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GANGLIOSIDE 9-O-ACETYL GD3 EXPRESSION IS UPREGULATED IN THE REGENERATING PERIPHERAL NERVE

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Abstract—Evidence accumulates suggesting that 9-O-acetylated gangliosides, recognized by a specific monoclonal antibody (Jones monoclonal antibody), are involved in neuronal migration and axonal growth. These molecules are expressed in rodent embryos during the period of axon extension of peripheral nerves and are absent in adulthood. We therefore aimed at verifying if these molecules are re-expressed in adult rats during peripheral nerve regeneration. In this work we studied the time course of ganglioside 9-O-acetyl GD3 expression during regeneration of the crushed sciatic nerve and correlated this expression with the time course of axonal regeneration as visualized by immunohistochemistry for neurofilament 200 in the nerve. We have found that the ganglioside 9-O-acetyl GD3 is re-expressed during the period of regeneration and this expression correlates spatio-temporally with the arrival of axons to the lesion site. Confocal analysis of double and triple labeling experiments allowed the localization of this ganglioside to Schwann cells encircling growing axons in the sciatic nerve. Explant cultures of peripheral nerves also revealed ganglioside expressing reactive Schwann cells migrating from the normal and previously crushed nerve. Ganglioside 9-O-acetyl GD3 is also upregulated in DRG neurons and motoneurons of the ventral horn of spinal cord showing that the reexpression of this molecule is not restricted to Schwann cells. These results suggest that ganglioside 9-O-acetyl GD3 may be involved in the regrowth of sciatic nerve axons after crush being upregulated in both neurons and glia.

Key words: sciatic nerve, dorsal root ganglia, regeneration, Schwann cell, gangliosides.

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Abbreviations: BSA, bovine serum albumin; DAL, days after lesion; DAPI, 4′,6-diamidino-2-phenylindole; DIV, days *in vitro*; DRG, dorsal root ganglia; ECM, extracellular matrix; GAP-43, growth-associated protein; GFAP, glial fibrillary acidic protein; MAG, myelin-associated glycoprotein; NF200, neurofilament 200; NGS, normal goat serum; PBS, phosphate-buffered saline; PF, paraformaldehyde; PNS, peripheral nervous system; TBS, Tris buffered saline; 9-O-acGD3, 9-O-acetyl GD3.

Mammalian peripheral nervous system (PNS) axons when severed are capable of regeneration unlike axons from the CNS. In the last few decades enormous effort has been directed to reveal cellular and molecular interactions within the PNS that justify the dramatic differences in regenerative capacity between these two systems. Evidence has accumulated to reveal that in PNS regeneration many molecules that participate in axon growth are re-expressed in the adult after lesion (Smith and Skene, 1997; Soares et al., 2005). Therefore molecules involved in axon growth during development, such as, cell adhesion molecules (L1, NCAM), extracellular matrix (ECM) molecules, integrins and the growth-associated protein GAP-43 appear to contribute to axon regeneration (for review see, Bulsara et al., 2002). Most importantly, since the work of Aguayo and co-workers (1990), PNS glial cells have been shown to be crucial for regeneration of both PNS and CNS axons (for review, Aguayo et al., 1990). The latter were shown to be capable of growing into peripheral nerve grafts and into artificial tubes lined with Schwann cells (for reviews, Bray et al., 1991; Anderson et al., 1998). The interaction between Schwann cells and growing axons appears to be central for PNS regeneration whereas reciprocally the interaction between axons and neighboring glia also appears important in the regulation of the molecular repertoire expressed by these glial cells (for review, Mirsky et al., 2002). In the PNS, support for axon growth has been thought to be mediated mainly by Schwann cell release of growth factors (Hansson et al., 1986; Glazner et al., 1994 for IGF-I effects) and the production by these cells of ECM molecules and CAMs that sustain axon extension by modulating adhesion (Mirsky et al., 2002). In recent years, gangliosides have been found to be implicated in PNS regeneration as receptors for tenascin-R (Probstmeier et al., 2001, GD3 ganglioside) and myelin-associated glycoprotein, MAG (GD1a and GT1b; Vyas et al., 2002; Yamashita et al., 2002). While the functional role of GD3 in regeneration is yet unknown, the interaction of GD1a or GT1b and MAG is thought to mediate inhibition of axonal extension. Several years ago, Constantine-Paton and colleagues (1986) described the expression of the 9-O-acetylated form of GD3 (9-O-acGD3) in CNS and PNS identified by a specific monoclonal antibody termed Jones. They found that this molecule was distributed in a spatio-temporally regulated manner during development such that it related with migration and axon growth (Mendez-Otero et al., 1988; Schlosshauer et al., 1988; Santiago et al., 2001). In PNS, a spatio-temporal correlation between the expression of 9-O-acetylated gangliosides and axon growth was described during development (Mendez-Otero et al.,

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1988). Mendez-Otero and Friedman (1996) revealed a role in axon extension for these molecules. Cultured dorsal root ganglion (DRG) neurons collapse their axon growth cones when Jones antibody is applied (Mendez-Otero and Friedman, 1996) and depolymerization of microtubules underlies growth cone collapse followed by Jones antibody application (Araujo et al., 1997). In this work, we investigated if the 9-O-acGD3 is reexpressed during nerve regeneration after sciatic nerve crush in adult rats and which cell populations could be involved in the tissue response.

EXPERIMENTAL PROCEDURES

Sciatic nerve crush lesion

All experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Committee for the Use of Experimental Animals of our institution approved the protocols and all efforts were made to minimize the number of animals used and their suffering. Fifty-one young adult male Lister rats (200–300 g body weight) had their right sciatic nerve exposed surgically under deep chloral hydrate (4%) anesthesia (1 mL/100 g body weight) at a mid-thigh level. At this region the nerve was crushed by means of a forceps previously cooled in liquid nitrogen. The process was repeated twice with a 20-s interval. At 3, 5, 6, 7, 8, 9, 10 and 14 days post-crush, the rats inhaled a lethal dose of ether and were perfused transcardially with 0.9% saline, followed by a buffered 4% paraformaldehyde (PF) solution, and 10% sucrose in PF. In all animals both sciatic nerves were removed and the non-operated one was used as a control. Additional controls submitted to sham surgery were performed. Sciatic nerves were dissected out such that the segment contained a portion of the nerve 3 mm proximal and 3 mm distal to the lesion. DRG at lumbar level 5 (L5 DRG) as well as a corresponding region of the lumbar spinal cord were dissected out. All pieces were immersed in a 30% buffered sucrose solution overnight for cryoprotection, then quickly frozen in liquid nitrogen and cut tangentially or transversally (nerves and DRG) and transversally (spinal cord) on a cryostat at 20 μm thickness. To block nonspecific binding sites, the sections were pre-incubated for 45 min, at room temperature, in a solution containing 10% normal goat serum (NGS, Sigma, St. Louis, MO, USA), 1% bovine serum albumin (BSA, Sigma) in 0.05 M Tris buffered saline (TBS). Sections were then incubated overnight with the primary antibodies in a solution containing 10% NGS, 1% BSA in TBS, at 4 °C. The following primary antibodies were used: mouse monoclonal IgM anti-9-O-acGD3 (1:100, Sigma), rabbit polyclonal against neurofilament 200 (NF200, 1:200, Sigma), rabbit polyclonal against GAP-43 (1:2500, kindly donated by Dr. L. I. Benowitz, Harvard Medical School, Boston, MA, USA), rabbit anti-βIII-tubulin (1:100, Sigma), mouse monoclonal IgG against glial fibrillary acidic protein (GFAP, 1:100, Sigma). We used the following secondary antibodies for double-staining: FITC conjugated goat anti-rabbit IgG (H+L) (1:100, Sigma), Cy3- or Cy5-conjugated goat anti-rabbit IgG (H+L) (1:1000, Jackson ImmunoResearch, West Grove, PA, USA) or Cy5- or Alexa Fluor 488–conjugated goat anti-mouse IgG gamma chain specific (1:200, Molecular Probes, Carlsbad, CA, USA) and Cy3 goat anti-mouse IgM mu chain specific (1:800 Sigma). For triple-label immunohistochemistry we used mouse IgM monoclonal anti-9-O-acGD3 (1:100 Sigma), mouse IgG monoclonal anti-GFAP (1:100, Sigma) and rabbit polyclonal anti-GAP-43 (1:2500, kindly donated by Dr. L. I. Benowitz, Harvard Medical School) and rabbit polyclonal anti-βIII-tubulin (1:100, Sigma), as primary antibodies. The secondary antibodies used were respectively Cy3-conjugated goat anti-mouse IgM (mu chain specific, 1:1000, Sigma), Cy5-conjugated goat anti-mouse IgG (gamma chain specific, 1:400, Sigma) and Alexa Fluor 488–conjugated goat anti-rabbit IgG (H+L) (1:200, Molecular Probes). Sections were incubated in the secondary antibodies for 2 h at room temperature. After rinsing in phosphate-buffered saline (PBS), the sections were kept for 15 min in a nuclear staining solution with 0.1% DAPI (4′,6-diamidino-2-phenylindole, Sigma) and mounted using N-propylgalate mounting medium. Corresponding controls containing only the secondary antibodies were performed. Slides were viewed with an Axiovert Zeiss fluorescence microscope equipped with rhodamine, fluorescein and DAPI filters or with a Zeiss LSM 510 META confocal microscope.

Explant cultures

Sciatic nerves obtained from young adult male rats that had been previously crushed 7 days earlier and normal sciatic nerves were dissected in sterile Gey's buffer solution. Explants were plated on poly-L-lysine (1 mg mL^{-1}, Sigma) coated glass coverslips. The sciatic nerve explants were covered with cooled Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and kept in a 5% CO/95% air incubator at 37 °C for 45 min before adding DMEM-F12 (Life Technologies, Sao Paulo, Brazil) culture medium with 1% fetal calf serum. Explants were cultivated for 3–7 days. Cultures were fixed with 4% PF for 15 min and immunoprocessed for 9-O-acGD3 and S-100beta. Explants were pre-incubated with goat serum for 45 min to block nonspecific binding sites, rinsed and then incubated overnight with monoclonal antibody against 9-O-acGD3 (1:100 in 10% NGS, 1% BSA in TBS, Sigma), and with rabbit polyclonal antibody against S100 (1:80 in 10% NGS, 1% BSA in PBS, Sigma). On the following day explants were rinsed and incubated for 1 h with 10% NGS, 1% BSA solution and Cy3-conjugated goat antibody against mouse IgM mu chain specific (1:2500, Jackson ImmunoResearch) and FITC goat antibody against rabbit IgG (H+L) (1:100, Sigma). After rinsing in PBS, the sections were kept for 15 min in a nuclear staining solution with 0.1% DAPI. Slides were viewed with an Axiovert Zeiss fluorescence microscope equipped with rhodamine, fluorescein and DAPI filters (Carl Zeiss, Oberkochen, Germany).

RESULTS

Time course of neurofilament expression after axotomy

Cryostat sections of crushed sciatic nerve were examined 3 mm proximal and distal to the lesion site for time-dependent changes in neurofilament expression compared with non-crushed sciatic nerve. Three days after lesion (DAL3), the proximal region of sciatic nerve showed no changes in neurofilament immunostaining compared with the non-crushed nerve (Fig. 1A and 1Q). Similar results were found in the proximal region at all ages post-lesion examined (Fig. 1A, 1E, 1I, 1M). In contrast, the distal region did not display any staining for neurofilament 3 days after crushing in all cases examined indicating the success of our nerve-crush surgery (Fig. 1C). Five days after crushing (DAL5), low levels of immunolabeling for neurofilament were detectable at the distal region (Fig. 1G). Seven days after crushing (DAL7), a significant increase in neurofilament expression was observed at the distal region of the sciatic nerve (Fig. 1K). At this time-point staining reached levels comparable to those observed in non-crushed nerves (Fig. 1Q) and in the proximal part (Fig. 1I). These immunostaining levels appeared to be maintained by DAL10 (M and O) and DAL14 (not shown).