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Progranulin expression in brain tissue and cerebrospinal fluid levels in multiple sclerosis

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Abstract

Background: Progranulin (PGRN) is a fundamental neurotrophic factor, and is also involved in inflammation and wound repair. PGRN may have pro- or anti-inflammatory properties, depending upon proteolysis of the anti-inflammatory parent PGRN protein and the generation of pro-inflammatory granulin peptides.

Objectives: Our objectives were as follows: (1) to evaluate the presence and distribution of PGRN in multiple sclerosis (MS) brain tissue, correlating it with demyelination and inflammation; (2) to evaluate cerebrospinal fluid (CSF) PGRN concentrations in patients with MS and controls, in relationship to the clinical features of the disease.

Methods: Our study involved the following: (1) neuropathological study of PGRN on post-mortem tissue of 19 MS and six control brains; (2) evaluation of PGRN CSF concentration in 40 MS patients, 15 non-inflammatory controls and five inflammatory controls (viral encephalitis).

Results: In active demyelinating lesions, PGRN was expressed on macrophages/microglia. In the normal-appearing white matter (NAWM), expression of PGRN was observed on activated microglia. PGRN was expressed by neurons and microglia in cortical lesions and in normal-appearing cortex. No expression of PGRN was observed in controls, except on neurons. PGRN CSF concentrations were significantly higher in patients with relapsing–remitting MS during relapses and in progressive MS patients, compared with relapsing–remitting MS patients during remissions and with non-inflammatory controls.

Conclusions: PGRN is strongly expressed in MS brains, by macrophages/microglia in active lesions, and by activated microglia in the NAWM; PGRN CSF concentrations in MS are correspondingly increased in conditions of enhanced macrophage/microglia activation, such as during relapses and in progressive MS.

Keywords

grey matter, inflammation, microglia, multiple sclerosis, neuroprotection, progranulin

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Introduction

Multiple sclerosis (MS) is the major inflammatory demyelinating disease of the central nervous system (CNS), predominantly considered an autoimmune white matter disease; however, grey matter pathology is extensive, may precede white matter pathology and occurs in part independently.[1,2] This makes conceivable that proteins previously studied for their role in neurodegenerative diseases might also play a role in MS.

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A potential candidate is progranulin (PGRN), a multifunctional protein with important roles in inflammation and tissue repair, and also a neurotrophic factor that enhances neuronal survival and axonal outgrowth.[3–5] Its importance as a trophic factor is underscored by the fact that PGRN is the only neurotrophic factor identified to cause human disease, namely frontotemporal lobe degeneration (FTLD), through null mutations and haploinsufficiency.[6]

PGRN is a cysteine-rich protein, containing seven granulin-like domains. Proteolytic cleavage of the PGRN protein by extracellular proteases, such as elastases, produces smaller fragments termed granulins (GRNs).[7] These fragments range in size from 6–25 kDa and have biological functions different from the parent PGRN protein. PGRN may have pro- or anti-inflammatory properties, depending upon the extent of regulated proteolysis of the anti-inflammatory parent PGRN protein and the generation of pro-inflammatory GRN peptides. PGRN has been demonstrated to be an important factor in wound healing and inflammation.[8,9] The addition of PGRN to a wound increases and prolongs infiltration of macrophages and neutrophils. Stimulation with PGRN causes proliferation and migration of fibroblasts and endothelial cells. Further studies have shown that PGRN and GRNs have different effects on epithelial cells and inflammatory cells. GRNs stimulate epithelial cells to secrete interleukin-8, a major chemoattractant for neutrophils and monocytes, whereas PGRN has no such effect. PGRN inhibits degranulation and oxidative bursts of leukocytes activated by TNFα. In contrast, GRN peptides have no inhibitory effect. These observations suggest pro-inflammatory and anti-inflammatory roles for GRNs and PGRN, respectively.[3]

Considering the roles of PGRN as a neurotrophic factor and in inflammation, we wished to assess the involvement of PGRN in MS brains, in correlation with key pathological features of MS such as demyelination and inflammation. We also wished to evaluate the correlations between cerebrospinal fluid (CSF) PGRN levels and clinical features of the disease in patients with MS.

Objectives

The aims of this study were to:

1. evaluate the presence and distribution of PGRN in MS brain tissue, correlating it with features such as demyelination and inflammation.
2. evaluate differences in CSF PGRN concentration between patients with MS and controls.
3. evaluate the relationship between CSF PGRN concentration and clinical features of the disease in MS patients.

Material and methods

Neuropathology

This part of the study was performed on formalin-fixed, paraffin-embedded material of 19 autoptic MS brains (eight relapsing–remitting (RR) MS, ten secondary progressive (SP) MS, one hyperacute MS) and of six control brains of patients without brain diseases. Material was obtained from the archives of the University of Turin and the University of Genoa.

In the present study, 66 tissue blocks were selected from coronal sections, basing on preliminary Luxol staining and myelin basic protein (MBP) immunostaining, including normal-appearing white matter (NAWM) (19 blocks), chronic white matter (WM) demyelinating lesions (12 blocks), active white matter demyelinating lesions (six blocks), normal-appearing grey matter (NAGM) (19 blocks) and chronic grey matter (GM) lesions (10 blocks).

Mean age of death in MS cases was 50.2 years (range 27–66 years). Mean duration of disease course was 15.1 years (range 6 months–30 years). Post-mortem interval was less than 36 h in all cases and controls (median 14 h, range 12–20 h). Mean age of death in control cases was 58.4 years (range 52–65). The cases were retrospectively defined as having RRMS or SPMS on the basis of hospital records.

Histology and immunohistochemistry

Consecutive 5 μm sections were obtained from each tissue block. Standard hematoxylin/eosin and Luxol stainings were obtained for each section.

Immunohistochemistry was performed with the following antibodies: MBP, HLA-DR, CD68, CD3, CD20, neurofilaments, PGRN (Table 1). After deparaffinization, sections were treated with 3% H_2O_2 for 10 min and then processed for antigen retrieval (Table 1). The sections were incubated with 10% normal serum for 30 min; they were later incubated overnight with the primary antibodies. After washing with TBS, the sections were incubated at room temperature for 30 min with the Envision complex (Dako, Glostrup, Denmark). Peroxidase labelling was visualized with 10% 3,3-diaminobenzidine (brown colour). Sections were counterstained with hematoxylin.

Double immunostaining was performed when necessary to identify a possible colocalization of antigens. Immunohistochemistry was performed as described above. First, peroxidase labelling for the first antibody

Table 1. Antibodies used for immunohistochemistry

Antigen	Antibody type	Source	Dilution and antigen retrieval
MBP (myelin basic protein)	Polyclonal	Dako (Dako, Glostrup, Denmark)	1:400; microwave 360 W 15 min in sodium citrate pH 6.0
HLA-DR (MHC II)	Monoclonal (clone CR343)	Dako (Dako, Glostrup, Denmark)	1:200; microwave 900 W 15 min in sodium citrate pH 6.0
CD 68 (monocytes/ macrophages marker)	Monoclonal (clone KP1)	Dako (Dako, Glostrup, Denmark)	1:100; microwave 360 W 15 min in sodium citrate pH 6.0
CD3 (T lymphocytes marker)	Polyclonal	Dako (Dako, Glostrup, Denmark)	1:50; microwave 360 W 15 min in TBS pH 9.0
CD20 (B lymphocytes marker)	Monoclonal (clone L26)	Dako (Dako, Glostrup, Denmark)	1:200; microwave 900 W 15 min in TBS pH 9.0
Progranulin (PGRN)	Polyclonal	R & D System (Minneapolis, MN, USA)	1:300; microwave 360 W 15 min in sodium citrate pH 6.0
Neurofilaments (NF)	Polyclonal	Dako (Dako, Glostrup, Denmark)	1:200; microwave 360 W 15 min in sodium citrate pH 6.0

was visualized with 10% 3,3-diaminobenzidine; after a further blocking with normal serum, immunohistochemistry for the second antibody was performed, with peroxidase labelling visualized with vector VIP (Vector Laboratories, Burlingame, USA) (dark purple colour).

Image acquisition

The sections were examined using either a Zeiss Axiophot microscope or a Zeiss Axio Imager.A1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were acquired using either a Nikon Digital Sight DS-DM camera (Nikon Corporation, Tokyo, Japan) or a Zeiss Axiocam MRc5 camera (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Evaluation of demyelination and inflammation

Demyelinating lesions were defined as sharply demarcated areas of complete loss of Luxol staining/MBP immunostaining, with relative sparing of axons demonstrated by neurofilament immunostaining.

Inflammation in demyelinating lesions was quantified according to Bø and Trapp,[10–12] assessing MBP and HLA-DR immunostaining; lesions were classified as: (1) active demyelinating lesions (thoroughly hypercellular), (2) chronic active demyelinating lesions (hypercellular border, hypocellular centre), (3) chronic inactive demyelinating lesions (hypocellular).

Assessment of HLA-DR and PGRN immun oreactivity in the NAWM

PGRN and HLA-DR immunostaining in the NAWM were evaluated assessing the number of HLA-DR positive cells and PGRN positive cells in 40× microscopic fields, in at least 10 fields for each tissue block, and expressed as number of elements/mm^2. All measurements were performed, on the digital images, by two independent observers, blinded on information on the case studied, using the Eclipse.Net software, version 1.16.6 (Laboratory Imaging s.r.o., Prague, Czech Republic). The density of HLA-DR positive cells and PGRN positive cells in the NAWM was compared between RRMS and SPMS brains, using the Mann–Whitney *U* test (BrightStat software, http://www.brightstat.com).

CSF study

This part of the study was performed on CSF samples stored at −80°C, obtained from the archives of the University of Turin. CSF samples from 40 patients with MS (diagnosed according to McDonald's criteria)[13] were selected, based on laboratory records: 20 RRMS patients during clinical remission, 10 patients with RRMS during clinical relapse, and 10 patients with SPMS. Relapses were defined as the appearance of new neurological symptoms, or worsening of previous symptoms, lasting more than 24 h, in the absence of hyperthermia or infections.[13] RRMS was defined by presence of relapses and absence of progression of disability between relapses.[14] SPMS was defined by progression of disability independently of relapses, after a RRMS phase.[14] As a non-inflammatory control group, archival CSF samples were selected from 15 patients either with non-inflammatory neurological diseases (including normal pressure hydrocephalus, cognitive impairment, headache, polyneuropathy), or with subjective symptoms with no conclusive evidence

of neurological disease. Furthermore, archival CSF samples were selected from five patients affected by viral encephalitis as an inflammatory control group.

MS and control CSF had been stored for research, with consent of the patients, after lumbar puncture performed either for diagnostic purposes or for intrathecal baclofen injection, always collecting for storage the final volume of the puncture and discarding samples showing blood contamination. All samples had been stored at −80°C in polypropylene tubes within 1 h of extraction.[15]

PGRN ELISA study

Determination of PGRN concentration in the CSF was performed using a commercial Human Progranulin ELISA kit (Adipogen Inc., Seoul, Korea) according to the instructions provided by the manufacturer. The dilution factor of the samples was 1:50. In order to avoid variations due to freezing/thawing cycles, PGRN ELISA was performed after the first thawing in all samples.

PGRN CSF concentrations were compared between the inactive RRMS group, active RRMS group and SPMS group, using a Kruskal–Wallis test with post-hoc comparison among groups (method after Conover) (BrightStat software, http://www.brightstat.com). PGRN CSF concentrations were compared between MS patients, inflammatory control patients and non-inflammatory control patients, using the Kruskal–Wallis test with post-hoc comparison among groups (method after Conover) (BrightStat software, http://www.brightstat.com). Correlations between PGRN CSF concentrations and clinical–demographic parameters (age and disease duration, CSF cell count) were evaluated using Spearman's Rank Correlation Test.

Results

WM and cortical lesions

In all MS brains, several partially confluent demyelinating lesions were observed in the WM. Several cortical lesions were observed in most MS brains, in many cases with a widespread pattern of subpial demyelination. No demyelinating lesions were found in control cases.

Inflammation in the NAWM

Inflammation in the NAWM, with widespread activation of microglia, displaying intense HLA-DR and CD68 immunoreactivity, and presence of occasional perivascular infiltrates of CD3-positive T lymphocytes, was consistently observed in MS brains. Mean density of HLA-DR positive microglia in the NAWM was higher in progressive MS ($82.08/mm^2 \pm SD$ 14.01) than in RRMS brains ($37.60/mm^2 \pm SD$ 7.46) ($p < 0.01$). No signs of inflammation were observed in the WM in control brains.

Distribution of PGRN immunoreactivity

In MS brains, PGRN immunoreactivity was consistently observed in immune cells such as macrophages and activated microglia, colocalizing with both HLA-DR and CD68 using double immunostaining; no colocalization with CD3 or CD20 was found. Weak PGRN immunoreactivity was also observed in neuronal cytoplasm both in control and MS cortex (Figure 1F and G).

In active demyelinating lesions, intense PGRN immunoreactivity was observed on myelin-laden macrophages and activated microglia (Figure 1A and B). In chronic active demyelinating lesions, PGRN immunoreactivity was observed in activated microglia at the lesion border.

PGRN immunoreactivity was observed on activated microglia also in chronic WM lesions and in the NAWM (Figure 1C and D); almost all CD68 positive cells also showed PGRN immunostaining. The density of PGRN positive microglia in the NAWM was higher in progressive MS ($75.30/mm^2 \pm SD$ 12.96) than in RRMS brains ($40.02/mm^2 \pm SD$ 8.65) ($p < 0.01$), paralleling the higher density of HLA-DR positive microglia in the NAWM in progressive MS brains.

In cortical grey matter (GM) lesions, PGRN immunoreactivity was observed on activated microglia and in neuronal cytoplasm (Figure 1G). A similar pattern of PGRN immunoreactivity was found in the NAGM. No differences in PGRN neuronal immunoreactivity was found between cortical lesions, NAGM and control cortex.

PGRN immunoreactivity was also observed on monocytes/macrophages in leptomeningeal inflammatory infiltrates in MS brains (Figure 1H). In controls, PGRN immunoreactivity was only observed in neuronal cytoplasm (Figure 1F); no PGRN immunoreactivity was found in the WM (Figure 1E).

CSF PGRN ELISA

Mean age in patients with MS was 40.4 years, median 36 years (range 22–65 years). Mean duration of disease course was 92 months, median 54 months (range 6 months–528 months). Male to female ratio was 0.62. Mean age in non-inflammatory control patients was 50.13 years, median 53 years (range 35–68). Male to female ratio was 0.53. (Table 2). Mean CSF cell count was $7.36/mm^2$ in MS patients (range 0–22), $2.3/mm^2$ in non-inflammatory controls (range 0–4), and $78.6/mm^2$ in inflammatory controls (range 38–205).

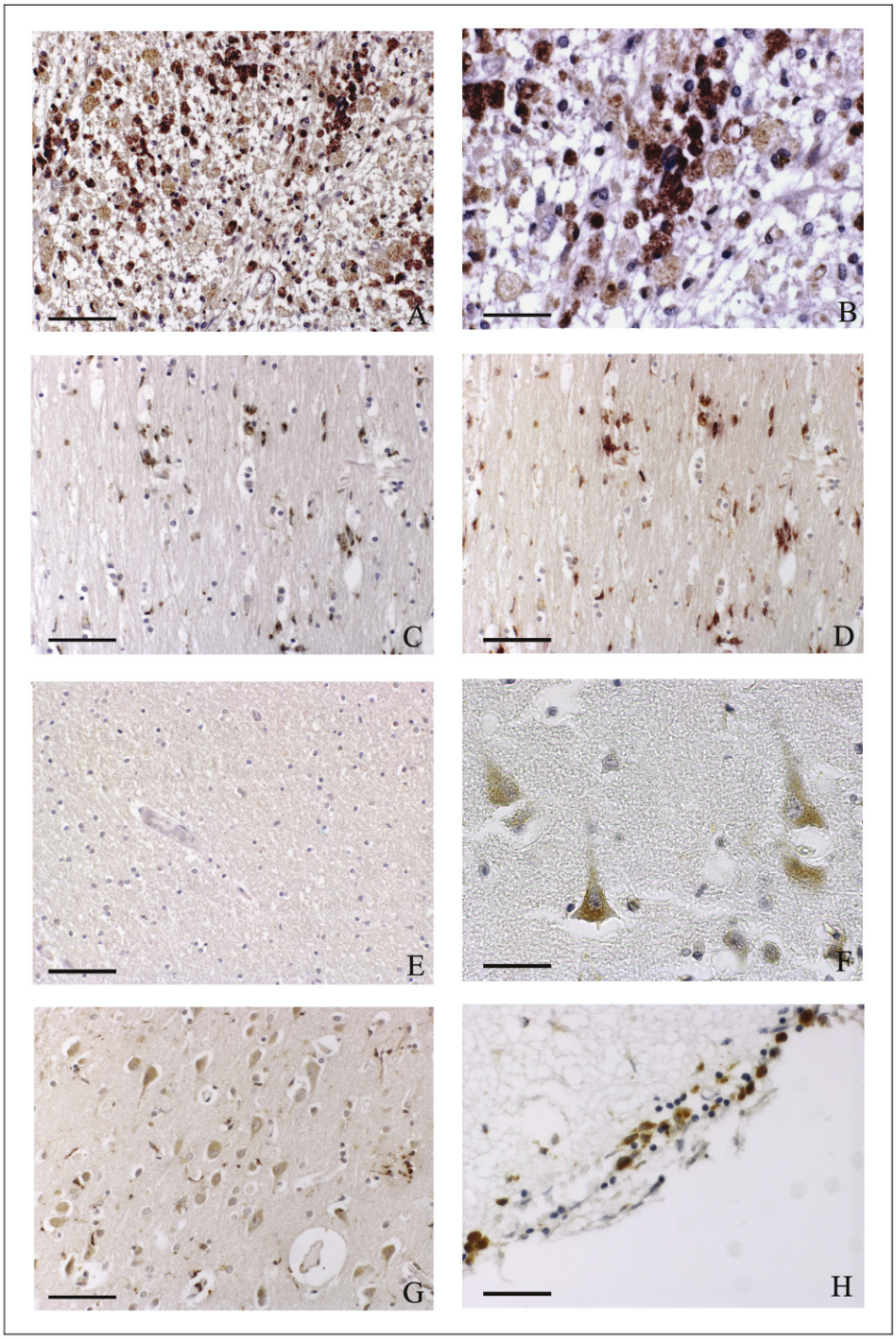

Figure 1. (A) Myelin-laden macrophages in active demyelinating lesion in multiple sclerosis (MS) brain, showing immunostaining for both progranulin (PGRN) and CD68 (double immunostaining PGRN (VIP)/CD68 (DAB), 20 ×; scale bar = 50 μm). (B) Myelin-laden macrophages in active demyelinating lesion in MS brain, showing immunostaining for both PGRN and CD68 (higher magnification of Figure 1A) (double immunostaining PGRN (VIP)/CD68 (DAB), 40×; scale bar = 25 μm). (C, D) Activated microglia in MS normal-appearing white matter (NAWM) (corpus callosum), showing immunostaining for both PGRN and HLA-DR (double immunostaining PGRN (VIP)/HLA-DR (DAB), 40×; scale bar = 25 μm); in image 1C a photograph was obtained only with PGRN immunostaining; subsequently the same microscopic field was photographed (image 1D) after performing HLA-DR immunostaining on the same slide. (E) Absence of PGRN immunostaining in control WM (frontal subcortical WM) (double immunostaining PGRN (VIP)/HLA-DR (DAB), 20×; scale bar = 50 μm). (F) PGRN immunostaining in neuronal cytoplasm, control cortex (frontal cortex) (PGRN immunostaining (DAB), 40×; scale bar = 25 μm). (G) PGRN immunostaining in neuronal cytoplasm and activated microglia, in a cortical lesion in MS brain (cingulate cortex); activated microglia shows also CD68 immunostaining (double immunostaining PGRN (DAB)/CD68 (VIP), 20×; scale bar = 50 μm). (H) PGRN immunostaining in macrophages/monocytes in inflamed leptomeninges over a cortical lesion, MS brain (cingulate cortex) (PGRN immunostaining (DAB), 40×; scale bar = 25 μm).

Table 2. Patients data for cerebrospinal fluid progranulin study

	Age	Disease duration	Male to female ratio
Multiple sclerosis	Mean 40.4 years	Mean 92 months	0.62
	Median 36 years	Median 54 months	
	Range 22–65 years	Range 6–528 months	
Non-inflammatory controls	Mean 50.13 years	NA	0.53
	Median 53 years		
	Range 35–68 years		

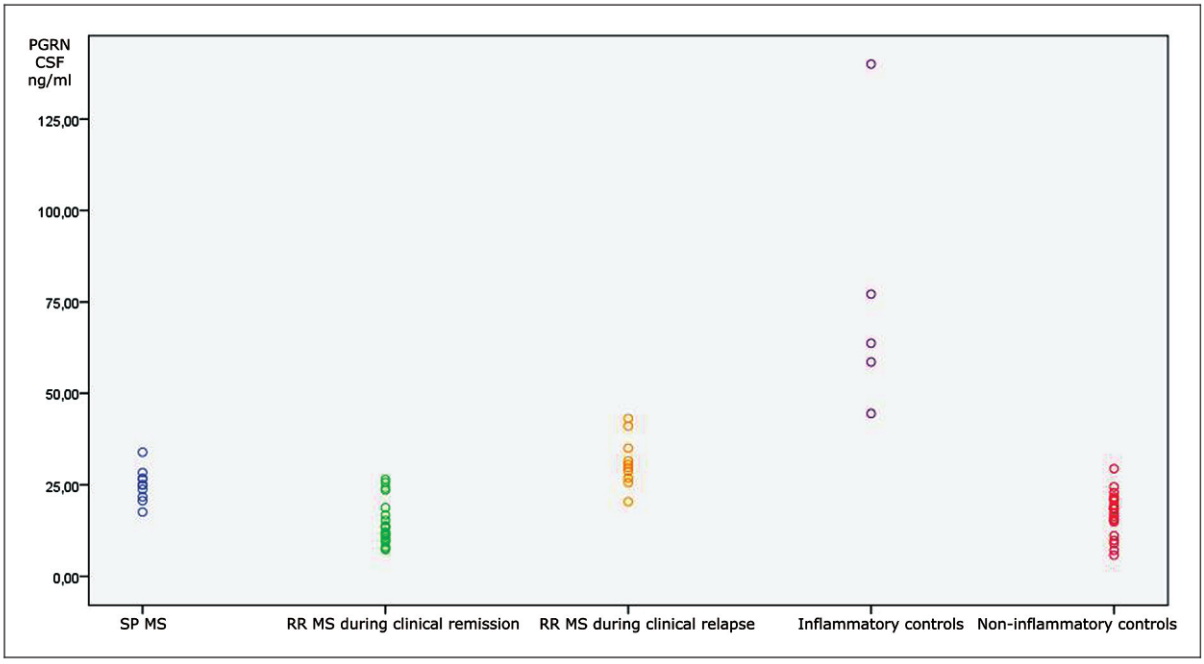

Figure 2. Scatter plot of progranulin (PGRN) cerebrospinal fluid (CSF) concentration in different multiple sclerosis patient groups, in inflammatory controls and in non-inflammatory controls. RRMS, relapsing–remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis.

No significant difference was found in mean PGRN concentration in the CSF between MS patients (mean 21.32 ng/ml ± SD 8.42) and non-inflammatory control patients (mean 17.15 ng/ml ± SD 4.92) (Figure 2). CSF PGRN concentration in inflammatory controls was greatly increased (mean 76.38 ng/ml ± SD 85.08), significantly higher if compared both with MS and non-inflammatory control groups ($p < 0.01$).

Mean PGRN concentration in the CSF was significantly higher in RRMS cases during clinical relapse (mean 31.26 ng/ml ± SD 5.62), and in progressive MS cases (mean 24.92 ng/ml ± SD 3.03), than in RRMS cases during clinical remission (mean 14.56 ng/ml ± SD 4.16) ($p < 0.01$). The difference in PGRN CSF concentration between RRMS cases during clinical relapse and progressive MS cases was not statistically significant (Figure 2).

No significant correlation was found between PGRN concentration in the CSF and age, nor in MS patients or in controls ($p = 0.12$ and $p = 0.48$, respectively). No significant correlation was found between PGRN concentration in the CSF and duration of disease ($p = 0.47$).

A weak but significant correlation ($r = 0.579$, $p < 0.05$) was found between PGRN CSF concentration and CSF cell count in patients with MS.

Discussion

In this study, we evaluate the presence and distribution of PGRN in MS and control brain tissue, correlating it

with features such as demyelination and inflammation. Moreover, we determine the concentration of PGRN in the CSF in patients with MS and controls, and we correlate it with disease subtype and disease activity.

Increased expression of PGRN is observed in brain tissue in all MS cases in our series. While in normal brain tissue neurons are the main source of PGRN, in MS brain tissue activated microglia and macrophages appear to be the predominant cellular sources of PGRN. The highest degree of PGRN expression in MS brains is observed in active demyelinating lesions.

Microglia and neurons are the major cell type that express PGRN in the CNS.[6,16,17] Trauma, infection and neurodegeneration are all accompanied by increases in PGRN mRNA expression.[3,4,18] Production of PGRN by activated microglia has also been described in brains of individuals suffering from neurodegenerative disorders,[16–18] including FTLD;[6,16] activated microglia and macrophages have been identified as a major source of PGRN in a model of traumatic CNS injury also.[19] These results are consistent with the notion that PGRN expression is involved in microglial proliferation and activation, implicating PGRN in neuroinflammation and potentially brain repair.[3,4]

It might be hypothesized that increased PGRN expression in MS brain tissue might play a role in protection of neurons and axons during brain inflammation. PGRN is thought to be critical in maintaining neuronal survival.[5] It has been recently observed that brains of PGRN-deficient mice display increased susceptibility to noxious stimuli and greater activation of microglia,[20] suggesting the importance of PGRN in neuroprotection during injuries to the CNS.

PGRN is also known as a modulator of inflammation. The full PGRN protein and the GRN fragments exert different effects on inflammation.[3] The extent of PGRN proteolysis and the resulting balance between full PGRN and GRN fragments are thus probably important in determining the final effects on inflammation in brain tissue. While our data show intense PGRN expression in MS brain tissue, correlating with inflammation, it remains to be determined whether the role of PGRN in MS is to enhance brain inflammation or to downregulate microglia activation and brain inflammation.

Paralleling the neuropathological data, CSF PGRN concentration appears to be increased in MS in phases of disease activity; this is scarcely surprising, given the intense expression of PGRN on macrophages and microglia in active demyelinating lesions. CSF PGRN concentration is also increased in patients with SPMS; this is likely related to the increased widespread microglial activation observed in progressive MS, as shown not only in the present study but also in previous studies on larger series.[1] In progressive MS, meningeal inflammation also has been described to be more common.[1,21] We have observed PGRN expression in meningeal inflammatory infiltrates in MS brains; these may represent an additional source of PGRN in the CSF. PGRN CSF concentration also weakly correlates with CSF cell count in MS patients.

A strong increase in PGRN CSF concentration was also observed in the inflammatory control group (patients affected by viral encephalitis), considerably higher than what was found both in the MS and non-inflammatory control groups. Increased PGRN CSF concentration does not appear to be specific for MS, but rather a general feature of CNS inflammation.

No significant difference was found in PGRN concentration in the CSF between MS patients and non-inflammatory control patient groups, as also observed in a recent study by De Riz et al.[22] A limitation of our study is that controls are represented by patients affected by non-inflammatory neurological diseases, or with subjective symptoms with no conclusive evidence of neurological disease, who underwent diagnostic lumbar puncture.

It is known that CNS inflammation may not always be harmful, but may also play a physiological neuroprotective function,[23] depending on complex mechanisms of balance between toxic and protective functions of the immune cells. Recent studies have hypothesized the simultaneous activation of protective and detrimental mechanisms of inflammation in MS brains, in a subtle balance that is crucial in determining the progression of the disease.[24]

Production of PGRN by microglia and macrophages may represent one of the mechanisms of neuroprotective inflammation in MS brains, influencing the survival of different neuronal cell populations in response to noxious stimuli. Furthermore, PGRN may be important in regulating inflammation in MS brain tissue.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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