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Progranulin haploinsufficiency reduces amyloid beta deposition in Alzheimer's disease model mice

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Abstract: Granulin (*Grn*) mutations were identified in familial frontotemporal lobar degeneration (FTLD) patients with TAR DNA-binding protein of 43 kd (TDP-43) pathology. *Grn* transcript haploinsufficiency is proposed as a disease mechanism that leads to the loss of functional progranulin (PGRN) protein. Thus, these mutations are strongly involved in FTLD pathogenesis. Moreover, recent findings indicate that *Grn* mutations are associated with other neurodegenerative disorders with tau pathology, including Alzheimer's disease. To investigate the influence of PGRN on amyloid beta (Aβ) accumulation, amyloid precursor protein (APP) transgenic mice were interbred with *Grn*-deficient mice, producing APP transgenic mice harboring the *Grn* hemizygote (APP/*Grn*$^{+/-}$). Brains were collected from 16–18-month-old APP and APP/*Grn*$^{+/-}$ mice and sequential extraction of proteins, immunoblotting and immunohistochemical analysis were performed. Immunohistochemical analysis showed that the number and area of Aβ plaque was significantly decreased in APP/*Grn*$^{+/-}$ mice as compared to APP mice. Immunoblotting analysis revealed that Aβ was reduced in the sarkosyl-insoluble fraction of 16–18-month-old APP/*Grn*$^{+/-}$ mice as compared with that of APP transgenic mice. Our data suggest that PGRN haploinsufficiency may decrease accumulation of Aβ.
Key words: Alzheimer's disease, amyloid beta (Aβ), granulin, haploinsufficiency, progranulin

Introduction

Progranulin (PGRN) is a growth factor which is encoded by a single gene on chromosome 17q21. It is a 593-amino acid, cysteine-rich protein with a signal peptide (17 amino acids) and highly conserved 7.5 tandem granulin repeats of a 12 cysteinyl motif. PGRN is involved in the regulation of multiple functions, including neuronal cell growth [7, 36], wound healing [11, 38] and inflammation [37]. It has also been strongly linked to tumorigenesis [26]. Moreover, it has a chemoattractive effect for microglia [28]. In 2006, granulin (*GRN*) null mutations were identified in familial frontotemporal dementia (FTD) linked to chromosome 17q21 with tau-negative, ubiquitin-positive inclusions. Many mutations, including frame shift by insertion and deletion or substitution of a nucleotide, have been reported, which generate premature termination codons. *GRN* transcript

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haploinsufficiency is the proposed disease mechanism that leads to the loss of functional PGRN protein. Premature stop codons are not translated into the mutant transcript, since translation is blocked by nonsense-mediated RNA decay. The mutation in the signal peptide may cause mislocalization of PGRN in a protein secretion pathway or PGRN loss of function by impairment of PGRN transport [1, 25]. Thus, these mutations are strongly involved in FTD pathogenesis.

GRN mutations causing loss of function have been confirmed in patients clinically diagnosed with Alzheimer's disease (AD) [3–6, 9, 16, 18–20, 30]. The rs5848 (3′UTR + 78C>T) variant in the 3′ untranslated region of *GRN* is known to reduce *GRN* mRNA levels in the brain and peripheral mononuclear cells in patients. The rs5848 variant was also found in AD [8] and associated with a risk for AD [21]. *GRN* mutations were also found in corticobasal syndrome, another tauopathy [1, 2, 23, 27, 31]. These findings suggest that decline or dysfunction of PGRN may cause tau abnormalities, leading to the formation of tau pathology by activation of cyclin dependent kinases (CDKs) [12], TYROBP network genes [32] or lysosomal dysfunction [33, 35].

There has been an interesting report by Minami *et al.* [24] on Aβ accumulation with *GRN* deficiency. They used APP^{high} LysM-cre^{+} $Grn^{flox/flox}$ mice and showed that PGRN reduction increased Aβ deposition in these mice model and that overexpression of PGRN by lentivirus decreased Aβ plaque load. Very recently, Takahashi *et al.* [32] reported that APP/PS1 mice harbouring the *Grn* homozygote knock-out (APP/PS1/$Grn^{-/-}$ mice) exhibited less Aβ pathology. These two reports suggested that PGRN null condition might affect Aβ pathology in mice. However, the influence of PGRN haploinsufficiency on Aβ accumulation is still unknown.

To investigate the influence of PGRN haploinsufficiency ($Grn^{+/-}$) on Aβ accumulation, we produced APP transgenic mice harboring the *Grn* hemizygote (APP/$Grn^{+/-}$) by interbreeding APP transgenic mice with *Grn*-deficient mice, and found that the PGRN haploinsufficiency decreases Aβ deposition.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, Labour and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science (Permit Numbers: 22–23 and 11–028). All experiments were performed under isofluran anesthesia and every effort was made to minimize suffering.

Animals

The APP transgenic mice (Tg2576) [15] were purchased from Taconic (Hudson, NY, USA). Granulin (*Grn*) deficient (knock-out: KO) mice were obtained from RIKEN Bioresource Center (Tsukuba, Japan), which was established by Kayasuga *et al*. [17]. *Grn*-KO ($Grn^{-/-}$) mice had been back-crossed to C57BL/6J mice for more than 10 generations. Male APP homozygote transgenic mice were interbred with female *Grn*-KO mice so that APP transgenic mice harboring the *Grn* hemizygote (APP/$Grn^{+/-}$) were produced. Control mice (APP hemizygote) were produced by interbreeding male APP homozygote transgenic mice and female C57BL/6J mice. We used three female APP mice and three female APP/$Grn^{+/-}$ mice (age range: 16–18-month-old) in this study. Minami *et al.* reported that PGRN expression level of $Grn^{+/-}$ mice was half of the wild type mice ($Grn^{+/+}$) [24]. The mice were reared in the animal facility of Tokyo Metropolitan Institute of Medical Science under conventional conditions at 24 ± 2°C and were maintained on a commercial diet (CE-2, Nihon CLEA, Shizuoka, Japan) *ad libitum*.

Mice were sacrificed under quick anesthesia with isofluran (Mylan Pharmaceutical Co., Ltd., Tokyo, Japan) and the brains were removed quickly. Brains of each group were cut in the sagittal plane and the left hemisphere was frozen and stored at −80°C for biochemical analyses. The right hemisphere was fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 36 h at 4°C. Brain blocks were then transferred to a maintenance solution of 20% sucrose in 0.01 M PBS, pH 7.4.

Sequential fractionation of brain extracts

Frozen left hemispheres (approximately, 0.2 g) were homogenized in 10 volumes of buffer H (10 mM Tris-HCl, pH 7.5, 0.8 M NaCl, 1 mM ethylene glycol bis-N, N, N', N'-tetraacetic acid, 1 mM dithiothreitol). The hemisphere included the olfactory bulb, cerebral cortex, striatum, thalamus, hypothalamus, cerebellum, midbrain,

pons, medulla oblongata and the upper part of the spinal cord. The method used for sequential fractionation of brain extracts was originally described by Greenberg *et al.* [10]. Briefly, each brain homogenate was centrifuged at 100,000 × g for 20 min at 4°C, and the supernatant was collected as the Tris-soluble fraction. The resultant pellet was homogenized in 10 volumes of buffer H, followed by an incubation for 30 min at 37°C with 1% Triton X-100. The homogenate was then centrifuged at 100,000 × g for 20 min at 4°C. The Triton X-100 insoluble pellet was sonicated in 5 volumes of buffer H, followed by an incubation for 30 min at 37°C with 1% sarkosyl and centrifuged at 100,000 × g for 20 min at 4°C. The pellet was sonicated in 1 volume of SDS-PAGE sample buffer [13].

Immunoblotting analysis

For immunoblotting, brain extracts from the mice were boiled for 5 min with SDS-PAGE sample buffer (60 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 0.025% bromophenol blue and 5% mercaptoethanol) and loaded onto a 10% acrylamide minigel. Loaded samples were electrophoresed for 45 min at 200 V with molecular weight markers (Bio-Rad, Hercules, CA, USA). Electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) for 60 min at 200 mA. The printed membranes were blocked with 3% gelatin for 30 min and then incubated in a primary antibody solution (6E10, 1:1,000, Covance, Dedham MA, USA or anti-α-tubulin, 1:10,000, Sigma, St. Louis, MO, USA) overnight at room temperature. Antibody labeling was performed by incubation with horse radish peroxidase-conjugated anti-mouse IgG (1:50,000, Bio-Rad) for 1 h. Following incubation with avidin-biotinylated horseradish peroxidase complex (ABC Elite, Vector Laboratories, 1:400), immunoreactivity was detected by the chemiluminescence method using a Super Signal West Dura (Thermo Scientific, West Palm Beach, FL, USA) and was visualized with LAS-4000 mini (GE Healthcare UK Ltd.). Densitometric analysis of Aβ level was performed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Analysis of Aβ deposition

For Aβ immunohistochemistry, sagittal sections from left hemisphere were cut serially on a freezing microtome at 30 μm thickness, collected in the maintenance solution, and immunostained as free-floating sections. Sections were incubated for 24 h with biotinylated anti-Aβ antibody (6E10). The antibody labeling was visualized by incubation with avidin-biotinylated horseradish peroxidase complex (ABC Elite, Vector Laboratories, 1:1,000) for 3 h, followed by incubation with a solution containing 0.01% 3,3′-diaminobenzidine (DAB), 1% nickel ammonium sulfate, 0.05 M imidazole and 0.00015% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6. Counter nuclear staining was performed with Kernechtrot stain solution (Merck, Darmstadt, Germany). The sections were then rinsed with distilled water, mounted on glass slides, treated with xylene, and coverslipped with Entellan (Merck).

Photographs were taken with a BZ-X710 (Keyence, Osaka, Japan). The dark-purple plaques were counted in the area of the cerebral cortex and hippocampus. Two sagittal sections from each mouse were subjected to counts of the Aβ plaque number and area by Keyence BZ-710.

Statistical Analysis of Aβ deposition

Data are presented as mean ± SE. The statistical significance of differences in the mean values between 2 populations was assessed with the Student *t*-test, whether variances were equal was determined by an F-test, and otherwise we used Mann-Whitney's *U*-test. $P<0.05$ was considered significant.

Results

Aβ deposition was decreased in APP/Grn$^{+/-}$ mice by immunohistochemical staining

Brains were collected from 16–18-month-old mice of APP or APP/*Grn*$^{+/-}$ and immunohistochemical staining was performed. Aβ deposition was visualized using an anti-Aβ antibody, 6E10. A 6E10 immunoreaction was observed in the cortex and hippocamups of APP mouse and APP/*Grn*$^{+/-}$ mouse (Fig. 1A). Two sagittal sections from each mouse were subjected to counts of the Aβ plaque number and area by Keyence BZ-710. The number of 6E10 positive Aβ plaques which were larger than 4 μm^2 in the cortex and hippocampus was significantly decreased in APP/*Grn*$^{+/-}$ mice (441 ± 5) compared with APP mice (978 ± 149) (P=0.0495 by Mann-Whitney's *U*-test) (Fig. 1B). The area of 6E10 positive plaques was also significantly decreased in APP/*Grn*$^{+/-}$ mice (181,358 ± 15,246 μm^2) compared with APP mice (412,777 ± 53,369 μm^2) (P=0.014 by Student *t*-test) (Fig. 1C).

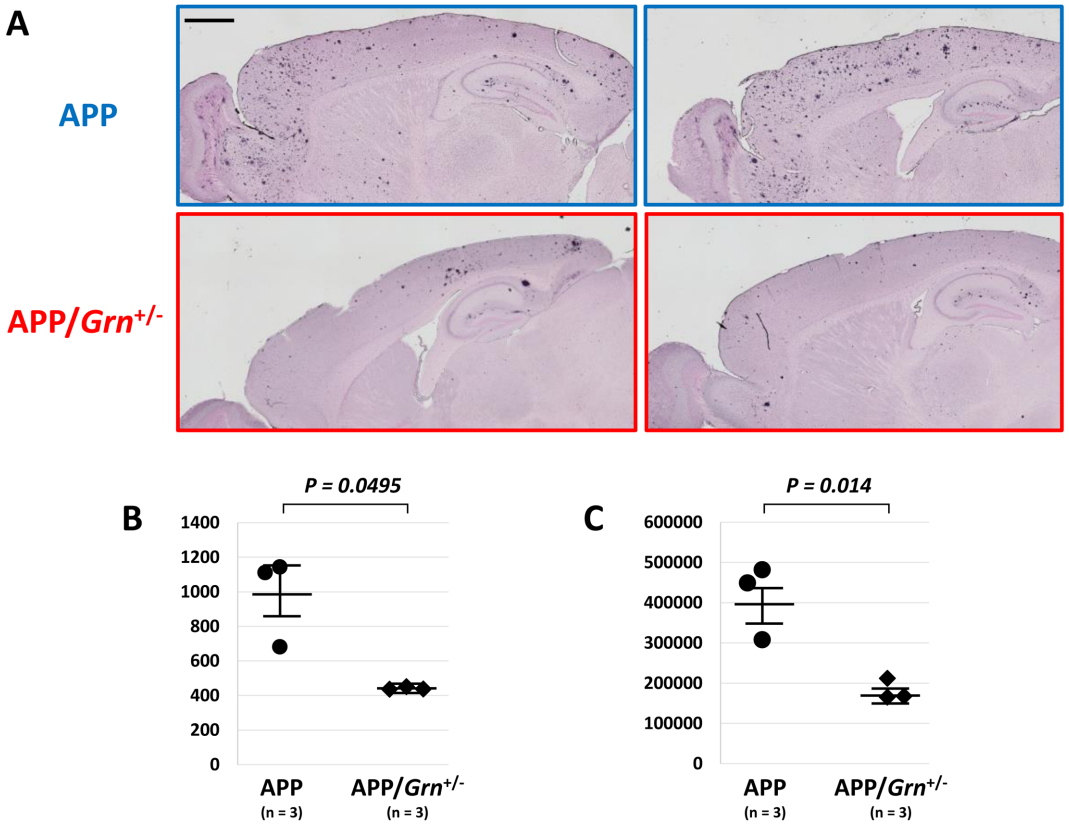

Fig. 1. Immunohistochemical analysis of Aβ deposition in 16-18-month-old APP or APP/*Grn*$^{+/-}$ mice. (A) Immunohistochemical analysis was visualized using the 6E10 antibody for detecting Aβ deposition in 16-18-month-old APP or APP/*Grn*$^{+/-}$ mice. The scale bar applies to all photomicrographs (1.0 mm). (B, C) A comparison of relative Aβ plaque number (B) and area (C) in 16-18-month-old mice. The Aβ plaque numbers and areas of Aβ deposition (μm^2) in the two different strains were compared. Two sagittal sections from each mouse were subjected to counts of the Aβ plaque number and area, then the average of them were plotted on the graph. $P<0.05$ was considered to represent a statistically significant difference.

Aβ deposition was decreased in the sarkosyl-insoluble fraction of APP/Grn$^{+/-}$ *mice*

Brains were collected from 16–18-month-old mice of APP or APP/*Grn*$^{+/-}$, then sequential protein extraction and immunoblotting were performed. Aβ in the sarkosyl-insoluble fraction was visualized by Western blotting using the 6E10 antibody. The results suggest that the level of sarkosyl-insoluble Aβ was decreased in the APP/*Grn*$^{+/-}$ mice (n=3) as compared with the APP mice (n=3) (Fig. 2A). The Aβ level in sarkosyl-insoluble fraction was significantly decreased in APP/*Grn*$^{+/-}$ mice (19,719.8 ± 936.7) compared with APP mice (35,148.3 ± 3,007.0) by the densitometric analysis. (P=0.009 by Student *t*-test) (Fig. 2B). The Aβ40 and Aβ42 levels in Tris-soluble fraction were no significant differences between APP and APP/*Grn*$^{+/-}$ mice by ELISA (data not shown).

Discussion

The results of the present study show that PGRN haploinsufficiency reduce Aβ deposition in the APP mice. It suggests that *GRN* mutations causing PGRN reduction may not be causative or risk factor for Aβ pathology. However, our previous report suggested that PGRN haploinsufficiency may cause tau abnormalities, leading to the formation of tau pathology by activation of CDKs [12]. Very recently, we have revealed that accumulation of phosphorylated tau was found in the brains of FTLD associated with *GRN* mutation [14].

These opposing effects of *GRN* deficiency against Aβ and tau might be explained as follows. Microglia produce PGRN and it suppresses hyper-activation of microglia by an autocrine effect [34]. PGRN deficiency may cause activation of microglia and they phagocyte extracellular

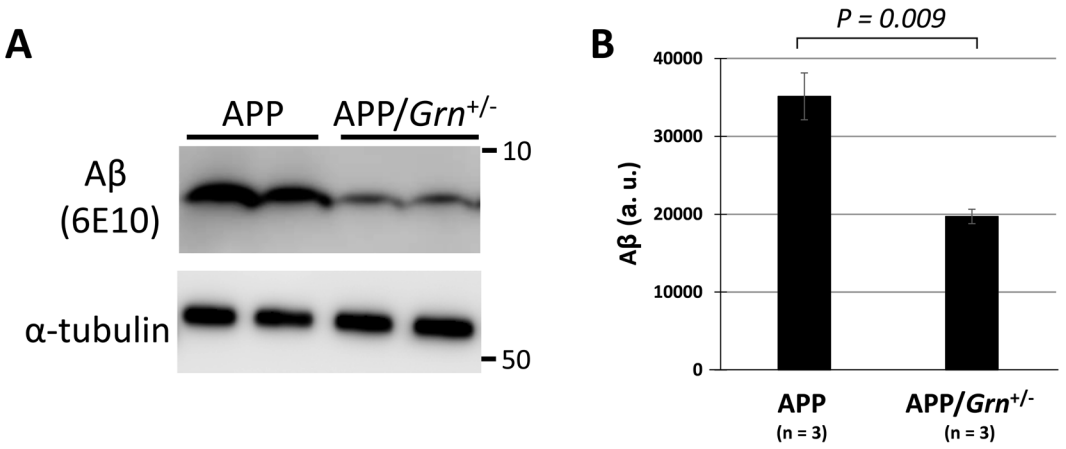

Fig. 2. Immunoblotting analysis of Aβ deposition for the sarkosyl-insoluble fraction in 16-18-month-old APP or APP/$Grn^{+/-}$ mice. (A) Immunoblotting analysis was visualized using the 6E10 antibody for detecting Aβ deposition in the sarkosyl-insoluble fraction of 16-18-month-old APP or APP/$Grn^{+/-}$ mice. Molecular weight markers are shown on the right (kDa). (B) Densitometric analysis of Aβ level in sarkosyl-insoluble fraction. $P<0.05$ was considered to represent a statistically significant difference. a.u., arbitrary unit.

Aβ. On the other hand, PGRN deficiency leads to lysosomal dysfunction in neuronal cells and may thus favor abnormal tau deposition. A schematic diagram of the opposing effects of *GRN* deficiency against Aβ and tau is shown in Fig. 3.

The inference of PGRN reduction on Aβ accumulation, has been reported by Minami *et al.* [24]. They used APP^{high} LysM-cre^{+} $Grn^{flox/flox}$ mice and showed that PGRN reduction increased Aβ plaque load in these mice model. The discrepancy between their results and ours might be explained by the difference in mouse strain as indicated in a recent study [32]. APP^{high} LysM-cre^{+} $Grn^{flox/flox}$ conditional mice were used in their study and LysM-cre mice lack endogenous *Lyz2*, which is markedly increased in $Grn^{-/-}$ mice [22, 29]. Microglia from $Grn^{-/-}$ mice showed upregulation of phagocytic activity [32], but phagocytic activitiy was down-regulated in APP^{high} LysM-cre^{+} $Grn^{flox/flox}$ mice [24].

Recently, Takahashi *et al.* [32] reported that global PGRN reduction induces microglial TYROBP network genes expression and increases AD risk by exacerbating neuronal injury and tau pathology, rather than by accelerating Aβ pathology [32]. They utilized APP/PS1-$Grn^{+/+}$, – $Grn^{+/-}$, and – $Grn^{-/-}$ mice. The APP/PS1- $Grn^{-/-}$ mice showed reduction of Aβ deposition compared with 16-month old APP/PS1-$Grn^{+/+}$ or APP/PS1-$Grn^{+/-}$ mice. Their study could not elucidate the effect of PGRN haploinsufficiency on Aβ deposition. However, global PGRN reduction decreased Aβ accumulation clearly. Their results supported our previous report [12] and this study.

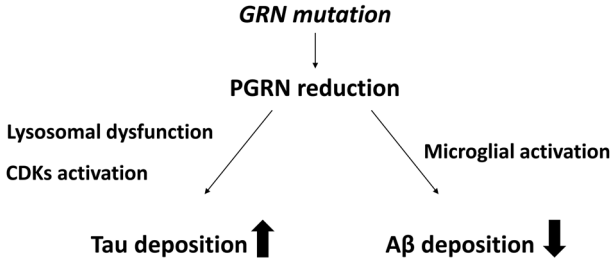

Fig. 3. Schematic diagram of the opposing effects of *GRN* deficiency against Aβ and tau. PGRN reduction caused by a *GRN* mutation may increase tau deposition in neuronal cells by lysosomal dysfunction or CDKs activation. PGRN reduction may decrease Aβ deposition by microglial activation.

Our results suggested that PGRN haploinsufficiency may reduce Aβ deposition and may not be causative or represent a risk factor for Aβ pathology.

Conflict of Interest

None declared.

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