Diffusely Abnormal White Matter in Multiple Sclerosis: Further Histologic Studies Provide Evidence for a Primary Lipid Abnormality With Neurodegeneration

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Abstract

Although multiple sclerosis (MS) lesions have been studied extensively using histology and magnetic resonance imaging (MRI), little is known about diffusely abnormal white matter (DAWM). Diffusely abnormal white matter, regions with reduced mild MRI hyperintensity and ill-defined boundaries, show reduced myelin water fraction, and decreased Luxol fast blue staining of myelin phospholipids, with relative preservation of myelin basic protein and 2',3'-cyclic-nucleotide 3'-phosphohydrolase. Because DAWM may be important in MS disability and progression, further histologic characterization is warranted. The MRI data were collected on 14 formalin-fixed MS brain samples that were then stained for myelin phospholipids, myelin proteins, astrocytes and axons. Diffusely abnormal white matter showed reduced myelin water fraction (~30%, p < 0.05 for 13 samples). Myelin phospholipids showed the most dramatic and consistent histologic reductions in staining optical density (~29% Luxol fast blue and ~24% Weil’s, p < 0.05 for 13 and 14 samples, respectively) with lesser myelin protein involvement (~11% myelin-associated glycoprotein, ~10% myelin basic protein, ~8% myelin-oligodendrocyte glycoprotein, ~7% proteolipid protein, ~5% 2',3'-cyclic-nucleotide 3'-phosphohydrolase, p < 0.05 for 3, 3, 1, 2, and 3 samples, respectively). Axonal involvement was intermediate. Diffusely abnormal white matter lipid and protein reductions occurred independently. These findings suggest a primary lipid abnormality in DAWM that exceeds protein loss and is accompanied by axonal degeneration. These phenomena may be important in MS pathogenesis and disease progression, which is prominent in individuals with DAWM.

Key Words: Brain, Diffusely abnormal white matter, Histopathology, MRI, Multiple sclerosis, Myelin.

INTRODUCTION

The most prominent and characteristic pathologic feature of multiple sclerosis (MS) is the presence of focal lesions in the central nervous system (CNS), referred to as plaques, which have been studied extensively with both histology for more than a century (1, 2) and with magnetic resonance imaging (MRI) for the last 3 decades (3, 4). Plaques exhibit inflammation, demyelination, and gliosis (5) and frequently show axonal damage and loss (6, 7), as well as remyelination (8). The advent of MRI has allowed for in vivo demonstration of MS pathology formerly only visible through postmortem examination. Magnetic resonance imaging is an exquisitely sensitive technique for detection of MS lesions (9), which are routinely documented and followed by means of conventional magnetic resonance (MR) techniques, particularly T2-weighted or proton-density MRI (10). However, despite the relative ease of imaging white matter (WM) lesions, it is clear from the poor correlation between total lesion burden and measures of disability (11, 12) that factors other than focal WM plaques contribute to clinical status and progression. Recent imaging advancements now allow for depiction of cortical gray matter lesions that may be closely linked to clinical manifestations and disease evolution (13–15). However, it is likely that substrates responsible for disability are not limited to focal abnormalities, and therefore, histology and MRI studies have expanded to include nonlesional WM.

Areas of MS WM that appear grossly normal, termed “normal-appearing white matter” (NAWM), have historically received less attention in pathology studies than lesions, although previous work suggests that similar mechanisms of damage occur in NAWM as those observed in plaques. Histopathologic findings have included diffuse gliosis, microglial activation, vascular fibrosis, perivascular cuffing by inflammatory cells, perivascular lipofuscin deposition, abnormal endothelial tight junctions, blood-brain barrier (BBB) breakdown, vessels containing proliferating endothelial cells, and sometimes demyelination (16–22). Electron microscopy shows increased numbers of lysosomes, particularly, in astrocytes (23–25). In addition, axonal loss is also observed in NAWM (26) and periplaque...
WM (27) attributable to Wallerian degeneration of axons destroyed in focal lesions (27, 28). Reductions in myelin basic protein (MBP) (29, 30) and, to a greater extent, myelin-associated glycoprotein (MAG) (31, 32) have also been reported in NAWM, but this is restricted to the immediate periplaque region.

Contrary to histology studies, MS NAWM has received a great deal of attention in recent imaging research. There is now substantial evidence from advanced MRI techniques that indicate that significant in vivo abnormalities exist in MS WM that appear normal by conventional MRI. Diffusion tensor imaging of NAWM shows higher apparent diffusion coefficient and reduced fractional anisotropy consistent with a loss of general tissue organization, whereas magnetization transfer imaging demonstrates a reduced magnetization transfer ratio indicative of structural, as well as water content, changes (33). Magnetic resonance spectroscopy experiments find increases in creatine, myo-inositol, choline, and lipid peaks and decreases in the axonal marker N-acetyl aspartate (33). Quantitative relaxation experiments find increases in l-carnitine, ubiquinol, and N-acetyl aspartate and decreases in myo-inositol and choline (34). Proton density and T2-weighted images demonstrate a reduced magnetization transfer ratio indicative of structural, as well as water content, changes (33). Magnetic resonance spectroscopy experiments find increases in creatine, myo-inositol, choline, and lipid peaks and decreases in the axonal marker N-acetyl aspartate (33). Quantitative relaxation experiments find increases in l-carnitine, ubiquinol, and N-acetyl aspartate and decreases in myo-inositol and choline (34).

In view of the potential clinical impact of nonlesional WM abnormalities on disability and progression, further studies are warranted to elucidate the pathology associated with DAWM. Here we report a more comprehensive histologic study that includes 2 lipid stains, 5 protein markers, as well as stains for astrocytes, gangliosides as identified by sialic acid residues, and axons. Comparisons were made between DAWM, NAWM, and WM lesions in formalin-fixed brain samples from primary progressive and secondary progressive MS subjects.

MATERIALS AND METHODS

Case Material

This study was approved by the Clinical Research Ethics Board of the University of British Columbia, and all cases had formal autopsy consent. A total of 14 brain slices (1 cm thick) in either the axial or coronal plane (6 axial, 8 coronal), fixed in 10% formalin, from 9 individuals with MS (4 secondary progressive [SP], 4 primary progressive [PP], 1 relapsing progressive; mean age, 62 years [range, 35–76 years]; 5 female, 4 male; mean disease duration, 26 years [range, 13–38 years]) (Table). Treatment histories were often incomplete because many of the cases (n = 7) were referred from other institutions. At least 1 individual (Case 8) was treated with Betaseron (for 10 months, 13 years before death).

MR Experiments

Brain samples were examined with a 32-echo T2 relaxation measurement. Samples were selected specifically based on the presence of DAWM from a larger tissue repository. Ten samples were imaged on a GE 1.5 T scanner using a transmit/receive head coil (TR [repetition time] = 3,000 milliseconds, 32 echoes with echo spacing 10 milliseconds, 8 averages, matrix = 256 × 256, 3 mm thick, in plane resolution = 586 × 586 µm, scan time = 102 minutes); the remaining 4 samples were imaged on a 7 T, 30-cm-bore, Bruker Avance MR scanner using a 7-cm inner diameter quadrature volume coil (TR/TE [time to echo] = 1500/6.673 milliseconds, 6 averages, and progress faster on disability measures (49, 50). Diffuse abnormalities of the spinal cord have also been observed, particularly in progressive types of MS and are associated with more severe disability (50–52). It is highly probable that many of the earlier studies of NAWM included DAWM in their assessments. Only a handful of studies have examined DAWM in vivo, but evidence supports the presence of abnormalities greater than those observed in NAWM but not as severe as in lesions. Magnetization transfer ratio is reduced in DAWM (53–57), as is MWF (56), whereas total water content and geometric mean T2 are increased (56). Although the exact nature of the pathologic processes responsible for DAWM is not known, histologic studies have shown evidence of BBB breakdown (58) as well as reductions in myelin and axons (58–60). Our preliminary published studies show that there is a selective loss or perturbation of myelin lipids, evident as reduced staining by the Luxol fast blue (LFB) stain, which is believed to recognize phospholipids (61–63), with relative preservation of myelin proteins MBP and 2’,3’-cyclic-nucleotide 3’-phosphohydrolase (CNP), as seen by immunohistochemistry, and variable involvement of axons demonstrable with the Bielschowsky technique (56, 64).

In view of the potential clinical impact of nonlesional WM abnormalities on disability and progression, further studies are warranted to elucidate the pathology associated with DAWM. Here we report a more comprehensive histologic study that includes 2 lipid stains, 5 protein markers, as well as stains for astrocytes, gangliosides as identified by sialic acid residues, and axons. Comparisons were made between DAWM, NAWM, and WM lesions in formalin-fixed brain samples from primary progressive and secondary progressive MS subjects.
matrix = 256 × 256, field of view = 6 cm, 1 mm thick, in plane resolution = 234 × 234 μm, scan time = 38 minutes).

**Tissue Embedding and Staining**

The tissue samples were embedded in paraffin blocks. These were sectioned into 10-μm-thick sections from 5 evenly spaced levels through the 3-mm tissue thickness that was scanned at 1.5 T and from the entire 1-mm tissue thickness scanned at 7 T. Representative sections were stained for phospholipid using LFB (65) and Weil’s stains (66), for axons using Bielschowsky impregnation, and with 1% Alcian blue stain (pH 2.5%) for carboxyl and sulfate-ester groups (67) comprising sialic acid residues present in gangliosides (which reside mainly in the axolemma and to a much lesser extent in the myelin sheath). Immunohistochemistry was used for the myelin proteins MBP, CNP, MAG, myelin-oligodendrocyte glycoprotein (MOG) and myelin proteolipid protein (PLP), and for astrocytes using glial fibrillary acidic protein (GFAP). Immunoreactivity was detected by the avidin-biotin complex immunoperoxidase technique using Nova Red (Vector Laboratories, Burlingame, CA) as the chromogen. All slides were then scanned using a backlit scanner and stored electronically as uncompressed TIFF (tagged image file format) images. For each brain slice, the histopathology slide corresponding to the center of the tissue thickness imaged by MRI was used for subsequent comparisons.

**MR and Histologic Analysis**

The digital histopathology images were first registered to the first echo MR image using a 10-point registration algorithm available in Image Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD). The registration process involved manually placing 10 anchor points on a reference MRI image and 10 location-matched anchor points on the corresponding histopathology image. The histopathology image was then spatially transformed by linear translation, scaling, and rotation to match the base MR image.

Regions of interest (ROIs) were outlined in NAWM, DAWM and WM lesions (plaques) on the first echo of the T2 relaxation data for each brain sample. Voxelwise T2 relaxation distributions were calculated using a non-negative least squares algorithm from the multi-echo T2 relaxation data (68, 69). The myelin water fraction was defined as the area of the short T2 component (<30 milliseconds at 1.5 T, <20 milliseconds at 7 T) (70, 71), divided by total T2 distribution; MWF maps (myelin maps) were created by displaying the MWF for every voxel in the image. The ROIs were mapped to the MWF images, and the mean MWF was determined for each ROI. The ROIs from the T2 relaxation measurement were then mapped on to the registered histopathology images. The mean optical density (OD) of the histologic stain in the ROI was determined using Image Pro Plus 5.1 (Media Cybernetics, Silver Springs, MD).

**Statistical Analysis**

Diffusely abnormal white matter and lesion MWF were compared to NAWM using a 2-tailed Student t-test for each sample (p < 0.05) and expressed as an average percentage change in MWF relative to NAWM. Diffusely abnormal white matter and lesion OD were compared to NAWM using a 2-tailed Student t-test for each sample (p < 0.05). Because histologic staining cannot be standardized between slices, results for DAWM and lesion were expressed as a percentage change in OD relative to NAWM. The percentage changes in MWF and histologic stains relative to NAWM were also compared between PP and SP tissue samples using a 2-tailed Student t-test (p < 0.05). Data normality was examined using a Shapiro-Wilk test (p < 0.05). Regression analysis between each phospholipid (LFB, Weil’s) and each protein (MAG, MBP, MOG, PLP, CNP) stain percentage change, as well as between the average phospholipid and average protein percentage change was examined with a Pearson correlation coefficient. All statistical analysis was carried out using Microsoft Excel 2003 (Redmond, WA) and SPSS 17.0 (Chicago, IL).

**RESULTS**

**Comparison of DAWM and Lesion to NAWM**

A total of 232 NAWM, 91 DAWM, and 83 WM lesion ROIs were drawn on the 14 MS brain samples (Table). Figures 1
and 2 demonstrate DAWM on MRI and the corresponding histologic features. Visual inspection shows more obvious reductions in the lipid-associated stains than in the myelin protein stains. Figure 3 summarizes mean MWF, LFB, Weil’s, Alcian blue, Bielschowsky, MAG, MBP, MOG, PLP, CNP, and GFAP optical density percentage difference between DAWM/lesion and NAWM. Diffusely abnormal white matter, characterized by an area of reduced intensity adjacent to the periventricular plaque on the proton density (arrows) and myelin water map, matches a region of reduced staining intensity on the Luxol fast blue, Weil’s, Alcian blue, Bielschowsky, and MAG stains.

FIGURE 1. Example of DAWM at 1.5 Tesla with corresponding myelin water map and histologic stains for phospholipids (Luxol fast blue, Weil’s), sialic acid groups (Alcian blue), axons (Bielschowsky impregnation), myelin proteins (MAG, MBP, MOG, myelin PLP, CNP), and astrocytes (GFAP). Diffusely abnormal white matter, characterized by an area of reduced intensity adjacent to the periventricular plaque on the proton density (arrows) and myelin water map, matches a region of reduced staining intensity on the Luxol fast blue, Weil’s, Alcian blue, Bielschowsky, and MAG stains.

FIGURE 2. Example of DAWM at 7 Tesla with corresponding myelin water map and histologic stains for phospholipids (Luxol fast blue, Weil’s), sialic acid groups (Alcian blue), axons (Bielschowsky impregnation), myelin proteins (MAG, MBP, MOG, myelin PLP, CNP), and astrocytes (GFAP). Diffusely abnormal white matter, characterized by an area of reduced intensity on the proton density (arrows) and myelin water map, matches a region of reduced staining intensity on the Luxol fast blue, Weil’s, Alcian blue, Bielschowsky, and, to a lesser degree, MAG stains. Several small plaques are seen within this region.
samples; range: −2% to −54%; p < 0.05 for 13 samples). The most dramatic and consistent histologic reductions were observed in the lipids (demonstrated by LFB [average decrease −29% across all samples; range: −14% to −45%], Weil’s [−24%; range: −12% to −35%], and Alcian blue [−15%; range: +7% to −46%]; p < 0.05 for 13, 14, and 8 samples, respectively), whereas there was lesser involvement of the myelin proteins (MAG [−11%; range: +2% to −29%], MBP [−10%; range: +3% to −37%], MOG [−8%; range: +6% to −17%], PLP [−7%; range: +6% to −27%], CNP [−5%; range: +8% to −18%]; p < 0.05 for 3, 3, 1, 2, and 3 samples, respectively). Axonal involvement, as reflected by the Bielschowsky technique, was intermediate (−15%; range: +4% to −44%; p < 0.05 for 7 samples). The astrocyte stain for GFAP showed almost no significant difference between DAWM and NAWM (−0.4%; range: +20% to −11%; p < 0.05 for 2 samples). Lesions, identified in 12 subjects, demonstrated larger reductions in MWF (−71%; range: −32% to −94%) and all histologic markers, with the exception of GFAP. Lesions also showed more frequent significant reductions in histologic staining than DAWM for individual samples (LFB: 12 samples; Weil’s: 10 samples; Alcian blue: 10 samples; Bielschowsky: 12 samples; MAG: 9 samples; MBP: 8 samples; MOG: 6 samples; PLP: 10 samples; CNP: 9 samples; GFAP: 2 samples). Panels A and B of Figure 4 depict average percentage difference of MWF and histologic stain OD for DAWM and lesion relative to NAWM. The SE for each mean percentage difference is shown in parentheses.

### Figures

**Figure 3.** Mean percentage difference of MRI-derived myelin measure and histologic staining optical density from DAWM and lesion relative to NAWM. The SE for each mean percentage difference is shown in parentheses.

**Comparison of PPMS and SPMS**

Comparing the 6 PP and 7 SP samples, significant differences in DAWM relative to NAWM were observed for MWF (PP: −18.2%; SP: −39.4%; p = 0.006) and Bielschowsky (PP: −6.4%; −23.6%; SP: p = 0.008), although there was a trend.
for Alcian blue (PP: −4.1%; SP: −21.8%; p = 0.08). Significant differences between PP and SP lesions were found for MWF (PP: −52.7%; SP: −81.2%; p = 0.01), LFB (PP: −44.1%; SP: −67.5%; p = 0.02), Weil’s (PP: −29.1%; SP: −57.0%; p = 0.02), Bielschowsky (PP: −29.4%; SP: −60.1%; p = 0.005); there were trends for Alcian blue (PP: −26.5%; SP: −43.1%; p = 0.07) and MBP (PP: −18.8%; SP: −38.4%; p = 0.07). Normality of data was confirmed.

Relationship Between Lipid and Protein Staining

Correlation analysis in DAWM found no significant correlations between any lipid staining percentage reductions and protein staining percentage reductions. Figure 5A demonstrates the lack of correlation between average phospholipid percentage reduction of LFB and Weil’s and average protein percentage reduction of all 5 protein stains ($R^2 = 0.007, p = 0.77$). In contrast, correlation analysis in lesions found that LFB reduction was significantly correlated with all protein reduction (MAG: $R^2 = 0.44, p = 0.02$; MBP: $R^2 = 0.56, p = 0.005$; MOG: $R^2 = 0.63, p = 0.002$; PLP: $R^2 = 0.39, p = 0.03$; CNP: $R^2 = 0.49, p = 0.01$). Lesion Weil’s reduction was also significantly correlated with reductions in all proteins (MAG: $R^2 = 0.63, p = 0.002$; MBP: $R^2 = 0.57, p = 0.005$; MOG: $R^2 = 0.72, p = 0.0005$; PLP: $R^2 = 0.63, p = 0.0002$; CNP: $R^2 = 0.72, p = 0.0004$). Lesion Alcian blue reduction was significantly correlated with PLP ($R^2 = 0.39, p = 0.03$) and CNP ($R^2 = 0.44, p = 0.02$) reductions. Figure 5B demonstrates the strong correlation between average phospholipid percentage reduction of LFB and Weil’s and average protein percentage reduction of all 5 protein stains for lesions ($R^2 = 0.68, p = 0.001$).

**DISCUSSION**

Multiple sclerosis DAWM demonstrated varying decreases in myelin water, phospholipids, sialic acid groups, myelin plasma proteins, and lipids demonstrated by Alcian blue staining.
proteins, and axons. Relative to NAWM, DAWM showed consistent and substantial decreases in MWF (93% of samples) and the phospholipid stain LFB and Weil’s (significantly reduced in 93% and 100% of samples, respectively). Alcian blue, staining the sialic acid groups of gangliosides, was also significantly reduced in DAWM (57% of samples). Axonal involvement was intermediate (significantly reduced in 50% of samples), and there was much less apparent involvement of the myelin proteins MAG, MBP, MOG, PLP, and CNP (significantly reduced in 21%, 21%, 14%, and 21% of samples, respectively). White matter lesions demonstrated consistent and more severe abnormalities than DAWM in myelin lipids, myelin proteins, and axons.

We identified DAWM on conventional MRI from formalin-fixed brain tissue. Although formalin fixation will alter tissue characteristics, including reducing relaxation time, image contrast is largely retained for proton density and T2-weighted imaging in MS tissue (72–74). In addition, previous work by Bergers et al (59) found that diffusely abnormal areas identified in situ corresponded to increased signal intensity on subsequently formalin-fixed tissue examined by high-resolution MRI; because their study also included tissue samples with no diffuse abnormalities, the authors concluded that it is highly unlikely that a postmortem artifact was present and responsible for the diffuse abnormality observed. The myelin water signal is also preserved with formalin fixation, and areas which appear diffuse on conventional imaging exhibited similar diffuse signal and levels of myelin water loss before and after fixation (70). Furthermore, similar levels of myelin loss, as measured by myelin water imaging, are observed in DAWM in vivo and in postmortem fixed tissue (56). In view of the aforementioned studies, it is reasonable to assume that DAWM identified in formalin-fixed tissue reflects DAWM in vivo. Nevertheless, an in-depth study of the effect of formalin fixation on DAWM is, to our knowledge, currently missing from the literature and would be a worthwhile endeavor.

A Primary Lipid Abnormality in DAWM

Our findings are consistent with a primary lipid abnormality or perturbation that exceeds both myelin protein loss and axonal degeneration in DAWM. If one assumes that the degree of the abnormality of a given histopathologic parameter is a reflection of its temporal profile of involvement in the disease process, it would follow that the lipid abnormality antedates the other abnormalities in DAWM. However, because this is a histopathologic study that produces only a single snapshot in time, it is not possible to draw definitive conclusions about temporal changes. This study builds on earlier studies in which we observed a selective loss of myelin lipids, measured by 1 marker (LFB), with relative preservation of 2 myelin proteins (MBP and CNP) in MS DAWM (56, 64). Moderate reductions in axonal density were found and a combined LFB-Bielschowsky stain showed individual Bielschowsky-positive axons without LFB staining in DAWM (64). To characterize DAWM abnormalities more fully in the current study, we used 3 lipid-related markers, as well as 5 myelin protein immunostains. We used LFB, believed to stain the phospholipid component in myelin (61) and Weil’s stain which also demonstrates myelin with the likely binding site being phospholipids (75). Alcian blue was used to demonstrate sialic acid residues, which are found in gangliosides present in the axolemma in greater amounts than myelin. In addition to the myelin proteins MBP and CNP, we also examined MAG, MOG, and PLP with immunohistochemistry.

Whereas both lipids and proteins contribute to the structure of the myelin, lipids are by far the major component of the bilayer that contains approximately 80% lipid and only 20% protein (76). Phospholipids make up approximately 44% of the total lipids in myelin (76) and play a role in biomembrane structure. The close association between MWF, a marker based on the short T2 component that is thought to represent water trapped between the closely apposed myelin membrane bilayers, and staining for phospholipids, is then, perhaps not surprising. Loss of myelin phospholipid is expected to result in reduced myelin volume, hence, a decrease in myelin water. A concept worthy of consideration is that the space formerly occupied by myelin phospholipid would be replaced by water, which could account for the increased water content found in DAWM on in vivo MRI studies (56). Other major myelin lipids include cholesterol (~30%), which is critical in the assembly and integrity of myelin, and glycolipids (~30%), which include cerebrosides, sulfatides, globosides, and gangliosides. However, because of the use of acetone for dehydration during histologic processing, cholesterol and glycolipids are largely extracted from the tissue, whereas phospholipids remain in place (67, 77). Therefore, although we see a reduction in staining for phospholipids in paraffin-embedded tissue, we cannot exclude the possibility of cholesterol and glycolipids also being affected in DAWM. The loss of myelin lipids will in all likelihood lead to functional abnormalities that might include disturbances in capacitance/resistance leading to interference of action potential propagation.

Myelin Proteins and Axons in DAWM

Diffusely abnormal white matter also showed alterations in myelin proteins but to a much lesser extent than myelin lipids. A variety of proteins contribute to the structure of CNS myelin, of which the largest component is PLP (~50%); PLP serves to maintain the extracellular spacing of compact myelin by electrostatic interactions with myelin lipids (78). Other important proteins include MBP (~30%), which is localized at the cytoplasmic surface of compact myelin, CNP (~4%), which is concentrated on the cytoplasmic side of the myelin lamellae, MAG (~1%) and MOG (<1%), which are located in noncompact myelin and are believed to be important in defining the structural integrity of the myelin sheath. Although levels of reduction were smaller for all proteins, these reductions may be important given the role of myelin proteins in bilayer structure and function. The most involved (albeit by a slim margin) was MAG, which is present in the inner mesaxon and interacts across the adaxonal space with ganglioside receptors (GD1a and GT1b) in the axolemma. Although not as evident in the statistical analysis of the group as a whole, in some instances the DAWM involvement of MAG could easily be detected by visual inspection of the histologic sections (Figs. 1, 2). In that regard, it is noteworthy that in DAWM there was a significant reduction in Alcian blue, a stain that in all likelihood recognizes the sialic acid groups of gangliosides.
which are more abundant in neuronal cell membranes than the myelin sheath. These findings raise the possibility that the lipid abnormality in DAWM also involves axolemmal gangliosides, which not only could impact their MAG ligand but also could be responsible for axolemmal dysfunction and the eventual axonal death that is reflected in the reduced Bielschowsky intensity. The present data support this hypothesis in that there was a strong correlation between reductions in Alcian blue and reductions in Bielschowsky (Fig. 6). Thus, in addition to the role of Wallerian degeneration in the pathogenesis of neurodegeneration in MS nonlesional WM (28), DAWM may reflect neurodegeneration, orchestrated through a lipidotoxic attack on myelin and axonal bilipid membranes. Indeed, MS patients who have DAWM tend to have more rapid and progressive clinical courses (49).

**Previous Biochemical Analysis of Nonlesional Tissue**

Our results are supported by a number of biochemical and histologic studies that document myelin loss in postmortem nonlesional WM. Biochemical analysis of normal-appearing brain tissue has found abnormalities in both lipids (25, 79–83) and, less frequently, in proteins (29, 84, 85). Cumings reported that apparently normal areas of MS brains showed the presence of cholesterol esters and a loss of phospholipids (79), a finding later confirmed by Gerstl et al (80, 81). In a fairly large study by Clausen and Hansen, myelin from so-called normal WM of brains from 22 patients with MS was compared to control data. The inositol and serine phosphoglyceride content and the content of sulfatide from MS myelin were lower than in normal adult myelin when related to dry weight of myelin (86). These findings are supported by Alling et al (82) who demonstrated significantly lower levels of serine phosphoglycerides and sulfatides of WM of MS brains. Rinne et al (25) observed reductions in both phospholipids and cerebrosides and Neu and Woelk (83) found decreased levels of phosphatidyl serine and phosphatidyl inositol. Protein abnormalities include reports of reduced MBP in normal-appearing tissue beyond the edge of a plaque (29, 32) and in NAWM (32, 85), as well as decreased CNP (84) and MAG (32) in NAWM. The methodology of the earlier biochemical studies have been called into question with respect to the possible inclusion of small plaques in the material assayed (87); however, given the now strong evidence of histologic abnormalities in DAWM, it is perhaps more likely that these earlier studies included areas of DAWM, which is typically unremarkable on gross examination. An interesting future area of research (which is beyond the scope of the current study) would be the biochemical assessment of lipid composition compared with protein composition in DAWM, NAWM, and control tissue samples.

**DAWM in PPMS and SPMS**

Although it seems that there may be some differences between PP and SPMS, given the small sample size of each group, these findings should be interpreted with caution. The PPMS subjects were slightly older than SPMS subjects, and average disease duration was longer for the SPMS group (35 vs 19 years). SPMS DAWM demonstrated greater reductions in MWF and axons compared to PPMS DAWM. Our observations are supported by in vivo studies which suggest that MR markers for myelin and axons are more severely affected in SPMS nonlesional WM (88). Furthermore, one recent study found that DAWM in SPMS exhibited significantly higher T1 and lower magnetization transfer ratio than DAWM in PPMS (89). Although it is certainly possible that the pathology giving rise to DAWM may be unique for different subtypes of MS (especially as emerging literature indicates less WM and more gray matter involvement in PPMS [90]), larger studies are required before definitive conclusions can be drawn. Plaques from PPMS were also more severely affected than PPMS lesions for MWF, myelin phospholipids, axons and MBP. This is consistent with previous work examining pathologic differences between PP and SPMS lesions which found significantly more inflammation in SPMS plaques than in PPMS (91). In addition, Bramow et al (92) showed that SPMS plaques had a higher load of active demyelination and higher grade inflammation, and lower remyelination capacity than PPMS.

**Relationship Between Myelin Lipids and Myelin Proteins**

Given that there was a range in both myelin lipid and myelin protein abnormalities in DAWM and lesions (Fig. 4), correlation analysis was used to determine if a more dramatic decrease in lipid was associated with a greater decrease in myelin proteins. Although a very strong association between myelin lipid and myelin protein reduction was found in lesions ($R^2 = 0.68, p = 0.001$), no relationship was observed in DAWM ($R^2 = 0.007, p = 0.77$) (Fig. 5). Although the dynamic range of DAWM abnormalities is not as large as those observed in lesions, the clear dissociation between phospholipids and myelin-protein stains only observed in DAWM suggests that myelin lipids and myelin proteins may be affected independently, or differently, or both, in this nonlesional WM.

**Possible Mechanisms of DAWM**

The underlying mechanism for the seemingly primary involvement of myelin lipids in DAWM is unknown. Histologic studies have found that diffuse abnormalities on T2-weighted MRI of MS tissue were associated with an increase in the...
serum protein fibrinogen, a marker for BBB damage, in areas of myelin pallor (58). Damage to the BBB may arise from inflammatory products like matrix metalloproteinases, reactive oxygen radicals, and proinflammatory cytokines secreted by activated glial cells and/or infiltrated leukocytes (93–97). Vos et al (58) also found that diffuse T2-weighted MRI abnormalities strongly coincided with the incidence of widened Virchow-Robin spaces, which contained infiltrating inflammatory cells. Therefore, it is possible that one or more of the molecular constituents present in the extracellular fluid as a result of the widespread BBB breakdown may be responsible for the initial perturbation of DAWM myelin lipids. It would be of interest to determine the inflammatory cellular constituents of DAWM and studies of this are currently underway.

Inflammatory infiltrates may lead to a dramatic increase in reactive oxygen species that can damage the myelin sheath directly by lipid peroxidation and promote further macrophage recruitment (98). Furthermore, certain by-products of lipid peroxidation, such as acrolein, a highly reactive aldehyde that can dissociate paranodal protein complexes, are thought to perpetuate oxidative stress (99). Indeed, increased lipid peroxidation has been observed in MS nonlesional WM. In a mass spectrometry postmortem study of freshly frozen brain tissue from 24 MS subjects, Wheeler et al (100) found large accumulations of both the lysine and histidine adducts of 4-hydroxynonenal (443% and 406%, respectively). They also observed a shift in the lipid composition of grossly NAWM to lower cholesterol and sphingolipid (sphingomyelin and ceramide) and higher phospholipid content in active MS. The pattern of disturbance in lipid composition suggests a metabolic defect that causes sphingolipids to be shuttled to phospholipid production. However, it is not clear whether NAWM, DAWM, or both were examined, and in view of our results, it is possible that this may be due to a compensatory phenomenon in NAWM. Because alterations in the lipid content of cellular membranes can have dramatic effects on cellular functions by altering the biophysical properties of the membrane, Wheeler et al (100) modeled the forces that govern interactions of opposing myelin bilayers. They considered 3 independent energies (van der Waals interactions, electrostatics, and thermal energy) and determined that the dominant destabilizing force resulted from an increased thermal undulation force, implying there is an increase in the magnitude of total repulsive pressure between myelin bilayers. An increase in the repulsive force between opposing bilayers could explain decompaction and disruption of myelin structure, which, together with free radical–mediated lipid peroxidation of the myelin sheath, could lead to the primary lipid abnormality observed in DAWM.

In summary, DAWM is characterized by a primary perturbation in myelin phospholipids with less involvement of myelin proteins and possibly axonal gangliosides. The underlying mechanism for the seemingly primary insult on myelin lipids in DAWM is unknown. These phenomena may be important in the pathogenesis of MS, particularly neurodegeneration, and may contribute to disease manifestations and clinical progression. Moreover, it raises the possibility of a primary role for lipid in the autoimmune or degenerative etiopathogenesis of MS, studies of which up to now have largely focused on myelin protein immunology and biology. Thus, examining the tissue changes underlying DAWM may identify substrates responsible for disability and progression, which could be a target for future therapies. The relationship between DAWM and the other pathologic features of MS, such as WM plaques, gray matter pathology, and the changes in NAWM, is unclear. Further studies are needed to understand how these anatomically diverse expressions of MS may be linked to one another and to the underlying pathogenesis of the disease.

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