Potential effects of progranulin and granulins against retinal photoreceptor cell degeneration

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Purpose: The authors previously reported that progranulin attenuated retinal degeneration. The present study focused on the role of progranulin and its cleavage products, granulins, in the pathogenesis of photoreceptor degeneration.

Methods: Photoreceptor degeneration was induced with excessive exposure of murine photoreceptor cells and the retinas of albino mice to white fluorescent light. Damaged photoreceptor cells and retinas were examined using a cell death assay, western blotting, and immunostaining.

Results: Even after proteolytic cleavage, treatment with progranulin or its cleavage products or both exerted protective effects on photoreceptors against light exposure. In the murine retina, the expression levels of granulins and the macrophage and microglia marker Iba-1 were increased at 48 h after light exposure. Additionally, progranulin and Iba-1 double-positive cells had accumulated in the outer nuclear layer, the primary location of photoreceptor cells.

Conclusions: These results suggest that progranulin or its cleavage products, granulins, or both may be therapeutic targets for age-related macular degeneration and other neurodegenerative diseases.

It has been established that excessive exposure to light can cause degeneration of photoreceptor cells through apoptotic and necrotic processes. Visual function relies heavily on photoreceptor cells, the loss of which is a leading cause of blindness in developed countries. An epidemiological study demonstrated that light exposure may be an important risk factor for progression of retinal degeneration during age-related macular degeneration (AMD) [1-3].

Progranulin (PGRN), also known as granulin-epithelin precursor (GEP) [4], proepithelin (PEPI) [5], acrogranin [6], and GP88/PC-cell derived growth factor (PCDGF) [7], is a multifunctional growth factor expressed by many cell types, including neurons and microglia in the central nervous system (CNS) [8]. It has been reported that PGRN is involved in multiple physiologic functions, such as wound healing [9], inflammation [10,11], tumorigenesis [12], and insulin resistance [13]. In 2006, mutations in the PGRN gene (GRN; Gene ID: 2896, OMIM: 138945) were discovered to be a cause of frontotemporal lobar degeneration (FTLD) with TAR DNA-binding protein 43 (TDP-43)-positive inclusions [14,15]. Several studies have shown that PGRN has a neuroprotective effect by promoting neurite outgrowth and cell survival [16], and protects against amyloid-β deposition and toxicity [17]. Another study reported that dysregulation of Wnt signaling may be a major pathway in Grn-deficient neuronal death [18].

Structurally, PGRN is a cysteine-rich protein consisting of 593 amino acids, with a molecular weight of 68.5 kDa, and is typically secreted in a highly glycosylated 88 kDa form. Notably, PGRN is composed of seven-and-a-half granulin domains, namely, p (paragranulin, a granulin half-domain); and granulins G, F, B, A, C, D, and E. After secretion, PGRN undergoes proteolytic cleavage at the linker domain. Neutrophil elastase (NE) [10], proteinase-3 (PR3) [11], matrix metalloproteinase 9 (MMP-9) [19], MMP-12 [20], MMP-14 [21], and a disintegrin-like and metalloproteinase domain 7 (ADAMTS-7) [22] can release individual granulin peptides. Additionally, secretory leukocyte protease inhibitor (SLPI) and high-density lipoprotein apolipoprotein A-I (HDL/Apo A-I) block PGRN from proteolytic conversion by elastase [10,23]. These reports highlighted the importance of the balance between PGRN and granulins controlled by SLPI or elastase.

As described above, PGRN and granulins have biologic effects, whereas the role of granulins in the CNS remains unclear. It has been reported that PGRN and granulins have opposing effects in some situations, including anti- and proinflammatory, growth promoting and inhibiting [10], and neuroprotective and neurotoxic effects [24]. Conversely, some studies have reported that PGRN and granulins exert similar effects (e.g., granulin E rescues neuronal cell survival similar to PGRN [16]). Thus, it remains controversial whether granulins contribute to pathogenesis, inert or protective, especially in the CNS.

In the present study, using a model of light-induced retinal damage, we focused on the effect of granulins on...
photo receptor degeneration. We previously reported that PGRN has a protective effect on photoreceptor cells in in vivo and in vitro models [25], which mimics the degenerative pathology of AMD or retinitis pigmentosa (RP). Although this finding is consistent with PGRN as a neuroprotective agent, it remains unclear whether granulins are capable of exerting protective effects similar to those of full-length PGRN. We examined whether PGRN and its cleavage products, granulins, have the same or opposite effects with regard to photoreceptor cell damage.

METHODS

Cell culture: The murine cone photoreceptor cell line 661W was a generous gift from Dr. Muayyad R. Al-Ubaidi (University of Houston, Houston, TX). The cells were cultured in Dulbecco’s modified Eagle medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Biosera, Kansas City, MO), 100 U/ml penicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 100 µg/ml streptomycin (Meiji Seika) in a humidified atmosphere of 5% CO₂, at 37 °C. Cultured adherent cells were trypsinized and passed every 2–3 days.

STR analysis: The 661W cells were authenticated with short tandem repeats (STR) profile and interspecies contamination testing (IDEXX BioResearch, Ludwigsburg, Germany). The sample was confirmed to be of mouse origin, and no mammalian interspecies contamination was detected. The genetic profile was verified to be consistent with a mixed FVB and C57BL/6J mouse strain of origin, and to carry this cell line’s unique transgene, HITI (Appendix 1) [26].

In vitro light-induced cell death assay: The 661W cells were seeded on 3 × 10⁴ cells/well in 96-well plates and subsequently incubated for 24 h at 37 °C; the medium was then replaced with 1% FBS. After incubation for 1 h, 500 ng/ml recombinant mouse PGRN, cleaved PGRN, phenylmethylsulfonyl fluoride (PMSF), or elastase + PMSF were added. The cells were then exposed to 2,500 lux of white fluorescent light (Nikon, Tokyo, Japan) for 24 h under 5% CO₂ at 37 °C. Cell death was measured using Hoechst 33,342 (Invitrogen, Carlsbad, CA) and propidium iodide (PI; Invitrogen). At the end of the light exposure, Hoechst 33,342 and PI were added to the medium to final concentrations of 8.1 and 1.5 µM, respectively, for 15 min. Images of stained cells were captured with an All-in-One BZ-X710 fluorescence microscope (Keyence, Osaka, Japan). The percentage of PI-positive cells was determined by distinguishing Hoechst 33,342 and PI fluorescence.

In vitro proteolytic reaction (for western blotting): Recombinant mouse PGRN (R&D systems, Minneapolis, MN) was cleaved using elastase (Type I porcine pancreatic elastase; Sigma-Aldrich, St. Louis, MO), diluted in 100 mM Tris-HCl and 960 mM NaCl. Recombinant PGRN (5 µg/ml) was mixed with elastase (0.1, 0.5, and 1.0 U/ml) and incubated for 1 h at 37 °C. Sample buffer (Wako Pure Chemical Corporation, Osaka, Japan) was added (sample:sample buffer = 3:1) and boiled for 5 min. All samples were analyzed with western blotting using polyclonal anti-mouse PGRN antibody (R&D Systems; dilution, 1:100).

Reagents for cell death assay: Recombinant mouse PGRN was cleaved using Type I porcine elastase. For the in vitro cell death assay, recombinant PGRN (10 µg/ml) was mixed with elastase (2.0 U/ml) and incubated for 1 h at 37 °C. An equal amount of PMSF (Nacalai Tesque, Kyoto, Japan), a protease inhibitor, at 1 mM (dissolved in dimethyl sulfoxide [DMSO], 0.1% final concentration) was added to the mixture to inhibit the activity of elastase, and the mixture was incubated for 15 min. The combination of PGRN (500 ng/ml) with elastase (0.1 U/ml), and PMSF (0.1 mM) was added to the culture medium, and the cell death assay was performed.

Removal of full-length PGRN: To remove full-length PGRN, 400 µl of cleaved PGRN (treated with PMSF) was directly applied to centrifugal filter devices (Amicon Ultra-15; 50,000 MW cutoff; Millipore, Billerica, MA). The mixture was centrifuged at 7,000 ×g for 20 min at 4 °C. Flow-through fractions were collected, and PBS (1X; 136.9 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄·12H₂O, 1.76 mM KH₂PO₄, pH 7.3) was added to attain a volume of 400 µl.

Mice: Male adult ddY mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and housed under controlled lighting conditions (12:12 light-dark). Eight-week-old ddY mice were used in the experiments, which were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University (Gifu, Japan).

Light-induced retinal damage in vivo: Before the mice were exposed to light, they were adapted to the dark for 24 h. Their pupils were dilated using 1% cyclopentolate hydrochloride (Santen Pharmaceuticals Co., Ltd., Osaka, Japan) 30 min before light exposure. Non-anesthetized mice were exposed to 8,000 lux of white fluorescent light (Toshiba, Tokyo, Japan) for 3 h in cages with a reflective interior. The temperature during the light exposure was maintained at 25 °C ± 1.5 °C. After light exposure, all mice were returned to darkness for 24 h and then placed in a normal light-dark cycle. At 24 and 48 h after light exposure, the eyes were enucleated, and the tissues were used for western blotting or immunostaining.
Western blotting: Retinas were rapidly frozen at −80 °C. Samples were then homogenized in radioimmunoprecipitation (RIPA) buffer containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich). Lysates were centrifuged at 12,000 × g for 10 min at 4 °C; the supernatants were used for the western blotting. Protein concentrations were measured by comparison with a known concentration of bovine serum albumin (BSA) using a bicinchoninic acid protein assay kit (1:2,000; Thermo Fisher Scientific, Waltham, MA). Sample buffer (Wako) was added (samples:sample buffer = 3:1) and boiled for 5 min.

The samples were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5–15% gradient gels (Wako), and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were incubated in blocking One P (Nacalai Tesque) for 30 min at room temperature. The membranes were then washed in TBS (Tris-buffered saline) containing 0.05% Tween-20 and incubated overnight with primary antibodies (sheep anti-PGRN, 1:100, R&D Systems; mouse anti-SLPI, 1:100, Santa Cruz, Dallas, TX; rabbit anti-Iba-1, Wako, 1:200; and mouse anti-β-actin, 1:2,000, Sigma-Aldrich) at 4 °C. All primary antibodies were diluted in Can Get Signal Solution 1 (TOYOBO, Osaka, Japan). Following primary antibody incubation, membranes were washed in TBS containing 0.05% Tween-20 three times, and incubated with secondary antibodies diluted in Can Get Signal Solution 2 (TOYOBO; horseradish peroxidase [HRP]-conjugated rabbit anti-sheep immunoglobulin [IgG; Thermo Fisher Scientific]; HRP-conjugated goat anti-rabbit IgG, 1:2,000, Thermo Fisher Scientific; HRP-conjugated goat anti-mouse antibody, 1:2,000, Thermo Fisher Scientific) for 1 h at room temperature. After incubation with secondary antibodies, the membranes were washed three times, and proteins were visualized using an imaging reagent (ImmunoStar LD, Wako) and device (LAS-4000, FUJIFILM, Tokyo, Japan).

Preparation of cryosections: Eyes were enucleated at 24 and 48 h after light exposure and fixed in 4% paraformaldehyde (PFA; Wako) in 0.1 M phosphate buffer (pH 7.4; Wako) overnight at 4 °C. Following fixation, the eyes were cryoprotected with 25% sucrose in 0.1 M phosphate buffer (pH 7.4) for 48 h at 4 °C. The eyes were then embedded in optimal cutting temperature (OCT) compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan) and rapidly frozen on dry ice. Sections (10 µm thick) were sliced on a cryostat and placed on glass slides (MAS COAT; Matsunami Glass Ind., Ltd., Osaka, Japan).

Immunostaining: After washing with PBS, the sections were blocked with 10% horse serum (Vector Labs, Burlingame, CA) for 1 h at room temperature, and then incubated overnight with primary antibodies (sheep anti-PGRN antibody, 1:50, R&D Systems; rabbit anti-Iba-1, 1:200, Wako) at 4 °C. The sections were washed with PBS and incubated with secondary antibodies (Alexa Fluor®647 donkey anti-sheep IgG, Thermo Fisher Scientific; Alexa Fluor®546 donkey anti-rabbit IgG, Thermo Fisher Scientific) for 1 h at room temperature. The sections were treated with Hoechst 33342 (1:2,000; Invitrogen) to stain nuclei, and were mounted in Fluoromount (Diagnostic BioSystems, Pleasanton, CA). A confocal microscope (LSM700; Zeiss, Oberkochen, Germany) or FLUOVIEW FV10i (Olympus, Tokyo, Japan) was used to capture representative data or for quantification analysis, respectively. For quantitative analysis, images were captured at 250 and 750 µm from the optic nerve head. The number of PGRN+ and Iba-1+ double-positive cells was counted within the area of the image (500 × 500 µm), and density was expressed as the number of PGRN+ and Iba-1+ double-positive cells per millimeter.

Statistical analysis: Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey’s test, or Dunnett’s test (SPSS version 24, IBM Corporation, Armonk, NY). A p value of less than 0.05 was considered statistically significant.

RESULTS

Cleaved PGRN exerts a protective effect against light-induced photoreceptor cell death: To investigate the effect of granulins, PGRN was proteolytically cleaved into granulins in vitro (Figure 1A). PGRN was converted into granulins by elastase in a concentration-dependent manner. The pattern of the lower molecular weight cleavage product was consistent with a previous report [20].

Light-induced photoreceptor cell death was then induced to investigate whether cleaved PGRN demonstrated a neuroprotective effect. Light exposure induced death in murine photoreceptor (i.e., 661W) cells. In this experiment, enzymatic activities of elastase were inhibited by PMSF after cleavage (Appendix 2), and then it was confirmed that anyatic activities of elastase were inhibited by PMSF after cleavage (Appendix 2), and then it was confirmed that any...
Figure 1. Protective effects of PGRN and cleaved PGRN treated with elastase against light-induced photoreceptor cell death. A: Progranulin (PGRN) was cleaved by elastase. Western blotting of recombinant PGRN and recombinant PGRN treated with 0.1, 0.5, and 1.0 Unit/ml elastase from the porcine pancreas. Recombinant PGRN was incubated with elastase for 1 h. The left and right images were detected with long and short exposure times, respectively. B: Representative images of cone photoreceptor cells (661W) stained with Hoechst 33,342 and propidium iodide (PI). C: 661W cells were incubated under light exposure. PBS, PGRN, cleaved PGRN ([P + E] + phenylmethylsulfonyl fluoride [PMSF]), PMSF, and elastase incubated with PMSF were added to the medium. The number of PI-positive cells was increased after light exposure. Recombinant PGRN and cleaved PGRN statistically significantly suppressed light-induced photoreceptor cell death. Data are shown as mean ± standard error of the mean (SEM; n = 5). **, p<0.01 versus control; ***, p<0.01 versus vehicle *, p<0.05 versus vehicle (Tukey’s test). P, PGRN; E, elastase. Scale bar = 100 µm.
the effect of granulins in the absence of full-length PGRN, full-length PGRN was first separated from this mixture using centrifuge filters (50 kDa cutoff), with separation confirmed with western blotting (Figure 2A), followed by investigation of the protective effect of granulins against photoreceptor cell damage. Intermediate poly-granulins and granulins attenuated photoreceptor cell death induced by excess light exposure, as well as full-length PGRN (Figure 2B). These data suggested that granulins and intermediate poly-granulins retained the active site that conferred protective action.

**Expression and cleavage of PGRN in the retina after light exposure:** Because the biologic function and cleavage of PGRN during photoreceptor degeneration are unclear, expression patterns in the retina after light exposure were examined (Figure 3A). Although western blotting revealed that the protein levels of the granulins were increased in the retina after light exposure compared with the non-irradiated control retinas, the levels of PGRN were not statistically significantly altered (Figure 3B–D). The specificity of the bands of PGRN and granulins were confirmed with Grn knockout (KO) mice [27-29] (Appendix 3).

Light irradiation also increased the ratio of granulins to PGRN (Figure 3E), and the expression level of SLPI at 48 h after exposure (Figure 3F). Moreover, we examined PGRN expression in the macrophages and microglia because PGRN is mainly secreted by macrophages, microglia, or neurons in the CNS [8]. In accordance with these data, time-dependent changes in the expression level of Iba-1 (a macrophage and microglia marker) was observed (Figure 3G). Taken together, these data suggested that PGRN and its cleavage product (i.e., granulins) are expressed in degenerative retinas after light exposure.

**PGRN is expressed by macrophages and microglia in the outer nuclear layer:** We previously reported that apoptotic cells were statistically significantly increased in the outer nuclear layer (ONL) at 48 h after excessive light exposure [30]. To investigate the localization of PGRN and granulins in the degenerative retina, retinal sections were immunostained with anti-PGRN antibody that can detect PGRN and granulins (Figure 3B) at the same time after light exposure (see Figure 4A). In the normal retina, the expression of PGRN and Iba-1 positive cells was not observed in the ganglion cell layer, inner nuclear layer, and ONL (Figure 4A,C). The number of PGRN‘ Iba-1‘ double-positive cells, however, was...
Figure 3. Expression of PGRN and granulins after excess light exposure. A: Schematic of the experimental protocol. Albino male mice were exposed to white light at 8,000 lux for 3 h. Then western blotting and immunostaining were performed 24 and 48 h after light exposure, respectively. B: The arrowhead and arrow indicate the progranulin (PGRN) and granulin bands, respectively. Quantification analysis shows the expression levels of granulins (C), granulins/PGRN (E), secretory leukocyte protease inhibitor (SLPI) (F), and Iba-1 (G) were increased in the retina after light exposure. The increasing tendency was observed in the PGRN expression level (D). Data are shown as mean ± standard error of the mean (SEM; n=5). *, p<0.05 versus normal (Dunnett’s test).
statistically significantly increased in the ONL at 24 or 48 h after light exposure (Figure 4A,B,D). Collectively, these data suggested that accumulated Iba-1+ macrophages and microglia in the ONL express PGRN or granulins or both in the degenerative retina.

**DISCUSSION**

Retinal degeneration, as observed in AMD and RP, primarily affects photoreceptor cells, and is a leading cause of blindness; however, there are few effective therapeutic strategies for these disorders. In the present study, we demonstrated that PGRN exerts a protective effect against photoreceptor cell damage, even after undergoing proteolytic cleavage into granulins. These findings support the therapeutic effects of PGRN and granulins in AMD or RP.

Potential roles for PGRN in the CNS have been proposed. PGRN exerts a neuroprotective effect in some diseases, including brain injury and neurodegeneration [18,31-34], and is known to be implicated as the primary genetic cause of FTLD [14,15]. Recently, it has been reported that complete loss of PGRN leads to neuronal ceroid lipofuscinosis, a
lysosomal storage disorder [35], which highlights the importance of PGRN in the brain.

However, granulins do not always exhibit the same effect as PGRN. Granulin E and PGRN promote neurite outgrowth and survival [16,36]. In contrast, expression of nematode granulin 2 and 3, most closely homologous to human granulins B and E, respectively, increase the levels of TDP-43, which has been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS) or FTLD, and exacerbate its toxicity [37]. These reports indicated that it is unclear whether individual granulins confer protective effects or lead to harmful effects in the CNS. Data from the present study demonstrated that PGRN and its cleavage products possess neuroprotective properties (Figure 1 and Figure 2). In addition, Tang et al. demonstrated that an engineered molecule consisting of three granulin fragments exhibited interaction with tumor necrosis factor receptor 2 (TNFR2) and suppressed TNF-α-mediated inflammation (an effect similar to PGRN) [38]. Therefore, it appears that granulins and intermediate poly-granulins may still retain the necessary active sites essential for their neuroprotective effects. Results from the present study suggest the potential for clinical applications of PGRN in neurodegenerative or inflammatory diseases by engineering smaller molecular weight derivatives.

Two types of cell surface receptors have been proposed as targets for PGRN: TNFR1/TNFR2 and sortilin [38,39]. PGRN blocks the binding of TNF-α to TNFR1 and TNFR2, and may regulate extracellular PGRN levels by interaction with sortilin. Despite its pleiotropic effects, it remains controversial whether these receptors truly have the ability to mediate signal transduction for its neurotropic actions [40]. We previously suggested hepatocyte growth factor (HGF) receptor as a novel target for PGRN, given that treatment with PGRN promotes phosphorylation of the HGF receptor in 661W cells [25]. Moreover, the differentiation of retinal precursor cells induced by PGRN was attenuated by SU11274, an HGF receptor inhibitor in primary retinal cells [27]. Neonatal (postnatal day 9) Grn−/− mice exhibited decreased phosphorylation of the HGF receptor [28]. These data suggest that the HGF receptor may be a pivotal target for PGRN in photoreceptor cell protection.

The balance between intact PGRN and granulins appears to be regulated by SLPI, which binds to PGRN or elastase, or both, and prevents cleavage [10]. In this study, we observed a statistically significant increase in granulin levels after light exposure (Figure 3B–E); the protein levels of SLPI were also increased after light exposure (Figure 3F). Other studies have demonstrated that PGRN and granulins act as anti- or proinflammatory cytokines, respectively, during peripheral inflammation [10,11]. Therefore, we hypothesized that SLPI may regulate the ratio of PGRN to granulins, and that a disruption in the control of this balance leads to tissue damage and inflammation related to AMD pathology. Light exposure causes retinal inflammation that involves the complement system [41]. The enhancement of SLPI and the increased tendency of PGRN at 48 h after light exposure may show that SLPI protects newly secreted PGRN from proteolytic conversion and results in recovery from inflammatory conditions. This concept is supported in mice lacking SLPI [10,42], or NE and PR-3 [11], which resulted in enhanced or decreased inflammation, respectively. However, details regarding the modulation of the “PGRN-granulin axis” remain largely unknown. A better understanding of the switching mechanism for the conversion of PGRN into granulins may lead to the identification of potential therapeutic targets in treating inflammation-related disorders.

Results of the present study suggested that PGRN plays a potentially crucial role in converting to granulins in progressive retinal degeneration in conditions such as AMD and RP. Further studies are needed to determine which active sites exert protective effects and whether granulins are also protective in vivo. In conclusion, the present results fill an important information gap in the literature and may contribute to the development of promising new therapeutic strategies targeting PGRN or studying the action of PGRN and granulins.

APPENDIX 1. STR ANALYSIS.
To access the data, click or select the words “Appendix 1.”

APPENDIX 2. INHIBITION OF ELASTASE’S CYTOTOXIC ACTIVITIES BY PMSF.
To access the data, click or select the words “Appendix 2.” PMSF inhibited the enzymatic activities of elastase. Data are shown as mean ± SEM. (n = 5). #, p < 0.01 versus control; **, p < 0.01 versus vehicle (Tukey’s test).

APPENDIX 3. COMPARISON OF THE OBSERVED PGRN/GRN BANDS OF RETINA BETWEEN LIGHT-DAMAGED ALBINO MICE AND GRN KO MICE.
To access the data, click or select the words “Appendix 3.” Arrowhead and arrow indicate the band of full-length PGRN and granulin, respectively.
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