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INDUCED NG2 EXPRESSING MICROGLIA IN THE FACIAL MOTOR NUCLEUS AFTER FACIAL NERVE AXOTOMY

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Abstract—Chondroitin sulfate proteoglycan (NG2) expressing cells, ubiquitously distributed in the CNS respond to injured or diseased neurons; however, their behaviors toward injured neurons have remained to be fully explored. In the present study, along with astrocytic and microglial responses, NG2 expressing cells reacted swiftly and robustly in the facial motor nucleus (FMN) subjected to axotomy. With time, hypertrophic NG2 expressing cells gradually adhered to and enwrapped the axotomized motoneurons. Tight encapsulations around axotomized motoneurons were eventually formed at 7, 14, and 28 days after axotomy. NG2 positive processes appeared to interpose between synapsin-1 immunoreactive nerve terminals and surfaces of axotomized motoneurons. Double labeling results showed that NG2 expressing cells encapsulating axotomized facial motoneurons were mainly microglia marked by OX42 and lectin; only a few of them were positive to platelet-derived growth factor-α receptor and none of them positive to ED-1. No Rhodamine particle was detected in the FMN ipsilateral to axotomy after venous injection of the particles. The results suggest that activated microglia in lesioned FMN were induced to express NG2 molecules. It is concluded that axotomized FMN showed two types of NG2 expressing cells namely constitutive NG2 cells and induced-NG2 expressing microglia.

Key words: axotomy, chondroitin sulfate proteoglycan, facial motor neurons, microglia, OX42.

Chondroitin sulfate proteoglycan (NG2) has been considered as a marker for a group of cells, which are ubiquitous in the gray and white matters in normal adult rodent brain (Dawson et al., 2003). These cells are used to be considered as oligodendrocyte precursor cells (OPCs), since many of them in the normal brain express platelet derived growth factor receptor α (PDGFR-α), a marker of OPCs (Nishiyama et al., 2002; Stallcup, 2002) and can differentiate to oligodendrocytes *in vitro* (Levine and Stallcup, 1987) or *in vivo* during normal development (Horner et al., 2000; Bu et al., 2004). However, there are some observations that seem inconsistent with this notion. NG2 expressing cells are distributed widely throughout the gray and white matters of the mature CNS, and their abundance does not necessarily match the abundance of oligodendrocytes or myelin (Nishiyama, 2007), suggesting that the cells expressing NG2 may have functions other than OPCs.

In fact, NG2 expressing cells possess capacity to respond to injuries or diseases in the CNS by undergoing hypertrophy (Levine et al., 2001) or extension of their processes at an early pathogenesis stage (Wu et al., 2008). Further studies have shown that recruited macrophages, but not resident microglia or astrocytes, could become positive to NG2 antibodies following hippocampal excitotoxicity (Bu et al., 2001) and spinal cord injury (Jones et al., 2002). These NG2 positive macrophage-like cells have been considered as blood-borne cells distinct from resident NG2 expressing cells (Bu et al., 2001) as all of these studies were done in the models which had impact on the integrity of the blood–brain barrier (BBB), including the *in vivo* model with lipopolysaccharide (LPS) stimulation (Gao et al., 2010). Blood-borne macrophages may accumulate in the CNS when BBB permeability increases. It is still unknown whether NG2 can be expressed on resident microglia if there is no damage on the BBB in adult CNS.

Peripheral facial nerve axotomy, without compromising the permeability of the BBB, not only results in axonal degeneration (Stoll et al., 1989) but also is followed by substantial corresponding neuronal deactivation in the cell body (Berkelaar et al., 1994; Villegas-Perez et al., 1988; Himes and Tessler, 1989; Mattsson et al., 1999) and reactions in surrounding glial cells (Torvik and Soreide, 1975). It is assumed that one way to manipulate reactions of axotomized neurons would be to modulate the functions of surrounding non-neuronal cells so as to improve nerve regeneration and functional restoration after facial nerve injury. This requires us to fully understand the pathological development after facial nerve injury. Responses of microglia, astrocytes, and oligodendrocytes have been addressed in details; however, information on responses of NG2 expressing cells in axotomized facial motor nucleus (FMN) appears to be lacking.

Although the exact function of NG2 expressing cells is still uncertain in the adult, it is clear that they can respond to various injuries and diseases in the CNS and interact

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Abbreviations: ABC, avidin–biotin complex; BBB, blood–brain barrier; ChAT, choline acetyltransferase; CNPase, 2′,3′-cyclic nucleotide-3′-phosphodiesterase; DAB, 3,3′-diaminobenzidine tetrachloride; DAPI, 4,6-diamidino-2-phenyl-indole; FMN, facial motor nucleus; GFAP, glial fibril acidic protein; OPC, oligodendrocyte precursor cells; PBS, phosphate buffered saline; PDGFR-α, platelet-derived growth factor receptor-α; TBS, Tris buffer saline.

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with other cells, for instance, forming synapses with neurons and respond to neuronal impulse (Wang and He, 2009). Hence, NG2 expressing cells may be one of the targeting candidates for modulation of the functions of non-neuronal cells. This study sought to determine the responses of NG2 expressing cells in axotomized FMN, a well-established model system without significant alteration in permeability of the BBB, as a step to fully explore the roles of these cells in CNS injuries and diseases.

EXPERIMENTAL PROCEDURES

Animal model

Adult male Sprague–Dawley rats (250~350 g, 6~8 weeks) were used in this study. All experiments were approved by the Institutional Animal Care and Use Committee, National University of Singapore (IACUC 122/08). The animals were maintained in a 12-hour day/night cycle and provided with food and water *ad libitum*.

The rats were deeply anesthetized with xylazine and ketamine (80 and 10 mg/kg, respectively; Sigma, USA). Under a dissecting microscope, the left facial nerve was exposed at its exit from the stylomastoid foramen and transected. A nerve segment of approximately 5 mm of the distal stump was removed to prevent connection between distal and proximal ends. The right facial nerve was untreated and used as the control. At 1, 2, 7, 14, and 28 days after the operation, six animals at each time point were deeply anesthetized and then perfused via the left ventricle with ice-cold Ringer's solution (pH 7.4) and 2% paraformaldehyde (pH 7.4). The brainstem with the cerebellum was removed and post-fixed in the same fixative for 4 h at 4 °C. The brainstem was then immersed in 20% sucrose of 0.1 M phosphate-buffered saline (PBS) over night at 4 °C. Frozen coronal sections of the brainstem containing FMN were cut at the thickness of 20–30 μm using a cryostat (Leica CM 3050).

In order to ascertain if any phagocytic cells in the axotomized FMN are recruited from the circulatory system, fluorescent dye, Rhodamine B isothiocyanate (Sigma, USA), which could be phagocytosed by macrophages (Xu et al., 1993), was i.v. injected in the animals with facial nerve axotomy. Under anesthesia, after left facial nerve axotomy, each rat was given an injection of 100 μl 1% Rhodamine via the right external jugular vein immediately. The rats were deeply anesthetized 7 days after operation. The blood was collected from the caudal vein for blood smear to verify Rhodamine-phagocytotic macrophages in the circulation. Then the rats were perfused and the brainstem, liver and spleen were removed and postfixed as described above.

Immunohistochemistry staining

For 3,3′-diaminobenzidine tetrachloride (DAB; Sigma, USA) staining, tissue sections including the FMN were first rehydrated in PBS (pH 7.4) for 10 min×3 times and then quenched with 0.3% H_2O_2 for 15 min to block endogenous peroxidase activity. They were then washed in PBS solution, and blocked with 5% normal goat or bovine serum in PBS with 0.1% TritonX-100 for 1 h at room temperature. Antibodies against NG2 (mouse monoclonal IgG, 1:500; Santa Cruz, USA), glial fibril acidic protein (mouse monoclonal IgG, GFAP, a marker for astrocytes, 1:1000; Chemicon International, USA), OX42 (mouse monoclonal IgG, a marker for microglia, 1:1000; Santa Cruz, USA), anti-2′,3′-cyclic nucleotide-3′-phosphodiesterase (mouse monoclonal IgG, CNPase, a marker for oligodendrocytes, 1:400; Chemicon International), anti-choline acetyltransferase (Goat Polyclonal IgG, ChAT, a marker for motoneurons, 1:100; BD Pharmingen, USA), were applied, respectively, to sections on the slides overnight at room temperature. The sections were incubated with respective biotinylated IgG (1:200; Vector Laboratories, USA) for 1 h at room temperature and then the secondary antibodies were localized using avidin–biotin complex (ABC; Vector Laboratories, USA) with 0.05% DAB as the peroxidase substrate. The sections were finally counterstained with Methyl Green or Cresyl Violet and coverslipped with permount after dehydration and cleaning. These slides were examined and photographed with a light microscope.

In order to determine the relationships between non-neuronal cells, double immunofluorescence labeling with anti-NG2, OX42, anti-lectin (a marker for microglial and endothelial cells), anti-ED1 (mouse monoclonal IgG, a marker for macrophages or phagocytic microglia, Chemicon International), anti-GFAP and PDGFR-α was carried out. For this purpose, tissue sections were first washed with 1×PBS for 10 min thrice and pre-incubated with 5% normal serum to block non-specific binding sites for 1 h. The rabbit polyclonal antibody anti-NG2 (1:100, Chemicon International, USA), anti-PDGFR-α (1:200, a marker for OPCs, Cell signaling Technology Inc, USA), and anti-Synapsin-1 (1:200, Chemicon International, USA) were doubly incubated with mouse monoclonal antibody anti-GFAP (1:200), anti-ED1 (1:100), OX42 (1:200) or NG2 (1:100), respectively, overnight at room temperature. The sections were then incubated with secondary antibodies goat anti-mouse IgG and goat anti-rabbit IgG conjugated with either FITC or Cy3 for 1 h for detecting the primary antibodies. Some sections at different time points were incubated with rabbit anti-NG2 (1:100) or PDGFR-α (1:200) for 2 h, followed by incubation of goat-anti-rabbit IgG conjugated with Cy3 (1:200, red) and FITC conjugated lectin from *Lycopersicon esculentum* (1:200; Sigma, USA) at room temperature for 1 h.

For animals injected with Rhodamine, immunofluorescence labeling using anti-NG2 (1:100), OX42 (1:100), and ED1 (1:100) respectively was carried out on coronal frozen sections of the brainstem (including FMN) at 30 μm thickness. Liver and spleen tissue sections were stained with the phagocytotic macrophage marker, ED1 (1:100). The relevant secondary antibodies conjugated with FITC were used to visualize the staining.

The sections were then coversliped using the mounting medium with 4,6-diamidino-2-phenyl-indole (DAPI, which stains nuclei bluish purple), and all images were captured using a laser-scanning confocal microscope (Olympus FluoView™ FV1000, Japan) at 408 nm (DAPI), 488 nm (FITC), and 568 nm (Cy3), respectively.

Cell counts

Anti-ChAT was used as a marker for the identification of cholinergic facial motoneurons in this study. Facial motoneurons were examined in the serial frozen coronal sections at 30 μm thickness with ChAT immunohistochemistry staining at 1, 2, 7, 14 and 28 days post-surgery (n=3 at each time point). The number of ChAT positive facial motoneurons on both sides were counted in every fifth section under a light microscope as described previously (Dai et al., 2000). The viable rate of the facial motoneurons was calculated as the L/R (L, the number of ChAT-positive motoneurons in the left transected side; R, the number of ChAT-positive motoneurons in the right control side).

Serial frozen coronal sections of the brainstem (20 μm thickness) at 1, 2, 7, 14 and 28 days post-surgery were double-immunostained with anti-NG2 and OX42 antibodies in combination with nuclear staining (n=3 at each time point). The number of NG2+/OX42+ and NG2+/OX42− cells in the FMN was obtained by counting DAPI-stained nuclei in every fifth section under a light microscope under a ×40 objective lens.

Western blot

Proteins were extracted from the dissected facial nuclei (n=3 for each-time-point) using a protein extraction kit (Pierce Biotechnol-