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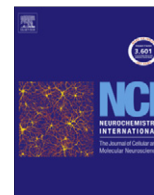

Axoskeletal proteins prevent oligodendrocyte from toxic injury by upregulating survival, proliferation, and differentiation in vitro

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ABSTRACT

Neurofilaments (NF) are detected in the cerebrospinal fluid of multiple sclerosis (MS) patients, and their concentration correlates with disease severity. We recently demonstrated that NF and co-isolated proteins increase the proliferation and differentiation of oligodendrocytes (OL) in vitro. If these proteins are released in the extracellular environment in MS, they might then regulate remyelination by OL. To test this hypothesis we took advantage of a paradigm of OL toxic injury using lysophosphatidyl choline (LPC), which decreases proliferation and differentiation of surviving cells, and destroys myelin-like membranes. In OL cultures that have been treated with LPC, NF fractions as well as tubulin (TUB) significantly improved recovery: the number of OL progenitors (OLP, A2B5+ cells) increased by 100% and their proliferation by 200%, whereas differentiated (CNP+) and mature (MBP+) cells increased by 150% compared to cultures treated with LPC alone. When added at the time of LPC treatment, NF and TUB protected OL from LPC toxicity; they increased OLP by 90%, as well as the number of CNP+ and MBP+ OL by 65–110%, respectively, compared to cultures treated only with LPC. These effects were specific since irrelevant proteins (actin, skin proteins) were ineffective. This demonstrates that NF and TUB protect OL and increase OLP proliferation, as well as their survival, when challenged with LPC, without delaying differentiation and maturation in vitro. Thus, NF and TUB delivered following axonal damage in MS could participate in the regulation of remyelination through this process.

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1. Introduction

Incomplete remyelination and axonal injury (e.g. Trapp et al., 1998) are the main pathological characteristics of multiple sclerosis (MS) a chronic, inflammatory and demyelinating disease of the central nervous system (CNS). In addition to demyelination axonal lesions also occur, such as abnormal non phosphorylated forms of neurofilaments (NF), as well as decrease in NF and β tubulin (TUB) (Trapp et al., 1998; Fressinaud et al., 2005), which might, in turn, render axons unproper for remyelination (Chang et al., 2002). Substantial axon loss occurs also in the normal appearing white matter (Evangelou et al., 2000), and axon damage is a major concern since it results in disability (Rudick et al., 1999; Wegner et al., 2006; Siffrin et al., 2010; Schirmer et al., 2011). Neuronal and axonal damage could result from demyelination (DeLuca et al., 2006), and experimentally, restoring remyelination capacity increases axon survival (Irvine and Blakemore, 2008), or from a distinct consequence of inflammation itself (Siffrin et al., 2010). One main data demonstrating axon lesions is the presence of NF, the intermediate filament proteins expressed specifically in neurons, in the cerebrospinal fluid (CSF) of MS patients (Lycke et al., 1998; Teunissen et al., 2005). The level of NFH correlates with relapse rate and disability (Kuhle et al., 2011; review in Gresle et al., 2011). Experimentally, a rapid degradation of NF occurs following axonal damage, and involves calcium activated proteases like calpain (Hall and Lee, 2005; Saatman et al., 2003). Recently, NF light chain positive macrophages have been identified in MS lesions, together with cells engulfing damaged axons (Huizinga et al., 2012). However, the appearance of NF or their proteolytic fragments during the evolution of MS are poorly documented, as well as their functions when they are present outside from the axonal compartment.

To determine whether alterations of proteins from the axonal cytoskeleton could be involved in MS remyelination failure we have recently analyzed the effects of these proteins on the development of OL in vitro (Fressinaud et al., 2012). Neurofilament

Abbreviations: BrdU, bromodeoxyuridine; CDM, chemically-defined medium; CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CNS, central nervous system; CSF, cerebrospinal fluid; MAP, microtubule associated protein; MBP, myelin basic protein; MS, multiple sclerosis; NF, neurofilaments; OL, oligodendrocyte; OLP, oligodendrocyte progenitor; TUB, tubulin.

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fractions, recovered by successive centrifugations from rat brain (Fasani et al., 2004), increase either the proliferation of OL progenitor (OLP) (2nd pellet, P2), or their differentiation and maturation (5th pellet, P5). Neurofilaments copurify with small amounts of other proteins, in particular TUB, the concentration of which decreases during the purification procedure (i.e. from P2 to P5). Purified TUB, and several purified or recombinant proteins (MAPs, tau, synapsin 2, spectrin βII), also increased OLP proliferation without delaying their maturation. Neurofilament fractions were ineffective on astrocyte cultures, whereas the other proteins also increased astrocyte proliferation in vitro (Fressinaud et al., 2012). Although these proteins are intracellular, we hypothesize that in the case of axon damage they might be released in the extracellular environment and regulate remyelination (Fressinaud et al., 2012). The present study was performed to determine whether they could rescue OL from toxic injury and/or prevent it from occurring. Lysophosphatidyl choline (LPC), was used to create a reproducible paradigm of OL injury in vitro (e.g. Fressinaud, 2005), and to test axon proteins effects under these conditions. In this model LPC is known to induce cell death, decrease OLP proliferation and differentiation, and finally to destroy myelin-like membranes (e.g. Fressinaud and Vallat, 1994). It has also been used in organotypic slice cultures (Defaux et al., 2010; Sheridan and Dev, 2012). LPC is well suited also to analyze the effects of molecules on the repair process (Fressinaud, 2005), and results obtained with growth factors have been confirmed in vivo (Allamargot et al., 2001; Jean et al., 2003), indicating its relevancy. The mechanism of LPC toxicity is partly unknown, but its membrane solubilizing properties probably account for the particular toxicity to OL (Defaux et al., 2010).

2. Material and methods

2.1. NF purification procedures

Three different batches of NF were purified from rat brain as previously described (Fasani et al., 2004; Fressinaud et al., 2012). These NF are predominantly phosphorylated, although there is a small proportion of poorly phosphorylated NF (Perrot et al., 2008). Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) for the care and use of laboratory animals. Briefly, after CO_2 inhalation the brains from 15 adult Sprague–Dawley rats were homogenized in MES buffer, and centrifuged at 100,000*g* for 1 h at +4 °C. The first pellet, containing mostly membranes and myelin, was removed. The supernatant was made 4 M glycerol and incubated for 2 h at +4 °C to prevent microtubule assembly, and then centrifuged as above. The resulting pellet (P2) was homogenised in MES buffer, and this procedure was repeated up to the 5th pellet to obtain P5 NF enriched fractions. The amount of proteins in each sample was determined. Proteins from each purification step were separated on a 7.5% acrylamide SDS–PAGE according to Laemmli (1970), and then transferred onto nitrocellulose membranes for immunoblotting analysis (Towbin et al., 1979). Primary antibodies (monoclonal mouse anti-NFH, -NFM, -NFL, -α, and -β TUB, 1:2000 dilution (Sigma)) were used to determine by Western blots the purity of NF proteins recovered. To serve as controls skin protein extracts from 3 newborn rats were similarly obtained by homogenising with Ultraturax™ in MES buffer.

2.2. Oligodendrocyte cultures

Cultures were performed as previously described (e.g. Fressinaud, 2005) using newborn Wistar rats (Breeding from the Faculty of Medicine, University of Angers). Briefly, brain hemispheres were freed from their meninges, and after several passages through a 1 mm diameter needle, grown in primary glial cell cultures enriched in OL, in Waymouth's medium supplemented with 10% calf serum (Gibco, Invitrogen Corp., Cergy Pontoise, France). After 3 weeks OL were dislodged from the astrocytic underlayer by flushing some medium over the cultures, and grown in secondary cultures. Contaminating microglial cells were removed by preplating on plastic Petri dishes before the subculture. OL recovered with the medium were seeded on poly-L-lysine precoated glass coverslips in multiwell dishes (24 wells), and grown in a chemically-defined medium (CDM) composed of Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, 4 g/L glucose, 5 μg/mL insulin, 10 μg/mL transferrin, 0.5 mg/mL bovine serum albumin, and 30 nM selenium. These cultures were at least 95% pure, and OL synthesize myelin-like membranes (Fressinaud et al., 1990, 1993).

Secondary OL cultures were kept for 24 h in CDM to allow cells to adhere and extend processes. On day 2 LPC (L-α-Lysophosphatidylcholine from egg yolk, >99% pure, 2.10^{-5} M final concentration, Sigma) was added for 24 h (except in control), then the medium was removed (day 3), and the wells were rinsed once with fresh medium. The LPC concentration used was based on previous reports (Fressinaud, 2005; Fressinaud and Vallat, 1994). After LPC removal, cells were grown further in CDM.

Since they were shown to have specific effects either on proliferation (NFP2), or on maturation (NFP5) (Fressinaud et al., 2012), NFP2 and NFP5 fractions were used to explore their properties on these aspects of OL development, respectively, when OL were challenged with LPC. For rescue experiments NF, or selected axonal and control proteins (see below), were added once just after LPC treatment (day 3). Proliferation was assessed by bromodeoxyuridine (BrdU) incorporation (10 μg/mL) for 24 h after LPC removal and during the time of treatment with proteins (day 3). Double immunocytochemistry of OLP with A2B5 and anti-BrdU antibodies was performed after 24 h (day 4). To explore differentiation and maturation, cultures were grown further for 2 days after LPC removal in fresh CDM alone (controls), or supplemented with axon proteins, until day 5. For prevention experiments NF or axonal proteins (see below) were added at the time of LPC treatment (day 2) and also removed after 24 h. Proliferation was assessed during the meantime by BrdU incorporation (day 2), and immunocytochemistry of OLP was performed after 24 h (day 3). Differentiation and maturation were analyzed in cultures grown further for 2 days after LPC and proteins removal and rinsing in fresh CDM alone, and immunocytochemistry was performed on day 5.

For each experiment, proteins were added either at the time of LPC treatment (prevention experiments) or immediately after it (rescue experiments), and concentrations were the same: (i) control (CONT): CDM alone; (ii) NF fractions (diluted in phosphate buffer saline pH 7.4 (PBS)): one treatment with NF fractions P2 or P5 (200 ng/mL final concentration); (iii) purified TUB (α and β tubulin from bovine brain, Cytoskeleton, Denver, CO, USA; diluted in PBS, 100 ng/mL final concentration); (iv) NFP2 or P5 were also used in association with TUB (using the same concentrations as above). The concentrations of NF (200 ng/mL), and TUB (100 ng/mL), were based on previous results of dose–response experiments (Fressinaud et al., 2012).

Finally, in order to confirm the specific effects of NF and TUB, control experiments were performed using in parallel several irrelevant proteins, such as actin (bovine cardiac muscle, 99% pure, 0.1 μg/mL, (Cytoskeleton)), and skin protein extracts (50 μg/mL final concentration). Actin was assayed because it belongs to another class of cytoskeleton proteins, and it has the ability to bind NFL (Hao et al., 1997). These proteins have also been used as control in previous report and had no effect on OL in CDM (Fressinaud et al., 2012).

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