Multiple therapeutic effects of progranulin on experimental acute ischaemic stroke

Masato Kanazawa,1,* Kunio Kawamura,1,* Tetsuya Takahashi,1 Minami Miura,1 Yoshinori Tanaka,2 Misaki Koyama,1 Masafumi Toriyabe,1 Hironaka Igarashi,3 Tsutomu Nakada,3 Masugi Nishihara,2 Masatoyo Nishizawa1 and Takayoshi Shimohata1

See Zhao and Bateman (doi:10.1093/awv123) for a scientific commentary on this article.

*These authors contributed equally to this work.

In the central nervous system, progranulin, a glycoprotein growth factor, plays a crucial role in maintaining physiological functions, and progranulin gene mutations cause TAR DNA-binding protein-43-positive frontotemporal lobar degeneration. Although several studies have reported that progranulin plays a protective role against ischaemic brain injury, little is known about temporal changes in the expression level, cellular localization, and glycosylation status of progranulin after acute focal cerebral ischaemia. In addition, the precise mechanisms by which progranulin exerts protective effects on ischaemic brain injury remains unknown. Furthermore, the therapeutic potential of progranulin against acute focal cerebral ischaemia, including combination treatment with tissue plasminogen activator, remains to be elucidated. In the present study, we aimed to determine temporal changes in the expression and localization of progranulin after ischaemia as well as the therapeutic effects of progranulin on ischaemic brain injury using in vitro and in vivo models. First, we demonstrated a dynamic change in progranulin expression in ischaemic Sprague-Dawley rats, including increased levels of progranulin expression in microglia within the ischaemic core, and increased levels of progranulin expression in viable neurons as well as induction of progranulin expression in endothelial cells within the ischaemic penumbra. We also demonstrated that the fully glycosylated mature secretory isoform of progranulin (~88 kDa) decreased, whereas the glycosylated immature isoform of progranulin (58–68 kDa) markedly increased at 24 h and 72 h after reperfusion. In vitro experiments using primary cells from C57BL/6 mice revealed that the glycosylated immature isoform was secreted only from the microglia. Second, we demonstrated that progranulin could protect against acute focal cerebral ischaemia by a variety of mechanisms including attenuation of blood–brain barrier disruption, neuroinflammation suppression, and neuroprotection. We found that progranulin could regulate vascular permeability via vascular endothelial growth factor, suppress neuroinflammation after ischaemia via anti-inflammatory interleukin 10 in the microglia, and render neuroprotection in part by inhibition of cytoplasmic redistribution of TAR DNA-binding protein-43 as demonstrated in progranulin knockout mice (C57BL/6 background). Finally, we demonstrated the therapeutic potential of progranulin against acute focal cerebral ischaemia using a rat autologous thrombo-embolic model with delayed tissue plasminogen activator treatment. Intravenously administered recombinant progranulin reduced cerebral infarct and oedema, suppressed haemorrhagic transformation, and improved motor outcomes (P = 0.007, 0.038, 0.007 and 0.004, respectively). In conclusion, progranulin may be a novel therapeutic target that provides vascular protection, anti-neuroinflammation, and neuroprotection related in part to vascular endothelial growth factor, interleukin 10, and TAR DNA-binding protein-43, respectively.

1 Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan
2 Department of Veterinary Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
3 Department of Centre for Integrated Human Brain Science, Brain Research Institute, Niigata University, Niigata, Japan
Correspondence to: Dr Takayoshi Shimohata, Department of Neurology, Brain Research Institute, Niigata University, 1-757 Asahimachi-dori, Chuoku, Niigata, Niigata 951-8585, Japan E-mail: t-shimo@bri.niigata-u.ac.jp

Keywords: progranulin; cerebral ischaemia; VEGF; IL10; TDP-43

Abbreviations: FTLD-TDP = TAR DNA binding protein positive frontotemporal lobar degeneration; OGD = oxygen glucose deprivation; TDP-43 = TAR DNA-binding protein 43; TGF-β = transforming growth factor beta; TNF-α = tumor necrosis factor alpha; tPA = tissue plasminogen activator

Introduction

Progranulin (PGRN) is a secreted N-linked glycoprotein growth factor implicated in tissue regeneration, wound repair, and inflammation (He et al., 2003a). Several in vitro studies have revealed that PGRN is classified into two isoforms according to its glycosylation status, the glycosylated immature isoform (58–68 kDa) and the fully glycosylated mature secretory isoform (~88 kDa) (Shankaran et al., 2008; Naphade et al., 2010; Alquézar et al., 2014).

In the CNS, PGRN is considered to play a crucial role in maintaining physiological functions and in the survival of neurons for the following reasons: first, primary cortical neurons derived from Pgrn knockout mice showed reduced neuronal survival (Kleinberger et al., 2010); second, PGRN has been shown to exhibit neurotrophic activity in vitro (Van Damme et al., 2008) and in vivo (Laird et al., 2010); and third, mutations in the Pgrn gene caused ubiquitin- and TAR DNA binding protein-positive frontotemporal lobar degeneration (FTLD-TDP), a progressive neurodegenerative disorder with an autosomal dominant inheritance pattern (Baker et al., 2006; Cruts et al., 2006).

Several studies raise the possibility that PGRN might be involved in ischaemic brain injury. First, PGRN might play a vascular protective role against focal cerebral ischaemia via suppression of blood–brain barrier disruption. Recent studies have demonstrated that intraventricular administration of recombinant PGRN could suppress cerebral oedema in a mouse transient focal cerebral ischaemia (suture model) (Egashira et al., 2013), and that Pgrn knockout mice may prompt post-ischaemic blood–brain barrier disruption (Jackman et al., 2013).

Second, PGRN has been shown to be involved in neuroinflammation after ischaemic brain injury. Previous studies have demonstrated that PGRN was induced in activated microglia after spinal cord injury (Naphade et al., 2010) and traumatic brain injury (Tanaka et al., 2013), indicating that the induction of PGRN reflects microglial responses. Similarly, in ischaemic brain injury, microglia have been shown to mediate expansion of the infarct via inflammation in the acute phase (Mabuchi et al., 2000; Yenari et al., 2010), while it might also contribute to endogenous anti-inflammatory protection in the subacute phase (Faustino et al., 2011). In this context, recent studies suggested that PGRN might suppress secretion of pro-inflammatory cytokines and recruitment of neutrophils associated with focal cerebral ischaemia (Tao et al., 2012; Egashira et al., 2013). However, a recent study showed that PGRN was not involved in neuroinflammation related to acute focal cerebral ischaemia (Jackman et al., 2013). Therefore, the role of PGRN in neuroinflammation after ischaemic brain injury is controversial.

Third, PGRN might exhibit protective effects on neuronal cells after acute focal cerebral ischaemia based on analyses using transgenic mice expressing PGRN (Tao et al., 2012) and Pgrn knockout mice (Jackman et al., 2013), although the mechanism of neuroprotection remains unknown. We previously demonstrated that ischaemic neuronal injury might be caused in part by cleavage and cytoplasmic redistribution of TARDBP (previously known as TDP-43), a key protein in FTLD-TDP and amyotrophic lateral sclerosis (Kanazawa et al., 2011b). Because PGRN can suppress the cleavage of TARDBP via inhibiting caspase 3 (Zhang et al., 2007; Kanazawa et al., 2011b), it raises the possibility that PGRN may prevent ischaemic neuronal injury via preservation of TARDBP functions.

Based on the abovementioned findings, we speculated that PGRN could exert a positive therapeutic effect via regulation of blood–brain barrier function, suppression of inflammation, and neuroprotection against acute focal cerebral ischaemia. Here, we investigated temporal changes in PGRN after focal cerebral ischaemia, including its cellular expression, glycosylation status, and sources of secretion. We also examined the role of PGRN in neuroinflammation after ischaemic brain injury and the mechanisms of neurovascular protective effects of PGRN. Moreover, we used a rat autologous thrombo-embolic model (Okubo et al., 2007), which shows cerebral oedema and haemorrhagic transformation when tissue plasminogen activator (tPA) is administered beyond the therapeutic time window.
(Kanazawa et al., 2011a; Kawamura et al., 2014) and demonstrated for the first time that intravenous administration of recombinant PGRN showed therapeutic effects on the volumes of cerebral infarct and oedema, haemorrhagic transformation, and prognosis.

Materials and methods

This study was carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Niigata University Administrative Panel on Laboratory Animal Care. All surgeries were performed under inhalation and intraperitoneal anaesthesia and according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Kilkenny et al., 2010).

Focal cerebral ischaemia

Transient acute focal cerebral ischaemia was induced using the intraluminal filament in male Sprague-Dawley rats weighing 300–370 g and wild-type (C57BL/6) and Pgrn knockout mice (C57BL/6 background) weighing 22–28 g (Kanazawa et al., 2011b). Briefly, anaesthesia was administered via inhalation of 1.5% halothane in a mixture of 70% nitrous oxide and 30% oxygen for rats and with intraperitoneal injection 10 mg/kg xylazine and 300 mg/kg chloral hydrate for mice. A nylon monofilament, with a diameter of 0.148 mm for rats and 0.074 mm for mice, was used for suturing. The tip of the nylon monofilament was rounded with heat. An 11-mm distal suture segment was coated with 0.350 mm diameter silicon for rats, and a 4-mm distal suture segment was coated with 0.185 mm diameter silicon for mice. Middle cerebral artery occlusion was performed by insertion of the suture via an external carotid artery into the internal carotid artery. After 90 min of ischaemia, the suture was withdrawn to restore blood flow. Body core temperature monitored with a rectal probe was maintained at 37.0 ± 0.5 °C, using a heating pad. To exclude the effects of anomalies of circle of Willis on the ischaemic size and oedema in Pgrn knockout mice, we investigated patterns of circle of Willis in both wild-type and Pgrn knockout mice using transcardial perfusion with carbon black suspended in saline (n ≥ 7) (Supplementary Fig. 1; Özdemir et al., 1999). The sample size was calculated before performing the experiments. We calculated the sample size needed to detect the difference in the amount of cerebral haemorrhage or cerebral oedema volume between the tPA with the recombinant PGRN group and tPA with the control group with 80% power (α = 0.05; one-sided analysis). Mice and rats were randomly assigned to various experimental groups, and analyses were performed by an investigator blinded to genotype and treatment.

Immunoblotting

The rats that survived for 10 min, 4 h, 10 h, 18 h, 24 h, and 72 h after reperfusion were euthanized with an overdose of halothane, followed by intracardiac perfusion with cold normal saline. We defined ischaemic penumbra as the region that was not rescued by reperfusion as described previously (Memezawa et al., 1992; Kanazawa et al., 2011b). The cortical tissues corresponding to the ischaemic core or penumbra were dissected for immunoblotting as described (Kanazawa et al., 2011b). Cortical tissues from the sham-operated rats were also dissected as controls. For whole-cell extracts, proteins from brain tissues were extracted as described previously (Shimohata et al., 2007). The proteins (50 μg) were subjected to tris-glycine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

For the whole-cell extracts in vitro, cells were harvested in cold cell lysis buffer (Cell Signaling Technology). Conditioned media were collected and concentrated (30×) using Amicon Ultra-0.5 tubes (Millipore). Proteins (5 μg) in the samples were separated by tris-glycine SDS-PAGE and were probed with primary antibodies (Supplementary Table 1), followed by a secondary horseradish peroxidase-conjugated antibody (1:10 000). Signals were detected by an enhanced chemiluminescence (Millipore) and semiquantified by densitometry (n = 5). The membranes were stripped and probed with an anti-β-actin or an anti-transferrin antibody to confirm even loading of proteins.

Immunofluorescence staining and confocal microscopy

Rats and mice euthanized with an overdose inhalation of halothane or intraperitoneal injection of xylazine chloral hydrate were sacrificed by transcardial perfusion with normal saline followed by perfusion with cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 24 h and 72 h after reperfusion. We stained free-floating sections as described previously (Kanazawa et al., 2011b) and mounted with Vectashield DAPI (Vector Laboratories). Information of the primary antibodies is described in Supplementary Table 1. The sections were examined under a confocal laser-scanning microscope (LSM510 META; Carl Zeiss). The results were confirmed in three independent samples.

Immunohistochemistry

Deeply anaesthetized rats and mice were perfused intracardially with cold 4% paraformaldehyde at 24 h and 72 h after reperfusion. The brains were removed and embedded in paraffin wax. Serial sections (4-μm thick) were cut from the paraffin blocks and stained using antibodies (Supplementary Table 1). The immunoreactive products were visualized using the ABC Vectastain kit (Vector Laboratories) and diaminobenzidine as the chromogen. The results were confirmed in three independent samples. To measure immunoreactivity, we used ImageJ software 1.37r (National Institutes of Health, Bethesda, MD, USA) as described elsewhere (Krishnamurthy et al., 2009). Briefly, images of the stained sections were visualized, examined blindly, and seven randomly chosen non-overlapping high-power fields (×400) covering the ischaemic cortex were analysed for both wild-type and Pgrn knockout mice (n = 21). For each image field, the total number of pixels was quantified on a grey scale (0–255). The levels of immunoreactivities were expressed as the average density (mean pixel value in an image field ± SEM) for each experimental group.
Measurement of the volume of the cerebral infarct and oedema

At 24 h after reperfusion, the rats and mice were given highly concentrated halothane and deeply anaesthetized. After transcardial perfusion with saline, the brains were extracted. The brains were cut into 3-mm slices and stained with 2% 2, 3, 5-triphenyltetrazolium chloride solution (#264310; BD, Franklin Lakes). After staining, the slices were photographed with a scanner (CanoScan LiDE 50; Canon Inc), and cerebral infarct volume and cerebral oedema volume were measured with ImageJ software 1.37v (National Institutes of Health, Bethesda, MD, USA) according to the method described by Swanson et al. (1990). These values were expressed as a proportion of the cerebral hemisphere occupied. The analyses were performed by an investigator blinded to the genotype and treatment.

Neurological evaluations

Neurological evaluations were conducted at 24 h after reperfusion with a 6-point neurological scale (Zausinger et al., 2000). Specifically, grade 5 indicated no neurological findings, grade 4 indicated an inability to move forward with the foot on the affected side, grade 3 indicated weak resistance to a force that was applied from the side on a level surface, grade 2 indicated turning to the affected side when pulled from behind on a level surface, grade 1 indicated spontaneously turning to the affected side, and grade 0 indicated an inability to move spontaneously or death.

Pgrn knockout mice

Pgrn knockout mice were produced by breeding heterozygous pairs in the Graduate School of Agricultural and Life Sciences, at the University of Tokyo, and genotyped using previously described protocols (Kayasuga et al., 2007). Additionally, Pgrn knockout mice purchased from RIKEN BioResource Centre (Tsukuba, Japan) were bred and maintained in the Department of Comparative and Experimental Medicine, Brain Research Institute, Niigata University. We used only homozygous pairs to obtain Pgrn knockout pups. The mice were maintained under controlled light (lights on, 05:00–19:00), temperature (23 ± 1°C), and humidity (55 ± 10%) and given free access to food and water.

C6 cell culture and analysis of glycosylation

Rat C6 cells were grown in Opti-MEM® (Gibco/Invitrogen) with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were grown in Opti-MEM® without FBS 24 h before the experiments. Cells were harvested in cell lysis buffer (Cell Signaling Technology) containing 1% Triton X-100 and inhibitors for proteases and phosphatases (Sigma-Aldrich). After centrifugation at 14,000 rpm for 5 min (4°C), the supernatants were collected. Additionally, conditioned media were also obtained for immunoblotting. To analyse glycosylation of PGRN, cell lysates and media were digested at 37°C with peptide-N-glycosidase F (PNGase F; New England Biolabs) according to manufacturer’s instructions, in triplicate.

Primary cell cultures

Primary murine neuronal cells were prepared from the neocortices of embryonic Day 17 C57Bl/6 mouse embryos (Takeuchi et al., 2005). Briefly, cortical fragments were dissociated into single cells in a dissociation solution (Sumitomo Bakelite) and resuspended in nerve culture medium (Sumitomo Bakelite). Neurons were plated onto poly-l-lysine-coated (P7405, Sigma-Aldrich) six-well dishes (10 × 10⁵ cells per well). The purity of these neuronal cultures was 95% as determined by NeuN-specific immunostaining (Takeuchi et al., 2005).

Primary murine microglia and astrocytes were obtained as previously described (Milner et al., 2008). Primary mixed glial cultures were established from the forebrains of postnatal C57Bl/6 mice by dissociating isolated cerebral cortices in papain and then growing the resulting cell suspension for 10 days in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS. After 10 days, the flasks were shaken for 15 min to remove the loosely attached microglia. The purity of these microglial cultures was 99% as determined by Mac-1 immunoreactivity in flow cytometry (Milner et al., 2008). For astrocytes, the flasks were then shaken overnight to remove the microglia and oligodendrocyte precursors. The remaining monolayer was determined as >95% astrocytes by glial fibrillary acidic protein (GFAP) immunoreactivity (Milner et al., 2008).

To investigate the effect of microglia and astrocytes on secretion of vascular endothelial growth factor (VEGF) from astrocyte after oxygen glucose deprivation (OGD), we cultured astrocytes with microglial conditioned media. Briefly, after OGD using primary murine microglia, we collected the microglial low-glucose conditioned media. Then, we removed the astrocytic high-glucose conditioned media and washed thoroughly with PBS. We finally exchanged the PBS for the collected microglial low-glucose conditioned media.

After overnight incubation under OGD condition, we measured the level of VEGF in the conditioned media using VEGF Quantikine® ELISA Kit (RRV00, R&D Systems), according to the manufacturer’s instructions (n = 6–7). IL10 levels in the conditioned media from microglia were also determined by a sandwich ELISA using the IL10 Quantikine® ELISA Kit (M1000B, R&D Systems), according to the manufacturer’s instructions (n = 5–6).

Oxygen–glucose deprivation

The standardized conditions for OGD have been described in detail elsewhere (Milner et al., 2008). Briefly, before induction of OGD, serum-containing media was removed from the cell cultures by washing twice thoroughly with PBS before adding serum-free high glucose medium (4.5 g/l), DMEM containing 4 mM L-glutamine, penicillin, and streptomycin, supplemented with N1 medium) or low-glucose medium (1.0 g/l, supplemented with DMEM). The cultures containing low-glucose medium were placed in a hypoxia chamber (Billups-Rothenberg), which was flushed with a mixture of 95% N₂ and 5% CO₂ for 1 h, and then closed for 18 or 24 h. Oxygen levels decreased to 0.1–0.4% at 4 h and was maintained throughout the experiment (del Zoppo et al., 2012).

To determine the effects of PGRN under OGD, we added full-length recombinant mouse PGRN produced from HEK293 cells (AG-40A-0080, AdipoGen) into conditioned media. The recombinant PGRN is N-glycosylated, as determined by a
PNase F assay (manufacturer’s datasheet). Because a FLAG-tag was attached to the C-terminus of the recombinant PGRN protein, localization of exogenous recombinant PGRN protein was determined by immunohistochemical staining with an anti-FLAG antibody (Supplementary Table 1).

The survival of cells in culture exposed to OGD was determined by lactate dehydrogenase (LDH) release into the culture supernatant using a kit assay in six-well format (Roche Diagnostics), according to the manufacturer’s instructions. The relative assessments of cytotoxicity were normalized by comparison with 100% cell damage (n = 5). The survival of neuronal cells in the culture exposed to OGD was determined using the propidium iodide method in which 33 μg/ml propidium iodide solution (Sigma) was added to each well, followed by incubation for 5 min (Osada et al., 2011).

Cell counting protocols
To determine the frequency of propidium iodide-positive neuronal cells after OGD conditions, we examined seven randomly chosen non-overlapping high-power fields (× 630) of primary neuronal cells incubated under OGD conditions for 24 h (n = 21) (Kanazawa et al., 2011b). To determine the frequency of microtubule-associated protein 2 (MAP2)-positive neurons with cytoplasmic redistribution of TARDBP, seven randomly chosen non-overlapping high-power fields (× 630) of primary neuronal cells under normoxic or OGD conditions during 24 h were examined (n = 7) (Kanazawa et al., 2011b). We also determined the appearance frequencies of microglia and neutrophils in the ischaemic tissue in wild-type and Pgrn knockout mice by examining seven randomly chosen non-overlapping high-power fields (× 400) (n = 21). These analyses were conducted blinded to the sample information.

Reverse transcription polymerase chain reaction
Primary microglia were collected as pellets after centrifugation at 13 000 rpm for 10 min. Total RNA was isolated from the samples using Nucleospin RNA XS (U0902A, Takara Bio), and the first-strand cDNA was synthesized with ReverTra Ace (TOYOBO). PCR reactions were performed with PrimeSTAR® Max DNA Polymerase (Takara Bio) and the following primers: TNF-α (forward, 5′- AAGAGGCACCT CCCCCAAAA -3′; reverse, 5′- GCTACAGGCTTGTCACT CGAA -3′), TGF-β (forward, 5′- TGGAGACACATGTTGGA ACTC -3′; reverse, 5′- CAGCAGCGGTATCCAAG -3′), IL-10 (forward, 5′- CTAAACGCCTCTTTATGCA -3′; reverse, 5′- TAAAACCTCTCTCATC -3′), and actin (forward, 5′- CATCCGAAAGACCTTATGCCAC -3′; reverse, 5′- ATGGAGCCACCGATCCACA -3′). The PCR reactions were carried out as follows: 35 cycles for amplification at 98°C for 10 s, 55°C for 5 s, and 72°C for 5 s. The genes of interest were normalized to actin (n = 5–7).

Quantitative real-time PCR
Primary microglia were collected as pellets after centrifugation at 13 000 rpm for 10 min. Total RNA was isolated from samples using Nucleospin RNA XS (U0902A, Takara Bio), and first-strand cDNA was synthesized from total RNA by ReverTra Ace (TOYOBO). Quantitative RT-PCR was performed with SYBR® Green Premix Ex Taq on a TP-850 Real-Time PCR Detection system (Takara Bio). We designed primer-pairs for IL10 as follows: (forward, 5′- ATTGTAAATCTGGTTGAGAAG -3′; reverse, 5′- CACAGGGGAGAAATCGATGACA -3′). PCR conditions were 30 s at 95°C for initial denaturing, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Expression levels of the target gene relative to housekeeping enzyme (Takara Bio) were determined using the ΔΔCT method (n = 3–4).

Thrombolytic treatment with tissue plasminogen activator and PGRN
Cerebral ischaemia was created using a model of focal embolic ischaemia, in which the animals had their middle cerebral artery occluded by autologous thrombi (Okubo et al., 2007; Kanazawa et al., 2011a). tPA was administered intravenously in the form of alteplase (Mitsubishi Tanabe Pharma Co.) at a dose of 10 mg/kg per animal at 4 h after cerebral ischaemia, because we previously demonstrated that this condition caused cerebral oedema, haemorrhagic transformation, and severe prognosis (Kanazawa et al., 2011a; Kawamura et al., 2014). We demonstrated that cerebral cortical blood flow decreased by 40% from baseline after ischaemia and tPA treatment 4 h after ischaemia improved it by >70% from baseline in this thrombo-embolic model (Kanazawa et al., 2011a). Immediately before tPA administration, 100 μg of FLAG-tagged recombinant PGRN or control protein (IgG; R5G10-048, OEM Concepts) was administered as a bolus through a catheter in the inguinal vein (n = 6–7). The volumes of the cerebral infarct and oedema, cerebral haemorrhage, neurological evaluations, and mortality ratio were examined. Cerebral infarct and oedema and cerebral haemorrhage measurements were performed in a randomized and blinded manner.

Statistical analyses
All data were presented as mean ± SEM. Differences in the parameters were analysed using one-way or two-way ANOVA, followed by Bonferroni’s post hoc test or unpaired t-test. Statistical analyses were performed using SPSS (ver.21; SPSS Inc.). Differences in the frequencies were assessed with Fisher’s exact test or Mann-Whitney’s U-test. All tests were considered statistically significant at a P-value < 0.05.

Results
Post-ischaemic changes of PGRN expression in neuronal cells, microglia and endothelial cells in rat cerebral cortex
To investigate the expression and localization of PGRN in the non-ischaemic cortex of Sprague-Dawley rats, we performed immunofluorescence staining using an anti-PGRN antibody. At first we tested specificity of the anti-PGRN antibody by immunoblotting and immunohistochemistry.
The cell lysates and brain tissue from Pgrn knockout mice did not show immunoreactivity against the antibody (Supplementary Fig. 1). Confocal microscopic studies revealed that the immunoreactivity for PGRN was detected only in cortical neurons (Fig. 1). PGRN was detected in a punctate pattern within neuronal cytoplasm, and double immunostaining revealed that PGRN was co-localized with marker proteins for the endoplasmic reticulum, Golgi apparatus, and lysosome [ERp57 (now known as PDIA3), Golgi-58k, and LAMP1, respectively]. We did not identify microglia or vessels that expressed PGRN.

We next investigated the effect of acute focal ischaemia on the expression and localization of PGRN using ischaemic Sprague-Dawley rats. Immunohistochemical analyses revealed that PGRN was not observed in the neurons from the ischaemic core (Fig. 2A), but observed in surviving neurons from the ischaemic penumbra (Fig. 2A). The expression pattern of PGRN in the surviving neurons from the ischaemic penumbra is diffused and strong, which is different from that of the non-ischaemic neurons (Fig. 2A). PGRN-positive microglia were increased at 24 h and markedly increased at 72 h after reperfusion, especially on the border of the ischaemic core and penumbra on the ischaemic core side (Fig. 2B). In addition, PGRN expression was observed in the von Willebrand factor-positive endothelial cells in the ischaemic penumbra at 24 h after reperfusion (Fig. 2C).

Temporal changes in PGRN expression and its glycosylated status in rat cerebral cortex after acute focal cerebral ischaemia

To investigate the effects of acute focal ischaemia on the expression and glycosylation status of PGRN, we next performed immunoblotting using whole-cell extracts of the ischaemic cortex of Sprague-Dawley rats. In the ischaemic core, the level of ~88 kDa PGRN gradually decreased after reperfusion, although it recovered at 72 h after reperfusion (Fig. 3A and B). On the other hand, the level of 58–68 kDa PGRN did not change until 18 h, but increased at 24 h and markedly increased at 72 h after reperfusion (Fig. 3A and B). In the ischaemic penumbra, the ~88-kDa PGRN decreased slightly, but not significantly, after reperfusion, while the 58–68 kDa PGRN moderately increased (Fig. 3A and B).

To confirm whether these two bands indicated secretory isoforms of PGRN, an in vitro study was performed. Cell lysates and conditioned media from rat C6 cells were subjected to immunoblotting, as this tumor cell line produces PGRN during growth (Liao et al., 2000). We identified a low molecular-weight band (58–68 kDa) in the cell lysates and a high molecular-weight band (~88 kDa) in the conditioned media (Fig. 3C). In addition, after digestion with PNGase F, which cleaves all N-linked oligosaccharides, the mobility of both bands from rat C6 cells changed to 58 kDa. A similar result was observed from an experiment using rat non-ischaemic brains (Fig. 3D). Therefore, it is considered that the high molecular-weight band (~88 kDa) was the secretory form of the fully glycosylated isoform of PGRN and that the low molecular-weight band (58–68 kDa) was the pre-secretory form of the glycosylated immature isoform of PGRN (Fig. 3D).

Production and secretion of the two isoforms of PGRN after OGD

To determine the potential cellular sources of the glycosylated immature isoform of 58–68 kDa PGRN, which markedly increased after reperfusion in ischaemic rats, immunoblot analyses of whole-cell extracts and conditioned media from primary murine neurons or microglia were performed. After 18 h incubation under OGD, both neurons and microglia were not dead as determined by the lack of change in LDH release from the cells (Fig. 4A). Under normoxic conditions, 58–68 kDa PGRN was observed in the neuronal cell extracts similar to C6 cell (Fig. 4B), and the ~88-kDa PGRN was observed in both neuronal and microglial conditioned media (Fig. 4C). We found that, after 18 h incubation under OGD, neurons produced ~88 kDa and 58–68 PGRN and secreted ~88 kDa PGRN, and the microglia produced ~88 kDa PGRN, and secreted both ~88 kDa and 58–68 kDa PGRN (Fig. 4C). Therefore, the glycosylated immature isoform of 58–68 kDa PGRN was secreted only from microglia.

Volumes of cerebral infarct and oedema, and outcome after ischaemia in Pgrn knockout mice

To investigate the roles of PGRN in acute focal cerebral ischaemia, we compared volumes of cerebral infarct and oedema between Pgrn knockout mice and wild-type mice, and evaluated the outcome by motor scale after middle cerebral artery occlusion. Before the comparison, we evaluated vascular anomaly of the circle of Willis (Ozdemir et al., 1999) of Pgrn knockout mice, and found no significant difference in vascular structure between wild-type and Pgrn knockout mice (Supplementary Fig. 2). There was also no significant difference in infarct size at 24 h after reperfusion between the two groups (P = 0.12) (Fig. 5A). However, cerebral oedema in the Pgrn knockout mice was larger than that in the wild-type mice at 24 h after reperfusion (P = 0.048) (Fig. 5B). In addition, motor scale at 24 h after reperfusion in Pgrn knockout mice was worse than that in the wild-type mice (P = 0.02) (Fig. 5C). There were no significant differences in cerebral oedema and motor scale at 72 h after reperfusion between the two groups (P = 0.95 and 0.75, respectively).
Figure 1 Analyses of PGRN expression in neuronal cells and microglia from rat non-ischaemic cerebral cortex. Microtubule-associated protein 2 (MAP2), endoplasmic reticulum stress protein 57 (ERp57, now known as PDIA3), Golgi-58k, lysosomal associated membrane protein 1 (LAMP-1), CD68/ED1 (green) / PGRN (red) / 4',6'-diamidino-2-phenylindole (DAPI; blue) triple labelling in the non-ischaemic cortices examined by confocal microscopy. MAP2, ERp57, Golgi-58k, LAMP-1, and CD68/ED1 are markers for neuronal cells, endoplasmic reticulum, Golgi apparatus, lysosome, and microglia, respectively. Scale bars = 10 μm.
Recruitment of neutrophils and microglia after ischaemia in Pgrn knockout mice

Because a recent study reported that recombinant PGRN treatment suppressed neutrophil recruitment into ischaemic mouse brain (Egashira et al., 2013), we investigated whether cerebral oedema in Pgrn knockout mice is associated with neutrophil recruitment. Immunohistochemistry using an anti-myeloperoxidase antibody revealed that there were no significant differences in the numbers of myeloperoxidase-positive neutrophils between the ischaemic wild-type and Pgrn knockout mice at 24 h and 72 h after reperfusion (Supplementary Fig. 3). We also found that there were no significant differences in the numbers of IBA1 (now known as AIF1)-positive microglia in the border of the ischaemic core and penumbra between the wild-type and Pgrn knockout mice at 24 h and 72 h after reperfusion (Supplementary Fig. 4).

VEGF expression in Pgrn knockout mice after ischaemia

We next investigated the possibility that cerebral oedema in Pgrn knockout mice was caused by VEGF expression, because VEGF is a potent angiogenic factor that regulates vascular permeability after focal cerebral ischaemia (Zhang et al., 2002). Additionally, a recent study, in which PDGF (platelet-derived growth factor) was used as an experimental agent to induce blood–brain barrier leakage, did not determine the VEGF protein level after ischaemia (Jackman et al., 2013). Although VEGF expression was undetectable in brains of sham-operated wild-type and Pgrn knockout mice, VEGF expression was observed in the peri-infarct area from brains of ischaemic wild-type and Pgrn knockout mice 24 and 72 h after reperfusion. At 72 h after reperfusion, the expression was much more prominent in Pgrn knockout mice than in wild-type mice ($P < 0.001$) (Fig. 5D). VEGF expression was observed
mainly in astrocytes and endothelial cells (but not in microglia) within ischaemic mice (Supplementary Fig. 5).

To confirm the increased level of VEGF in ischaemic Pgrn knockout mice, we performed an in vitro study. We first compared VEGF secretion after OGD from microglia and astrocytes between wild-type and Pgrn knockout mice (Fig. 5E). The levels of VEGF from microglia of Pgrn knockout mice were higher than those of wild-type mice.
P50.001), although the levels of VEGF from astrocytes were not different between wild-type and Pgrn knockout mice (P = 1.00). To investigate the effect of microglia on VEGF secretion from astrocytes, microglial condition media of wild-type and Pgrn knockout mice were added to the astrocytes of wild-type and Pgrn knockout mice, respectively. Interestingly, the levels of VEGF from the astrocytes with microglial conditioned media of knockout mice was higher than that of wild-type under OGD (P < 0.001). In addition, the levels of VEGF from astrocytes with microglial conditioned media of knockout mice was higher than that from microglia alone (P = 0.013) or astrocytes alone (P = 0.014) of Pgrn knockout mice (Fig. 5E). These results suggest that lack of PGRN or unknown humoral factors from ischaemic microglia of Pgrn knockout mice might synergically promote VEGF secretion from the astrocytes.

### The effects of VEGF expression by recombinant PGRN treatment after ischaemia in vitro and in vivo

To investigate the effect of recombinant PGRN on VEGF expression in vitro, we compared VEGF levels in astrocytes with microglial conditioned media for wild-type mice treated with vehicle controls and recombinant PGRN. The level of VEGF in astrocytes cultured in microglial conditioned media was decreased by recombinant PGRN (10 μg/ml) compared to vehicle treatment (23.0 ± 4.8% reduction; P = 0.046, n = 3–6). We previously reported that delayed tPA treatment 4 h after ischaemia promoted expression of VEGF in vivo (Kanazawa et al., 2011a). We investigated the effect of recombinant PGRN on VEGF expression using this thrombo-embolic model. For this purpose we administered 100 μg recombinant PGRN or vehicle along with tPA treatment 4 h after ischaemia. The co-administration of recombinant PGRN and tPA decreased the expression of VEGF compared to co-administration of vehicle and tPA (P = 0.046) (Supplementary Fig. 6).

### Comparison of cytokine response of microglia between wild-type and Pgrn knockout mice after OGD

To investigate the effect of PGRN on neuroinflammation after acute ischaemia, we compared mRNA levels of several cytokines in microglia from wild-type and Pgrn knockout mice, under normoxic and OGD conditions, because the balance between pro-inflammatory and anti-inflammatory microglial phenotypes is considered to influence expansion of cerebral infarct (Hu et al., 2012). We found that there were no differences in the mRNA levels of pro-inflammatory cytokines (TNF-α and TGF-β) between wild-type and Pgrn knockout mice, while the mRNA level of anti-inflammatory cytokine IL10 in the Pgrn knockout microglia was lower than that in the wild-type microglia after OGD.
Figure 5  Comparison of ischaemic outcomes and VEGF expression between wild-type and Pgrn knockout mice. These panels show volumes of the cerebral infarct (A) and cerebral oedema (B) at 24 h and 72 h after reperfusion (all, n = 9), and outcome evaluated by a 6-point neurological scale score (C) at 24 h after reperfusion [wild-type (WT) n = 29, knockout (KO) n = 22]. Volumes of cerebral infarct and oedema are expressed as proportions on the ischaemic side of the cerebral hemisphere. *P < 0.05, **P < 0.01. (D) VEGF expression in the sham-operated and ischaemic brains (at 24 h and 72 h after reperfusion) from the wild-type and Pgrn knockout mice. The results were confirmed in triplicate. A secondary-only antibody control confirms that extracellular stainings of VEGF after ischaemia were not non-specific (data not shown). At 72 h after reperfusion, the expression of VEGF was higher in ischaemic brains of Pgrn knockout mice than in those of wild-type mice (**P < 0.001) (n = 21). Scale bars = 200 μm. (E) The levels of secretory VEGF from microglia, astrocytes, and astrocytes with microglial conditioned media of wild-type and Pgrn knockout mice after OGD (n = 6–7). *P = 0.01, **P < 0.01.
We also found that *Il10* mRNA levels in the *Pgrn* knockout microglia were lower than that in the wild-type microglia after OGD by real-time PCR (*P* = 0.001) (Fig. 6C). The level of *Il10* after 18 h incubation under OGD from microglial conditioned media of *Pgrn* knockout mice (10.3 ± 3.1 pg/ml) was lower than that from microglial conditioned media of wild-type mice (37.4 ± 12.2 pg/ml) (*P* = 0.040).

We also investigated the expression of TNF-α, TGF-β, and IL10 in brains from ischaemic wild-type and *Pgrn* knockout mice 24 and 72 h after reperfusion. Although the expressions of TNF-α and TGF-β in the ischaemic brains were not different between wild-type and *Pgrn* knockout mice (*P* = 0.262 and *P* = 0.228, respectively) (Supplementary Fig. 7A and B), IL10 expression was lower in the ischaemic brains of *Pgrn* knockout mice than in those of wild-type mice (*P* < 0.001) (Supplementary Fig. 7C). IL10 was expressed mainly in the extracellular parenchyma.

**Effects of PGRN on neuronal cell death and cytoplasmic redistribution of TARDBP**

We investigated the neuroprotective effect of PGRN on murine primary neuronal cells under OGD condition. Twenty-four hours incubation under OGD promoted neuronal cell death as evaluated by a lactate dehydrogenase assay (Fig. 7A), and the percentage of propidium iodide-positive neuronal cells after 24-h incubation under OGD was 8.4 ± 3.7% (*n* = 21) (Supplementary Fig. 8). The attached neuronal cells that showed cytoplasmic redistribution of TARDBP under the OGD conditions were confirmed to be living as they were propidium iodide-negative (Fig. 7B). However, we found that the recombinant PGRN (5 μg/ml) suppressed the neuronal cell death compared to the control (*P* = 0.045) (Fig. 7C). Immunocytochemistry confirmed that these surviving neurons incorporated the exogenously administered...
Figure 7  Suppression of neuronal cell death and cytoplasmic redistribution of TARDBP (TDP-43) under OGD conditions by recombinant PGRN. (A) Cytotoxicity of neuronal cells between normoxic and OGD conditions at 18 h and 24 h (n = 5). * \(p < 0.01\). (B) Confocal microscopic observation of TDP-43 (now known as TARDBP, green)/propidium iodide (PI) (red)/DAPI (blue)-triple labelling in neuronal cells under OGD conditions. Magnification is double in the small inset. Cytoplasmic redistribution of TDP-43 was observed in surviving neurons under the OGD conditions. These neuronal cells were propidium iodide-negative. Scale bar = 50 \(\mu m\). (C) Recombinant PGRN (rPGRN) alleviated cytotoxicity at 24 h under OGD conditions (n = 6). * \(p < 0.05\). (D) MAP2 (green)/FLAG (red) / DAPI (blue) triple labeling in neurons between normoxic and OGD conditions by confocal microscopy. FLAG-tagged recombinant PGRN was co-localized in surviving neurons under OGD condition (arrow). Scale bars = 20 \(\mu m\). (E) MAP2 (green)/TDP-43 (red)/DAPI (blue) triple labelling in neurons between normoxic and OGD conditions with or without recombinant PGRN treatment by confocal microscopy. Arrow indicates cytoplasmic redistribution of TARDBP at 24 h under OGD conditions. Scale bars = 20 \(\mu m\). (F) The frequencies cytoplasmic redistribution of TARDBP in MAP2-positive neuronal cells between normoxic and OGD conditions. recombinant PGRN blocked cytoplasmic redistribution of TARDBP at 24 h under OGD conditions. * \(p < 0.05\), ** \(p < 0.01\) versus normoxic condition (n = 7).
FLAG-tagged recombinant PGRN (Fig. 7D). We also found that the neuronal cells, which incorporated the exogenous FLAG-tagged recombinant PGRN, showed a decrease in the cytoplasmic redistribution of TARDBP (Fig. 7E): the frequency of cytoplasmic redistribution of TARDBP in the neurons treated with recombinant PGRN under OGD was lower than that treated with the vehicle control (48.0 ± 35.4% versus 86.9 ± 10.4%; \( P = 0.010 \)) (Fig. 7F).

**Therapeutic effects of recombinant PGRN against delayed tPA treatment for acute focal cerebral ischaemia**

Finally, we investigated therapeutic effects of recombinant PGRN using a rat autologous thrombo-embolic model (Okubo et al., 2007), because this model shows cerebral oedema and haemorrhagic transformation when tPA is administered beyond the therapeutic time window (4 h) (Kanazawa et al., 2011a; Kawamura et al., 2014). The group treated with tPA and intravenous administration of recombinant PGRN at 4 h after reperfusion showed therapeutic effects on the cerebral infarct volume (Fig. 8A), oedema volume (Fig. 8B), haemorrhage (Fig. 8C), motor scale (Fig. 8D), and mortality ratio (Fig. 8E) compared to the group treated with tPA and control (\( P = 0.007, 0.038, 0.007, 0.004 \) and 0.026, respectively).

**Discussion**

We demonstrated for the first time the dynamic changes in PGRN in vivo in the neurons, microglia, and endothelial cells after ischaemia, including decreased level of PGRN.
expression in neurons within the ischaemic core, increased level of PGRN expression in the surviving neurons, as well as induction of PGRN expression in microglia and endothelial cells in the ischaemic penumbra. Immunoblot analyses demonstrated that the fully glycosylated mature secretary isoform (~88 kDa) decreased, while the glycosylated immature isoform (58–68 kDa) markedly increased after reperfusion. In addition, in vitro experiments revealed that the glycosylated mature isoform was secreted from neurons and microglia, and the glycosylated immature isoform was secreted only from microglia. Consistent with this finding, immunohistochemical analyses demonstrated a marked increase in PGRN-positive microglia at 72 h after reperfusion in ischaemic rats. These findings suggest that the marked increase in the glycosylated immature isoform of 58–68 kDa PGRN after reperfusion was caused by microglia.

We next demonstrated that PGRN has multiple therapeutic effects against ischaemic brain injury. First, we found that PGRN could attenuate blood–brain barrier disruption after acute focal cerebral ischaemia. We demonstrated for the first time that PGRN is expressed in the endothelial cells in the ischaemic penumbra. A previous report has shown that PGRN is induced in the capillary endothelium of wound granulation tissue and promotes the mitosis and migration of adult dermal microvascular cells (He et al., 2003b), although PGRN is not expressed in the healthy endothelium (Daniel et al., 2000). We speculated that the expression of PGRN in endothelial cells may be involved in the vascular protection or repair against ischaemic injury. Indeed, cerebral oedema volume in Pgrn knockout mice was larger than that in wild-type mice after focal cerebral ischaemia. Regarding the mechanism by which PGRN regulates vascular permeability, we considered that cerebral oedema is not caused by recruitment of neutrophils and microglia based on the findings from immunostainings using wild-type and Pgrn knockout mice. A very recent study demonstrated the involvement of the PDGF receptor pathway using Pgrn knockout mice (Jackman et al., 2013). We proposed another possibility that PGRN might regulate vascular permeability via VEGF pathway, because we observed more prominent expression of VEGF in Pgrn knockout mice than in wild-type mice after focal cerebral ischaemia. In addition, Pgrn knockout microglia itself secreted VEGF and the conditioned media from Pgrn knockout microglia synergically stimulated VEGF secretion from Pgrn knockout astrocytes after OGD. Although we could not identify the specific molecules that stimulate VEGF expression, a recent study demonstrated that PGRN might inhibit cerebral oedema via NF-κB (Egashira et al., 2013), which activates VEGF transcription (Yoshida et al., 1998). PGRN may regulate vascular permeability by inhibiting microglial production of molecules that activate NF-κB-VEGF signalling pathway.

In breast cancer, high levels of PGRN result in increased VEGF production (Tangkeangsirisin et al., 2004), while in this study of ischaemia, depletion of PGRN increased VEGF expression. Furthermore, we showed clear increases in PGRN expression in vessels of the penumbra (Fig. 2C), which is similar to what has been reported in peripheral wounds (He et al., 2003b). Because elevated levels of PGRN have a significant biological effect on vessel growth that might be independent of VEGF (Toh et al., 2013), some of the effects of Pgrn knockout on penumbral blood vessels might be mediated directly by PGRN loss rather than via increased VEGF expression (Jackman et al., 2013). Further studies are needed to clarify the mechanism underlying PGRN-mediated vascular permeability.

Second, PGRN may suppress neuroinflammation after acute focal cerebral ischaemia via the anti-inflammatory cytokine, IL10. We demonstrated that the number of PGRN-positive microglia (CD68/ED1-positive cells) increased in the ischaemic core of Sprague-Dawley rats, and that the primary cultured-microglia from Pgrn knockout mice showed a decrease in the levels of IL10 under OGD, although levels of inflammatory cytokines such as TNF-α and TGF-β were not changed. Consistent with this finding, previous studies reported that the level of IL10 in glial cells from PGRN-overexpressing transgenic mice was higher than that from wild-type mice (Tao et al., 2012), and that the macrophage/microglia from Pgrn knockout mice released less IL10 than from wild-type mice when exposed to bacterial lipopolysaccharide (Yin et al., 2010). However, findings on pro-inflammatory cytokines are controversial and inconclusive. It has been demonstrated that levels of other pro-inflammatory cytokines (IL1B, IL6 and TNF-α) in glial cells from PGRN-overexpressing transgenic mice were lower than those from wild-type mice (Tao et al., 2012), and that they were increased in the macrophage/microglia from Pgrn knockout mice compared to wild-type mice when mice exposed to bacterial lipopolysaccharide (Yin et al., 2010). In contrast, a very recent study demonstrated that mRNA levels of IL1b, Il6, TNF-α (now known as Tnf), and TGF-β (now known as Tgfb1) (Il10 not examined) after ischaemic brain injury were not associated with Pgrn knockout condition as seen in our results (Jackman et al., 2013). We consider, based on our findings, that PGRN may suppress neuroinflammation via IL10 not by inhibiting pro-inflammatory cytokines.

Third, we confirmed that PGRN might play a neuroprotective role against acute focal cerebral ischaemia in vitro and in vivo. Neuronal PGRN decreased in the ischaemic core where neuronal death occurred, and increased in the ischaemic penumbra where neuronal survival occurred after acute focal cerebral ischaemia. In vitro studies demonstrated that the neuronal cells and microglia produced and secreted PGRN after ischaemia, and that exogenous recombinant PGRN could suppress neuronal cell death under OGD. Although PGRN has been shown to have neurotrophic effects, the exact mechanism remains unknown (Van Damme et al., 2008; Laird et al., 2010). We consider that the neurotrophic effects of PGRN might be explained in part by the inhibition of abnormal cytotoxic
redistribution of nuclear TARDBP (Zhang et al., 2007, 2009). Stated another way, the decreased level of PGRN might cause loss of function of TARDBP in neurons, resulting in neuronal damage.

Finally, we demonstrated that intravenously administered recombinant PGRN reduced volumes of cerebral infarct and oedema, suppressed haemorrhagic transformation, and improved motor outcome with delayed tPA treatment resulting in haemorrhagic transformation that is similar to human stroke, in a rat autologous embolic model (Kanazawa et al., 2011a; Kawamura et al., 2014). A previous study reported that intraventricular administration of recombinant PGRN reduced cerebral infarct and oedema in a mouse focal suture ischaemic model (Egashira et al., 2013), although this model and injection route poorly reflected the clinical practice. Our findings indicate the possibility that recombinant PGRN could be used as a novel neurovascular protective drug with anti-inflammatory effect after delayed tPA treatment.

Because of the importance of protein glycosylation in mediating a wide range of biological processes, characterization of glycan structures is necessary to understand the structure–function relationship (Songsrirote et al., 2010). Therefore, we chose the full-length glycosylated recombinant PGRN protein. We could not evaluate the impact of glycosylated status of the PGRN on neuronal cells because it is technically hard to use deglycosylated PGRN after PNGase assay because of reagent toxicities for cells. Further studies are needed to determine the impact of glycosylation status for recombinant PGRN on this effect.

In conclusion, we demonstrated dynamic changes of expression and localization of PGRN after acute focal cerebral ischaemia. PGRN may be a novel therapeutic target that provides vascular protection, anti-neuroinflammation, and neuroprotection related in part to VEGF, IL10 and TARDBP, respectively.

Acknowledgements

We thank Dr Riuko Ohashi (Department of Cellular Function, Division of Cellular and Molecular Pathology, Niigata University) for her support in the confocal imaging studies.

Funding

This work was supported by a Grant-in-Aid for Scientific Research, a grant from Naito Foundation, Takeda Science Foundation, Japan Cardiovascular Research Foundation, Japan Brain Foundation, Yujin Memorial Grant, Japan Medical Association (TS), and a Grant-in-Aid for Scientific Research, a grant from SENSIN Medical Research Foundation, Uehara Memorial Foundation (MK).

Supplementary material

Supplementary material is available at Brain online.

References


