Olig genes are upregulated in oligodendrocyte precursor cells in the injured central nervous system

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Summary. The Olig gene family encodes basic helix-loop-helix (bHLH) transcription factors and plays a critical role in oligodendrogenesis during development. We examined whether the Olig genes are involved in pathological conditions such as the injured brain or spinal cord by *in situ* hybridization. Olig1 mRNA was detected in cells surrounding necrotic tissue. The signal intensity of Olig1 mRNA gradually increased 2 to 7 days and decreased by 14 days after injury. Olig2 mRNA was expressed in a similar pattern and was co-localized with Olig1 mRNA. Similar results were obtained for the expression of Olig1 and Olig2 mRNAs in spinal cord injury. When we compared the mRNA expression pattern of Olig1 with that of NG2 proteoglycan and platelet-derived growth factor receptor α (PDGFR α), markers for oligodendrocyte precursor cells (OPCs), by double labeling *in situ* hybridization, Olig1 mRNA was co-localized with mRNAs for NG2 proteoglycan and PDGFR α 7 days after brain injury, suggesting that Olig mRNAs were expressed in OPCs. These findings suggest that the Olig transcription factors

regulate differentiation of oligodendrocytes in the injured CNS as well as in the developing CNS.

Introduction

Glial cells respond to central nervous system (CNS) injury and are transformed into a population of reactive cells, including reactive astrocytes, remyelinating oligodendrocytes and activated microglia (Rabchevsky *et al.*, 2001). Oligodendrocyte precursor cells (OPCs), consisting of a 5–8% glial cell population in the intact adult CNS, are increased in number and recruited to a region surrounding necrotic tissue 3 to 5 days and decreased 7 to 10 days after injury (Levine *et al.*, 2001). In the injured CNS, NG2 is upregulated in OPCs identified by markers such as NG2 proteoglycan, platelet-derived growth factor receptor α (PDGFR α) and basic fibroblast growth factor receptor (bFGFR) (Stallcup *et al.*, 1987; Levine *et al.*, 2001).

As its name implies, OPCs are equivalent to the oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells that can develop into either oligodendrocytes or type 2 astrocytes *in vitro* (Lee *et al.*, 2000). In an *in vivo* developmental situation, however, O-2A progenitors preferentially differentiate into oligodendrocytes. A question then arises: Do O-2A progenitors or OPCs in the injured sites differentiate into oligodendrocytes or astrocytes or both? In certain demyelinating diseases such as multiple sclerosis (MS), incomplete remyelination is attributed to either a shortage or differentiation failure of

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OPCs (Wolswijk, 2002). Recent evidence suggests that OPCs in the MS lesion preferentially differentiate into astrocytes, but not into oligodendrocytes, due to an activated Notch signal (John *et al.*, 2002). In this regard, differentiation mechanisms of OPCs in the adult injured brain are of particular interest.

The Olig gene family encodes basic helix-loop-helix (bHLH) transcription factors. Olig1 and Olig2, are expressed in the spinal cord and forebrain during development (Zhou *et al.*, 2000), while Olig3 is not detected in the CNS (Takebayashi *et al.*, 2000). Ectopic expression of Olig1 gene in progenitor cells promotes oligodendrocyte differentiation (Lu *et al.*, 2001). Gene-disruption studies revealed that Olig1 and Olig2 govern the early differentiation process of oligodendrocytes (Lu *et al.*, 2002; Zhou *et al.*, 2002). Although the pivotal roles of Olig genes in oligodendrocyte development have been established, the expression and functions of Olig genes in the adult brain remain enigmatic. Previous studies have shown that Olig2 is upregulated in OPCs under pathological conditions in the adult brain (Fancy *et al.*, 2004; Buffo *et al.*, 2005; Talbott *et al.*, 2005; Tatsumi *et al.*, 2008) and that Olig2-positive progenitor cells differentiate into oligodendrocytes in demyelinated lesions (Islam, 2008). However, but it remains uncertain whether Olig1 is involved in pathological conditions. To investigate the mechanisms regulating such glial cell responses, we examined spatio-temporal expression patterns of Olig mRNAs in the injured CNS using markers for OPCs.

Materials and Methods

Animal experiments and tissue preparation

Adult male ICR mice were used in our experiments and all surgical procedures were approved according to the guidelines for animal experiments established by the institutional committee of Fukushima Medical University School of Medicine. Animal experiments were based on previous reports (Iseki *et al.*, 2002; Hagino *et al.*, 2003). Briefly, surgical procedures were performed under inhaled anesthesia of diethyl ether. Cryo-injury model was inflicted by placing a lead probe pre-chilled with liquid nitrogen on the cranium for 1 min. Spinal cord injury model was made by the same technique as the brain injury after laminectomy at the eighth thoracic vertebra. Thirty mice were sacrificed under deep ether anesthesia at 2, 4, 7 and 14 days after surgery and perfused transcardially with phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA). The brains or spinal cords were dissected, post-fixed in 4% PFA for 24 h at 4°C, and immersed in 30% sucrose in diethyl

pyrocarbonate-treated PBS. They were embedded in an OCT compound (Tissue Tek, Torrance, CA, USA) and stored at -80°C until use. Coronal sections (10 µm thick) of the brains or sagittal sections of the spinal cords (10 µm thick) were cut on a cryostat and mounted onto silane-coated glass slides. Sections were then subjected to *in situ* hybridization.

In situ hybridization

In situ hybridization using a digoxigenin-labeled cRNA probe was performed as described previously (Nikaido *et al.*, 2001; Iseki *et al.*, 2002). Digoxigenin-labeled cRNA probes (antisense and sense probes) were made by *in vitro* transcription using Olig1, Olig2, NG2 and PDGFR α . The NG2 and PDGFR α cDNAs were obtained from IMAGE consortium cDNA library. The Olig1 and Olig2 cDNAs were kindly provided by Dr. Zhou Q (Zhou *et al.*, 2000). Genbank accession numbers for the cDNAs were as follows; A1019805 for NG2 and BE629400 for PDGFR α . We confirmed the validity of the cDNAs by sequencing and no cross-reactivity between the probes by *in situ* hybridization on mouse embryo sections.

Double-labeling fluorescence in situ hybridization

Double-labeling *in situ* hybridization was performed as described previously (Iseki *et al.*, 2002). Sections were hybridized with digoxigenin-labeled Olig2, NG2 or PDGFR α cRNA probes and FITC-labeled Olig1 cRNA probes. The Olig2, NG2 or PDGFR α mRNAs were visualized using a rhodamine-conjugated anti-digoxigenin antibody (1:100 dilution, Roche Products, Indianapolis, IN, USA). Olig1 mRNA was detected using an anti-FITC antibody conjugated to horseradish peroxidase (1:500, Roche Products) and was amplified using FITC-conjugated tyramide (TSA Fluorescence System; NEN Life Science Products, Boston, MA, USA). Double-labeled immunofluorescent sections were examined using an Olympus Fluoview FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Results

Expression of Olig gene mRNAs in CNS injury

Cryoinjury to the brain disrupted the blood brain barrier and induced edema followed by necrosis in our experimental model. Olig1 mRNA was increased in intensity primarily in the margin surrounding the necrotic region 7 days after injury (Fig. 1A, C). Similarly, Olig2 was also weakly expressed in the marginal region 7 days after injury

Fig. 1. *In situ* hybridization of Olig mRNAs at 7 days after CNS injury. (A–F) Messenger RNA transcripts for Olig1 (A) and Olig2 (D) are upregulated in the margin (arrows) of necrotic tissue (asterisks) of the injured brain. Higher magnification of the marginal zone (C for Olig1, F for Olig2) shows labeled cells (arrowheads). Incubation with sense probe for Olig1 gene (B) or Olig2 gene (E) generated no detectable signals. (G–I) Confocal images of double-labeling *in situ* hybridization show that Olig1 mRNA (G) is co-localized with Olig2 mRNA (H) in one and the same cells (arrows). (J, K) In spinal cord injury, Olig1 mRNA is similarly upregulated at 7 days after insult in the margin (arrows) of necrotic tissue (asterisk). Higher magnification shows labeled cells (K, arrowheads). Scale bars: 1 mm (A, B, D, E, J), 100 μm (C, F, K), 50 μm (I).

Fig. 2. Time-course of *in situ* hybridization of Olig1 mRNA expression after brain injury. Sections at 2, 4, 7 and 14 days after brain injury at low (A, B, C, D) and high (E, F, G, H) magnifications, respectively. The signal intensity for Olig1 mRNA gradually increases in the margin of the necrotic tissue (asterisks) from 2 to 7 days. It decreases by 14 days. High magnification shows labeled cells (arrowheads). Scale bars: 1 mm (A–D), 100 μ m (E–H).

(Fig. 1D, F). Control experiments with labeled sense strand probes gave no detectable signals in adjacent sections, confirming the specificity of the hybridization signals in this study (Fig. 1B, E). To examine whether Olig1 and Olig2 mRNAs are coexpressed or not, we performed double-labeling fluorescence *in situ* hybridization. Figures 1G–I showed that Olig1 and Olig2 mRNAs are co-localized in one and the same cell (Fig. 1G–I). In spinal cord injury, the expression pattern of Olig1 mRNA was observed in a similar fashion to that in brain injury (Fig. 1J, K).

We next examined the time course of Olig1 mRNA expression; we performed *in situ* hybridization at time points of 2, 4, 7, and 14 days after injury. Olig1 mRNA positive cells were detected on days 2–14 (Fig. 2). On day 2 to 4, Olig1 mRNA was weakly expressed in cells surrounding the necrotic tissue and reached a maximum level on day 7 (Fig. 2A–C). On day 14, necrotic tissue was largely absorbed where Olig1 mRNA expression was detected at low intensity (Fig. 2D).

Olig1 mRNA is coexpressed with those of NG2 and PDGFR α

In the developing brain, NG2 and PDGFR α serve as well-known markers for OPCs. To investigate whether Olig1 is expressed in OPCs, we compared the expression pattern of Olig1 mRNA with that of NG2 or PDGFR α by double labeling *in situ* hybridization. Olig1 mRNA was colocalized with NG2 proteoglycan core protein mRNA (Fig. 3A–C).

Furthermore, Olig1 mRNA was also coexpressed with PDGFR α mRNA in one and the same cell (Fig. 3D–F). These results suggest that Olig1 is expressed in OPCs in the injured brain.

Discussion

The present study reveals that Olig1 mRNA is coexpressed with Olig2 mRNA in OPCs that are labeled for either NG2 mRNA or PDGFR α mRNA in CNS injury. Previous studies have shown that NG2 and PDGFR α are reliable markers for OPCs *in vivo* (Levine *et al.*, 2001) and that mRNAs for NG2 and PDGFR α are detected in not only the developing CNS but also in the white and gray matters of the adult CNS (Nishiyama *et al.*, 1996), suggesting that OPCs remain in the adult CNS. In addition, it is shown that NG2-positive OPCs are increased in number and reach a peak at 7 days after CNS injury (Levine, 1994; Jones *et al.*, 2002) and that PDGFR α mRNA expression is upregulated in a similar manner and peaks at 7 days after injury (Cook *et al.*, 1998). In this regard, we reveal that the mRNA expression time course of Olig1 closely resembles that of NG2 and PDGFR α . In the developing CNS, genes for Olig and PDGFR α are detected at the earliest phase of the oligodendrocyte lineage, which is followed by NG2 expression (Lu *et al.*, 2000; Zhou *et al.*, 2000; Alberta *et al.*, 2001). We detected upregulation of Olig1 mRNA as early as 2 days after injury, suggesting that in the injury paradigm Olig

Fig. 3. Confocal images showing double-labeling *in situ* hybridization for Olig1 mRNA and oligodendrocyte precursor cell marker mRNAs at 7 days after brain injury. Olig1 mRNA is colocalized with both NG2 proteoglycan core protein mRNA (A–C) and PDGFR α mRNA (D–F) in one and the same cell (arrows). Scale bars: 50 μ m (C, F).

genes may be an early-phase regulator of OPCs as in the developing CNS.

During development, Mash1, Hes5, and Id4 are shown to be involved in the differentiation of neural stem cells into OPCs. Among them Hes5 and Id4 inhibit oligodendrocyte differentiation (Kondo *et al.*, 2000a; Kondo *et al.*, 2000b; Raff *et al.*, 2001). Although we examined the expression of these bHLH genes in our injury model, we found that their expressions are below the detection level (data not shown). Since Id1, Id2 and Id3 are reported to be expressed in spinal cord injury (Tzeng *et al.*, 2001), these genes may substitute for Hes5 or Id4 in our injury model. These findings suggest that adult OPCs and/or the surrounding environment might be somewhat different between pathological conditions and developing brain. Another discrepancy between the adult injury paradigm and developmental situation is the expression of sonic hedgehog (shh), which, in embryonic CNS, is necessary and sufficient for the activation of Olig genes in OPC differentiation (Lu *et al.*, 2000; Alberta *et al.*, 2001). However, we hardly observed the expression of shh and its receptors (Patched and Smoothened) in the injured brain (data not shown). The lack of shh expression may be ascribed to insufficient oligodendrocyte differentiation

and poor remyelination in our injury model, which is frequently found in the adult CNS lesion (Alberta *et al.*, 2001).

Recently, Olig-deficient mice have shed light on the functional roles of Olig genes (Alberta *et al.*, 2001; Lu *et al.*, 2002; Zhou *et al.*, 2002). In Olig1 and Olig2 double-deficient mice neither OPCs labeled by PDGFR α and Sox10 nor mature oligodendrocytes labeled by myelin basic protein (MBP) and PLP/DM20 are detected in the brain and spinal cord (Zhou *et al.*, 2002). In Olig2-null mice, on the other hand, OPCs labeled by PDGFR α , Sox10, and Olig1 exist in the forebrain and hindbrain, although no OPCs are detected in the spinal cord. Furthermore, in Olig1-null mice both OPCs and mature oligodendrocytes are generated (Lu *et al.*, 2002). Taken these results together, it is suggested that Olig2 are required for OPC differentiation in the brain and spinal cord although Olig1 can compensate for the lack of Olig2 in oligodendrocytes in the brain but not in the spinal cord. Therefore, OPC differentiation may be controlled by Olig1 and Olig2 in a differential manner in different CNS regions. In this context, our present finding that shows coexpression of Olig1 and Olig2 mRNAs in OPC of the injured brain suggests that both Olig1 and Olig2 are

required for the OPC proliferation and differentiation after injury. It should also be noted that Olig genes are good markers for OPCs in addressing the mechanisms of injury-response in the adult brain.

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