Multiple Sclerosis: Neurofilament Pathology in Spinal Motor Neurons

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Abstract

Objective: While traditionally a disorder of myelin, in multiple sclerosis (MS) neuronal and axonal damage has in recent years become an important topic of clinical relevance. To address this, alterations in neurofilament phosphorylation, known markers of neuronal health, were investigated in anterior horn cells in MS spinal cord tissue for signs of motor neuron damage.

Methods: Spinal cord tissue was examined from 13 MS and 6 control patients. Fresh frozen sections were labelled with antibodies against phosphorylated and non-phosphorylated epitopes of neurofilament H (NF-H) and analyzed by light microscopy.

Results: In MS, increased expression of phosphorylated NF-H in spinal motor neuron perikarya (abnormal for neurons) occurred in 61.5% of cases, mostly in chronic active lesions, with the strongest immunoreactivity at the lumbar level. Inflammatory activity was common in chronic active but rare in chronic silent lesions. In one case with an acute lesion, we saw swollen axons positive for non-phosphorylated NF-H, a pathologic marker in axons, but no signs of perikaryal damage. Expression of non-phosphorylated NF-H in spinal motor neuron perikarya was similar in both MS and controls.

Conclusion: In line with previous studies, our findings implicate anterior horn cell damage as a common feature in MS. We propose that underlying mechanisms may involve reduced synaptic input and/or retrograde degeneration, subjects which remain to be investigated.

Keywords: Multiple sclerosis; Neuronal damage; Anterior horn cells; Spinal cord; Neurofilaments

Introduction

Multiple sclerosis (MS) is the leading cause of non-traumatic neurologic disability among young adults in Europe and North America [1]. A major component of its pathology is destruction of myelin and oligodendrocytes against a background of inflammation, sometimes leading to reversible neurological dysfunction. However, irreversible disability in MS is usually attributed to neuronal damage [2,3], the reason for the recent focus of attention on this topic in MS research. Axonal damage [2-5], neuronal apoptosis [6,7] and neuronal loss [8] in grey and white matter lesions have been described both in MS and its widely-used animal model, experimental autoimmune encephalomyelitis (EAE). In spinal cord, substantially reduced nerve cell counts were found both in EAE [9] and MS [10-12].

In the search for molecular markers of neuronal pathology, neurofilament phosphorylation has proven useful. Neurofilaments (NF) are a major structural component of the neuronal cytoskeleton, particularly prominent in large neurons with long axons [13]. In healthy neurons, neurofilaments in perikarya are non-phosphorylated in contrast to those of axons in which neurofilaments are phosphorylated [14]. Thus, phosphorylated NF in damaged neuronal perikarya and non-phosphorylated NF in injured axons are well established indicators of pathology [15,16].

The phenomenon of neuronal NF phosphorylation has been used to visualize axonal damage in the MS brain [4], as well as axonal and motor neuron perikaryal damage in EAE spinal cord [17,18]. Interestingly, recent reports on the spinal cord in MS that examined NF phosphorylation in spinal cord neurons, emphasized axonal changes [12,19]. In the present study on spinal cord in MS, we have investigated NF phosphorylation in perikarya of anterior horn cells and were impressed by the frequency of anterior horn cell (motor neuron) anomalies in active lesions and the degree of neuronal loss at later stages.

Material and Methods

Tissue material

We examined early autopsy, fresh frozen spinal cord tissue from 13 MS patients (9 female, 4 male, mean age 48.3 ± 11.6 years) and 5 control cases (2 male with other neurological diseases (OND), mean age 52.5 ± 21.5 years; 1 female, 2 male with non-neurological diseases, mean age 67.0 ± 28.7 years) (Table 1). MS lesions were classified in acute, chronic active or chronic silent according to standard criteria [20]. Briefly, “acute” refers to fresh white matter lesions with parenchymal and perivascular collections of infiltrating cells, edema, ongoing myelin and axonal damage, astroglial hypertrophy, microglial reactivity and an indistinct margin; “chronic silent” covers total loss of myelin, depletion of axons, scarring fibroblastic astrogliosis, microglial reactivity and a sharp edge to the lesion; “chronic active” lesions are mostly areas of damaged white matter showing glial scarring, myelin loss and axonal depletion with a sharp edge along which more recent activity consisting of inflammatory changes and myelin loss, is seen. Spinal cords were obtained with a post-mortem interval of 9.0 ± 5.8 h in MS patients and 4.2 ± 1.2 h in control cases. Segments of fresh spinal cord 0.5-1 cm in length were embedded end-on in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA), snap-frozen on dry ice and kept at -80°C until use. Transverse sections 10 μm thick were cut on a Cryostat (Thermo Electron Corporation, Pittsburgh, PA, USA).

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USA) and stored on glass slides at -20°C until use. The procedure for obtaining and processing human tissue was approved according to an Institutional Review Board protocol (IRB number 89-31) (Table 1).

### Immunohistochemistry

As primary antibodies, we used SMI-31, a mouse monoclonal immunoglobulin G (IgG) binding a phosphorylated epitope of neurofilament H, abbreviated in the figures as "P-NF-H" (BioLegend, San Diego, CA, USA), and SMI-32, a mouse monoclonal IgG binding a non-phosphorylated epitope of neurofilament H, abbreviated in the figures as "Non-P-NF-H" (BioLegend), both diluted 1:1000 in Tris buffered saline containing 0.1% Tween 20 (TBS/Tween). Biotinylated goat anti-mouse IgG, diluted 1:300 was used as secondary antibody (Vector Laboratories, Burlingame, CA, USA). Binding was revealed by an avidin-biotin-peroxidase method (Vectastain ABC solution, Vector Laboratories, 1:100), and visualized with 3,3′-diaminobenzidine (DAB) solution containing 0.5 mg/ml DAB and 0.1% hydrogen peroxide in TBS.

Sections were dried at room temperature for 30 min, then fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) for 5 min and washed 3 times, 5 min each, in TBS/Tween. Endogenous peroxidase activity was blocked by incubation with 0.1% hydrogen peroxide for 30 min. Sections were washed twice and incubated in 10% normal goat serum (NGS). After 30 min, excess serum was removed and sections were incubated with primary antibody (1:1000 in 2% NGS in TBS/Tween), overnight at 4°C. Sections were washed twice, incubated with biotinylated goat anti-mouse IgG (1:300 in 2% NGS) for 90 min and then after 3 washes incubated in ABC solution (1:100) for 90 min. Sections were then washed and incubated with DAB solution (0.5 mg/ml) in TBS containing 0.1% hydrogen peroxide for 10 min to visualize reaction product. Negative controls omitting the primary antibody or using an irrelevant antibody were used. Sections were washed in distilled H₂O, dehydrated in ethanol and xylene and cover slipped.

### Section Analysis

Hematoxylin and eosin-stained and immuno-stained sections were analyzed by light microscopy (Axioskop, Carl Zeiss, Thornwood, NY, USA). We concentrated on perikarya in lamina IX, the area where anterior horn cells are situated in the spinal cord. Cases showing positive immunostaining for SMI-31, i.e., abnormally phosphorylated NF-H, in neuronal perikarya were counted as positive. Cases not showing this reaction were counted as negative.

For the neuron count of a subgroup of MS cases, 5 serial sections of each patient stained with Nissl stain (0.075 % cresyl violet) were examined with stereological methods using StereoInvestigator software (MicroBrightfield Inc., Williston, VT, USA). In the anterior horn as the region of interest, all neuronal perikarya were counted, measured and grouped according to their size into motor neurons (diameter ≥ 24 μm) and interneurons (diameter ≥ 10-24 μm) [21]. For evaluation, total numbers of motor neurons per patient were compared. Further details of the procedure can be found in an earlier publication [11].

Images were captured with a digital camera (Axiocam and software by Carl Zeiss); GraphPadPrism software was used for graph production.

### Results

Established, gliotic lesions devoid of myelin and displaying variable degrees of axonal degeneration were common in all cases with chronic lesions but inflammatory changes (reflecting ongoing demyelination), were only seen in chronic active lesions. Such changes comprised perivascular cuffs and scattered lipid-laden macrophages in the white matter (mainly anterior or lateral columns) and occasional collections of small lymphocytes within the meninges.

By immunocytochemistry, clear differences were seen between MS and control cases in the expression of phosphorylated NF-H in anterior horn cell perikarya. Specifically, eight of thirteen MS patients showed widespread phosphorylated (abnormal) NF-H positivity compared to none of the controls (3 healthy, 2 OND: olivopontocerebellar degeneration and polyglucosan inclusion disease). Expression of non-phosphorylated (normal) NF-H in anterior horn cell perikarya was positive in all MS cases except one and in all controls (Figures 1a and 2).

Phosphorylated NF-H (abnormal) immunoreactivity of anterior horn cell perikarya was seen in all three cases with chronic active lesions regardless of spinal cord level, but in only five of nine cases with chronic silent lesions, and none was seen in the single
Figure 1: Neurofilament phosphorylation in spinal motor neuron perikarya in MS patients (left) and controls (right). Note that in MS patients there are abnormally phosphorylated NF-H in approximately two thirds of the group compared to none in the control group. Pathologic phosphorylation in perikarya depending on lesion type: chronic active (left), chronic silent (middle) and acute (right).

Figure 2: Spinal cord sections displaying immunoreactivity in perikarya against normal, non-phosphorylated epitopes of neurofilament H (Non-P-NF-H, left column) and abnormal, phosphorylated epitopes of neurofilament H (P-NF-H, right column). In the upper row are two images of a case of MS; The middle row, images for a neurologically healthy control (HC), and in the lower row, images of a case of polyglucosan inclusion disease, an OND control. Note that anterior horn cell perikarya contain prominent expression of phosphorylated NF-H (thick arrows) only in MS, while in both controls, perikarya remain non-reactive (thin arrows).
case with an acute lesion examined (Figure 1b). Perikarya expressing phosphorylated NF-H were found at all levels of spinal cord. The highest intensity was found at lumbar levels. At thoracic and cervical levels, immunoreactivity was less pronounced. This pattern was particularly evident within one individual case where sections of multiple levels of spinal cord were available (Figure 3). In a subgroup of 4 lumbar patients, the neuron count was compared between cases showing abnormal NF-H phosphorylation in perikarya (positive) and those that did not (negative). In this quantitative comparison, the negative case had a neuron count of only 72.1% of the mean neuron count of the positive cases (figure not shown).

In the one case with an acute lesion, in which spinal cord sections showed scattered perivascular cuffs of lymphocytes in the white matter and an abundance of lipid-laden macrophages, axons with increased diameter were common, indicative of incipient degeneration. Some of these swollen axons expressed non-phosphorylated NF-H, consistent with ongoing damage (Figure 4). Neurofilaments in perikarya in this acute lesion were normal and non-phosphorylated. None of the other MS cases (with chronic active or silent lesions), displayed overt evidence of ongoing axonal damage such as swellings, spheroids or manifestations of neurofilament pathology.

**Discussion**

In this short report, we have documented high levels of phosphorylated NF-H immunoreactivity in anterior horn cell perikarya in fresh frozen, early autopsy spinal cord tissue from a group of 13 cases of MS. About two thirds of the group displayed this phenomenon. Similar changes have been reported previously in spinal motor neuron perikarya in amyotrophic lateral sclerosis (ALS), where damage to lower motor neurons is a characteristic feature [22]. In our study, we also examined a case of ALS as a positive control, which indeed showed phosphorylated NF-H immunoreactivity in perikarya (not shown).

In other OND, in which spinal motor neurons are spared, one might not anticipate such changes. Accordingly, our OND control cases of olivopontocerebellar degeneration and polyglucosan inclusion disease did not show increased phosphorylation of NF-H in anterior horn cell perikarya, nor did neurologically healthy controls. Thus, we conclude that damage to spinal motor neuron perikarya indicated by abnormally increased NF phosphorylation is not a rare but rather a common feature of MS pathology.

In our single case with an acute MS lesion, we found pathologic NF-H only in axons, but not in perikarya. This might indicate that in early stages of lesion formation, axonal degeneration precedes motor neuron perikaryal damage. Correspondingly, Bannerman et al. found in mice on day 21 post-sensitization for EAE a large number of disrupted axons of which a significant majority displayed pathologic NF, but only the occasional anterior horn cell was positive for abnormal (phosphorylated) NF-H [18].

Of the cases with chronic active lesions, all samples revealed phosphorylated (pathologic) NF in perikarya of anterior horn cells compared to a little more than half of the cases with chronic silent...
lesions. Schirmer et al. also reported a number of phosphorylated NF-H positive neurons in anterior horn cells of MS patients and noted that they were more pronounced in early and active lesions [12]. Considering the significant depletion of spinal motor neurons in MS that has been described in previous studies [10-12], it is likely that in non-immunoreactive areas of spinal cord, most damaged neurons have disappeared, although some may also have recovered. This hypothesis is supported by our comparison of the motor neuron count in a lumbar subgroup of 4 MS cases. 3 cases showing abnormal NF-H phosphorylation in perikarya (positive) had a notably higher motor neuron count than a single case that did not show abnormal NF-H phosphorylation (negative). Keeping in mind the low number of cases, this finding of course needs to be interpreted carefully, but it may suggest that abnormal NF phosphorylation indicates a damage neurons cannot survive on the long run. Schirmer et al. [12] describe upregulation of c-Jun in anterior horn neurons in or next to active and early inactive lesions, which may lead to cell death. Further studies are needed to explore whether the neurons with abnormally phosphorylated NF-H are indeed on the death pathway.

Mechanisms underlying these neuronal changes are currently under debate. Schirmer et al. attributed the damage and loss of neuronal cell bodies mainly to retrograde degeneration and stress reactions related to inflammation, demyelination and axonal damage in close proximity to perikarya [12]. In our chronic active MS cases, there was a correlation between inflammation and neurofilament immunopathology, but whether this inflammatory environment may have caused retrograde degeneration in the neurons cannot be stated with certainty. A decrease in synaptic input due to demyelination and/or damage to afferent axons was offered as an explanation for the neuronal apoptosis seen in cortical lesions [6] and this factor could also be causally related to damage to and loss of spinal motor neurons. Assuming that afferent fiber length (and therefore probability of lesions somewhere along the route) correlates with extent of anterior horn cell damage at respective levels, one might account for the greatest predisposition for damage occurring in lumbar samples compared to samples from more proximal levels.

The scientific application of the neurofilament profile is not limited to post-mortem neuropathologic studies in MS research, inasmuch as soluble NF levels in the cerebrospinal fluid (CSF) have been shown to be useful as biomarkers. NF-L levels have been found to reflect acute axonal damage and NF-H chronic irreversible damage, therefore implying prognostic value for disease progression or disability [23]. In addition, immunosuppression therapies using Natalizumab or Fingolimod have been evaluated using soluble NF-L and NF-H levels in CSF with interesting results [24-26]. Thus, NF changes may become a useful measure in clinical trials and therapy in MS.

**Conclusion**

The present findings further implicate involvement of anterior horn cell perikarya in MS pathology as a common phenomenon. We propose that this occurs as a consequence of axonal damage to afferent and/or efferent fibres at early stages of lesion development and ultimately leads to substantial motor neuron loss in the spinal cord that may contribute to the irreversible disability common to chronic stages of the disease. Clearly, there is need for additional research in this field to elucidate further the mechanisms underlying this previously de-emphasized feature. The clinical and therapeutic implications of the phenomenon also remain subjects for future study.
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