Published in final edited form as:

Neurosci Lett. 2016 August 3; 627: 222–232. doi:10.1016/j.neulet.2016.05.028.

Novel pathologic findings in patients with Pelizaeus-Merzbacher disease

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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.

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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.

Keywords

Pelizaeus-Merzbacher disease; Proteolipid Protein; Immunocytochemistry; Electron microscopy; Hypomyelination; Myelin and axon pathology

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 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 hypomyelinated 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 *PLP1* 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 1980's, 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.

Materials and Methods

PLP1 duplication

All the *PLP1* duplication autopsy tissue used in this study was personally collected by the late James Garbern MD, PhD within 12 hours 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.

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.

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 4µm thick sections and

subsequently stained with routine hematoxylin (HE), Luxol-fast blue (LFB), or Bielschowsky silver.

Immunocytochemistry

The paraffin-embedded tissue was cut into 5µm 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% H₂O₂ for 5 minutes at room temperature. Epitope antigen retrieval was required, so the tissue was treated with 0.1M citrate buffer and heated in the microwave for 10 minutes on high power, then cooled to room temperature for approximately 20 minutes. The tissue was rinsed briefly in distilled water, then blocked for non-specific antibody binding by incubating in 5% serum/0.5% BSA in 1× 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 1× PBS and incubated overnight at 4°C. The tissue was washed 3× over 5 minutes with distilled water. Biotinylated secondary antibody (Vector Labs, Burlingame, CA) was diluted in 1× PBS and applied at 1:500 for MBP (mouse) and PLP (Rat). 500µl of 3-amino-9-ethylcarbazole [5] substrate (Vector labs) was applied and tissue was incubated for 30 minutes. The tissue was washed 3× in distilled water and then counterstained in Mayer's Hematoxylin for 1 minute and washed in tap water. The tissue was then rinsed once in 0.2% 100% NH₄OH 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 1mm × 1mm × 1mm 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 49hrs in an oven. 1µm semi-thin sections were cut and stained with Toluidine Blue for light microscopy or 0.1µm ultra-thin sections were counter-stained with lead citrate and uranyl acetate for electron microscopy. Images from the ultra-thin sections were obtained with a JEOL 1010 microscope.

G-ratio Analysis

G-ratios were calculated from scanned electron micrographs using ImageJ software. The inside and outside circumferences of the myelin sheaths were traced and Feret's Diameters were calculated by ImageJ and the g-ratios plotted against the axonal diameters. The purpose of this study was not intended to be quantitative but rather to demonstrate abnormally thin and thick myelinated fibers compared to normal.

Results

Autopsy tissue

Autopsy tissue of brain cerebrum and corpus callosum was obtained from a complete deletion of *PLP1* and *PLP1* duplication patients and plastic embedded to obtain high-resolution fiber pathology (see Materials and Methods). Only one PMD patient with a *PLP1* deletion is described in this study, because we know of, and have access to tissue only from this one PMD *PLP1* deletion. While the neuropil shows freezing and thawing artifacts, myelin sheaths and many axons retain good cytoarchitectural features sufficient to describe fiber pathology. The adequacy of this tissue to demonstrate fiber pathology is shown in electron micrographs wherein myelin sheaths of many fibers show normal compaction and g-ratios. Moreover, microtubules and neurofilaments are distinguishable in many of the axons (Figure 2, 8). The quality of preservation of the human tissue used here is comparable to or better than that used in published studies of multiple sclerosis [2] and PMD tissue [20, 47, 68].

Myelin and Axonal Pathology in the absence of PLP—In the PMD patient with a *PLP1* deletion, myelin sheaths exhibited a wide-range of defects. In semi-thin sections, myelin is often of thinner caliber than normally found. The myelin sheaths appear well-compacted around a small percentage of axons, particularly those of small diameter (Figure 1). Myelin sheaths, especially large diameter fibers, show splitting and decompaction of myelin in semi-thin sections (Figure 1). This observation is verified by ultrastructural findings that show all the myelin lamellae surrounding an axon are uncompacted (Figure 2). Together, these findings reinforce the structural importance of PLP in maintaining an intact myelin sheath. Somewhat surprisingly, many of the myelin sheaths are abnormally thick in relation to axonal diameter, suggesting oligodendrocytes are metabolically functional and attempting to make myelin (Figures 1-2). The presence of both abnormally thin and thick myelin sheaths is confirmed by analyzing the g-ratios in both the *PLP1* null and duplications patients (Figure 3). For the *PLP1* null patient, decompacted fibers were not included in the analysis as they would bias the g-ratios and only well-compacted myelin of different diametered fibers were included. The combination of both abnormally thin and thick myelin sheaths described here in both the PMD *PLP1* null and duplication cases have been described in numerous demyelination and remyelination studies (see Discussion for significance of these findings).

Many other fibers are undergoing degeneration, evidenced by dissolution of myelin and abnormally shrunken axoplasm. Still other fibers have swollen axons with few organelles, microtubules, and neurofilaments and axoplasm (Figures 1-2). These axons are often surrounded by abnormally thin sheaths. Myelinated fibers, sectioned longitudinally (Figures 1B-C) and serial sections (Figures 4A-B), exhibit zones of fiber constriction and fiber transection. Serial sections (Figures 4A-B) of an individual fiber confirm that a fiber may be both transected and constricted along its length. The serial sections suggest that fiber degeneration remains an active process up to the time of death. Interestingly, we have little evidence of cells directly opposed to these fibers, more suggestive of neuronal degeneration than active phagocytosis. Bielschowsky silver stained sections and hematoxylin/eosin/Luxol

fast blue stained sections confirm the axonal spheroids, transections and fiber constrictions (Figure 5). The absence of PLP is a strong indication that its molecular properties are necessary and required for the overall health and maintenance of axons. In the surrounding neuropil, proliferation and hypertrophy of astrocytic processes and astrogliosis was preserved in some regions, and likely contributed to the grey background stain (Figure 2C).

Myelin and Axonal Pathology in PMD patients with duplications—Fiber pathology in PMD patients with *PLP1* duplications exhibit some similarities as the deletion, but they also exhibit unique differences of the examined corpora callosa and adjacent cortical tissue. We examined tissue from 3 PMD patients with duplications using semi-thin or ultrathin sections (Figures 6-9). All 3 cases exhibited extensive degeneration of fibers that included involution of myelin into the space previously occupied by axons, myelin balls, ballooning of fibers, constriction and/or transection of fibers (Figures 6-7, 9). Most strikingly, demyelination of axons was easily observed (Figure 7A-B). Other axons expand into giant myelin balls that can be visualized with antibodies to myelin proteins (Figure 6A-B), with H-E staining (Figure 6C), in Toluidine Blue semi-thin sections (Figures 6F-G, 7C) and ultrastructurally (Figure 8B,E). Demyelination took several forms: in some fibers, an internode was surrounded on each side by demyelinated segments (Figure 7B) in other fibers, an internode was followed for a considerable distance by a demyelinated segment (Figure 7A). The ultrastructure of the fiber shown in Figure 8A exhibited a demyelinated segment, thus confirming the demyelination and also showing degeneration of the adjacent myelin sheath and axoplasm containing a variety of inclusions. At the paranode, the myelin has noticeably thinned, but transverse bands remain on one side of the paranode. At the electron microscopic level, myelin adjacent to a demyelinated segment appears relatively normal (Figure 8B). This observation suggests that both degeneration and survival of oligodendrocytes exists within small territories. Involution of myelin into axoplasm (Figures 6G, 7C, 8B,E) begins modestly but apparently progresses so that axoplasm is frequently lost. Large, swollen ballooned axons are common (Figures 6H-I, 8C, 9B) and contain abundant debris that seems derived from axoplasm whereas axoplasm in other axons is lucent. Similar findings have been described in a mouse model with a duplication of the human *PLP1* gene [11].

Discussion

PMD is a genetically heterogeneous disorder caused by point mutations, deletions and/or duplications of the gene encoding the major CNS myelin protein, PLP. The pathological description of PMD started in the early 19th century and since then, relatively few electron microscopic studies have been done on human patients to characterize the pathological differences in *PLP1* duplications and *PLP1* deletions. In this neuropathology study, we describe for the first time unique differences between 3 PMD patients with *PLP1* duplications compared to 1 patient with a deletion.

Deletion of PLP

Patients with genomic deletions of *PLP1* exhibit a mild clinical phenotype and near normal longevity. Based on longevity, the prediction is that myelin wrapping and amount of myelin

is relatively normal in the first decades of life but then gradually deteriorates. This inference is based mainly on animal studies of *Plp1* null mice wherein their behavior is nearly normal for essentially the first year and then gradually deteriorates. As for fiber composition, mature *Plp1* null mice exhibit axonal swellings and fiber degeneration that is accompanied by splitting and decompaction of adjacent lamellae [42, 43]. Decompaction of myelin in rodents is predicted based upon biochemical studies showing PLP functions as an “adhesive strut” to maintain compacted myelin [6, 30]. However, decompaction in humans has never been demonstrated. And, in agreement with the observations in the *Plp1* null mice, the myelin shows a similar splitting of lamellae [8, 9, 30]. In the *Plp1* null patient described here, the number of lamellae surrounding many axons appears normal or even increased, indicating the initial wrapping of myelin was normal. Together, these findings also demonstrate that the technical aspects of myelin preservation, fixation and sectioning are intact in our work in spite of using human autopsy tissue. In both rodents and humans, fiber pathology seems to preferentially affect long motor and sensory tracts indicating a length-dependent axonopathy [20]. The animal pathology provides a plausible explanation for many clinical observations that includes spastic quadriparesis affecting the lower extremities. Within that same study that included PMD patients with duplications, proton MR spectroscopy (MRS) revealed a decrease in NAA/Creatine (Cr) ratio levels, a strong indicator for axonal damage and swellings. In support of NAA/Cr as a marker for axonal damage, decreased NAA/Cr in the brains of patients with MS correlated well with clinical disability making these metabolites reliable markers for longitudinal monitoring [12, 13, 17].

Difficult to resolve from MR and most light microscopic techniques, electron microscopy revealed both large diameter fibers with abnormally thick and seemingly well-spiraled myelin and axons surrounded by abnormally thin myelin. These observations are based upon analysis of g-ratios where we find skewed g-ratios higher than (thinner than normal myelin sheaths) and lower than (thicker than normal) myelin sheaths. In our *PLP1* null and duplication cases, we found g-ratios as low as 0.2 and as high as 0.9. These values are considerably at variance from normal human white matter where the g-ratio ranges from 0.55 to 0.75 [2]. Interestingly, higher than normal g-ratios were only found in the human *PLP1* deletion patient, indicative of thinner than normal myelin sheaths, and suggestive of attempts at remyelination. Conversely, thinner than normal g-ratios indicative of thicker myelin sheaths were mainly found in the patients with *PLP1* duplications, suggestive of continued myelination. While remyelination is generally manifested by abnormally thin sheaths, abnormally thick myelin sheaths have also been described in demyelination/remyelination studies [16, 18, 44] and in human white matter diseases [2]. Abnormally thick myelin sheaths have been carefully documented in a remyelination model, the basis for which may be elevated activation of the ERK MAP kinase pathway [29]. In this regard, constitutively active Akt in oligodendrocytes leads to hypermyelination and a corresponding decrease in the g-ratio [36]. The importance of documenting these findings is twofold: first, it strongly suggests that some oligodendrocytes in the *PLP1* mutants remain metabolically active and might be amenable to drug therapies to prevent axonal degeneration. Second, abnormally thick and thin fibers are known to alter axonal conduction rates and may contribute to the spasticity of these patients [63].

In children, hypomyelination and demyelination is difficult to distinguish from active myelination. Since our autopsy material is from adults, we have a baseline as to normal levels of myelin, and can conclude that fiber degeneration is extensive in both *PLP1* null and duplication patients. In agreement with our findings, mild increases in choline levels, indicative of myelin break down products, have been described with MR spectroscopy [25]. Hence, both demyelination and remyelination could be explained by the presence of newly generated oligodendrocytes and degenerating oligodendrocytes. Because of the paucity of fibers in the neuropil, we presume that microglia/macrophages removed the degenerated myelin debris at some point during the disease's natural history. However our 1µm serial section analysis does not show close apposition of phagocytic cells to degenerating fibers. Rather, an individual fiber undergoes sequential degeneration preceded by narrowing of the axoplasm with contiguous myelin and then presumably pinching of the constricted segment leading to transection of the fiber.

Overexpression of PLP (Duplication)

The 3 most prominent findings in the *PLP1* duplication patients are segmental demyelination, clumps of degraded myelin and axonal degeneration. Segmental demyelination has not been previously reported in patients with PMD and was a common finding in our patients. Clumps of degraded myelin lacking normal periodicity were also a common observation in our study and have been previously identified as 'myelin balls' using EM (Watanabe, 1973) and Sudan III staining [47]. The 'myelin balls' observed in the early literature (Watanabe, 1973), however are different than those identified in this study, since neither disintegration of myelin nor axon pathology were found or associated with 'myelin balls' in these earlier studies. Often, the myelin was degenerating but in some images, the myelin seemed relatively normal indicating degeneration proceeded over a protracted time period. No matter how the degeneration proceeds, conduction velocity is certain to be altered. The selective demyelination of some fibers and numerous myelin balls strongly suggests that oligodendrocyte death is increased and, indeed, we previously showed that at any given time in the mice with *Plp1* duplications that apoptosis is 4× that of age-matched wild-type mice [10].

Why is there evidence of active demyelination in the *PLP1* duplication, but not in the *PLP1* deletion? Although we have no direct evidence to explain this at the moment, the answer must be due to the cellular consequences of either the absence or overexpression of *PLP1*. Since *PLP1* is thought to act as a molecular strut to hold compact myelin together at the intraperiod line, its absence likely weakens the myelin structure and produces myelin splitting. These structural changes are then transmitted to the axon, possibly causing axonal degeneration. In contrast, in the *PLP1* duplication, myelin is more actively degenerating, suggesting that myelin structure is more significantly disrupted, and produces a more active attempt by the cell to remove the damage. There is also likely a more active inflammatory response as well, including both macrophages and microglia.

The pathologic elucidation of PMD is characterized as a dysmyelinating disorder [39, 45, 54, 59, 66], likely on the pathogenic basis of a metabolic abnormality in myelin without inflammation. Historically, descriptions of PMD pathology never discussed inflammation as

a contributory factor to the pathology, particularly neuronal degeneration. The basis for this conclusion was reasonable as PMD is an inherited disorder with its origins in the CNS. More recently, transgenic mice with an increased copy number of native *Plp1* were shown to have extensive microglial activity and up-regulation of two pro-inflammatory markers as based upon message levels, TNF- α and IL-6 [53]. The wide spread activation of multiple cytokines and chemokines, presumably produced mainly by microglia and astrocytes, is likely a response to several factors including the demyelination and insertion of PLP into mitochondria when the gene is duplicated [3]. The effects of an inflammatory response cannot be ignored and must be included as a factor contributing to the pathology in PMD. The inflammatory process that accompanies the immune-related attack on myelin (demyelination) is the picture that is emerging from the PMD studies suggesting an inflammatory response much akin to that of MS. The inflammatory changes and axonal pathology found in MS has been well documented and is likely responsible for the irreversible neurologic impairment [7, 57]. Increased microglial activity found in areas of demyelinating lesions in MS [58] has been shown to correlate with the disease severity [4, 21] and this finding may be a confounding factor influencing the disease severity and overall clinical disability in PMD.

Quantitative MRI neuroimaging studies have proven instrumental in describing the axonal and myelin pathology in white matter diseases at a gross level [15]. However, MRI does not explain the basis for the fiber loss. Is it due to apoptosis of an oligodendrocyte and subsequent segmental demyelination of those axons myelinated by a particular oligodendrocyte or is it due to death of a neuron and all its myelin internodes? In our study, the segmental demyelination strongly argues for individual death of oligodendrocytes. However, whether the atrophic and transected axons are due to focal axonal degeneration or anterograde axonal degeneration is unclear. The answer may be a combination of the above, and results from a diffusion tensor imaging study may shed some light on this issue. In PMD patients with *PLP1* mutations, there is a significant reduction in fractional anisotropy, a measure of fiber integrity and directionality [32]. The axons most vulnerable to these pathologic changes remain elusive, but small caliber axons appear relatively unaffected compared to the degenerative changes affecting seemingly larger size fibers. In both *PLP1* duplications and null patients, many small diameter fibers appear to have normally compacted myelin. This observation is somewhat surprising because small diameter fibers are strongly PLP positive, suggesting they should be highly susceptible [24]. But, if PLP functions as an adhesive molecule, increased wt-PLP in the duplications may explain their normal appearance.

Conclusion

Our study shows pathologic findings that are unique to each mutation, and not previously reported in humans with a *gain and loss of PLP1* mutations. Based upon the differences in fiber degeneration, we can deduce that the molecular and cellular pathways leading to myelin damage and loss are different in patients with *PLP1* deletions and *PLP1* duplications. We also recognize the importance of performing a study that examines inflammation in PMD autopsy tissue. Describing the central pathologic differences between *PLP1* mutation types in humans is worthy of attention because it should lead to a more refined discussion on

future therapies [55]. For example, in the *PLP1* deletion patient, extensive myelin, albeit of abnormal composition, is present and is likely indicative of numerous functional oligodendrocytes. In contrast, in the *PLP1* duplication patients, extensive loss of myelin and, presumably, oligodendrocytes is present. The hallmark of axonal damage and active segmental demyelination in the PMD duplication patients versus the hallmark of myelin decompaction in the PMD null patient is important not just for understanding the pathogenesis of the disease, but for designing the integration of stem cell, gene and drug therapies based upon the cellular pathology.

Acknowledgments

The authors would like to acknowledge the late James Y. Garbern, MD, PhD (2011) for his relentless pursuit to advance PMD research in developing new treatments for his patients and family affected by PMD.

Funding: This research was supported by NIH NS38236 and the European Leukodystrophy Association (RS).

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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

Figure 1.

Fiber pathology of a *PLP1* null syndrome patient visualized with 1 μ m Toluidine Blue sections. (A) Myelinated fibers in varying degrees of demyelination fill this field. Some fibers exhibit large axonal swellings (asterisks) surrounded by thin myelin sheaths; myelin around other axons exhibits decompaction and splitting of myelin lamellae (arrowhead). (B-C) Axons, sectioned longitudinally, show numerous zones of constriction (arrowheads), possibly indicating a fiber transection in C (arrow). A possible node is indicated in B (arrow). (D) Several axons (arrowheads) of varying diameter have myelin sheaths that are thinner than normal, but relatively well-compacted, suggestive of active remyelination. (A-D) Toluidine Blue. Mag. Bar 10 μ m.

Figure 2.

Ultrastructure of myelin abnormalities in *PLP1* null syndrome. (A-C) Axons (arrow) in the process of degeneration exhibit shrunken axoplasm accompanied by intracellular debris and/or an abundance of neurofilaments. The cytoplasm of the large axons in A-B (arrowheads) is abnormally dense and contains microtubules and an abundance of neurofilaments. Note that the ratio of myelin diameter to axon diameter is much thicker than normal. Astrocyte processes likely account for the abundant grey background staining (asterisks). (A-C) electron micrograph. Mag. Bar 2 μ m.

Figure 3.

G-ratios were calculated from transverse circular profiles of normally appearing, well-compacted myelin of a *PLP1* duplication and null patient. In the duplication patient, abnormally thin myelin sheaths predominate over abnormally thick myelin sheaths. In the null patient, both abnormally thin and thick fibers are present in addition to fibers of normal thickness.

Figure 4.

(A-B). One-micron plastic serial sections from the *PLP1* null patient. Individual fibers can be consecutively followed from adjacent section to section. Some fibers are transected (T) or constricted (C). Red arrows and lines identify structures that can be matched to the same zone of a fiber. Transection is defined as a zone of a fiber in which both myelin and axoplasm are not clearly visible. Constriction is defined as a zone in which myelin is present but axoplasm is sharply narrowed compared to immediate surrounding axoplasm. In the set of serial sections to the right, the fiber labeled with a T at the left side of the picture may also be a nodal region but it is difficult to determine with certainty. Mag. Bar 10 μ m

Figure 5.
(A-B). In the *PLP1* null syndrome, multiple, large axonal swellings (arrows) are visualized with the Bielschowsky silver stain. (C) An axon terminates in a large swelling (see inset) visualized with hematoxylin and eosin counterstained with Luxol Fast Blue. Mag. Bar A: 10 μ m; B: 100 μ m; C: 50 μ m.

Figure 6.

Myelin pathology in two PMD patients with duplications, case 1 (A-F) and case 2 (G-I). Myelin basic protein (A) and proteolipid protein (B) immunocytochemistry visualized with immunoperoxidase staining. (A-C) Aggregate clumps of degraded myelin product (arrowheads) are often described as “myelin balls”. The myelin balls are diffusely distributed and characterize the late onset demyelinating process. Semi-thin 1µm Toluidine Blue sections show several zones of constriction (arrowheads in D-E) of affected axons. (F) Myelin balls (arrowheads) show clumps of myelin, and in (G), a myelinated segment expands into a large myelin ball (asterisk) and then appears to continue on for a short distance. A thinly myelinated sheath (arrowheads), continue as an unmyelinated axon or is at a node. (H-I) Large swollen axons (asterisks) are surrounded by smaller diameter fibers, the majority of which appear normal, whereas others are degenerating. (A-B) immunoperoxidase stain; (C) hematoxylin and eosin; (D-I) Toluidine blue. Mag. Bar 10µm.

Figure 7.

Segmental demyelination in case 2 (duplication) with over-expression of *PLP1*. (A-C) Longitudinally sectioned axons (asterisks) show segmental demyelination (arrowheads). (B) A myelinated internodal segment is surrounded on both sides by unmyelinated regions. (C) Possible nodes (arrows) with abnormal paranodes or short demyelinated internodes illustrate the segmental demyelination. Involution of myelin into a ball previously occupied by an axon (arrowhead). (A-C) Toluidine blue. Mag. Bar 10µm.

Figure 8.

Ultrastructural features of fiber pathology with over-expression of *PLP1* (case 2). (A) This fiber exhibits a degenerating myelin sheath and axoplasm at one end whereas at the other end, the fiber is unmyelinated at the nodal and paranodal zone (arrows). Transverse bands between the axolemma and oligodendrocyte process (arrowhead) are still intact. Mitochondria are lacking at the nodal-paranodal junction. Some axons have abnormally thick myelin. Reactive astrocytes (asterisk) (A and D) are identified by an arrangement of relatively thick bundles of filaments (arrows) later seen in C and D that are interspersed between intact and degenerating fibers. (B and E) Ongoing axonal degeneration and demyelination resulted in large, irregular whorls of myelin membrane (asterisks). A degenerating axon (E) with an abnormally thick myelin sheath is indicative of active myelination. (C) A large swollen axon (arrowhead) with a thin myelin sheath is observed and contains an accumulation of membranous organelles. (C) Microglial cells (asterisk). (A-E) electron microscopy. Mag. Bar 2 μ m.

Figure 9.

Fiber pathology in case 3 (duplication) with over-expression of *PLP1*. (A-B) Multiple sites of fiber constriction (arrowheads); (B), Ballooned axoplasm (asterisk) is surrounded on both sides by myelin. (C) This fiber exhibits both involution of myelin into axoplasm and bulging into surrounding neuropil (arrowhead). (A-C) Toluidine blue. Mag. Bar 10 μ m.