into the skeletal muscle was observed early in the course of exercises such as downhill running and prolonged running [15,16]. We analyzed the number of macrophages in the skeletal muscle by immunofluorescence staining, and observed a 4.9-fold increase in macrophage infiltration into the muscle 24 h after exhaustive exercise. Additionally, F4/80 mRNA expression in the muscle was also enhanced by exhaustive exercise. Therefore, our results showed that macrophage infiltration was pronounced in the skeletal muscle after exhaustive exercise. A recent study reported that MCP-1 modulates the activation and infiltration of macrophages in the muscle, and that MCP-1-deficient mice show fewer infiltrating macrophages relative to control mice following cardiotoxin-induced muscle injury [19]. In this study, we observed that exhaustive exercise increased MCP-1 mRNA expression in the muscles. Therefore, the exhaustive exercise-induced elevation of macrophage infiltration in the muscle might be regulated by upregulation of MCP-1.

Recent findings showed that macrophages induce muscle injury through production of inflammatory cytokines. Although clodronate liposome has been frequently used to deplete macrophages from the muscle, clodronate liposome injection reduced injured myofibers and TNF-α mRNA levels in the muscle following freeze treatment [13]. In this study, we observed that injection of clodronate liposome before exhaustive exercise reduced macrophage content and F4/80 mRNA expression in muscle following exhaustive exercise. Thus, our clodronate liposome injection protocol provided an opportunity for reducing macrophages in the skeletal muscle after exhaustive exercise. Our findings from histological analysis of myofiber-membrane lesions with IgG staining demonstrated that myofiber injury occurred as late as 24 h after exhaustive exercise. Surprisingly, macrophage depletion by clodronate liposome injection resulted in decreases in IgG-positive fibers after exhaustive exercise. Therefore, our data strongly suggest that muscle injury by exhaustive exercise is attenuated when macrophage infiltration is blocked. We also observed that the content of F4/80-positive macrophages was correlated with the percentage of IgG-positive injured fibers ($r=0.886$; $p<0.01$). The findings from our macrophage depletion model support the hypothesis that macrophage infiltration into the skeletal muscle after exhaustive exercise regulates muscle injury.

Macrophages in the skeletal muscle induce inflammatory response through the production of pro-inflammatory cytokines [12], and excessive inflammatory response can directly injure myofibers [20]. In humans, the plasma concentrations of pro-inflammatory cytokines appears elevated after prolonged exercise [7,8]. Moreover, protein and gene expression of TNF-α were elevated in the muscle after exhaustive exercise in rats [9,10]. Interestingly, anti-inflammatory drug treatment reduced plasma levels of CK and LDH following eccentric exercise [11,21–23]. Therefore, it is possible that muscle injury by exhaustive exercise is influenced by mediators released from activated macrophages, including pro-inflammatory cytokines. In the present study, we found that macrophage depletion decreased the mRNA levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in the skeletal muscle after exhaustive exercise. Therefore, macrophage infiltration is likely to be a primary cause of local inflammation in the muscle following exhaustive exercise. This study also showed that alteration in pro-inflammatory cytokine mRNA levels in the muscle was similar to the altered pattern of injured myofibers. Therefore, induction of inflammation by macrophage infiltration may play a key role in muscle injury following exhaustive exercise.

Depletion of macrophages can attenuate the inflammatory response in the early phase after trauma-induced muscle damage. However, macrophage depletion leads to an elevation of fat accumulation in the regeneration phase. Muscle satellite cells play an important role in muscle regeneration after the muscle injury. Interestingly, Summan et al. [24] showed that TNF receptor deficient mice presented reduced activated satellite cells after muscle injury. Therefore, mediators secreted by macrophages might be required for regeneration after muscle injury. In this study, it is unclear whether macrophage depletion impaired muscle regeneration. Future study is necessary to investigate the effect of macrophage depletion on regeneration after exhaustive exercise induced muscle injury.

In summary, we have demonstrated that macrophage depletion markedly reduces the number of injured myofibers and expression of pro-inflammatory cytokines in the muscle. Taken together, our results provide evidence that macrophages play a critical role in increasing muscle injury by regulating inflammation.

Information about the contributions of each author

Noriaki Kawanishi; I designed research, analyzed data (all analyses), performed statistical analyses, and wrote this manuscript
Mizokami Tsubasa; he analyzed data (histological analysis)
Niihara Hiroyuki; he analyzed data (PCR analysis)
Koichi Yada; he supported animal experiment and sampling
Katsuhiko Suzuki; he had primary responsibility for final content as the supervisor

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.11.022.

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