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Selective expression of L-serine synthetic enzyme 3PGDH in Schwann cells, perineuronal glia, and endoneurial fibroblasts along rat sciatic nerves and its upregulation after crush injury*

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Summary. Non-essential amino acid L-serine functions as a highly potent, glia-derived neurotrophic factor, because it is a precursor for syntheses of proteins, other amino acids, membrane lipids, and nucleotides, and also because its biosynthetic enzyme 3-phosphoglycerate dehydrogenase (3PGDH) is preferentially expressed in particular glial cells within the brain. Here we pursued 3PGDH expression in peripheral nerves and its change after crush injury. In the pathway of rat sciatic nerves, 3PGDH was selectively expressed in non-neuronal elements: Schwann sheaths and endoneurial fibroblasts in sciatic nerves, satellite cells in dorsal root ganglia, and astrocytes and oligodendrocytes in the spinal ventral horn. In contrast, 3PGDH was immunonegative in axons, somata of spinal motoneurons and ganglion cells, and endoneurial macrophages. One week after crush injury, 3PGDH was upregulated in the distal segment of injured nerves, where 3PGDH was intensified in activated Schwann cells and fibroblasts. 3PGDH was still negative in activated macrophages, which were instead associated or surrounded by activated Schwann cells with intensified 3PGDH. These results suggest that in the peripheral nervous system, these non-neuronal cells synthesize and may supply L-serine to satisfy metabolic demands for maintenance and regeneration of peripheral nerves and for proliferation and activation of macrophages upon nerve injury.

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Introduction

L-Serine is important not only as a building block of proteins, but also as a material for syntheses of other amino acids (glycine, D-serine, and L-cysteine), membrane lipids (phosphatidylserine and sphingolipids), heme, and nucleotides. In the human body, L-serine is derived from dietary intake, *de novo* synthesis from glycolytic intermediate 3-phosphoglycerate (i.e., phosphorylated pathway), conversion from glycine, and degradation of proteins and lipids (Snell, 1984; for a review, see de Koning *et al.*, 2003). Emerging evidence highlights that *de novo* synthesis of L-serine and its supply to neurons are crucial for brain development and function (for a review, see Furuya and Watanabe, 2003). In *in vitro* studies, application of L-serine and glycine to cultured neurons greatly promotes their survival and differentiation (Savoca *et al.*, 1995; Mitoma *et al.*, 1998a; Furuya *et al.*, 2000). When neurons are cultured in the absence of L-serine, levels of sphingolipids and phosphatidylserine are drastically decreased, and L-threonine, instead of L-serine, is abnormally recruited for phosphoglyceride synthesis (Mitoma *et al.*, 1998b). Enrichment of L-serine and glycine in astrocyte-rich conditioned medium suggests glia to be the source (Mitoma *et al.*, 1998a; Furuya *et al.*, 2000; Verleysdonk and Hamprecht, 2000). The physiological importance of L-serine biosynthesis is further

evidenced from inherited 3PGDH deficiency in humans; the patients who exhibit marked decreases in the L-serine and glycine levels in the plasma and cerebrospinal fluid are afflicted with severe neurological disorders, including congenital microcephaly, dysmyelination, seizures, and psychomotor retardation. These individuals show beneficial effects by oral treatment of the deficient amino acids (Jaeken *et al.*, 1996; de Koning *et al.*, 1998, 2002; Klomp *et al.*, 2000).

Recently, we have demonstrated in the mouse central nervous system (CNS) that the radial glia/astrocyte lineage and olfactory ensheathing glia preferentially coexpress 3-phosphoglycerate dehydrogenase (3PGDH), the initial step enzyme of the phosphorylated L-serine pathway, and ASCT1, a transporter of small neutral amino acids, including L-serine (Yamasaki *et al.*, 2001; Sakai *et al.*, 2003). In contrast, neurons and microglia in the brain lack 3PGDH expression (Sugishita *et al.*, 2001; Yamasaki *et al.*, 2001). The distinct cellular system should play a fundamental role in the brain, by which neurons and microglia can survive more safely against energy loss by saving their own glucose for preferential use in energy production and by receiving L-serine for anabolic metabolisms from nearby glial cells. In the present study, we aimed to clarify the cellular system of L-serine biosynthesis in the peripheral nervous system (PNS) and its change after nerve injury. To this end, we selected adult rat sciatic nerves and examined the cellular expression of 3PGDH along the nerve pathway. Here we show that 3PGDH is selectively expressed in Schwann cells, perineuronal glia, and endoneurial fibroblasts, and that it is upregulated after injury. In contrast, neurons, nerves, and macrophages do not express 3PGDH even while undergoing Wallerian degeneration.

Materials and methods

Animals

In the present study, we used female WKA rats at 8–10 weeks of age, which were treated according to the guidelines regarding the Care and Use of Laboratory Animals of Hokkaido University School of Medicine. Under deep pentobarbital anesthesia (100 mg/kg of body weight, i.p.), rats were perfused transcardially with 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB, pH 7.2) for light microscopic immunohistochemistry and fluorescent *in situ* hybridization or with 4% paraformaldehyde/0.1% glutaraldehyde in PB (pH 7.2) for immunoelectron microscopy. Fixed tissues were immersed in 30% sucrose/PB before sectioning. For immunoblot and isotopic *in situ* hybridization, tissues were freshly obtained and immediately frozen in powdered dry ice. The fixed tissues and fresh frozen tissues were subjected to cryostat sectioning (20 μm in thickness). Under pentobarbital anesthesia (40 mg/kg of body weight, i.p.), the left sciatic nerve was exposed and crushed distal to the sciatic notch in a standardized way for 60 sec with a fine forceps. After survival of 1–4 weeks, the left (injured) and right (control) sciatic nerves were examined by immunofluorescence and isotopic *in situ* hybridization.

Antibody

The production and specificity of affinity-purified rabbit and guinea pig anti-mouse 3PGDH antibodies have been reported previously (Yamasaki *et al.*, 2001). We also used commercial mouse anti-rat ED1 antibody (MCA341R; Serotec, Oxford, UK), mouse anti-neurofilament-160 (NF160; N5264, Sigma, St. Louis, MO, USA), rabbit anti-S-100β (74–92, RY330; Yanaihara Institute, Fujinomiya), and rabbit anti-bovine glial fibrillary acidic protein (GFAP; L1812, DAKO, Carpinteria, CA, USA). Biotinylated, Cy3-conjugated, or fluorescein isothiocyanate-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). For immunoperoxidase with rabbit primary antibodies, a Histofine SAB-PO (R) kit was used (Nichirei, Tokyo).

Immunoblot

The post-nuclear fraction was obtained from the spinal cord, dorsal root ganglion, and the sciatic nerve as reported previously (Sakai *et al.*, 2003). A hundred micrograms of protein samples were fractionated by 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Germany). Blotted membrane was incubated with 3PGDH antibodies at 1 μg/ml in phosphate-buffered saline containing 0.1% Tween 20 and 0.5% skimmed milk, and visualized with an ECL chemiluminescence detection system (Amersham).

Immunohistochemistry

All immunohistochemical incubations were performed at room temperature. For immunoperoxidase, sections were incubated with 10% normal goat serum for 20 min, rabbit anti-3PGDH antibody (1 μg/ml) overnight, biotinylated goat anti-rabbit IgG for 1 h and streptavidin-peroxidase complex for 30 min, followed by visualization with 3,3'-diaminobenzidine. For immunofluorescence, sections immunoreacted with rabbit and mouse primary antibodies (1 μg/ml for each) were incubated with a mixture of fluorescence-labeled species-specific secondary antibodies for

2 h. Photographs were taken with a light microscope (AX-70, Olympus, Tokyo) equipped with a digital camera DP11 (Olympus) or with a confocal laser scanning microscope. (Fluoview, Olympus). For immunoelectron microscopy, sections stained by immunoperoxidase were treated with 0.5% osmium tetroxide for 15 min and 2% uranyl acetate for 20 min, dehydrated using graded alcohols, and embedded in Epon 812. Electron micrographs were taken with an H7100 electron microscope (Hitachi, Tokyo).

In situ hybridization

For isotopic *in situ* hybridization, two antisense oligonucleotides were synthesized to detect the rat 3PGDH mRNA. The sequences were 5'-TACTCATCAGTGATAGCCTCG ACCCCTGCTGCCGGAAGATCCTGC-3' and 5'-TACAG CTGCTGTCCTACCAGACCTCCAAGGTGTCTGACGG AGACA-3', which corresponded to nucleotide residues 94–138 or 1537–1581 of the rat 3PGDH cDNA (GenBank accession no. X97772). They were labeled with ^{33}P-dATP using terminal deoxyribonucleotidyl transferase (BRL, Bethesda, MD, USA). Sections were processed for prehybridization, hybridization, washing, and exposure to an X-ray film for 6 weeks, as reported previously (Yamasaki *et al.*, 2001). For fluorescent *in situ* hybridization, a digoxigenin-labeled antisense cRNA probe was used to detect mouse proteolipid protein (PLP) mRNA and visualized using an HNPP Fluorescent Detection Set (Roche), as reported previously (Yamasaki *et al.*, 2001).

Results

Cellular expression along sciatic nerve pathway

The specificity of 3PGDH antibodies was examined by immunoblot using protein samples from the lumbosacral spinal cord, dorsal root ganglia, and sciatic nerves of adult female rats. In each sample, the rabbit (Fig. 1A) and guinea pig (data not shown) antibodies recognized a single protein band at 57 kDa, as expected. Using these antibodies, we applied immunohistochemistry to sciatic nerves (Fig. 1B–F), dorsal root ganglia (Fig. 1G–I), and the ventral horn of the lumbosacral cord (Fig. 1J–L).

In the sciatic nerve, immunoperoxidase labeled fibrous structures running parallel to the longitudinal axis (Fig. 1B). By double immunofluorescence, 3PGDH (green, Fig. 1C) was detected on both sides of the NF160-immunopositive axons (red), and the two fluorescences were separated from each other by a narrow immunonegative space. When examined for S-100β, a marker for Schwann cells and satellite cells, 3PGDH was overlapped with S-100β in cells having oval large nuclei (arrows in Fig. 1D). 3PGDH was also detected in S-100β-negative flattened cells having spindle-shaped small nuclei (arrowheads in Fig. 1D). By immunoelecron miscroscopy, diffuse immunoreaction products for 3PGDH were detected in the Schwann sheath overlying the immunonegative myelin sheath (Fig. 1E, F). In addition, 3PGDH was detected in endoneurial fibroblasts, which were not connected to the myelin sheath and were apart from the Schwann sheath with loose connective tissues (Fig. 1F). By double immunofluorescence for a macrophage marker ED-1, 3PGDH was not detected in ED-1-positive macrophages (data not shown).

The dorsal root ganglion contains a number of primary sensory neurons sending central and peripheral axon branches to spinal nerves, including the sciatic nerve. At a low magnification, intense immunostaining for 3PGDH was seen as a large ring-like structure surrounding the somata of immunonegative ganglion cells, and moderate immunostaining was detected as a small ring-like structure surrounding the immunonegative axons (Fig. 1G). By immunofluorescence, the latter structure was judged to be the Schwann sheath around NF160-positive axons (arrows in Fig. 1H), and the former, S-100β-positive satellite cells (arrows in Fig. 1I).

In the ventral cord, no significant immunostaining for 3PGDH was observed inside the somata and dendrites of motoneurons. Rather, 3PGDH was detected along the surface of the neuronal somata and dendrites, and also in small cells dispersed in the gray and white matters (Fig. 1J). Some of these 3PGDH-positive cells were overlapped with an astrocyte marker GFAP in perikarya and star-like processes, yielding a yellowish fusion color (arrows in Fig. 1K). Others were negative to GFAP, had larger somata than GFAP-positive astrocytes, and were often attached to the cell bodies of motoneurons (arrowheads in Fig. 1K and L). By double staining with fluorescence in situ hybridization, the cells attached to motoneurons were shown to express PLP mRNA (red, Fig. 1L), thus being identified as perineuronal oligodendrocytes. Therefore, in the sciatic nerve pathway of adult rats, 3PGDH is selectively expressed in perineuronal cells and elements, i.e., Schwann sheath and endoneurial fibroglasts in sciatic nerves, satellite cells in the dorsal root ganglion, and astrocytes and oligodendrocytes in the ventral horn.

Changes after sciatic nerve injury

Next, we examined changes of 3PGDH expression after nerve injury (Fig. 2). One week after crush injury, longitudinal sections of injured and control sciatic nerves were simultaneously processed for *in situ* hybridization with ^{33}P-lableled antisense oligonucleotide probes and X-ray film

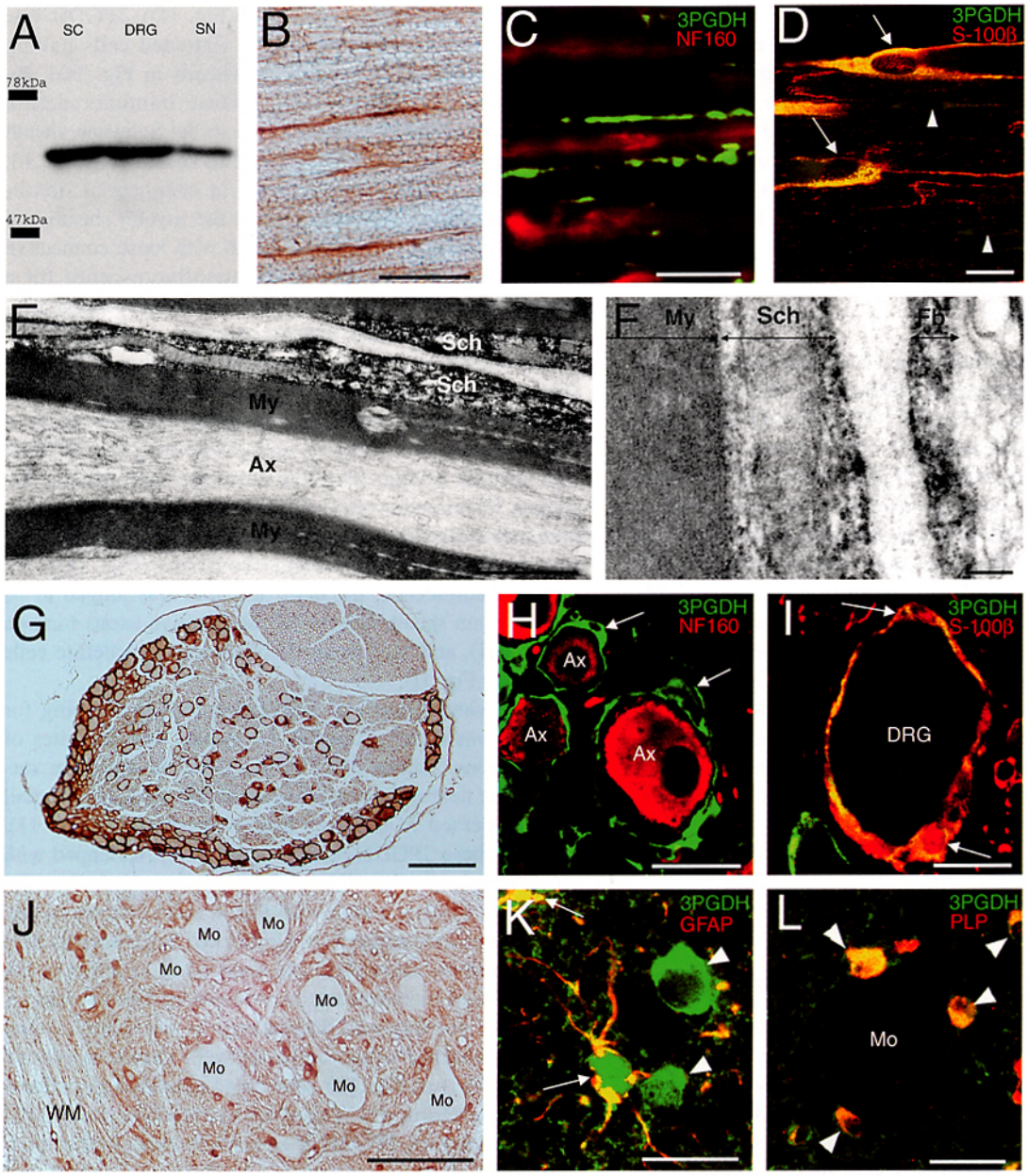


Fig. 1. Immunoblot (**A**) and immunohistochemistry for the sciatic nerve (**B**–**F**), dorsal root ganglion (**G**–**I**), and spinal ventral horn (**J**–**L**) of adult female rats. **A:** Immunoblot for the spinal cord (SC), dorsal root ganglion (DRG), and sciatic nerve (SN). The size of marker proteins is indicated to the left. **B, E, F, G, J:** Immunoperoxidase with rabbit 3PGDH antibody. **E** and **F** are electron micrographs showing the 3PGDH localization in Schwann sheath (Sch) and endoneurial fibroblast (Fb), but not in myelin sheath (My) and axon (Ax). In **J**, cell bodies of motoneurons (Mo) in the ventral horn are immunonegative. **C, H:** Double immunofluorescence for 3PGDH (green) and NF160 (red). Arrows indicate 3PGDH-positive Schwann sheath (**H**). **D, I:** Double immunofluorescence for 3PGDH (green) and S-100 β(red). Arrows indicate 3PGDH expression in S-100 β-positive Schwann cells (**D**) or satellite cells (**I**) enwrapping immunonegative soma of the ganglion cell (DRG). **K:** Double immunofluorescence for 3PGDH (green) and GFAP (red). Arrows and arrowheads indicate 3PGDH expression in GFAP-positive or -negative cells, respectively. **L:** Immunofluorescence for 3PGDH (green) and fluorescent *in situ* hybridization for PLP mRNA (red). Arrowheads indicate perineuronal oligodendrocytes expressing 3PGDH and PLP mRNA. Scale bars: 200 μm (B), 10 μm (C, H), 20 μm (D, I, K, L), 500 μm (E), 100 μm (F, J), 250 μm (G)

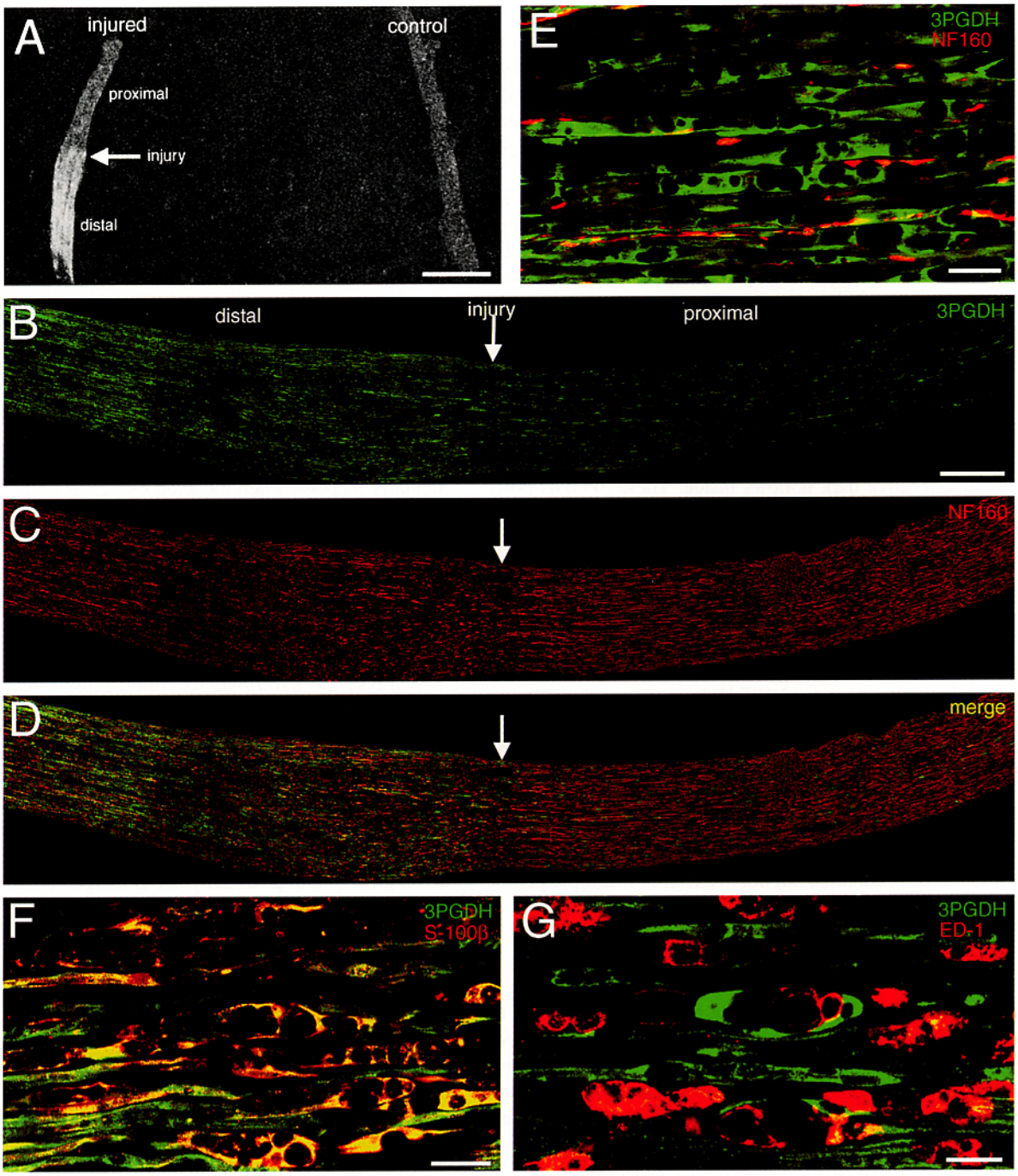


Fig. 2. Upregulation of 3PGDH in the sciatic nerve 1 week after crush injury. **A:** *In situ* hybridization for 3PGDH mRNA to injured (left) and intact (right) nerves. An arrow indicates the injured point. This photograph is a negative image printed directly from X-ray film. **B–D:** Double immunofluorescence for 3PGDH (green, *B*) and NF160 (red, *C*). *D* is a merged view of *B* and *C*. Arrows indicate the injured point. **E–G:** High power views of double immunofluorescence for 3PGDH with axon marker NF160 (red, **E**), Schwann cell marker S-100 β (red, **F**), or macrophage marker ED-1 (red, **G**). Scale bars: 3 mm (A), 500 μm (B), 20 μm (E–G).

autoradiography (Fig. 2A). Signal intensity for 3PGDH mRNA was augmented in the injured sciatic nerve distal to the injured point (Fig. 2A, left). The specificity was confirmed with a similar result by use of another nonoverlapping probe and by blank autoradiogram when hybridization was carried out in the presence of excess unlabeled oligonucleotides (data not shown).

Changes at the protein level were demonstrated by immunofluorescence in the distal segment one week after crush injury. Compared to the proximal segment, the distal segment was characterized by intensified immunofluorescence for 3PGDH (green, Fig. 2B–D) and by decreased and fragmented immunfluorescence for NF160 (red), suggesting 3PGDH upregulation during or after Wallerian degeneration. At a higher magnification, marked changes of 3PGDH-positive structures were clear (Fig. 2E). In the distal segment, 3PGDH was detected in longitudinal arrays of cuboidal or cylinder-like structures, often containing immunonegative dark hollows inside. These 3PGDH-positive structures were negative for NF160. The cuboidal and cylindrical structures having dark hollows were shown to coexpress S-100β (i.e., activated Schwann cells), while those having smooth thin processes, small nuclei, and no hollows were low or negative to S-100β (fibroblasts) (Fig. 2F). ED-1-positive macrophages were rounded in shape and remarkably increased in number (Fig. 2G). They were still immunonegative for 3PGDH, but were associated with or surrounded by 3PGDH-positive Schwann cells and fibroblasts (Fig. 2G). Such changes were seen at 2 and 4 weeks after injury, with the maximal immunofluorescence level of 3PGDH one week after injury (data not shown). These results suggest that 3PGDH is selectively upregulated in response to nerve injury in Schwann cells and endoneurial fibroblasts, but not in axons or macrophages.

Discussion

In the present study, we examined cellular expression of L-serine biosynthetic enzyme 3PGDH along the sciatic nerve pathway. We have shown selective expression in distinct populations of non-neuronal supporting cells: Schwann cells and endoneurial fibroblasts in the sciatic nerve, satellite cells in the dorsal root ganglion, and astrocytes and oligodendrocytes in the ventral cord. Furthermore, crush injury induces upregulation of 3PGDH at the transcription and translation levels in, at least, some of these supporting cells.

Local L-serine synthesis for axon elongation and regeneration

Schwann cells are the myelin-forming glia in the PNS. They promote axon regeneration after peripheral nerve injury and also promote regeneration of CNS nerves when transplanted into the spinal cord (Richardson *et al.*, 1980; Weidner *et al.*, 1999). It has been pointed that this trophic activity is mediated, at least in part, by trophic factors and cytokines released from Schwann cells (Heumann *et al.*, 1987; Bergsteinsdottir *et al.*, 1991; Sendtner *et al.*, 1992; Curtis *et al.*, 1994; Murwani *et al.*, 1996; Skundric *et al.*, 1997). Endoneurial fibroblasts are also known to secrete neurotrophins and cytokines that promote neurite growth and neuronal survival (Acheson *et al.*, 1991; Murwani and Armati, 1998). Since L-serine is a precursor for membrane lipids (Furuya *et al.*, 1995; Mitoma *et al.*, 1998b: Yamashita *et al.*, 1999), demands for L-serine should particularly increase when the axon elongates during development and regenerates after injury. Therefore, 3PGDH expression in Schwann cells and endoneurial fibroblasts suggests that L-serine functions as one such intercellular mediator to support growth, maintenance, and regeneration of peripheral nerves.

Local L-serine synthesis for macrophage activation in response to injury

During Wallerian degeneration of peripheral nerves, macrophages drastically increase in number (Mueller *et al.*, 2003). They are activated morphologically and functionally to phagocytose degenerating myelin, express various cytokines, secrete free radicals, provide trophic support for axon growth, and potentially act as antigen-presenting cells (Brück, 1997; Hirata *et al.*, 1999; Mueller *et al.*, 2003). The explosive proliferation and dynamic cytodifferentiation should increase the demands for L-serine in macrophages. However, they are still devoid of 3PGDH expression during Wallerian degeneration. Rather, a number of Schwann cells and fibroblasts upregulate 3PGDH and surround activated macrophages. From the distinct cellular expression and regulation, it is conceivable that L-serine synthesized by local Schwann cells and fibroblasts may be supplied to macrophages to satisfy their metabolic necessity. It is also known that activated macrophages secrete mitogen factors to promote the proliferation of Schwann cells and fibroblasts (Martinet *et al.*, 1986; Baichwal *et al.*, 1988). Therefore, these non-neuronal cells in the PNS appear to cooperate with each other to lead injured nerves to successful regeneration.

L-serine synthesis by perineuronal oligodendrocytes

Previously, we have revealed in the mouse corpus callosum that 3PGDH is expressed in oligodendrocytes but the immunohistochemical level is apparently lower than that in adjacent astrocytes (Yamasaki *et al.*, 2001). In the present study, quite intense 3PGDH is detected in perineuronal oligodendrocytes that express PLP mRNA. Most of the white matter oligodendrocytes (i.e., interfascicular oligodendrocytes) are the myelinating glia, while perineuronal oligodendrocytes are assumed as the non-myelinating glia in the normal state (Ludwin, 1979, 1984). Although their biochemical signature and role remain to be completely established, perineuronal oligodendrocytes resemble astrocytes in that both show structural affinity to neuronal cell bodies and express glutamine synthetase (D'Amelio *et al.*, 1990), an enzyme that is involved in the glutamate-glutamine cycle to maintain the homeostasis of excitatory synaptic transmission and neuronal survival. From the present finding, L-serine synthesis in perineuronal oligodendrocytes and its supply to nearby neurons is likely to be one of their roles common to astrocytes.

Through our present and previous molecular-anatomical investigations (Furuya *et al.*, 2000; Yamasaki *et al.*, 2001; Sakai *et al.*, 2003), L-serine metabolism stands on an elaborate neuroglial relationship and glioglial interplay in both the CNS and PNS. In future studies, the physiological importance of this cellular system needs to be tested experimentally by developing animal models with cell type-specific control of 3PGDH expression.

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