Expression of Neurofilament Proteins in the Hypertrophic Granule Cells of Lhermitte-Duclos Disease: An Explanation for the Mass Effect and the Myelination of Parallel Fibers in the Disease State

ANTHONY T. YACHNIS, M.D., JOHN Q. TROJANOWSKI, M.D., PH.D., MICHAEL MEMMO, B.S., AND WILLIAM W. SCHLAEPFER, M.D.

Abstract. The expression of neurofilament (NF) proteins was examined in the surgical specimen from a 42-year-old woman with Lhermitte-Duclos disease. Hypertrophic granule cell neurons of the dysplastic tissues were reactive with monoclonal antibodies, including antibodies to each of the three human NF subunits. Furthermore, antibodies to dephosphorylation-dependent epitopes on NF proteins stained the cell bodies of hypertrophic granule cells, whereas antibodies to phosphorylation-dependent epitopes stained the enlarged and myelinated axons of the hypertrophic granule cells. Enzymatic dephosphorylation of this tissue abolished axonal staining with phosphorylation-dependent antibodies and uncovered determinants recognized by antibodies to the dephosphorylated state of NF proteins. The NF protein immunoreactivity of hypertrophic granule cells was indistinguishable from that of large, NF-rich neurons in control human cerebellum, suggesting that a normal pattern of expression and phosphorylation of NF proteins occurs in hypertrophic granule cells in Lhermitte-Duclos disease. An increased expression of NF proteins by cerebellar granule cells may account for many of the observed alterations of Lhermitte-Duclos disease, including the hypertrophy of the granule cells and enlargement of their axons, leading to the myelination of parallel fibers within the molecular layer of the cerebellum. Attention should now be directed at the underlying mechanisms which lead to the coordinated up-regulation of the three NF genes and whether or not additional gene products or cell types are altered in Lhermitte-Duclos disease.

Key Words: Granule cells; Lhermitte-Duclos disease; Neurofilament disease; Parallel fibers.

INTRODUCTION

Lhermitte and Duclos (1) first described a malformation of circumscribed regions of the cerebellar cortex featuring enlarged and thickened folia containing abnormal ganglion cells. To date, 42 cases of Lhermitte-Duclos disease (dysplastic gangliocytoma of the cerebellum) have been reported in the literature; 13 of these, including the present report, have been surgical specimens (2, 3). While adults, ranging in age from 17 to 59 with a mean age of 34 years, are most commonly affected (2), the disease has been reported in a newborn (4). Patients usually come to clinical attention because of increased intracranial pressure secondary to obstructive hydrocephalus caused by obstruction of the aqueduct of Sylvius. Surgical resection has been the treatment of choice (3, 5, 6).

The cellular changes in Lhermitte-Duclos disease have been well described in...
previous studies (2, 7–10). The small neurons of the cerebellar internal granular layer undergo a graded hypertrophy with a concomitant increase in diameter of their axons which project into the molecular layer. These axons become myelinated causing an abnormal diffuse hypermyelination of the molecular layer. These changes are accompanied by the disappearance of Purkinje cells, loss of afferent fibers to the cerebellar cortex and effacement of the cerebellar cortical architecture.

Ambler et al (2) noted that the dysplastic malformation of Lhermitte-Duclos disease offers a unique opportunity to study regulatory mechanisms that underlie the abnormal differentiation and expression of cerebellar tissues, including those factors which determine the number, size and shape of neurons as well as patterns of myelination. Many of the alterations in Lhermitte-Duclos disease are related to changes in the size and shape of granule cell neurons, including their hypertrophy, axonal enlargement and the myelination of their axons in the molecular layer of the cerebellum (2). The size and shape of granule cells, like all neurons (11), are regulated by cytoskeleton elements, especially by the number and distribution of NF (12–14) and, perhaps, by the state of NF protein phosphorylation (15). Indeed, it is possible that alterations of NF metabolism may account for many of the changes of the cerebellar granule cells that are encountered in Lhermitte-Duclos disease.

The present communication re-explored the alterations of Lhermitte-Duclos disease with immunocytochemical probes to NF proteins. These probes were selected from several large libraries of monoclonal antibodies (MAb) (16–19), according to their abilities to immunostain human tissues (20), to immunoreact monospecifically with each human NF subunit (21) and to recognize differentially phosphorylated forms of the large (NF-H) and mid-sized (NF-M) subunits in human tissues (21) that are differentially enriched at varying proxiomodist regions along the neuraxis (21). Our study demonstrates that the hypertrophied granule cells of Lhermitte-Duclos disease are highly immunoreactive to NF proteins and that the specificity and distribution of NF immunoreactivities (e.g. to phosphorylated vs dephosphorylated epitopes on NF-M and NF-H) are very similar to those of large NF-rich control human neurons (e.g. Purkinje cells). Our findings indicate that the abnormalities that typify Lhermitte-Duclos disease may represent an increased expression of NF proteins by cerebellar granule cells.

MATERIALS AND METHODS

Case Report: The patient is a 43-year-old woman who was in good health until one year before admission when she began to develop occipital headaches that radiated bifrontally. Four months before admission, she noticed a decreased sense of equilibrium with a tendency to fall to the left. Shortly thereafter, she developed neck pain. Medical evaluation at that time included a computed tomographic scan of the neck which was negative and she was treated conservatively. Several weeks before admission, she developed decreased visual acuity on the left. Evaluation at another hospital included a CT scan of the head which showed evidence of obstructive hydrocephalus. On admission to our institution, general examination showed that the patient had an enlarged head and neck pain on flexion. Neurologic examination was significant for decreased visual acuity on the left, a tendancy to fall to the left, and hyperreflexia on the left with a left extensor plantar response. Computed tomographic scan revealed a diffuse, non-enhancing left cerebellar mass. A left craniotomy was performed with subtotal resection of a poorly-defined left cerebellar mass. The patient tolerated surgery well, was discharged in good condition and was doing well at the time of writing this report.

Methods: Bouin’s-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) and luxol fast blue for histologic study. Immunohistochemical studies were performed on 6-μm-thick paraffin sections from the surgical specimen and from normal human
TABLE 1
Specificity of Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Normal cerebellum (autopsy)</th>
<th>Hypertrophic cerebellum (surgical specimen)</th>
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<tr>
<td></td>
<td>Perikarya</td>
<td>Axons</td>
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<td>RMS12</td>
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<td>RMO3</td>
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<td>TA51</td>
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<td>RMD020</td>
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<td>TA50</td>
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The phosphorylation state of the epitopes recognized by these MAbs, as described by Schmidt et al. (21), is denoted as follows: P(ind) indicates that recognition of the determinant with the MAb is unaffected by enzymatic dephosphorylation with alkaline phosphatase; P(+), the MAb no longer binds to the epitope following the same enzyme treatment; P(-), epitopes only recognized by an MAb following enzymatic dephosphorylation. All MAbs to the two large NF proteins bind to determinants in the C-terminal (peripheral domain of NF-H and NF-M except RMO194 which binds to a core determinant of NF-H. Immunoreactivity was graded from 0-3 (0 = negative; 3 = most intense). In normal cerebellum, perikaryal staining refers to Purkinje perikarya; axonal staining refers to white matter and to axon collaterals of basket cell neurons. In the abnormal cerebellum, the hypertrophic granule perikarya were graded as were the hypertrophic axons in the vicinity of these enlarged neurons.

cerebellum; a library of MAbs to specific NF epitopes were used as previously described (21). Specificities of the MAbs used in this study are shown in Table 1. Further, a MAb specific for human glial filament protein (GFAP) was used to assess the integrity of radial glial fibers in the diseased and control cerebellar tissue. The enzymatic dephosphorylation of slides prior to the application of the MAbs was conducted exactly as described by Schmidt et al. (21).

RESULTS

Most of the tissues revealed effacement of normal cerebellar cortical architecture and its replacement by a bilayered pattern. The outer layer contained myelinated fibers in parallel arrays (Fig. 1A). The inner layer was sharply demarcated and contained granule cells in varying stages of hypertrophy (Fig. 1B–D). In sections of transition from normal cerebellum (Fig. 1B), small neurons with dark nuclei were admixed among larger neurons with vesicular nuclei, prominent nucleoli and discernible perikarya. Purkinje cells were conspicuously absent from transitional regions.

Immunoperoxidase studies comparing reactivities of NF epitopes in disease vs control human cerebellum are summarized in Table 1. Similar patterns of immunoreactivities, including the presence of each NF subunit, the presence of posttranslational phosphorylation and the cellular distributions of differentially phosphorylated isoforms, were present in hypertrophied and control tissues.
Antibodies to dephosphorylated NF-H (e.g. RMDO20), that recognize native NF in perikarya of control neurons (21, 22), stained the perikarya and neurites in proximity to the hypertrophied granule cells (Fig. 1C, D). In transitional regions of disease tissues (Fig. 1C), expression of NF proteins could be detected at very early stages of neuronal hypertrophy as a perinuclear rim of immunoreactivity. In large hypertrophied neurons (Fig. 1D), NF proteins were present throughout the perikarya and in adjoining neurites. Perikarya of hypertrophied neurons were also stained with MAb to NF-M and NF-L (Table 1). Occasionally, very large neurons and distended neurites could be visualized by their NF immunoreactivity (Fig. 1D).

Antibodies to phosphorylated epitopes on NF-M or NF-H (e.g. RMO24), that immunoreact selectively with large myelinated axons of control cerebellum (21, 22), heavily stained the myelinated axons of the dystrophic cerebellar cortex (data not shown) as well as neurites adjacent to the hypertrophied granule cells (see Fig. 3C). These antibodies, defined by their strong dependency on NF phosphorylation (19) and by their lack of reactivity with NF in the perikarya of CNS neurons (22), showed slight to mild immunostaining of perikarya of some hypertrophied granule cells (data not shown).

Figure 2 demonstrates the effect of dephosphorylation of tissue sections with alkaline phosphatase probed with a dephosphorylation-dependent antibody, RMDO20. Before dephosphorylation, this monoclonal antibody stains neuronal perikarya and large dendritic processes of normal Purkinje cells (Fig. 2A). After alkaline phosphatase treatment, there is prominent staining of Purkinje and basket cell axons (Fig. 2B). Dendritic staining is reduced. Figure 2C, D demonstrates the effect of dephosphorylation on immunoperoxidase staining of abnormal hypertrophic granular cells in Lhermitte-Duclos disease.

Figure 3 shows the effects of dephosphorylation when tissues are probed with a phosphorylation-dependent antibody. Immunostaining of axons in control (Fig. 3A) and diseased (Fig. 3C) cerebellum are not present (Fig. 3B, D) when the respective tissues are dephosphorylated. Enzymatic dephosphorylation removes the immunoreactivity of axons adjacent to hypertrophic neurons (Fig. 3C, D) and in the myelinated outer layer of diseased cerebellum (data not shown).

Staining of radial glia in the molecular layer of control cerebellum by antibodies specific for GFAP is shown in Figure 4A. In Lhermitte-Duclos disease, the processes of radial glia maintain their parallel alignment but are reduced in their number, are thicker and assume an irregular or corkscrew appearance (Fig. 4B, C, D).

DISCUSSION

Lhermitte-Duclos disease exhibits striking morphological alterations of cerebellar cortex that have been interpreted as a malformation (1), as a neoplasm (7) or as a dysplastic condition (2). Regions of transition between normal and abnormal cerebellum support the view of a malformed cortex with the granule cell neuron as the major cell type affected by this disease (2). Other features of the lesion, such as the pattern of growth, its occurrence focally in mature tissues and the proliferative histological features of the lesion have favored a neoplastic, possibly hamartomatous, condition. The present study has not resolved this issue, but has, instead, attempted to address the nature of mechanisms that may underly some prominent abnormalities in this disease.

This report documents a close correlation between the level of NF expression and the hypertrophy of the granule cell neurons in diseased tissues. Furthermore, the NF protein immunoreactivities in hypertrophied granule cell neurons are indistinguish-
Fig. 1. Hypertrophic cerebellum stained with luxol fast blue for myelin (A), hematoxylin and eosin (B), and with MAb RMO254 (C, D). The arrowheads in A and B indicate the junction between the molecular layer (above) and the granule cell layer (below). Note the heavily myelinated molecular layer in A, the moderately hypertrophied granule cell neurons in B and the absence of Purkinje cells at the interface of the molecular and granule cell layers in A and B. Two different regions of the granule cell layer are shown in C and D; the granule cell neurons are more hypertrophic in D compared to C, but both contain granule cells with cytoplasmic NF protein immunoreactivity (curved arrows in C and D). Hypertrophic NF protein-positive processes are prominent in D. The sections in C and D were lightly counterstained with hematoxylin. A, ×80; B, ×165; C and D, ×325.
Fig. 2. Normal human cerebellum (A and B) and the granule cell region of the hypertrophic cerebellum (C and D) probed with MAb RMDO20. The sections in A and C were incubated in buffer without alkaline phosphatase prior to application of the MAb, while those in B and D were incubated in buffer with alkaline phosphatase prior to the application of the MAb. Treatment of the sections with alkaline phosphatase enables RMDO20 to bind to NF epitopes in processes that are only weakly reactive or completely negative in the sections incubated with buffer alone, although Purkinje cell dendrites are strongly immunoreactive even in untreated normal cerebellum (A). In contrast, perikaryal NF protein immunoreactivity is evident in treated and untreated sections. Numerous hypertrophic processes are seen in D. All of the sections were lightly counterstained with hematoxylin. A–D, ×165.

able from those seen in large neurons of normal cerebellum used as a control most notably with reference to their cytochemical immunoreactivities to each of the three human NF proteins (21) and to the localization of NF protein immunoreactivities that can be distinguished by differential states of NF protein phosphorylation (19, 22, 23). The latter findings suggest that a normal pattern of NF protein metabolism
Fig. 3. Normal human cerebellum (A and B) and the granule cell region of the hypertrophic cerebellum (C and D) probed with MAb HO14. Sections in A and C were incubated in buffer without alkaline phosphatase prior to application of the MAb, while those in B and D were incubated with alkaline phosphatase before application of the MAb. Treatment of the section with alkaline phosphatase (B and D) completely abolishes the strong NF protein immuno-reactivity seen in the processes of sections treated with buffer alone (A and C). Numerous hypertrophic processes are seen in C. All of the sections were lightly counterstained with hematoxylin. A and C, ×165; B and D, ×80.

is established in hypertrophied granule cells, unlike the aberrant patterns of NF protein metabolism in some neoplastic conditions (24). The presence of all three NF proteins in hypertrophied granule cells, including those of smaller size with limited perikarya, also indicates the expression of the “mature” rather than the “immature” form of NF in the altered granule cells. The “immature” form of NF proteins, consisting of NF-L and NF-M but lacking the NF-H subunit, is expressed during the initial phase of embryogenesis, prior to the formation and establishment of synaptic connections (25). Thus, the expression of the “mature” form of NF by
Fig. 4. Normal human cerebellum (A) and the hypertrophic cerebellum (B–D). All sections were probed with the MAb (2.2B10) specific for GFAP and counterstained with hematoxylin. Arrowheads in each figure indicate the junction between the molecular (above) and granule cell (below) layers. Glial fibrillary acidic protein-positive radial glial fibers are numerous, ordered and delicate in the molecular layer of normal cerebellum (A). In contrast, the molecular layer of the hypertrophic cerebellum contains reduced numbers of GFAP-positive radial glial fibers (A and B), and they frequently appear disordered (D) and enlarged (curved arrow in B). Note the normal density of GFAP-positive astrocytes in the granule cell layer and subjacent white matter in normal cerebellum, and the comparatively scant number of GFAP-positive astrocytes among the hypertrophic granule cells (lower portion of 3B–D) of the present case of Lhermitte-Duclos disease. Treatment of the sections with alkaline phosphatase before application of this MAb did not alter the distribution of GFAP. A–D, ×165.
hypertrophied granule cell neurons may represent the response of neurons that have already established functional interneuronal circuits.

Myelination of the molecular layer is a striking and distinctive feature of Lhermitte-Duclos disease that is closely associated with the hypertrophy of granule cells and the myelination of their axons (2). The enlargement of the diameter of axons results from an increase in their NF content (12–14) and this is believed to be a major determinant of the degree of axonal myelination (26). It is likely that an increase of NF could also underlie the myelination of hypertrophied granule cell parallel fibers. Thus, an altered state of cytoskeletal metabolism with increased NF expression could account for the major structural alterations of Lhermitte-Duclos disease, including the hypertrophied appearance of the granule cells as well as the enlargement and aberrant myelination of their axons.

The notion that an altered state of NF metabolism is causally related to the development of Lhermitte-Duclos disease raises some interesting questions regarding the nature of this disease entity, especially its pathogenesis. First, what are the initiating factors or signals that turn on NF metabolism in granule cell neurons, a cell type that normally expresses limited, if any, NF protein (27)? Is there a derepression of factors that ordinarily limit the transcription, processing or translation of NF proteins in granule cell neurons? Does the altered state of NF metabolism represent an internal derangement of the granule cells; why, then, in contiguous neurons? And why are the changes initiated among neurons in the outer layer of the granule cells (2, 7–9)? Alternatively, do the changes reflect an atypical response to some extrinsic signals, possibly from transsynaptic sites, such as the loss of Purkinje cells (2, 4, 8) or the deafferentation (2, 9) that occurs in affected regions of cerebellum? Is the diminished density or apparent loss of granule cells in regions of altered cortex (6) indicative of a diminished viability among hypertrophied neurons? For example, are the occasional mitoses among hypertrophied granule cells (2) another form of a reactive response in neurons? Finally, are the hypertrophic changes with enhanced NF expression limited to cerebellar granule cells? Or could the increased brain weights of afflicted individuals (2) reflect a more generalized increase of NF in other brain nuclei, a change which might remain undetectable by conventional methods of examination?

The documentation of an increased level of NF protein metabolism in the hypertrophied granule cells of Lhermitte-Duclos disease serves to redirect our inquiry into the nature of this disease. Indeed, model systems where NF metabolism is up-regulated may have special relevance for this disease entity. For example, neurite-free, NF-poor adrenal chromaffin cells “transdifferentiate” into NF-rich, neuron-like cells with numerous long neurites upon growth in special culture media (28). In fact, several other phenotypic properties of chromaffin cells are responsive to growth factors (29–32), all indicative of a plasticity inherent in a mature differentiated cell. It is possible that the alterations in the phenotypic properties of granule cells noted here in Lhermitte-Duclos disease may also represent a form of “transdifferentiation” that results from exposure to an altered microenvironment in vivo. Indeed, Lhermitte-Duclos disease appears to represent a highly relevant clinical paradigm for studies which probe the regulation and expression of cytoskeletal protein genes.

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