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EXACERBATED INFLAMMATORY RESPONSES RELATED TO ACTIVATED MICROGLIA AFTER TRAUMATIC BRAIN INJURY IN PROGRANULIN-DEFICIENT MICE

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Abstract—Progranulin (PGRN), a multifunctional growth factor, appears to play a role in neurodegenerative diseases accompanied by neuroinflammation. In this study, we investigated the role of PGRN in neuroinflammation, especially in the activation of microglia, by means of experimental traumatic brain injury (TBI) in the cerebral cortex of mice. The expression of GRN mRNA was increased in association with neuroinflammation after TBI. Double-immunohistochemical study showed that PGRN-immunoreactive (-IR) cells were mainly overlapped with CD68-IR cells, suggesting that the main source of PGRN was CD68-positive activated microglia. To investigate the role of PGRN in inflammatory responses related to activated microglia, we compared the immunoreactivity and expression of ionized calcium-binding adaptor molecule 1 (Iba1), CD68, and CD11b as markers for activated microglia between wild-type (WT) and GRN-deficient (KO) mice. The number of Iba1- and CD11b-IR cells and gene expression of Iba1 and CD11b were not significantly different between WT and KO mice, while the number of CD68-IR cells and CD68 expression in KO mice were significantly greater than those in WT mice. Double-immunohistochemical study showed that CD68-IR microglia were also IR for TGFβ1, and TGFβ1 expression and Smad3 phosphorylation in KO mice were elevated compared to WT mice. Moreover, double-immunostaining between phospho-Smad3 and glial fibrillary acidic protein suggested increased TGFβ1–Smad3 signal mainly by astrocytes. The levels of protein carbonyl groups, which reflect protein oxidation, and laminin immunoreactivity, which is associated with angiogenesis, were also significantly increased in KO mice compared to WT mice. These results suggest that PGRN is produced in CD68-positive microglia and suppresses excessive inflammatory responses related to activated microglia after TBI in mice. © **2012 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: progranulin, traumatic brain injury, neuroinflammation, microglia, CD68; TGFβ1.

INTRODUCTION

Progranulin (PGRN), also known as granulin epithelin precursor, protein carbonyl groups (PC) cell-derived growth factor, proepithelin, and acrogranin, is an autocrine growth factor (Bateman and Bennett, 2009) that encodes a 68.5-kDa protein containing 7.5 tandem granulin motif repeats (Baba et al., 1993), cleaved by serine proteases to generate granulins (Zhu et al., 2002; Kessenbrock et al., 2008). We have identified PGRN as one of the factors involved in sex differentiation of the brain (Suzuki et al., 1998; Kayasuga et al., 2007), and adult neurogenesis (Chiba et al., 2007; Asakura et al., 2011). PGRN is also related to tumorigenesis (He and Bateman, 2003; Bateman and Bennett, 2009), early embryogenesis (Daniel et al., 2000), wound healing (He et al., 2003), inflammation (Zhu et al., 2002; Kessenbrock et al., 2008), host defense (Yin et al., 2010) and cartilage development and degradation (Xu et al., 2007; Liu, 2009; Feng et al., 2010; Guo et al., 2010). Additionally, mutations in the PGRN gene were identified as one of the major factors causing frontotemporal lobar degeneration (FTLD) in humans (Baker et al., 2006; Cruts et al., 2006). PGRN deficiency also increases the risk of developing Alzheimer's disease (AD) (Brouwers et al., 2008), and modifies the course of amyotrophic lateral sclerosis (ALS) (Sleegers et al., 2008). Since these neurodegenerative diseases are accompanied by neuroinflammation, PGRN may have a role in the process of neuroinflammation.

Neuroinflammation is characterized by activated microglia and astrocytes (Zhang et al., 2010). Microglia, the resident innate immune cell in the brain, has been implicated as an active contributor to neuron damage in neurodegenerative diseases (Block et al., 2007). Activated microglia release cytotoxic factors such as nitric oxide (NO) and reactive oxygen species and secrete pro-inflammatory cytokines that can potentially damage neurons, oligodendrocytes or extracellular

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Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; EDTA, ethylenediaminetetraacetic acid; FTLD, frontotemporal lobar degeneration; GFAP, glial fibrillary acidic protein; HE, hematoxylin and eosin; HPRT, hypoxanthine phosphoribosyltransferase; Iba1, ionized calcium-binding adaptor molecule 1; IR, immunoreactive; KO, GRN-deficient; NCL, neuronal ceroid lipofuscinosis; NO, nitric oxide; PBS, phosphate-buffered saline; PBST, PBS-containing Triton X-100; PC, protein carbonyl groups; PGRN, progranulin; pSmad3, phospho-Smad3; SCI, spinal cord injury; TBI, traumatic brain injury; TBST, TBS-containing Tween 20; TDP-43, TAR DNA-binding protein; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptors; WT, wild-type.

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matrix structures. Activated microglia also enhance angiogenesis (Ling et al., 2009; Jantaratnotai et al., 2010; Welser et al., 2010). Additionally, activated microglia is a predominant source of increased TGFβ1 after injury (McTigue et al., 2000; Doyle et al., 2010). TGFβs are pleiotropic cytokines with central roles in neuroprotection, vascular remodeling, immune suppression, immune homeostasis and repair after injury (Pepper, 1997; Dhandapani and Brann, 2003; Bertolino et al., 2005; Li et al., 2006). In contrast to its neuroprotective properties, TGFβ1 has been also implicated in neurotoxic events (Wyss-Coray et al., 1997). Interference with TGFβs and downstream Smad2/3 signaling in innate immune cells mitigated AD pathology (Town et al., 2008).

Interestingly, PGRN levels increase after traumatic brain injury (TBI) or spinal cord injury (SCI), and activated microglia predominantly express PGRN (Pereson et al., 2009; Naphade et al., 2010; Wang et al., 2010; Byrnes et al., 2011). Macrophages or microglia from GRN-deficient (KO) mice secrete higher levels of proinflammatory cytokines against immunological challenges than those from wild-type (WT) mice (Yin et al., 2010; Martens et al., 2012). Furthermore, gliosis in aged mice was facilitated by PGRN deficiency (Ahmed et al., 2010; Ghoshal et al., 2012). These findings suggest PGRN is involved in the activation of microglia. In this study, mice were subjected to experimental TBI, which is accompanied by neuroinflammation (Barreto et al., 2009; Schachtrup et al., 2010), to investigate the role of PGRN in inflammatory responses related to activated microglia.

EXPERIMENTAL PROCEDURES

Animals

In this study, 8- or 9-week-old male C57BL/6J mice were used. Mice purchased from Charles River Laboratories (Yokohama, Japan) were used for investigation of distribution, localization and expression levels of PGRN. WT mice and KO mice produced from breeding heterozygous pairs in our laboratory (Kayasuga et al., 2007) were used for comparison between WT and KO mice. They were genotyped using previously described protocols (Kayasuga et al., 2007). Mice were maintained under controlled lighting (lights on, 05:00–19:00), temperature (23 ± 1 °C), and humidity (55 ± 10%), and given free access to food and water. All animal procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agricultural and Life Sciences, The University of Tokyo.

TBI

TBI, a model that induces the activation of microglia and astrocytes, was performed as described previously (Barreto et al., 2009; Schachtrup et al., 2010). Mice were anesthetized by intraperitoneal administration of xylazine hydrochloride (8 mg/kg) and chloral hydrate (300 mg/kg) dissolved in sterile saline, and subsequently placed in a stereotaxic apparatus (Narishige, Tokyo, Japan). An incision was made in the scalp and the cranium was exposed. The periosteum was cleaned from the skull, a hole was drilled over the right cerebral hemisphere with a dental drill, and the dura mater was removed with a needle tip. A stainless steel cannula, with a 0.5-mm outer diameter, was used to make a longitudinal stab wound in the right hemisphere. The cannula was positioned at 1.3 mm lateral to the midline, and 1 mm posterior to bregma, and introduced into the brain until the tip reached a depth of 2 mm. The cannula was then shifted 2 mm caudally (bregma −3 mm), and then shifted back 2 mm rostrally to its initial position. Finally, the cannula was removed from the brain, and the scalp wound was sutured.

Histology and immunofluorescent staining

Mice were transcardially perfused with saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2) under anesthesia. Brains were removed from the skull, postfixed in 4% paraformaldehyde overnight, immersed in 10% sucrose in PBS for 24 h, 20% sucrose for 24 h and 30% sucrose for 48 h for cryoprotection, and cut into 30-μm sections on a cryostat. Hematoxylin and eosin (HE) staining was used to assess the magnitude of injury and to evaluate injury size. The injury size was estimated as described previously (Glover et al., 2012). In brief, three HE-stained sections equally spaced at 180 μm were used. Images were acquired using a BX-53 microscope equipped with a DP-73 digital camera (Olympus, Tokyo, Japan). The digitized images were analyzed using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). The cavity area was defined at the injured area. The injured area in each section was calculated, and the sum of three sections was presented as the injury size.

Immunofluorescent staining was carried out on free-floating sections. The sections processed for immunofluorescent staining were from alternating sections of HE-stained brains. All washes were done in 0.03% Triton X-100 PBS (PBST), all incubations with primary antibody were done in 0.3% PBST containing 1% bovine serum albumin (BSA), and all incubations with secondary antibody or streptavidin were done in 0.3% PBST. Free-floating sections were incubated in the blocking solution (Block Ace, Snow Brand Milk Products, Sapporo, Japan) for 2 h, and then sections were incubated with primary antibody (anti-PGRN, 1:1000, R&D Systems; anti-ionized calcium-binding adaptor molecule 1 (Iba1), 1:1000, Wako Pure Chemical Industries Ltd., Osaka, Japan; anti-glial fibrillary acidic protein (-GFAP), 1:1000, Dako, Glostrup, Denmark; anti-CD68, 1:1000, Serotec, Oxford, UK; anti-CD11b, 1:1000, Millipore, Billerica, MA, USA; anti-phospho Smad3 (-pSmad3), 1:500, Cell Signaling Technology, Danvers, MA, USA; anti-laminin, 1:500, Sigma, St. Louis, MO, USA) at 4°C for 60 h. Then, sections were washed three times. When immunostaining for PGRN, sections were incubated with biotin conjugated donkey anti-sheep IgG (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 3 h, and then washed three times. Next, sections were incubated with streptavidin (1:500, Invitrogen, Carlsbad, CA, USA) at room temperature for 3 h in the dark. When immunostaining for pSmad3, sections were incubated with biotin-conjugated goat anti-rabbit IgG (1:1000, Vector Laboratories, Burlingame, CA, USA) at room temperature for 3 h, and then washed three times. Next, sections were incubated with streptavidin (1:1000, Invitrogen, Carlsbad, CA, USA) at room temperature for 3 h in the dark. When immunostaining for other antigens, sections were incubated with Alexa Fluor Dye-conjugated secondary antibodies (1:500, Invitrogen) at room temperature for 3 h in the dark. When double immunostaining between PGRN and NeuN, Iba1, CD68, CD11b, and GFAP, the sections were incubated with anti-PGRN (1:1000) and one of the following primary antibodies: anti-NeuN (1:500, Millipore), anti-GFAP (1:1000), anti-Iba1 (1:500), anti-CD68 (1:500), and anti-CD11b (1:500) at 4 °C for 60 h. Sections were washed three times, and incubated with biotin-conjugated donkey anti-sheep IgG (1:500)

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