The Journal of Veterinary Medical Science

FULL PAPER
Physiology

Progranulin deficiency leads to prolonged persistence of macrophages, accompanied with myofiber hypertrophy in regenerating muscle

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J. Vet. Med. Sci.
80(2): 346–353, 2018
doi: 10.1292/jvms.17-0638

Received: 25 November 2017
Accepted: 7 December 2017
Published online in J-STAGE: 18 December 2017

ABSTRACT. Skeletal muscle has an ability to regenerate in response to injury due to the presence of satellite cells. Injury in skeletal muscle causes infiltration of pro-inflammatory macrophages (M1 macrophages) to remove necrotic myofibers, followed by their differentiation into anti-inflammatory macrophages (M2 macrophages) to terminate the inflammation. Since both M1 and M2 macrophages play important roles, coordinated regulation of their kinetics is important to complete muscle regeneration successfully. Progranulin (PGRN) is a pluripotent growth factor, having a protective role against the inflamed tissue. In the central nervous system, PGRN regulates inflammation by inhibiting the activation of microglia. Here we used muscle injury model of PGRN-knockout (PGRN-KO) mice to elucidate whether it has a role in the kinetics of macrophages during muscle regeneration. We found the prolonged persistence of macrophages at the late phase of regeneration in PGRN-KO mice, and these macrophages were suggested to be M2 macrophages since this was accompanied with an increased CD206 expression. We also observed muscle hypertrophy in PGRN-KO mice at the late stage of muscle regeneration. Since M2 macrophages are known to have a role in maturation of myofibers, this muscle hypertrophy may be due to the presence of increased number of M2 macrophages. Our results suggest that PGRN plays a role in the regulation of kinetics of macrophages for the systemic progress of muscle regeneration.

KEY WORDS: inflammation, macrophage, progranulin, regeneration, skeletal muscle

Skeletal muscle is the most abundant tissue of the body, and has high regenerative capacity to make new myofibers once it is damaged by injury or under some pathological circumstances such as muscular dystrophy [6]. Cells responsible for muscle regeneration are normally quiescent muscle stem cells, called satellite cells. In response to muscle injury, satellite cells become activated, and proliferate as myoblasts [20]. These myoblasts then differentiate and eventually fuse with each other to form myotubes. Thus formed myotubes mature to become newly regenerated muscle fibers [5]. Prior to satellite cell activation, degenerated fibers recruit massive infiltration of monocytes at the injury site [17]. These monocytes primarily differentiate into proinflammatory macrophages (M1 macrophages) that have the ability to remove the necrotic myofiber debris through phagocytosis [7, 12]. As the process of muscle regeneration advances, they switch their phenotype, named M2 macrophages, to repress inflammation and begin to secrete anti-inflammatory cytokines like TGF-β and IL-10 [1, 8]. *In vitro* and *in vivo* studies show M2 macrophages enhance proliferation and differentiation of myoblasts [1, 16]. However, excess number of M2 macrophages may hyper-activate fibroblasts, leading to skeletal muscle fibrosis [15, 23]. Thus, it is important to elucidate the factor that regulates the kinetics of macrophages in the course of muscle regeneration.

Progranulin (PGRN) is a glycoprotein with the molecular mass of approximately 68.5 kDa [4, 19, 26]. It contains seven and a half copies of granulin motifs characterized by 12-cysteine residues [27]. PGRN is a pluripotent growth factor [2] and involved in tumorigenesis, wound healing, inflammation and early embryogenesis [3, 18]. PGRN regulates inflammation by inhibiting the activation of microglia, the tissue resident macrophage in the central nervous system [22]. Another study shows that bone marrow derived-macrophages obtained from PGRN-knockout (KO) mice become easily over-activated and secrete higher levels

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of inflammatory cytokines [25]. Therefore, there is a possibility that PGRN regulates inflammation by controlling the nature of macrophages also in skeletal muscle regeneration.

There are some reports about the involvement of PGRN in skeletal muscle. A previous study showed PGRN promoted myotube hypertrophy through PI3K/Akt/mTOR pathway, using immortalized mouse myoblast cell line C2C12 cells [10]. However, another experiments suggest PGRN inhibits myotube formation *in vitro* and knockdown of PGRN enhance myogenesis in neonatal mice [24]. These findings suggest PGRN may play a role in skeletal muscle, but its effect on myogenesis *in vivo* is still controversial.

In this study, we used a muscle injury model of PGRN-KO mice to examine whether PGRN is involved in the regulation of inflammation, especially the kinetics of macrophages during muscle regeneration.

MATERIALS AND METHODS

Animals

Adult female (2- to 5-month-old) C57BL/6J wild-type (WT) and PGRN-deficient (PGRN-KO) mice [11] of the same genetic background, bred in our laboratory were used. They were maintained in our laboratory under controlled environmental conditions; 23 ± 1°C with a photoperiod of 12-hr light and 12- hr dark (lights on at 0700 hr). Animals were fed commercial chow *ad libitum*. All animal experiments in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals of The University of Tokyo, and were approved by the Institutional Animal Care and Use Committee of The University of Tokyo (P13-842).

CTX injection

Mice were anesthetized by isoflurane, and 100 *µl* of cardiotoxin (CTX; Sigma-Aldrich, St. Louis, MO, U.S.A.; 10 *µ*g/m*l* in ultrapure water) was injected into the TA muscle to induce degenerative/regenerative response of myofibers [21]. On the indicated day after the injection (indicated as days post injection, DPI), the mice were sacrificed and their TA muscle was obtained. The TA muscle was snap-frozen in liquid nitrogen-cooled iso-pentane, and stored at −85°C until use.

Histological analyses

Transverse frozen sections (7–8 *µ*m thickness) of the mouse TA muscle were prepared by cryostat, and were subjected to histological analyses.

For hematoxylin-eosin (HE) staining, sections were fixed with 4% paraformaldehyde in phosphate buffered saline (4% PFA/PBS). After washing with running tap water for 5 min, sections were stained with hematoxylin. After washing with running tap water for 10 min, the sections were immersed in eosin solution for 6 min, then dehydrated and mounted with Multi Mount 220 (Matsunami Glass Inc., Ltd., Osaka, Japan).

For immunohistochemistry, the sections were fixed with 4% PFA/PBS for 15 min at room temperature (RT). After washing with PBS, the sections were blocked with 10% normal donkey serum (NDS) in PBS for 20 min, then incubated overnight with primary antibodies at 4°C. The following primary antibodies were used: anti-PGRN (Sheep, AF2557, R&D Systems, Minneapolis, MN, U.S.A., 1:200 dilution), anti-CD68 (Rat, AbD Serotec, Oxford, U.K., 1:200 dilution), and anti-laminin (Rabbit, L9393, Sigma, St. Louis, MO, U.S.A., 1:200 dilution). The sections were washed with PBS and incubated with secondary antibody for 1 hr at RT. The following secondary antibodies were used; Alexa Fluor488-labeled anti-sheep IgG (Invitrogen, Carlsbad, CA, U.S.A., 1:500 dilution), Alexa Fluor594-labeled anti-rat IgG (Invitrogen, 1:500 dilution), Alexa Fluor594-labeled anti-rabbit IgG (Invitrogen, 1:500 dilution). All the antibodies were diluted with 10% NDS/PBS. Cell nuclei were counterstained with Hoechst 33258. Fluorescent signals were observed with a fluorescent microscope.

For quantitative analyses of myofiber diameters, 15 fields were randomly chosen in the sections stained with anti-laminin antibody. Photos were taken under a fluorescence microscope (BX51; Olympus; Tokyo, Japan) equipped with a digital camera (DP73; Olympus). The minimal Feret's diameters were calculated using ImageJ software (ver.1.47; National Institutes of Health, Bethesda, MD, U.S.A.).

Quantitative RT-PCR

TA muscles were homogenized using a Shake Master (ver. 1.0, Bio Medical Science Inc.; Tokyo, Japan), and RNA was isolated with TRIzol (Invitrogen) and reverse-transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using a Light Cycler 2.0 (Roche Diagnostics, Roche, Basel, Switzerland) with Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan). The following primer sets were used for qRT-PCR: *Hprt* (forward, 5′-AGTCCCAGCGTCGTGATTAGCCAT-3′; reverse, 5′-CTTGAGCACACAGAGGGCCACAAT-3′), *Grn* (forward, 5′-GGTTGATGGTTCGTGGGGATGTTG-3′; reverse, 5′-AAGGCAAAGACACTGCCCTGTTGG-3′), *Cd206* (forward, 5′-CTAACTGGGGTGCTGACGAG-3′; reverse, 5′-GGCAGTTGAGGAGGTTCAGT-3′). The expression level of each gene was analyzed by the crossing point method.

Flow cytometry

TA muscles were dissociated in Ca^{2+} free Hanks' balanced salt solution (Sigma-Aldrich) containing 0.1% collagenase type 2 (Worthington, Columbia, NJ, U.S.A.), 0.1% fetal bovine serum, 0.1% trypsin inhibitor (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and 0.01% ATP for 1 hr at 37°C. After enzymatic treatment, the dissociated cells were filtered and counted. The cells

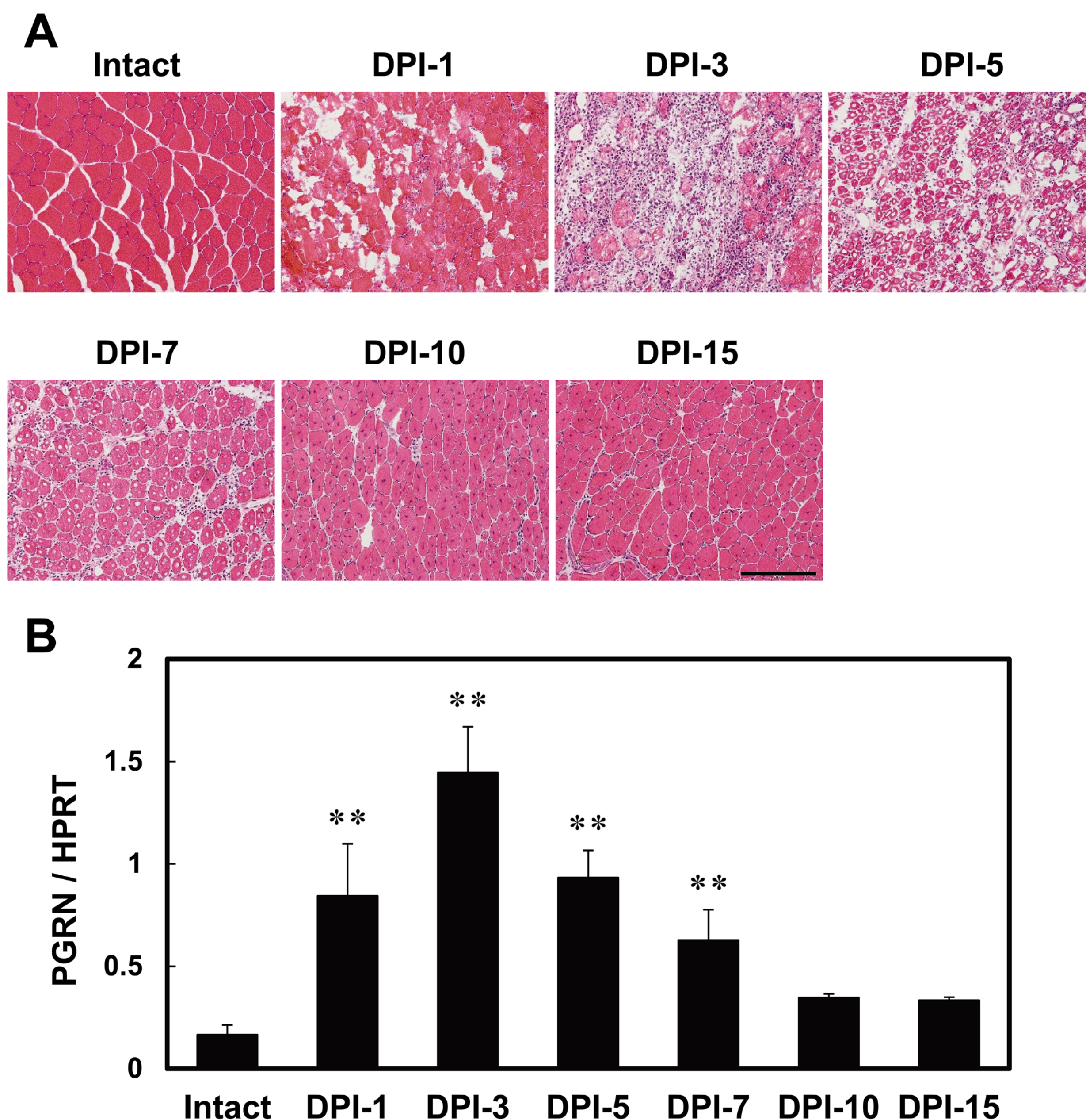

Fig. 1. Expression pattern of PGRN during muscle regeneration after CTX injection. (a) Histological analysis of TA muscles in wild type (WT) mice after CTX injection. Frozen sections of mouse TA muscles untreated (Intact) and obtained at DPI-1, 3, 5, 7, 10 and 15 were stained with hematoxylin and eosin (HE). Scale bar=100 μm. (b) Quantitative RT-PCR analysis of PGRN expression levels in TA muscles of WT mice obtained at DPI-1, 3, 5, 7, 10 and 15, and of untreated (Intact). Graphed data are expressed as mean ± SEM. **, *P*<0.01 vs. Intact.

(1×10^6 cells/m*l*) were fixed with 1% PFA/PBS for 30 min at RT and permeabilized with 0.1% Tween/PBS for 15 min at RT, followed by incubation with PE-conjugated CD45 and Alexa Flour647-conjugated CD68 Ab (1:200 diluted) for 1 hr at RT in PBS. After washing, Stained cells were analyzed with a BD FACSVerse^{TM} (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.). The absolute counts of cells were determined with Flow-Count fluorospheres (Beckman Coulter, Brea, CA, U.S.A.).

Statistical analyses

Unpaired student's *t*-tests and one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test were used to evaluate statistical differences between groups. For the distribution of myofibers, median values were compared using the Wilcoxon rank sum test. *P*-values less than 0.05 were considered statistically significant. Graphed data represent means ± SEM.

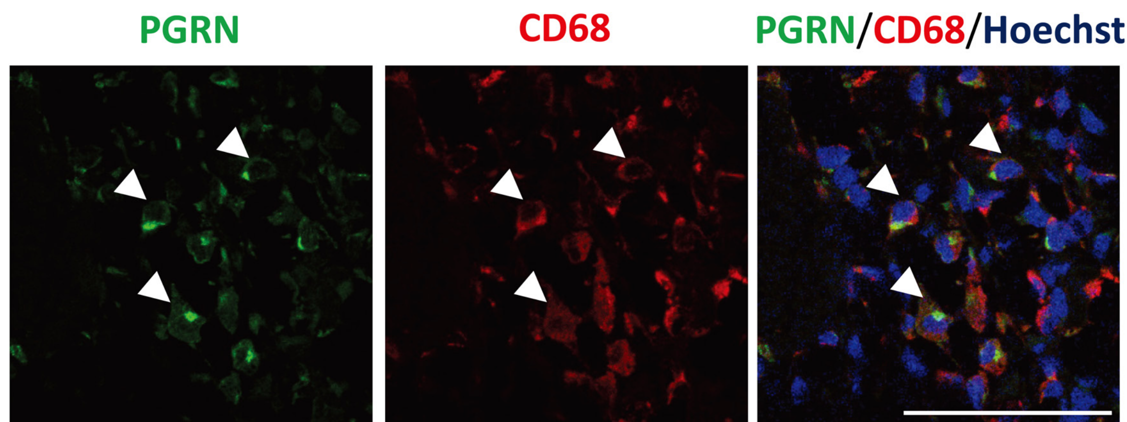

Fig. 2. PGRN distribution during muscle regeneration after CTX injection. Frozen sections of TA muscles of WT mice obtained at DPI-3 were subjected to immunohistochemistry of PGRN and CD68. White arrowheads indicate PGRN and CD68-positive nuclei. Scale bar=100 μm.

RESULTS

PGRN expression is transiently increased during muscle regeneration

To confirm that CTX induces degenerative/regenerative response of myofibers, histological analyses were performed on mouse TA muscles after CTX injection. HE staining on sections revealed that at DPI-1, the degenerative change of myofibers occured, and the infiltration of mononucleated cells, presumably inflammatory cells, was observed (Fig. 1A). The number of mononucleated cells present in the degenerated area peaked at DPI-3 (Fig. 1A). At DPI-5 and DPI-7, newly formed multinucleated myotubes appeared, indicating the initiation of muscle regeneration (Fig. 1A). At DPI-10, immature myofibers with central nuclei were observed mainly, but the regeneration was yet to be completed (Fig. 1A). Regeneration of myofibers was almost completed at DPI-15 (Fig. 1A).

We next examined PGRN mRNA expression during regenerative process of skeletal muscle. The PGRN expression increased from DPI-1, and peaked at DPI-3, but decreased to the same level as intact at DPI-10 (Fig. 1B). These results indicate that PGRN expression increases transiently upon skeletal muscle injury, and suggest the involvement of PGRN in muscle regeneration.

PGRN is localized in macrophages during muscle regeneration

From the above results that both the number of inflammatory cells and the PGRN expression peaked at DPI-3, we suspected that the cells expressing PGRN are infiltrating inflammatory cells. In addition, previous reports showed that PGRN is mainly and strongly expressed in macrophages during tissue repair [25]. Thus, we performed immunohistochemistry for PGRN and pan-macrophage marker, CD68. As shown in Fig. 2, almost all the PGRN-positive cells were CD68-positive, indicating that infiltrating macrophages are the source of PGRN expression.

PGRN deficiency increases the diameter of the regenerated myofibers

To examine whether PGRN has a functional role in muscle regeneration, we performed histological analyses on TA muscles of WT and PGRN-KO mice after CTX injection. HE staining revealed that there was no difference in the temporal changes of muscle regeneration between WT and PGRN-KO mice (Fig. 3A). However, we noticed that the diameters of regenerated myofibers of PGRN-KO mice appeared larger than those of WT mice. Thus, we quantitatively compared the diameter of newly formed myotubes (DPI-5) and regenerated myofibers (DPI-14) on laminin-stained sections (Fig. 3B). There was no difference in the diameters of myotubes at DPI-5 (Fig. 3C), while those of regenerated myofibers of PGRN-KO mice were significantly larger than those of WT mice at DPI-14 (Fig. 3C). These results suggest the role of PGRN in the maturation of regenerated myofibers.

PGRN deficiency leads to an increase of M2 macrophages in regenerating muscle

Since PGRN is expressed in macrophages in CTX-injured muscle (Fig. 2), we examined whether the PGRN deficiency affects the kinetics of macrophages during muscle regeneration. The flow cytometric analysis revealed that the number of $CD45^{+}CD68^{+}$ macrophages was decreased from DPI-5 to DPI-14 in both WT and PGRN-KO mice, and there was no difference in their number between WT and PGRN-KO mice at DPI-5 (Fig. 4B), while the number in PGRN-KO mice was significantly higher than that in WT mice at DPI-14 (Fig. 4B). These kinetics were in agreement with the previous study where it was shown that 65–78% and almost 100% of macrophages present at the middle and late phase of muscle regeneration are anti-inflammatory type (corresponding to M2 macrophages), respectively [1]. Thus, it was suggested that the macrophages whose number was higher in PGRN-KO mice at DPI-14 are M2 macrophages. In agreement with this, the expression level of CD206, an M2 macrophage marker, was higher in the muscle of PGRN-KO mice at DPI-14, while there was no difference at DPI-5 (Fig. 4C). These results indicated that PGRN deficiency leads to an increase of M2 macrophages in the late phase of muscle regeneration.

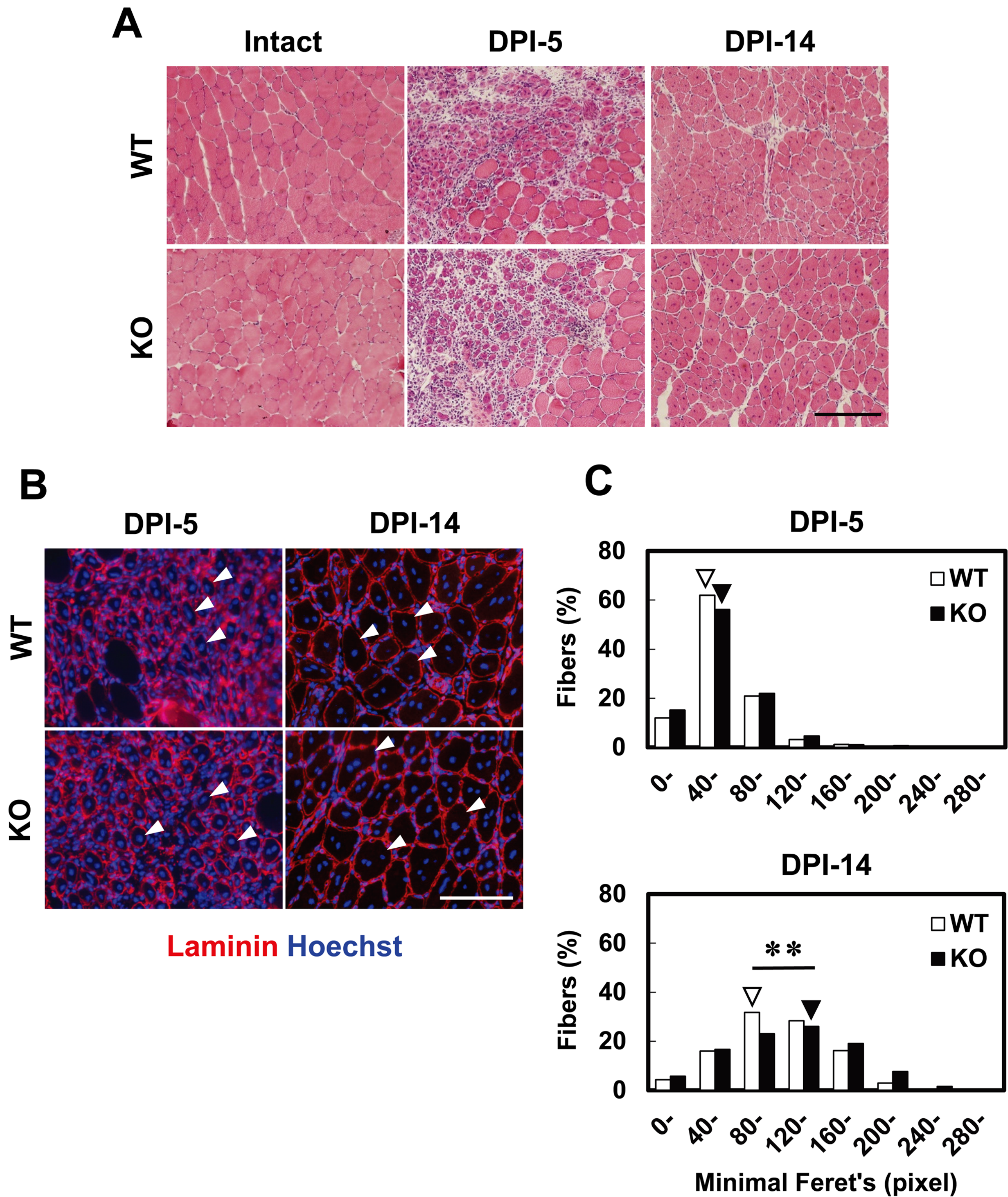

Fig. 3. Histological analysis of TA muscle of WT and PGRN-KO mice after CTX injection. Frozen sections of TA muscles of WT and PGRN-KO mice obtained at DPI-5 and 14, and of untreated (Intact) were (a) stained with HE, and (b) subjected to immunohistochemistry of laminin. White arrowheads indicate regenerating myofibers with central nuclei. Scale bar=100 μm. (c) Relative distributions of the diameters of regenerating myofibers with central nuclei in TA muscles of WT and PGRN-KO mice. Arrowheads indicate median values. **, P<0.01.

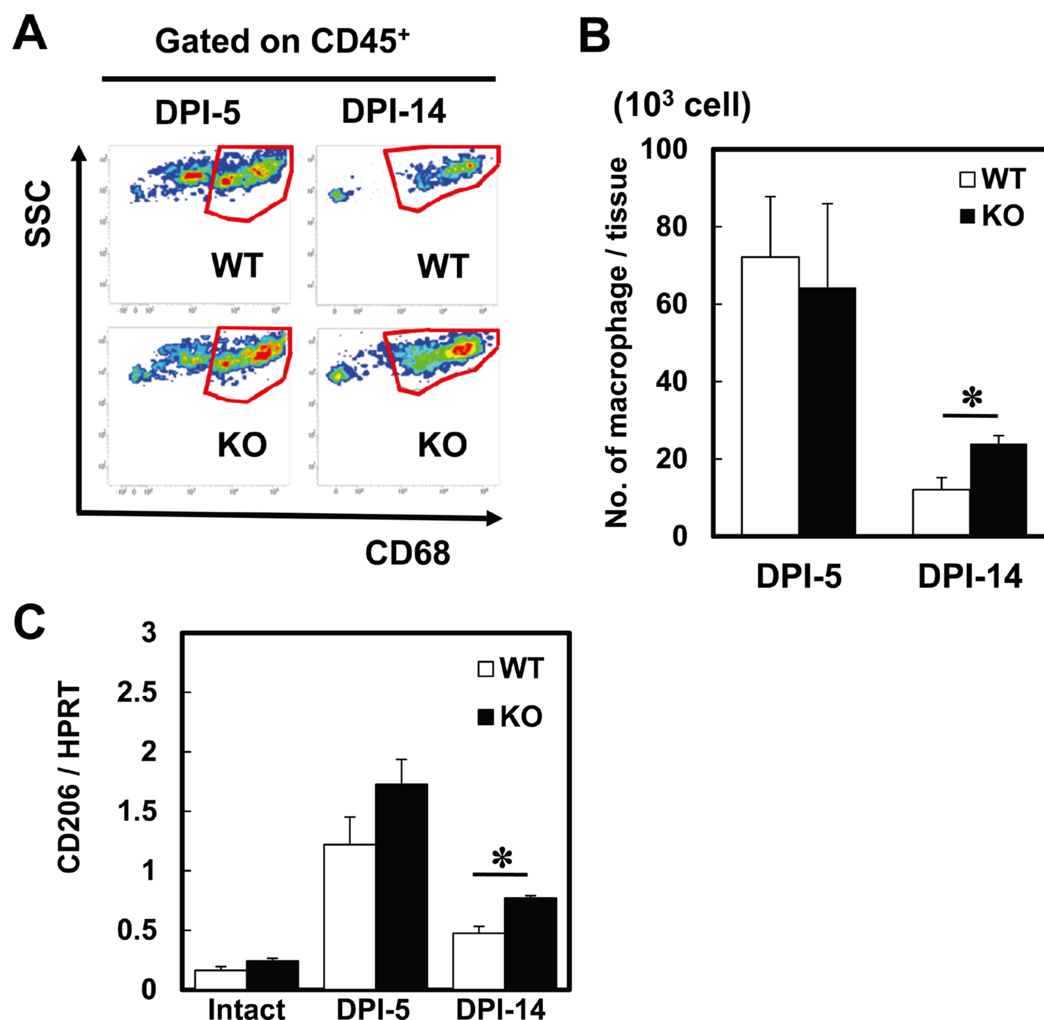

Fig. 4. Kinetics of macrophages during muscle regeneration in WT and PGRN-KO mice. $CD45^+$ cells present in TA muscles of WT and PGRN-KO mice obtained at DPI-5 and 14 were analyzed for macrophage marker (CD68) expression by flow cytometry. (a) Representative examples of FACS analysis at each time point. Red gate represents macrophage populations. (b) Results are expressed as the total number of $CD45^+$ $CD68^+$ cells isolated from muscle and represent means + SEM. (c) Quantitative RT-PCR analysis of M2 macrophage marker, CD206 expression levels in TA muscles of WT and PGRN-KO mice obtained at DPI-5 and 14, and of untreated (Intact). Graphed data are expressed as mean + SEM. *P<0.05.

DISCUSSION

In the present study, we showed that PGRN deficiency leads to an increase in the number of macrophages in the late phase of muscle regeneration, which is accompanied by the hypertrophy of skeletal muscle fibers.

The number of M1 and M2 macrophages is regulated by several mechanisms such as recruitment (infiltration) to the site of muscle injury, proliferation, differentiation and clearance. Previous study has shown that amongst the infiltrated macrophages followed by muscle injury, macrophages of inflammatory type (M1 macrophages) never proliferate, whereas those of anti-inflammatory type (M2 macrophages) actively enter into cell cycle [1]. In addition, some of the infiltrated M1 macrophages differentiate into M2 macrophages and then proliferate, and the total number of macrophages reaches plateau at DPI-4~7. After DPI-7, macrophages are cleared from the tissue, accompanied with the termination of inflammation [1]. We found no difference in the number of macrophages at DPI-5, which may reflect the total number of infiltrated and proliferating macrophages. In addition, there was no difference in CD206 expression, an M2 macrophage marker, between WT and PGRN-KO mice at DPI-5. These results suggest that recruitment, proliferation and differentiation are not affected by PGRN deficiency.

Little is known about the mechanism that leads to the clearance of once infiltrated and proliferated macrophages in regenerating muscle. The higher number of remaining M2 macrophages in PGRN-KO mice compared to WT mice at DPI-14 suggest the involvement of PGRN in the clearance of macrophages after the middle phase of muscle regeneration. The most possible

mechanism is an apoptotic cell death of macrophages. Previous study demonstrated the presence of TUNEL-positive macrophages during rat skeletal muscle regeneration, and suggested the apoptosis of macrophages [9]. However, PGRN is known to inhibit apoptosis in a variety of cell types [13, 14], and this is contradictory to our result. Further studies will be required to elucidate the exact mechanism by which PGRN deficiency results in the higher number of remaining macrophages at the late phase of muscle regeneration.

The current results suggest the role of PGRN in the maturation of regenerated myofibers, rather than formation of myotubes. In terms of the effect of on myotube formation, PGRN is shown to be both stimulatory [10] and inhibitory [24] using C2C12 mouse myoblast cell lines. However, in the present study, there was no difference in the diameters of newly formed myotubes between WT and PGRN-KO mice at DPI-5, suggesting that the involvement of PGRN in myotube formation during muscle regeneration is unlikely. It should be noted that C2C12 cell line express PGRN endogenously [10, 24], while in our immunohistochemical analysis, we did not find PGRN expression in skeletal muscle cells. Thus, the absence of the effect of PGRN deficiency on myotube formation in the present study may be due to the difference in the nature between C2C12 cell lines and skeletal muscle myoblasts.

Our results suggest the correlation between the higher number of remaining M2 macrophages and enhanced maturation of regenerated myofibers at DPI-14 in PGRN-KO mice. Previous study demonstrated that the depletion of anti-inflammatory macrophages (possibly M2 macrophages) results in the reduced diameters of regenerated myofibers, suggesting that these macrophages play a role in myofiber growth [1]. As discussed above, PGRN may regulate the clearance and/or apoptosis of M2 macrophages at the late phase of muscle regeneration, and its deficiency may result in the increased number of remaining M2 macrophages. It is possible therefore that thus remained M2 macrophages may lead to an enhancement of myofiber growth in PGRN-KO mice.

In conclusion, our data demonstrate that PGRN deficiency leads to a persistence of macrophages (possibly M2 type), and leads to the hypertrophy of myofibers. Although M2 macrophages contribute to tissue repair, excess number of M2 macrophages is known to lead to hyper-activation of fibroblasts, resulting in the fibrosis of skelatal muscle [15, 23]. We propose a novel role of PGRN in regulation of inflammation through its effect in clearance of macrophages, thus contributing to systematic regeneration process of skeletal muscle.

ACKNOWLEDGMENTS. This study was supported by the Japan Society for the Promotion of Science KAKENHI Grants 23228004 and 17H03930 to MN and 16H05041 to KY.

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