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Research paper

Novel pathologic findings in patients with Pelizaeus-Merzbacher disease

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HIGHLIGHTS

- Characterizing the pathology of PMD patients with PLP1 deletion and Duplication.
- Examining and comparing two pathologies using electron microscopy and light microscopy.
- Active segmental demyelination and axonal degeneration in duplication patients.
- Decompaction and splitting of myelin and axonal pathology in PLP1 deletion.

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ABSTRACT

Pelizaeus-Merzbacher disease (PMD) is an X-linked inherited hypomyelinating disorder caused by mutations in the gene encoding proteolipid protein (PLP), the major structural protein in central nervous system (CNS) myelin. Prior to our study, whether hypomyelination in PMD was caused by demyelination, abnormally thin sheaths or failure to form myelin was unknown. In this study, we compared the microscopic pathology of myelin from brain tissue of 3 PMD patients with PLP1 duplications to that of a patient with a complete PLP1 deletion. Autopsy tissue procured from PMD patients was embedded in paraffin for immunocytochemistry and plastic for electron microscopy to obtain highresolution fiber pathology of cerebrum and corpus callosum. Through histological stains, immunocytochemistry and electron microscopy, our study illustrates unique pathologic findings between the two different types of mutations. Characteristic of the patient with a PLP1 deletion, myelin sheaths showed splitting and decompaction of myelin, confirming for the first time that myelin in PLP1 deletion patients is similar to that of rodent models with gene deletions. Myelin thickness and g-ratios of some fibers, in relation to axon diameter was abnormally thin, suggesting that oligodendrocytes remain metabolically functional and/or are attempting to make myelin. Many fibers showed swollen, progressive degenerative changes to axons in addition to the dissolution of myelin. All three duplication cases shared remarkable fiber pathology including swellings, constriction and/or transection and involution of myelin. Characteristic of PLP1 duplication patients, many axons showed segmental demyelination along their length. Still other axons had abnormally thick myelin sheaths, suggestive of continued myelination. Thus, each type of mutation exhibited unique pathology even though commonality to both mutations included involution of myelin, myelin balls and degeneration of axons. This pathology study describes findings unique to each mutation that suggests the mechanism causing fiber pathology is likewise heterogeneous.

1. Introduction

In 1885, Friedrich Pelizaeus first identified a genetic disorder in five boys in a single family with nystagmus, spasticity of the limbs and developmental delay [38]. Twenty-five years later in 1910, Ludwig Merzbacher independently found that all affected members of

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this family shared a common ancestor [33]. He further described the neuropathology of 14 affected individuals within this family, all descended from a common female ancestor. Together, Pelizaeus and Merzbacher identified the X-linked inheritance, the neonatal neurological deficits and the hypo-myelinated pattern of the central nervous system pathology that now characterizes the disease, Pelizaeus-Merzbacher disease (PMD) more than a century later.

PMD is now known to be caused by mutations affecting the proteolipid protein 1 gene (*PLP1*) located on the X-chromosome. This gene encodes proteolipid protein (PLP) the major structural protein in compact myelin [8,14,30,37]. More than 100 point mutations have been identified within the *PLP1* coding region that cause a wide spectrum of clinical abnormalities. The clinical features of PMD describing the aggressiveness of the disease and how the severity depends on the nature of the *PLP1* mutation and gene expression has been well studied in animal models [22,26,35,51,55,64,67].

The most common form of PMD, representing 60–70% respectively, has been shown to be due to X-chromosomal duplications that include the *PLP 1* gene [26,34,50]. Animal studies have demonstrated that increased gene dosage of *Plp1* and presumably increased *PLP1* gene expression in humans is the cause of this disease [27,50,65]. This form of PMD is moderate in severity and described in the literature as the classical form of PMD; neurological impairment manifests in the first five years of life with nystagmus, hypotonia, spasticity, ataxia and cognitive impairment.

PMD is also caused by loss of function mutations, a result of either large genomic deletions or mutations that truncate the translation of the protein, where no or little PLP is produced [41]. *PLP1* does not appear to be necessary for myelin formation and myelin is well preserved in these individuals, although they demonstrate a progressive axonal degeneration. This form of PMD has a relatively mild early clinical course that evolves into severe spastic quadriparesis, ataxia and cognitive impairment during early adolescence. Unique to this distinctive form is the mild demyelinating peripheral neuropathy [19,56].

Prior to the era of molecular biology and genetic testing in the 1980s, PMD patients were diagnosed based upon their clinical phenotype and fairly unique CNS pathology compared to other leukodystrophies [20,28,31,40,47,60–62,68]. Autopsy tissue of human PMD patients from known gene duplications were often those of children [23,40,49], and light microscopic descriptions highlighted differences in pathology between patients. The only consistent, uniform finding in humans with PMD that has been derived from both, neuroimaging and histologic studies is that there is an overall deficit in myelin, demonstrating that PMD is a classic hypomyelinating disorder [46,48,52].

While PMD has been described as a hypomyelinating disease, the basis for the decrease in myelin is unclear. Whether myelin formation is impeded (dysmyelination), the sheaths are abnormally thin (hypomyelinated), or normal myelin is formed and then degenerated (demyelinated) has not been clarified. Our study focuses upon fiber pathology of the CNS from adult patients with *PLP1* duplications and a *PLP1* deletion. We describe for the first time that the basis for the hypomyelination in these two types of mutations are unique, a finding that has important implications for future therapies for PMD.

2. Materials and methods

2.1. PLP1 duplication

All the *PLP1* duplication autopsy tissue used in this study was personally collected by the late James Garbern MD, PhD within 12 h of death, immediately frozen, and stored in −80 °C conditions. Two of the three patients, cases 1 and 3 were brothers of a family previously described using Nissl stains [49], and who expired at 47 and 54 years of age, respectively. The date of autopsy was 08/2001 and 07/2001, respectively. They had spastic quadriparesis and never ambulated independently. Voluntary movements were slow with rigidity compromised by the severe spasticity. Both had understandable but dysarthric speech. Case 2 was previously reported as a cousin to cases 1 and 3 and exhibited a similar clinical course and expired at age 50.

2.2. PLP1 null mutation

This patient had a complete deletion of the *PLP1* gene and flanking genes on the X-chromosome and is the same patient described by Raskind and coworkers [41]. He had severe spastic quadriplegia during the last five years of life and was confined to a wheelchair. He lost speech 2 years before he expired at 47 years of age from aspiration pneumonia. As far as we are aware, the only available brain tissue from null patients is the one described here.

3. Histology

Corpora callosa (CC), adjacent cerebra and striata were procured from frozen PMD autopsied brains until tissue processing when the frozen tissue was placed in 10% formalin. The CC were then embedded in paraffin blocks and cut into 4 μm thick sections and subsequently stained with routine hematoxylin (HE), Luxol-fast blue (LFB), or Bielschowsky silver.

3.1. Immunocytochemistry

The paraffin-embedded tissue was cut into 5 μm thick sections and analyzed for myelin basic protein (MBP) and proteolipid protein (PLP) using immunoperoxidase staining techniques. The tissue was deparaffinized in xylene (3 changes) and rehydrated through a series of graded ethanol (100, 95, 70 and 50%) to distilled water. The tissue was blocked for endogenous peroxidase activity with 3% H_2O_2 for 5 min at room temperature. Epitope antigen retrieval was required, so the tissue was treated with 0.1 M citrate buffer and heated in the microwave for 10 min on high power, then cooled to room temperature for approximately 20 min. The tissue was rinsed briefly in distilled water, then blocked for non-specific antibody binding by incubating in 5% serum/0.5% BSA in 1X PBS (Goat for monoclonal MBP and Horse for polyclonal PLP). The primary polyclonal antibody (PLP 1:200) and primary monoclonal antibody (MBP 1:500) was diluted to the optimal concentration in 1X PBS and incubated overnight at 4 °C. The tissue was washed 3x over 5 min with distilled water. Biotinylated secondary antibody (Vector Labs, Burlingame, CA) was diluted in 1X PBS and applied at 1:500 for MBP (mouse) and PLP (Rat). 500 μl of 3-amino-9-ethylcarbazole [5] substrate (Vector labs) was applied and tissue was incubated for 30 min. The tissue was washed 3x in distilled water and then counterstained in Mayer's Hematoxylin for 1 min and washed in tap water. The tissue was then rinsed once in 0.2% 100% NH_4OH and then briefly in tap water. The tissue was then cover slipped using a water-based mounting media.

For semi-thin and ultra-thin microscopy, small blocks of frozen tissue was chipped out, immediately post-fixed in 4% paraformaldehyde, and embedded in Araldite plastic several days afterwards using routine procedures. Tissue was rinsed over-night in PBS, and small blocks of tissue approximately 1 mm × 1 mm × 1 mm were osmicated, dehydrated in a graded series of ethanol over several hrs, placed in a 1:1 mixture of propylene oxide and ethanol, 100% propylene oxide, placed in pure Araldite until the evening when re-embedded in pure Araldite and cured for 49 h in an oven. 1 μm semi-thin sections were cut and