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Enhanced expression of netrin-1 protein in the sciatic nerves of Lewis rats with experimental autoimmune neuritis: Possible role of the netrin-1/DCC binding pathway in an autoimmune PNS disorder

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Abstract

Netrin-1 is a chemotropic factor that plays an important role as a survival factor in the adult nervous system. To investigate whether netrin-1 is involved in autoimmune injury of the peripheral nervous system (PNS), the temporal expression of netrin-1 protein was analyzed in the sciatic nerves of Lewis rats with experimental autoimmune neuritis (EAN). Western blot analysis revealed a significant increase in the level of netrin-1 protein in the sciatic nerves of rats on days 11 to 24 post-immunization (p.i.) compared to controls; netrin-1 expression declined by day 30 p.i. Immunohistochemistry revealed that netrin-1 protein was expressed weakly in Schwann cells and vessels in the sciatic nerves of normal and CFA-immunized control rats. In the sciatic nerves of EAN-affected rats, netrin-1 immunoreactivity was increased mainly in the cell membrane and extracellular matrix of OX42-positive macrophages and S100-positive Schwann cells at the peak and recovery phases of EAN. Moreover, the putative netrin-1 receptor, deleted in colorectal cancer (DCC), was expressed mainly in axons, some macrophages, and Schwann cells in EAN-affected sciatic nerves, although the level of protein expression did not change significantly over the course of EAN. We suggest that a significant increase in netrin-1 expression contributes to host cell survival and axon regeneration to counter autoimmune injury and inflammation, which may play a role in recovery from EAN-induced paralysis.

Keywords: Experimental autoimmune neuritis; Macrophage; Netrin; Schwann cell

1. Introduction

Experimental autoimmune neuritis (EAN) is a T cell-mediated autoimmune disease of the peripheral nervous system (PNS) that is used as a model of human demyelinating diseases such as Guillain–Barré Syndrome (Gold et al., 2000). The clinical course of EAN is characterized by weight loss, ascending progressive paralysis, and spontaneous recovery. It has been proposed that intra- and extracellular survival factors produced in EAN-affected sciatic nerves are involved in the pathogenesis of EAN. Recently, we observed that the expression of extracellular signal-regulated kinase (ERK) increased markedly in sciatic nerves during EAN (Ahn et al., 2004). ERK appears to act as a survival transduction molecule during repair of the rat sciatic nerve and recovery following autoimmune injury and inflammation. Moreover, it has been reported that expression of a low-affinity nerve growth factor (NGF) receptor (p75 NGFR) increased in the sciatic nerves of EAN-affected rats, suggesting that NGF induction in the adult PNS is related to axonal regeneration (Conti et al., 1995).

Recently, in addition to growth factors and their receptors, it has become apparent that some chemotropic molecules that are involved in cell migration and axon growth in the developing nerve system influence axon

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regeneration and cell survival in the adult PNS (De Castro, 2003; Madison et al., 2000; Manitt and Kennedy, 2002). Netrins are a family of secreted proteins that direct the migration of cells and axon growth cones during neural development (De Castro, 2003). Netrins function either as short- or long-range cues; under some circumstances, netrins act close to the surface of the cells that produce them, while in other cases, netrins act at a distance (Manitt et al., 2001). Two classes of receptors mediate cell responses to netrins, i.e., the deleted in colorectal cancer (DCC) family and the UNC-5 homologue family (Hong et al., 1999). Among the netrins, netrin-1 is an important survival factor that acts via the putative receptor DCC (Llambi et al., 2001). Although the function of netrin in the embryonic nervous system has been studied extensively, netrin-1 is expressed constitutively in the adult mammalian nervous system (Madison et al., 2000; Manitt et al., 2001). In the adult nervous system, the majority of netrin-1 protein is not freely soluble, but is associated with membranes and extracellular matrix (De Castro, 2003). Moreover, in adults, netrin-1 is expressed by multiple types of neurons and by myelinating glia, specifically oligodendrocytes in the CNS and Schwann cells in the PNS (Madison et al., 2000; Manitt et al., 2001).

Although a previous report implied that there was a significant increase in PNS netrin-1 mRNA expression in the 2 weeks that followed nerve transection and repair (Madison et al., 2000), there has been no detailed analysis of the expression of netrin-1 and DCC protein in the injured PNS. Furthermore, nothing is known of the temporal changes in netrin-1 protein expression in the PNS of animals with autoimmune inflammatory PNS injury such as EAN. Therefore, we examined temporal changes in netrin-1 protein expression during the course of EAN and confirmed which host and inflammatory cell phenotypes relate to the netrin-1/DCC binding pathway in EAN-affected nerves.

2. Materials and methods

2.1. Animals

Lewis rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) and were bred in our animal facility. Sixty female rats aged 7–12 weeks and weighing 160–200 g were used in the experiments. All experiments were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23, revised 1996).

2.2. Induction of experimental autoimmune neuritis

Active EAN was induced in the rats as described previously (Ahn et al., 2004; Moon et al., 2005). Each rat was injected in both hind footpads with an emulsion that contained 100 μg of SP26, a neuritogenic peptide homologous to amino acids 53–78 of bovine myelin P2 protein (Shimadzu, Kyoto, Japan), and Freund's complete adjuvant (CFA; *M. tuberculosis* H37Ra, 5 mg/ml). Each rat was treated with 50 ng of pertussis toxin (Sigma, St. Louis, MO) on days 0 and 2 p.i. Ten control rats were immunized with CFA only. Rats were monitored for clinical signs of EAN, and the clinical progression of EAN was divided into seven stages: grade (G) 0, no signs; G1, floppy tail; G2, mild paraparesis; G3, severe paraparesis; G4, tetraparesis; G5, moribund condition or death; and R0, recovery (Matsumoto et al., 2000).

2.3. Tissue sampling

To study the expression of netrin-1 in rats with EAN, on days 11, 16, 24, and 30 after injection, 10 rats each were killed under ether anesthesia before 5 cm of the sciatic nerve was removed bilaterally. Sciatic nerves were obtained from CFA-immunized control rats on day 16 p.i. The sciatic nerves of 4 rats were removed and were frozen at −70 °C for protein analysis. To prepare frozen sections, 3 rats were sacrificed at each stage p.i., and the sciatic nerves were snap-frozen in optimal temperature compound (OCT; Sakura, Tokyo, Japan). Sections (8 μm thick) were cut in a cryostat and were stored at −70 °C until used. In addition, the sciatic nerves of 3 rats were embedded in paraffin wax after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4.

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

DNA fragmentation was detected using in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) performed according to the manufacturer's instructions (ApopTag® In Situ Apoptosis Detection Kit; Intergen, Purchase, NY). Colocalization of the TUNEL reaction product and either R73- or OX42-immunoreactivity was examined using double immunofluorescent labeling in the same tissue section. Briefly, following the TUNEL reaction in which fluorescein isothiocyanate (FITC)-labeled anti-digoxigenin antibody was used, a secondary antibody [tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-mouse IgG (1:50 dilution; Sigma)] was applied to the same tissue section to identify different types of cell.

2.5. Western blot analysis

Each sciatic nerve was dissected free, minced, homogenized, and lysed in a buffer that contained 40 mM Tris–HCl (pH 7.4), 120 mM NaCl, and 0.1% Nonidet P-40 (polyoxyethylene [9] p-t-octyl phenol) supplemented with the protease inhibitors leupeptin (0.5 μg/ml), PMSF (1 mM), and aprotinin (5 μg/ml). Equal amounts of protein (60 μg/20 μl) were loaded in each lane of an 8% polyacrylamide gel. Electrophoresis was then performed under denaturing conditions. After electrophoresis, the proteins were electro-