Progranulin Promotes Functional Recovery in Rats With Acute Spinal Cord Injury via Autophagy-induced Anti-inflammatory Microglia Polarization.

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Research Article

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Abstract

Since microglia-associated neuroinflammation plays a critical role in the progression of acute spinal cord injury, modulation of microglial activation has been suggested as a potential therapeutic strategy. Progranulin has been reported to exert neuroprotective effects by attenuating neuroinflammation, but whether these effects are due to the modulation of microglial polarization and the underlying mechanism remain unclear. Here, we investigated the effect of progranulin on microglial polarization and analyzed the crosstalk between microglial autophagy and polarization. We found that progranulin could reduce proinflammatory cytokine production in the lesion site and promote locomotor functional recovery after acute spinal cord injury. In vitro, we found that progranulin could activate microglia to an anti-inflammatory phenotype and express IL-10. Moreover, progranulin-mediated enhancement of anti-inflammatory microglial polarization was attributed to the protection of lysosomal function and the enhancement of autophagic flux. Above all, progranulin exerts anti-inflammatory effects by protecting lysosomal function to enhance microglial autophagy to induce M2 microglial polarization and ultimately improves neurological function after acute spinal cord injury. These results suggest that targeting autophagy-lysosomal pathway to modulate microglial polarization to reduce neuroinflammation is a potential treatment for spinal cord injury.

1. Introduction

Traumatic spinal cord injury (SCI) has a very serious impact on the central nervous system, usually leading to irreversible sensory, motor, and autonomic dysfunction[1]. Increasing evidence has shown that the changed microenvironment in spinal cord tissue plays an important role in SCI, particularly the inflammatory response[2]. Microglia act as vital regulators of inflammation and immune responses in SCI. Microglia are extremely adaptable cells and are able to transform to functionally different phenotypes in response to changes in their microenvironment[3]. Classically activated (M1) microglia are considered to be proinflammatory and cytotoxic, which cause tissue necrosis and axon retraction after SCI and may activate astrocytes to cause scar formation, while alternatively activated (M2) microglia have anti-inflammatory and neuroprotective effects in SCI. IL-4 can reduce tissue damage and dysfunction by activating microglia to an anti-inflammatory phenotype[4]. Therefore, activating microglia to differentiate into the M2 type has become a potential method to reduce inflammation after SCI. However, how to increase M2 microglia has become another problem that plagues scholars.

The growth factor progranulin (PGRN) regulates wound healing and promotes the growth of blood vessels and tumors[5]. With the deepening of the research on PGRN, scholars have discovered that it is not only a growth factor but also a neurotrophic factor that promotes the differentiation and growth of neurons. In patients with frontotemporal dementia due to mutations in the GRN gene, the expression of PGRN was found to be significantly reduced[6]. In subsequent studies, it was found that the expression of lysosomes and PGRN is closely related in neurodegenerative diseases[7]. In the PGRN defect model, the function of the lysosome is significantly impaired[8]. The relationship between the two may be the transport of PGRN to the lysosome through the sorting receptor sortilin and the colocalization of the two

in the lysosomal membrane protein LAMP1[9]. The results show that PGRN plays an important regulatory role in the production and function of lysosomes. Studies have shown that upregulating the expression of PGRN is beneficial to protect Alzheimer's mice from spatial memory deficits and the loss of hippocampal memory neurons, which is related to PGRN's resistance to amyloid β deposition and its toxicity[10]. Although the effect of PGRN on lysosomes remains to be verified, the above studies have shown that high expression of PGRN is conducive to the stability of lysosomal function in central nervous system diseases, which is of positive significance for the treatment of central nervous diseases. However, it is unclear whether PGRN can exert a neuroprotective effect after spinal cord injury by protecting lysosomal function.

Autophagy is an evolutionarily conserved and important cellular function of eukaryotes. It is a lysosome-dependent catabolic pathway that degrades misfolded proteins, protein aggregates and damaged organelles[11]. Under starvation conditions, autophagy provides nutrients to maintain cell function through the degradation of macromolecules, organelles, proteins and final products[12]. The latest study found that the p62 protein and LC3 accumulated in secondary injury after SCI, indicating that autophagy flow is damaged after SCI[13]. This situation will gradually recover 7 days after the occurrence of SCI, which indicates that there is a potential mechanism to promote the recovery of autophagy after SCI in vivo[14]. Actively researching this self-protection mechanism is of vital importance for preventing early (within 7 days) injury of SCI. This discovery provides a new idea for the treatment of SCI.

Studies have shown that PGRN secreted by activated microglia after SCI is greatly increased, and its existence plays an important role in the survival and repair of neurons after SCI[15]. Another scholar's study found that when the expression of PGRN was downregulated after SCI, neuronal inflammation and cell necrosis were aggravated.[16] The peak concentration of PGRN secreted by activated microglia is 5 to 7 days after SCI, which highly overlaps with the aforementioned time to regulate the recovery of autophagic flux after SCI[15, 17, 18]. Therefore, we hypothesized that microglia protect the function of lysosomes and the integrity of autophagy by upregulating PGRN expression to protect and repair neurons. Herein, we designed a series of experiments to verify the hypothesis that PGRN could improve locomotor function by inducing anti-inflammatory M2 microglial polarization by regulating autophagy.

2. Materials And Methods

Cell culture

BV-2 microglial cells (Chinese Academy of Sciences, Shanghai) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (Gibco, Grand Island, NY) and 1% streptomycin/penicillin and maintained at 37 °C under 5% CO₂. Cells were divided into the following three groups: control group, 20 μ g/L recombinant progranulin (PGRN, R&D Systems) group, and 200 μ g/L PGRN group. The culture medium was changed once a day, and the control group did not receive any treatment.

Flow cytometry analysis

To investigate the microglial polarization phenotype, the cells were centrifuged, and the supernatant was removed. Cells were incubated with F4/80-eFluor 450 (1:150, eBioscience) and CD163 antibodies (1:100, Sigma, USA) for 30 min on ice. Cells were analyzed by flow cytometry (Beckman Coulter). Flow cytometry data were analyzed using FlowJo software (Tree Star).

Spinal cord contusion model and treatment

The female SD rats (220-250 g) used in this experiment were raised in the Experimental Animal Center of the Sixth People's Hospital of Shanghai. All experimental procedures were in accordance with the regulations of the animal ethics committee. The rat spinal cord contusion model was established as described previously. The brief procedure is as follows. After anesthesia with 10% chloral hydrate, an incision was made in the middle of the back of the rat to expose the T9-11 segment, and then a laminectomy was performed to expose the T10 spinal cord. An Infinite Horizon Impact Device (10 g weight, 30 mm vertical height, 10 g × 30 mm) was used for moderate spinal cord contusions. The sham operation group only underwent laminectomy without spinal cord contusion. One milliliter of 20 µg/L or 200 µg/L PGRN was administered by intraperitoneal injection 30 min after surgery and then once a day for seven days. The sham group and the SCI group received 1 ml of normal saline. The bladder was emptied twice a day postoperatively, and antibiotics were given.

Locomotor function assessment

Basso, Beattie and Bresnahan (BBB) scores were used to evaluate rat hindlimb function[19]. The test time points included 0, 1, 7 and 14 days in this experiment. Two measurements were taken to obtain an average value for each rat. Two trained examiners who were blinded to the experimental conditions independently performed the tests to obtain the score as previously described.

Hematoxylin-eosin (H&E) staining and histological analysis

Seven days after the spinal cord injury, a 1-cm-long spinal cord segment with the injury site as the center was removed to prepare a paraffin section. HE staining for histological evaluation was performed according to the manufacturer's instructions. Spinal cord cross-sections were observed under a light microscope (Nikon, Japan).

Immunofluorescence staining

Spinal cord sections were blocked with 5% bovine serum albumin (BSA) and then incubated with the following primary antibodies overnight at 4 °C: NeuN, IBA1, Arg1 and iNOS (Abcam, USA). After washing with PBS 3 times, the sections were incubated with conjugated secondary antibodies for 2 hours at 37 °C. Nuclei were incubated with DAPI, and images were acquired under a fluorescence microscope.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells or 1 cm length segments from the injured spinal cord epicenter using TRIzol reagent (Invitrogen). RT-qPCR was performed according to the manufacturer's instructions after reverse transcription. The mRNA levels (Arg1, CD163, CD206, IL-10, IL-1 β and iNOS) were detected. GAPDH was used for normalization, and reactions were repeated in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

Rats were sacrificed 7 days after SCI, spinal cord tissue was dissected 0.5 cm above and below the site of injury, placed in precooled PBS buffer, and homogenized. The supernatant was collected by centrifugation at 3000 revolutions/min (r/min) for 20 min at 4 °C. For cultured cells, the supernatant was collected after treatment under the same conditions as described above, and the supernatant was carefully collected by centrifugation at 2000 r/min for 20 min. The expression of TNF- α , IL-10, IL-6 and IL-1 β in the spinal cord homogenate and cell supernatant was quantitated according to the instructions of the ELISA kit (eBioscience USA) using a standard curve.

Transmission electron microscopy

The cells were collected by centrifugation, the culture medium was removed, and the electron microscope fixative solution was added for fixation for 4 h at 4 °C. After centrifugation, the cells were washed 3 times with PBS and then fixed with 1% osmic acid at room temperature for 2 h in the dark. The tissues were dehydrated, embedded and sectioned and then stained with 2% uranyl acetate saturated alcohol solution and 2.6% lead citrate solution. Finally, the sections were observed with a transmission electron microscope (Hitachi, SUHT7700).

Tandem Ad-LC3-GFP-RFP confocal microscopy

Ad-LC3-GFP-RFP adenovirus (Yazhai Biology, China)-transfected BV2 cells were cultured in a 24-well plate at 37 °C in a 5% CO₂ environment and divided into PBS, 20 μ g/LPGRN, and 200 μ g/LPGRN. Rapamycin and chloroquine were used as controls to observe changes in autophagic flux. After treatment, they were fixed with formaldehyde and finally observed with a laser confocal microscope (OLYMPUS). The yellow spots represent autophagosomes, and the red spots represent autolysosomes.

Western blot

For cultured cells, following a similar protocol, cells were lysed with RIPA lysis buffer on ice for 30 min, and whole cell extracts were obtained after the supernatant was collected by centrifugation at 12000 rpm for 20 min at 4 °C. The protein concentration in the supernatant was measured with a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). SDS-PAGE loading buffer was added to the supernatant and heated at 100 °C for 5 min. A normalized amount of protein (30 μ g) was loaded on 10% SDS-PAGE gels for electrophoresis. The proteins were then transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membrane was blocked with skimmed milk powder at room temperature for 2 h and then incubated overnight with the following primary antibodies: LC3 II/I (1:1000, Abcam), Beclin1 (1:1000, Abcam), P62 (1:1000, Abcam), LAMP1 (1:1000, Abcam), CTSD (1:2000, Abcam) and β -actin

(1:1000, Abcam) at 4 °C. After washing with Tris-buffered saline Tween (TBST) 3 times (10 min each), the membrane was incubated with a secondary antibody at room temperature for 1 h and again washed with TBST 3 times (10 min each). After enhanced chemiluminescence (ECL; Thermo Fisher Scientific, USA) was added, proteins on the membrane were detected by an ECL Western blotting detection system (Thermo Fisher Scientific, USA), and gray value analysis was performed.

Statistical analysis

All data are from three independent experiments. The results are presented as the mean $\pm$ SEM. Statistical differences were determined by one-way analysis of variance (ANOVA) and Tukey's post-hoc analysis. Two-way ANOVA followed by Bonferroni post hoc comparison test was used to analyze BBB scores. Differences were considered significant at $P < 0.05$.

3. Results

3.1 PGRN promoted anti-inflammatory type BV2 microglial polarization in vitro.

To assess the effect of PGRN on microglial polarization in vitro, we designed a series of experiments for validation. First, flow cytometry was used to evaluate the expression of the microglial M2 surface marker CD163 after PGRN treatment, and it was found that the expression of anti-inflammatory microglia in the 200 $\mu\text{g/L}$ PGRN group was significantly increased compared with that in the PBS group (Figure 1A and 1B). Then, qPCR was used to detect the mRNA expression of the anti-inflammatory microglial markers Arg1, CD163, CD206 and IL-10. The expression of the above mRNA in the 200 $\mu\text{g/L}$ PGRN group was significantly increased compared with that in the other two groups (Figure 1C-1F). Finally, we used ELISA to detect the expression of the anti-inflammatory factor IL-10 in the supernatant of the three groups of cells. The results showed that the expression of IL-10 in the 200 $\mu\text{g/L}$ PGRN group was significantly higher than that in the other two groups (Figure 1G). We demonstrate in vitro that PGRN can significantly activate microglial polarization toward an anti-inflammatory phenotype.

3.2 PGRN improved locomotor function and tissue structure after SCI.

First, we used HE staining to evaluate tissue repair on the 7th day after SCI and found that the 200 $\mu\text{g/L}$ PGRN group significantly alleviated the loss of nerve tissue caused by secondary SCI (Figure 1A). Then, we assessed the number of neurons at the SCI site with NeuN staining. The fluorescence intensity of the 200 $\mu\text{g/L}$ PGRN group was significantly higher than that of the other two groups (Figure 2B and 2C). Finally, the BBB score was used to evaluate the therapeutic effect of PGRN on spinal cord injury. We found that on days 7 and 14 after SCI, the 200 $\mu\text{g/L}$ PGRN group had significantly improved hindlimb motor function compared with the SCI group (Figure 2D). Taken together, PGRN can significantly improve histopathology and neurological function after SCI.

3.3 PGRN promoted microglial polarization from the M1 to M2 phenotype in vivo.

To assess whether PGRN can attenuate the inflammatory response at the site of spinal cord injury and explore its effect on microglial polarization. On the 3rd day after spinal cord injury, the ELISA results showed that 200 µg/L PGRN reduced the expression of proinflammatory factors (IL-1β, IL-6 and TNF-α) and increased the expression of the anti-inflammatory factor IL-10 at the site of spinal cord injury (Figure 3A-3D). To further analyze the effect of PGRN on microglial polarization, we analyzed the mRNA expression levels of M1 type (iNOS and IL-1β) and M2 type (Arg1 and CD206) macrophages in the four groups by qPCR. These results indicated that the mRNA levels of iNOS and IL-1β were significantly increased and that the mRNA levels of Arg1 and CD206 were significantly decreased at the site of spinal cord injury. Treatment with 200 µg/L PGRN significantly reversed this change, indicating that PGRN can induce the polarization of microglia to the M2 phenotype after spinal cord injury to exert an anti-inflammatory effect (Figure 3E-3H). Immunofluorescence staining showed that the expression of iNOS+IBA1+ microglia was significantly increased in the spinal cord injury site, while the expression of Arg1+IBA1+ microglia was significantly decreased. Treatment with 200 µg/L PGRN significantly increased the number of Arg1+IBA1+ microglia and decreased the number of iNOS+IBA1+ microglia (Figure 3I and 3J). The results showed that PGRN reduced the inflammatory response at the site of spinal cord injury by inducing microglial polarization toward the M2 phenotype.

3.4 PGRN could enhance microglial autophagic flux by improving lysosomal function.

To explore the effect of PGRN on microglial autophagy, we observed by electron microscopy that the number of autophagosomes in microglia in the 200 µg/L PGRN group was significantly increased compared with that in the PBS group (Figure 4A). To detect the integrity of autophagic flux, we used Ad-LC3-GFP-RFP adenovirus to transfect microglia in which yellow fluorescence represented autophagosomes. When autophagosomes fused with lysosomes, the green fluorescence was quenched, so the red fluorescence represents autolysosomes, and the autophagic flux was observed by confocal microscopy. The results showed that the fluorescence of autophagosomes and autolysosomes in the 200 µg/L PGRN group was significantly increased compared with that in the PBS group (Figure 4B and 4C). We used western blotting to detect autophagic flux (Beclin1, LC3II/I and P62) and lysosomal functional proteins (CTSD and LAMP1) to investigate whether PGRN promotes autophagy by enhancing lysosomal function (Figure 5A-5F). The results showed that the protein levels of Beclin1 and LC3II/I in the 200 µg/L PGRN group were significantly increased and the level of P62 protein was decreased compared with those in the PBS group, indicating that PGRN not only increased autophagosomes but also significantly enhanced autophagic flux. At the same time, we found that the expression of the lysosomal functional proteins CTSD and LAMP1 was also significantly increased. These results suggest that PGRN is able to enhance autophagic flux by protecting lysosomal function.

4. Discussion

In the present study, we first investigated the relationship between PGRN and microglial polarization in spinal cord injury and explored the potential mechanism in vivo and in vitro. We found that intraperitoneal injection of PGRN could significantly reduce proinflammatory cytokines and increase anti-inflammatory

cytokines that could protect neurons and inhibit tissue loss to improve functional recovery after SCI. In vitro, we found that Bv2 cells cultured with PGRN were activated to an anti-inflammatory M2 phenotype by enhancing autophagic flux by protecting lysosomal function. In summary, we confirmed that exogenous PGRN treatment could attenuate neuroinflammation by inducing autophagy to activate resident microglia to an anti-inflammatory M2 phenotype, which could improve locomotor function.

Reducing neuronal death in secondary injury after SCI has always been a hot spot and focus of scientific and clinical research. In recent years, the continuous deepening of research on autophagy, lysosomes and upstream regulatory factors has provided a novel opportunity and approach for the repair and treatment of SCI. The occurrence of autophagic flux is inseparable from normal lysosome functions[20]. Although autophagy substrates increase after SCI, lysosomal dysfunction leads to the destruction of the integrity of the autophagic flux[21]. Therefore, maintaining the integrity of autophagic flux by protecting lysosome functions may be an effective strategy for alleviating secondary injury.

Microglia are the main innate immune cells in the central nervous system and play an important role in neuroinflammation. Microglia are rapidly activated to a proinflammatory M1 phenotype in response to SCI, which can release proinflammatory cytokines to aggravate secondary damage[22]. M2 polarization of microglia can produce anti-inflammatory cytokines to alleviate neuroinflammation[23]. Dunxin Han et al confirmed that plasma hemopexin could ameliorate secondary injury by shifting proinflammatory M1 microglia to anti-inflammatory M2 microglia[24]. PGRN is a multifunctional neurotrophic factor and is associated with the inflammatory response[25]. In addition, the expression of PGRN is significantly increased in activated microglia after SCI[18]. PGRN deficiency aggravates neuroinflammation and cell apoptosis in spinal cord injury, resulting in tissue damage and consequent locomotor function loss[16]. In this study, we found that PGRN could induce Bv2 cells to express markers of anti-inflammatory M2 microglia in vitro. Treatment with PGRN significantly reduced tissue damage and promoted locomotor functional recovery in SCI rats.

The impairment of microglial autophagic flux in inflammatory diseases suggests that impaired microglial autophagy may contribute to the dysregulated inflammatory response[26]. However, the effect of autophagy on microglial polarization is controversial. Zi-Wei Hu et al. found that FTY720 could switch microglia cultured with LPS from the proinflammatory M1 phenotype to the anti-inflammatory M2 phenotype by suppressing autophagy[27]. Vinpocetine mediates BV2 cells treated with oxygen-glucose deprivation to an anti-inflammatory M2 phenotype by enhancing autophagic flux[28]. The difference in microglial polarization by autophagy may be related to the experimental design or the state of microglia. The absence of PGRN could significantly impair lysosomal function in microglia[29]. In this study, we found that PGRN increased LAMP1 and CTSD expression to protect lysosomal function in microglia, which could augment microglial autophagic flux to exert anti-inflammatory activities.

5. Conclusion

In summary, we found that PGRN could induce microglia to express anti-inflammatory cytokines by enhancing autophagy. For the first time, we indicated that PGRN could protect lysosomal function to repair damaged autophagic flux, which could improve locomotor functional recovery. Therefore, maintaining the integrity of autophagic flux by preserving lysosomal function in the early stages of acute spinal cord injury may serve as a potential therapeutic target for spinal cord injury.

Declarations

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Conflicts of interest:

The authors declare that they have no competing interests.

Availability of data and material:

Not applicable.

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Not applicable.

Authors' contributions:

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All authors contributed equally to this work, and all authors read and approved the final manuscript.

Ethics approval:

All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and all procedures were approved by the Animal Research Committee of the Sixth People's Hospital at Shanghai Jiao Tong University.

Consent to participate:

Not applicable.

Consent for publication:

All data generated or analyzed during this study are included in this published article.

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Figures

Figure 1

Characterization of PGRN in inducing anti-inflammatory M2 microglial polarization. A-B. Flow cytometry analysis of M2 microglia and quantitative analysis. C-F. mRNA expression of M2 microglia. G. The concentration of IL-10 by ELISA. * $p < 0.05$; $n = 3$.

Figure 2

The effects of PGRN on spinal cord injury in rats. A. HE analysis (scale bar=100 μ m). B-C. NeuN staining and fluorescence quantification analysis (scale bar=100 μ m). D. The result of BBB scores. * p <0.05; n =3.

Figure 3

The effects of PGRN on microglial polarization in vivo. A-D. Enzyme-linked immunosorbent assay (ELISA) of proinflammatory cytokines. E-H. The mRNA expression of Arg1, CD206, IL-1 β and iNOS by q-PCR. I-J: Representative fluorescence images of Arg1/iNOS (red) and IBA1 (green) (scale bar=100 μ m). *p<0.05; n=3.

Figure 4

The effects of PGRN on microglial autophagy. A. The representative image of TEM. scale bar:5 μ m (low) and 2 μ m (high). B. Microglial autophagy analysis of Ad-LC3-GFP-RFP adenovirus-transfected BV2 cells by confocal microscopy. Red and green fluorescence (yellow) represent autophagosomes, and red fluorescence represents autolysosomes (scale bar=20 μ m). C. Relative fluorescence intensity of RFP and

GFP. * indicates comparison with the PBS group, $p < 0.05$; # indicates comparison with the RFP group, $p < 0.05$; $n = 3$.

Figure 5

PGRN protects lysosomal function to enhance microglial autophagic flux in vitro. A. Western blot analysis of lysosomal function and autophagy markers. B-F. Quantitative analysis of different proteins in

microglia. *p<0.05; n=3.