

Original Article

Effects of progranulin in cell model of osteoarthritis and LPS-induced inflammation

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Abstract: Rheumatoid arthritis (RA) is a type of common chronic autoimmune disease that damages various body joints and extra-joint organs, thus severely affecting the life quality of patients. Due to the complicated pathogenesis, so far no effective drugs have been utilized against RA. Progranulin represents a chondrogenesis factor, which prevents the bone degradation via antagonizing inflammation in bone tissues. However, the effect of Progranulin on RA and related mechanism has not been illustrated. Rat arthritis model was generated and treated with 5 μ M and 10 μ M of Progranulin. The indexes of weight increasing rate, swelling of toes and arthritis index were determined to evaluate the treatment efficacy. Lipopolysaccharide (LPS) was used to induce inflammation on *in vitro* model of RAW264.7 cells. MTT was used to measure tumor cell proliferation, whilst ELISA was applied to detect levels of inflammatory cytokines including tumor necrosis factor-alpha (TNF- α) and interleukin-1beta (IL-1 β). NF- κ B expression was measured by Western blot. Progranulin treatment increased body weight of rats, decreased toe swelling and arthritis index ($P < 0.05$ compared to control group) in a dose dependent manner ($P < 0.05$). Progranulin treatment inhibited proliferation of RAW264.7 cells induced by LPS, decreased TNF- α , IL-6 and p-p65 expressions ($P < 0.05$ compared to control group). Such effects became more potent as the dosage elevated ($P < 0.05$). Progranulin alleviated arthritis symptoms through impeding the secretion of inflammatory cytokines, suppressing inflammatory cell proliferation and down-regulating the activation of NF- κ B signal pathway.

Keywords: Progranulin, rheumatoid arthritis, NF- κ B, inflammation, proliferation

Introduction

Rheumatoid arthritis (RA), as a type of chronic autoimmune disease, commonly occurred with unknown reasons. RA mainly caused injury in multiple joints and extra-joint organs of the body, posing a severe impact on patients' life [1, 2]. RA often occurred in females, the incidence of whom was 2~3 fold higher than that of males. RA can be found at any age, but more popular in elder people [3]. RA was characterized as systemic disorder with inflammatory synovitis, and was manifested as morning stiffness, multi-joint, symmetrical and invasion arthritis in small joints of hand and foot. RA frequently affected extra-joint organs such as heart or kidney, and may lead to joint deformation or dysfunction in severe cases [4]. Pathological changes of RA primarily include proliferation of synovial inner membrane, abundant infiltration of mesenchymal inflammatory cells, angiogenesis of micro-vessels, formation of vascular pannus, and destruction of carti-

lage or bone tissues, with occurrence of rheumatoid factor (RF) [5, 6]. Due to the damage in multiple joints and atrophy of skeletal muscle, along with long disease duration and high morbidity, the treatment of RA drew extensive focus from both scientists and clinicians [7, 8].

The pathogenesis mechanism of RA consisted of genetic factors, risk genes, and viral or bacterial infections, in addition to abnormal immune or endocrine functions, trauma or fatigue [9, 10]. The development of RA also involved the dys-regulation of autoimmune functions, further causing thickening of joint synovial tissues, destructing joint cartilage and bone tissues [11, 12]. The proliferation of inflammatory cells elicited the secretion of inflammatory factors, leading to abnormality of cell/humoral immunity, dysregulated focal or systemic immune functions, which aggravated the injury caused by RA [13, 14]. Progranulin (PRGN), as chondrocyte generating factor, contributed to an anti-inflammatory role in protecting bone tis-

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sues [15, 16]. The effect of Progranulin on RA and related mechanism, however, has not been illustrated yet.

Materials and methods

Experimental animals

Healthy male Wistar rats (2 month old, SPF grade, body weight 250 ± 20 g) were purchased from Laboratory Animal Center in Shandong University and were kept in an SPF grade animal facility. Animals were kept under temperature ($21 \pm 1^\circ\text{C}$) and humidity (50-70%) with 12 h light/dark cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Ningbo No 6 Hospital (Ningbo, Zhejiang, China).

Major materials and equipment

RAW264.7 cell line (TIB-71™) was purchased from ATCC cell bank (US). 10% hydrate chloral was purchased from Zhaohui Pharm (China). Progranulin was purchased from R&D System (US). LPS was purchased from Sigma (US). PVDF was purchased from Pall Life Science (US). Western blot reagents were purchased from Beyotime (China). ECL reagent was purchased from Amersham Bioscience (US). Rabbit anti-mouse p-p65, p65, β -actin monoclonal antibody, goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG secondary antibody were purchased from Cell signaling (US). DMEM culture medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (US). DMSO and MTT powders were purchased from Gibco (US). Trypsin digestion solution was purchased from Sigma (US). ELISA kits for TNF- α and IL-6 were purchased from R & D (US). Surgical microscope instruments were purchased from Suzhou Medical Instrument (China). KQ-250DE ultrasound was purchased from Kunshan Ultrasound Equipment (China). YLS-7C toe volume meter was produced by Zhongshi Dichuang (China). Forma Steri-Cycle CO₂ incubator was purchased from Thermo Fisher (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US). Other common reagents were purchased from Sangon (China).

Animal grouping and treatment

Wistar rats were randomly assigned into four groups (n = 5): control group; model group,

which was prepared for arthritis model by complete Freund's adjuvant (CFA); 5 μM and 10 μM Progranulin treatment groups, which received tail vein injection of 5 μM and 10 μM Progranulin, respectively, at 7 days post modeling and for 24 consecutive days.

Preparation of rat adjuvant arthritis model

Rat RA model was established based on the following literature [4]. In brief, liquid paraffin and sterile lanolin were mixed at ratio of 2:1 to prepare adjuvant. Bacillus calmette-guerin was heated at 58°C for 1 hr, and was mixed with 10 mg/ml adjuvant for ultrasonic emulsification to prepare CFA. 0.1 ml CFA was injected intracutaneously on left posterior toes for 25 consecutive days.

Analysis of body weight increasing rate in arthritis rats

After treatment, all groups of rats were weighted and the increasing rate of body weight was calculated.

Arthritis index of rats

Arthritis index (AI) of rats was evaluated as previously reported [4], using a score range 0 to 4 for the assessment of normal joint, no swelling and inflammation. 1: swelling of toe joint and/or reddish. 2: reddish of 3 to 4 toe joints or 1 major joint. 3: more than 4: joints having reddish affecting ankle joints. AI was summarized for all joints.

Toe swelling rate of arthritis rats

Rat toe volumes were measured before and after treatment from all groups with the calculation of toe swelling rate (%), which was equal to the ratio between the decrease of toe volume after treatment and volume before model preparation.

Cell culture and LPS induction of inflammatory cell model

Cryopreserved RAW264.7 cells were resuscitated at 37°C for centrifugation at 300 g for 5 min. The supernatant was removed to 50 ml culture flask, which contained 4 ml fresh DMEM medium for 37°C incubation with 5% CO₂ in a humidified chamber for 24-48 h. Cells were seeded into 6-well plate at 1×10^5 cells per

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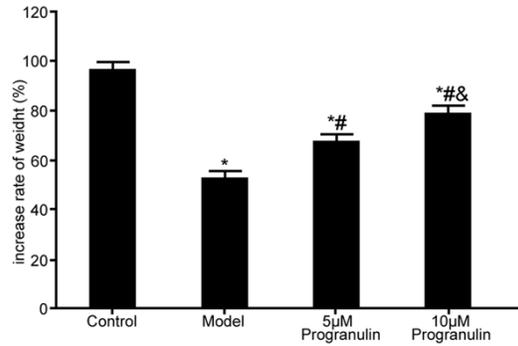


Figure 1. Effects of Progranulin on weight gain of arthritis rats. *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to model group; &, $P < 0.05$ compared to 5 µM Progranulin group.

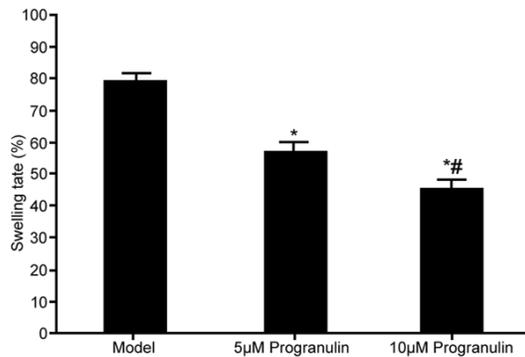


Figure 2. Effects of Progranulin on toe swelling of arthritis rats. *, $P < 0.05$ compared to model group; #, $P < 0.05$ compared to 5 µM Progranulin group.

cm² density with high glucose DMEM/medium (containing 100 U/ml penicillin, 100 µg/ml streptomycin, 10% FBS) and were cultured at 37°C with 5% CO₂. Culture medium was changed every 3 days. Cells were passed when paving 80%~90% of dish bottom. During 1:2 passage, cells were rinsed in D-Hanks solution, and were digested for 5~10 min using trypsin-0.02% EDTA buffer. Cells at log-growth phase with 2~5th generation were selected for experiments, and were randomly assigned into three groups: inflammatory group, which was cultured in medium containing 1 µg/ml LPS for 24 h; and 5 µM or 10 µM Progranulin treatment group, which received Progranulin treatment after LPS treatment. All cells were cultured for 48 h for further experiments.

MTT for the effect of Progranulin on LPS-induced inflammatory cell proliferation

RAW264.7 cells (5×10^3 cells per well) at log-growth phase were inoculated into 96-well

plate containing DMEM/F12 medium with 10% FBS. After 24 h culture, the supernatant was discarded. Cells were randomly assigned into model group (without progranulin treatment), 5 µM Progranulin treatment group, and 10 µM Progranulin treatment group as above mentioned. After 48 h treatment, 20 µl sterile MTT was added into each well in triplicates. After 4 h incubation, the supernatant was discarded, followed by the addition of 150 µl DMSO per well for 10 min shake incubation until crystal violet completely resolved. A microplate reader was used to measure absorbance (A) values at 570 nm of each well for calculating proliferation rate. Each experiment was repeated for more than three times.

ELISA for TNF-α and IL-6 expression in supernatant

All samples were measured for TNF-α and IL-6 expression by ELISA kit, following manual instruction of test kit. In brief, 96-well plate was added with 50 µl serially diluted samples, which were used to plot standard curves. 50 µl test samples were then added into test wells in triplicates. After washing for 5 times, liquids were discarded to fill with washing buffer for 30 sec vortexing. The rinsing procedure was repeated for 5 times. 50 µl enzyme labelling reagent was then added into each well except blank control. After gentle mixture, the well was incubated for 30 min at 37°C. Chromogenic substrates A and B were sequentially added (50 µl each), followed by dark incubation at 37°C for 10 min. The test plate was then mixed with 50 µl quenching buffer as the blue color turned into yellow. Using blank control well as the reference, absorbance (A) values at 450 nm wavelength were measured by a microplate reader within 15 min after adding quenching buffer. Linear regression model was then plotted based on the concentration of standard samples and respective A values. Sample concentration was further deduced based on optical density (OD) values using the regression function.

Western blot for NF-κB protein expression

Cell proteins were firstly extracted. In brief, cells were lysed with lysis buffer on ice for 15~30 min, followed by ultrasound rupture (5 s × 4) and centrifugation (4°C, 10000 g, 15 min). Supernatants were kept, quantified for protein contents, and were stored at -20°C for further Western blotting. Proteins were then separated

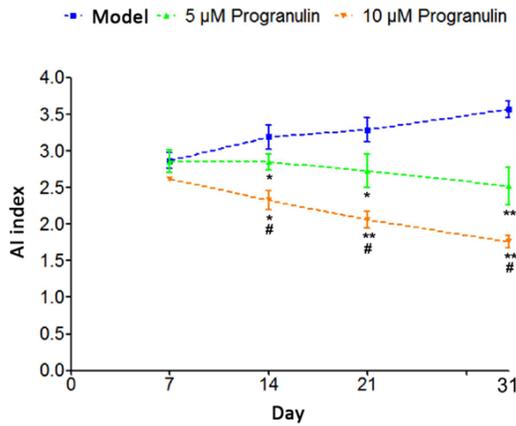


Figure 3. Effects of Progranulin on AI index of arthritis rat. *, P < 0.05 compared to model group; #, P < 0.05 compared to 5 μM Progranulin group.

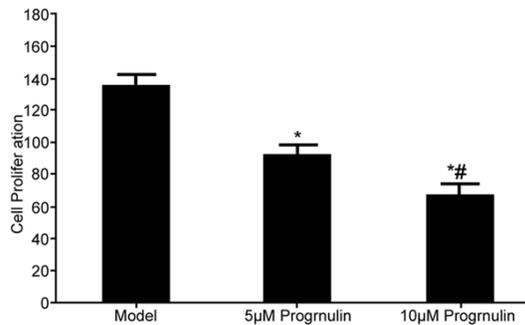


Figure 4. Effects of Progranulin on proliferation of inflammatory cells induced by LPS. *, P < 0.05 compared to model group; #, P < 0.05 compared to 5 μM Progranulin group.

using 10% SDS-PAGE gel, and were transferred to PVDF membrane using semi-dry method (100 mA, 1.5 h). Non-specific background was removed by 5% defatted milk powder at room temperature for 2 h, followed by incubation with anti-p-p65 monoclonal antibody (1:500), anti-p65 monoclonal antibody (1:1000) or anti-β-actin monoclonal antibody (1:2000) at 4°C overnight. On the next day, the membrane was rinsed in PBST, and incubated with secondary antibody for 1 h dark incubation. ECL reagent was used to develop the membrane for 1 min, followed by exposure under X-ray for observing results. Protein imaging analysis system and Quantity One software were used to scan X-ray films for observing band density. Each experiment was repeated for four times (N = 4).

Statistical processing

SPSS19.0 was used for analysis. Measurement data were presented as mean ± standard deviation (SD). Comparison of means among multiple groups was performed by one-way analysis of variance (ANOVA). A statistical significance was defined when P < 0.05.

Results

Effects of progranulin on body weight of arthritis rats

The increasing rate of body weight was significantly retarded in arthritis model rats (P < 0.05 compared to control group). Progranulin treatment significantly increased the body weight gain (P < 0.05 compared to model group). With higher drug dosage, the promoting effects on body weight became more significant (P < 0.05, **Figure 1**).

Effects of progranulin on tow swelling rate in arthritis rats

No significant morphological changes were observed among different groups except the discrepancy at toe swelling and of weight gain. Toe swelling rate of arthritis rats was 79.21 ± 13.35%. In contrast, after the treatment of Progranulin, tow swelling was statistically relieved (**Figure 2**, P < 0.05 compared to model group). With higher dosage of 10 μM Progranulin, the swelling was further alleviated (**Figure 2**).

Effects of progranulin on AI of arthritis rats

From day 7 to day 31 after modeling, joint swelling in arthritis rats was gradually deteriorated with enlarged joint cleft and deformation, indicating higher index of AI. However, the treatment of Progranulin significantly reduced the value of AI (P < 0.05 compared to model group) and 10 μM Progranulin presented more obvious effect of AI decline (**Figure 3**).

Effects of progranulin on proliferation of LPS-induced inflammatory cells

MTT assay was used to measure the effect of different concentrations of Progranulin on proliferation of LPS-induced inflammatory cells. Results showed that Progranulin treatment suppressed the proliferation of inflammatory

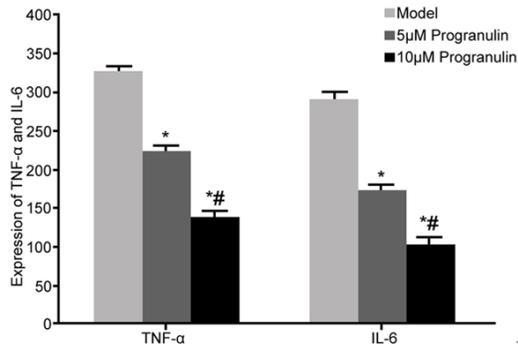


Figure 5. Effects of Progranulin on secretion of inflammatory cytokines. *, $P < 0.05$ compared to model group; #, $P < 0.05$ compared to 5 μM Progranulin group.

Effects of progranulin on secretion of inflammatory cytokines from LPS-induced inflammatory cells

ELISA was used to test the effect of Progranulin on the levels of inflammatory cytokines TNF- α and IL-6 in LPS-induced inflammatory cells. Results indicated that Progranulin significantly suppressed the secretion of inflammatory cytokines TNF- α and IL-6 ($P < 0.05$ compared to model group). Moreover, the inhibitory effect was more potent as the concentration of Progranulin increased (**Figure 5**).

Effects of progranulin on NF- κ B expression in LPS-induced inflammatory cells

We further used Western blot to measure NF- κ B signaling pathway at protein level. Our data demonstrated that Progranulin obviously impeded the expression of p-p65 with no remarkable changes in the level of p-65 ($P < 0.05$ compared to model group). Also, in the group of 10 μM Progranulin, the level of p-p65 was even down-regulated (**Figure 6**).

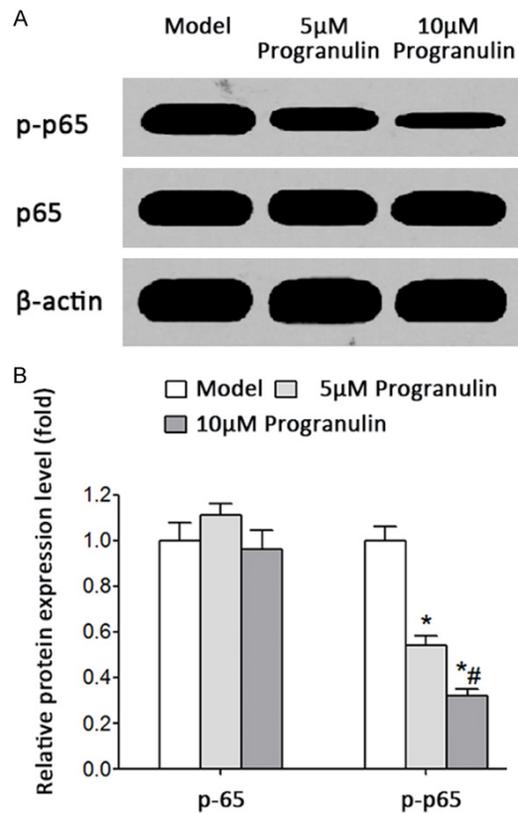


Figure 6. Effects of Progranulin on NF- κ B signaling pathway. A. Effects of Progranulin on NF- κ B protein expression in LPS-induced inflammatory cells; B. Analysis of the effect of Progranulin on NF- κ B protein expression in LPS-induced inflammatory cells. *, $P < 0.05$ compared to model group; #, $P < 0.05$ compared to 5 μM Progranulin group.

Discussion

The complicated pathogenesis and various risk factors hampered the development of the successful therapy of RA and major challenge also existed that currently no effective treatment has been validated against RA. The development of molecular biology, immune therapy, biological reagent and gene therapy provided promising basis for the therapy of arthritis inflammation [17, 18].

In this study, we successfully established RA model of rats and found that the weight gain in model group was obviously retarded compared to control group. In addition, pathological features after LPS treatment included synovial hyperplasia, inflammation of sub-synovial tissues, cartilage damage and irreversible joint injury, all of which were similar with pathological features and clinical symptoms of human RA, suggesting reliable preparation of arthritis model of rats [4, 19].

Progranulin was found widely expressed in immune cell lineage, epidermal and nervous system [20]. Previous evidence showed that progranulin inhibited degradation of chondrocyte oligomerized matrix protein during arthritis pro-

cells in a dose dependent manner ($P < 0.05$ compared to model group) (**Figure 4**).

gression [21]. In mouse inflammatory arthritis model, Progranulin can antagonize TNF- α secretion [22]. However, the function and mechanism of Progranulin in RA has not been fully elucidated. Our study demonstrated that Progranulin treatment increased the body weight gain, suppressed AI and tow swelling rate on LPS-induced arthritis rat model.

Our data also showed the inhibitory role of Progranulin on the proliferation of inflammatory cells and the secretion of the inflammatory cytokines. As the nuclear transcription factor, NF- κ B participated in body defense, stress response, tissue injury, cell differentiation and apoptosis, and exerted pivotal function in modulating inflammation and immune response [23]. Previous finding showed critical roles of NF- κ B in multiple phages of the development of RA, as growing level of NF- κ B can be observed in synovial tissues at both early and late phase [24]. This study demonstrated that Progranulin could alleviate RA via suppressing NF- κ B signaling pathway and inhibiting secretion of inflammatory factors.

Conclusion

Progranulin can alleviate arthritis symptoms (slowly increase rate of weight gain, alleviate toe swelling, and decrease high AI index) possibly via suppressing NF- κ B signal pathway and inflammatory cytokines, which provided a theoretical basis for the therapy of RA.

Disclosure of conflict of interest

None.

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