Introduction

Progranulin (PGRN) is a glycosylated protein released by a variety of cells. It contains a signal peptide and 7.5 tandem repeats of highly conserved granulin motifs each with 12 cysteine residues (Bateman et al., 1990; Shoyab et al., 1990; Bhandari et al., 1992; Plowman et al., 1992; He and Bateman, 2003). PGRN is widely expressed (Bhandari et al., 1993; Daniel et al., 2000) and has been implicated in many processes, such as development, tumor proliferation, wound healing, and inflammation (Bateman and Bennett, 1998; He and Bateman, 2003; Ahmed et al., 2007). In peripheral tissues, extracellular proteases, such as elastase, were shown to be able to cleave PGRN into several GRNs (GRN A–F and paragranulin), which probably have separate functions (Zhu et al., 2002; He and Bateman, 2003). In models of wound healing, secreted leukocyte protease inhibitor (SLPI) prevents PGRN processing through inhibition of elastase enzymatic activity and by binding PGRN and thus sequestering it from elastase (Zhu et al., 2002; He and Bateman, 2003). Little is known about the role of PGRN in the central nervous system. PGRN is widely expressed during early neural development (Daniel et al., 2003) but later on its expression becomes restricted to defined neuronal populations, such as cortical and hippocampal pyramidal neurons and Purkinje cells (Daniel et al., 2000). It has been implicated in the sexual differentiation of the brain (Suzuki and Nishiahara, 2002). PGRN is up-regulated in activated microglial cells (Baker and Manuelidis, 2003; Baker et al., 2006; Mackenzie et al., 2006; Mukherjee et al., 2006) but not in astrocytes or oligodendrocytes.

Recently, mutations in the progranulin (PGRN) gene were found to cause familial and apparently sporadic frontotemporal lobe dementia (FTLD). Moreover, missense changes in PGRN were identified in patients with motor neuron degeneration, a condition that is related to FTLD. Most mutations identified in patients with FTLD until now have been null mutations. However, it remains unknown whether PGRN protein levels are reduced in the central nervous system from such patients. The effects of PGRN on neurons also remain to be established. We report that PGRN levels are reduced in the cerebrospinal fluid from FTLD patients carrying a PGRN mutation. We observe that PGRN and GRN E (one of the proteolytic fragments of PGRN) promote neuronal survival and enhance neurite outgrowth in cultured neurons. These results demonstrate that PGRN/GRN is a neurotrophic factor with activities that may be involved in the development of the nervous system and in neurodegeneration.

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Abbreviations used in this paper: ALS, amyotrophic lateral sclerosis; AM, acetyoxymethyl; CSF, cerebrospinal fluid; FTLD, frontotemporal lobe dementia; GFAP, glial fibrillary acidic protein; GRN, granulin; NF-H, neurofilament heavy chain; PGRN, progranulin; SLPI, secreted leucocyte protease inhibitor.
Reduced PGRN-induced neuronal survival is thought to cause neurodegeneration. Missense mutations in PGRN have been identified as well. They were found in some patients with FTLD, with or without amyotrophic lateral sclerosis (ALS; Spina et al., 2007; van der Zee et al., 2007), and in rare ALS patients (Schymick et al., 2007; Sleeegers et al., 2008). The pathogenetic nature of these missense mutations remains to be demonstrated. It was recently shown that several of these missense mutations reduce the release of PGRN and thus also give rise to insufficient availability of PGRN (Shankaran et al., 2008).

Results and discussion

PGRN protein levels in the CSF from patients and controls

To measure PGRN protein levels, we developed an ELISA assay using a monoclonal PGRN antibody to coat 96-well plates, a polyclonal biotinylated PGRN antibody to detect the signal, and human recombinant PGRN as standard (Fig. 1 A). PGRN protein was detectable in CSF and was measured in the CSF from three patients carrying the Ser82fs mutation in PGRN and from 24 controls. The optical densities were 0.08 ± 0.04 and 0.23 ± 0.02 for patients and controls, respectively (P = 0.004). This corresponded to 2.2 ± 1.0 and 6.2 ± 0.6 ng/ml in patients and controls (P = 0.04; Fig. 1 B). The clearly reduced levels in patients with the Ser82fs mutation are in agreement with the predicted nonsense-mediated mRNA decay caused by the premature translation termination, resulting in reduced protein levels.

Effect of PGRN on neuronal survival

Although PGRN was shown to induce proliferation of PC-12 (rat adrenal gland pheochromocytoma) and SK-N-DZ (human brain neuroblastoma) cells (Daniel et al., 2000) and to inhibit apoptosis of tumor cells (He et al., 2002; Tangkeangsirisin et al., 2004), the effects of PGRN on neurons have not been established. To explore the neurotrophic potential of PGRN, we studied the effects of recombinant GRN E (aa 494–594 of PGRN, a functional proteolytic fragment of PGRN) and full-length PGRN (only lacking the first 18 aa, which code for the signal peptide) in monocultures of rat motor and cortical neurons.

To reduce potential confounding effects from other growth factors, neurons were treated with GRN E or PGRN in serum-free medium with no other recombinant growth factors added. Motor neurons isolated from the ventral spinal cord were seeded at low density allowing reliable quantification of survival by counting experiments (Van Den Bosch et al., 2004; Van Damme et al., 2007). GRN E was added to these cultures on day 1 in culture and was found to support neuronal survival in a dose-dependent manner (Fig. 2 A). This neurotrophic effect was still visible after 1 wk, a time point at which only a limited number of neurons in these serum-free and neurotrophin-free cultures survive (Fig. 2 B). The maximal dose of GRN E increased neuronal survival by 64% on day 2 and by 101% on day 6.
Survival and axonal outgrowth, respectively, supports the hypothesis that different GRNs and their precursors may have distinct biological functions (Zhu et al., 2002). Recently, PGRN-deficient mice have been generated (Kayasuga et al., 2007). These mice develop normally and do not show behavioral or motor abnormalities until 11 wk of age. However, it remains to be seen whether these animals develop axonal loss or neurodegeneration as they age.

Our results demonstrate that PGRN/GRN is a neurotrophic factor that enhances neuronal survival and axonal outgrowth. It supports the hypothesis that a relative lack of PGRN in patients with PGRN mutations may alter the integrity of neurites and lead to neurodegeneration. Mechanisms other than the lack of neurotrophic effect resulting from PGRN shortage may play a role in PGRN-associated neurodegeneration as well. In a recent study, PGRN knockdown was found to induce caspase-dependent cleavage of TDP-43 with accumulation of TDP-43 fragments (Zhang et al., 2007), similar to what is seen in FTLD and ALS (Neumann et al., 2006).

Although insufficient trophic support for neurons has been an appealing hypothesis for the pathogenesis of neurodegenerative disorders and the premise for many therapeutic strategies (Vande Velde and Cleveland, 2005), PGRN is the only neurotrophic factor identified to cause human disease through null mutations and haploinsufficiency. Therefore, it is warranted to investigate the therapeutic potential of PGRN for neurodegenerative disorders.

**Figure 3.** Inhibition of PGRN by SLPI abolishes its neurotrophic properties. (A and B) Effect of SLPI (ng/ml) on the neurotrophic properties of PGRN (ng/ml) in motor neurons (A; n = 3–4; *, P < 0.001) and cortical neurons (B; n = 6; *, P < 0.01). Error bars show mean ± SEM.

**Figure 4.** Effect of PGRN/GRN on soma size and neurite outgrowth. (A–D) NF-H staining of motor neuron (A and B) and cortical culture (C and D) treated with GRN E (B), PGRN (D), or control (A and C; Bar, 50 μm). (E and F) Effect of 100 ng/ml GRN E or PGRN (in the absence and presence of 100 ng/ml SLPI) on soma size (E) and maximal neurite length (F; n = 109–476; *, significantly different from control [P < 0.001]; #, significantly different from GRN E [P < 0.03]). Error bars show mean ± SEM.
A motor neuron–enriched neuronal population was obtained by centrifugation from rat embryos and dissociated enzymatically and mechanically. Afterward, bound PGRN was detected using biotinylated polyclonal anti-PGRN antibody (R&D Systems) were loaded. Afterward, bound PGRN was recognized, not recombinant GRN E or PGRN cleavage products obtained by elastase treatment (1 U/ml for 30 min at 25°C; Athens Research & Technology).

Neuronal cultures
Monocultures of cortical and motor neurons were prepared from 14-day-old Wistar rat embryos as previously described (Van Damme et al., 2003, 2007; Van Den Bosch et al., 2004) and plated in culture dishes (40,000 cells per dish for spinal cultures) or 96-well plates (10,000 cells per well) coated with poly-ornithine and laminin. Ventral spinal cords were dissected from rat embryos and dissociated enzymatically and mechanically. A motor neuron–enriched neuronal population was obtained by centrifugation on a 6.5% Optiprep cushion. For cortical cultures, the cortex was dissociated by trypsinization and trituration but the density centrifugation was omitted. The culture medium consisted of L15 supplemented with 0.2% sodium bicarbonate, 3.6 mg/ml glucose, 20 mM progesterone, 5 μg/ml insulin, 0.1 mM putrescine, 0.1 mg/ml conalbumin, 30 nM sodium selenite, and horse serum. No horse serum was added in the serum-free medium. Because partial recombinant human PGRN (coding for GRN E) was dissolved in buffer containing reduced glutathione, this was added to the corresponding controls as well. The relative survival was calculated as the number of cells on day 2 or 6 in culture divided by the number of cells on day 1. Survival of cortical neurons was quantified using a calcein assay (Bozyczko-Coyne et al., 1993; Lin et al., 2001). On day 1 in culture, neuronal cultures seeded in 96-well plates were treated with PGRN/GRN or control buffer. Neuronal survival was quantified 24 h later. On day 2 in culture, cells were collected with 2 μM calcein-AM in the culture medium for 1 h at 37°C. After replacement of the medium with PBS, fluorescence was measured on a microplate reader (excitation, 480 nm; emission, 535 nm). After subtraction of background signal from wells without cells, the values obtained were normalized to untreated control wells. For every condition, the mean of at least five wells was taken (considered as n = 1).

Immunocytochemistry
Immunostainings were performed as previously described (Van Damme et al., 2007). Cells were fixed for 20 min using 4% PFA. Cultures were incubated with NF-H antibody (1:400; rabbit polyclonal), NeuN antibody (1:1,000; mouse monoclonal), GFAP antibody (1:1,000; mouse monoclonal), and O4 (1:500; mouse monoclonal) overnight. The appropriate Alexa594- and Alexa488-labeled antibodies (1:500) were used to develop the stain.

Quantification of neurite length
Motor and cortical neuronal cultures were exposed to recombinant PGRN/GRN in serum-free medium immediately after seeding for 24 h and stained for NF-H on day 1 in culture. Photographs of representative areas of the culture were taken on an upright microscope (DMRB; Leica) at a magnification of 10 (0.3 NA; PL Fluotar; Leica) at room temperature using a camera (DMX 1200; Nikon). The size of the cell soma and the longest neurite of more than 100 neurons per experimental condition were measured on the obtained digital pictures using Lucia imaging software (version 4.60; Laboratory Imaging).

Materials and statistics
Media and additives were obtained from Invitrogen. All other chemicals were obtained from Sigma-Aldrich. Partial recombinant human PGRN (aa 494–502, coding for GRN E and flanking regions) was obtained from Abnova Corporation. Full-length recombinant human PGRN (aa 18–593) was obtained from R&D Systems. NF-H and GFAP antibodies were obtained from Sigma-Aldrich. NeuN antibody was obtained from Millipore and anti-oligodendrocyte marker O4 was obtained from R&D Systems. Alexa-labeled secondary antibodies were obtained from Invitrogen.

Mean data are shown as mean ± SEM and Student's t tests were used to calculate significance. When more than two groups were compared, a one-way analysis of variance with Tukey-Kramer multiple comparisons was used.

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