Neuronal and Axonal Loss in Normal-Appearing Gray Matter and Subpial Lesions in Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a demyelinating and neurodegenerative disease of the CNS. Multiple sclerosis lesions include significant demyelination of the gray matter, which is thought to be a major contributor to both physical and cognitive impairment. Subpial (Type III) lesions are the most common demyelinated cortical lesions. We investigated neurodegenerative features of subpial lesions in cerebral cortex samples from 11 patients with MS and 6 nondemented non-MS controls. There were no significant differences in neuron and axon density between normally myelinated normal-appearing gray matter (NAGM) and Type III MS lesions. Neurons were 11.2% smaller in Type III lesions than in NAGM in the cingulate cortex only; Type III lesions contained 25.4% fewer NeuN-positive neurons compared with control cortex. Neurons in MS NAGM were 13.6% smaller than those in control cortex. Finally, the same regions, immunostained with anti-SMI312 antibodies, showed reduced axon densities in Type III lesions compared with controls. There were no significant differences in neuron and axonal changes, but they had no consistent differences in neuronal and axonal alterations. This suggests that neurodegeneration in the cerebral cortex of patients with MS may be independent of cortical demyelination.

Key Words: Axonal loss, Gray matter pathology, Multiple sclerosis, Neurodegeneration, Neuronal loss, Subpial lesions.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS that predominantly occurs in young adults and often causes substantial disability across time (1). Multiple sclerosis has long been regarded as a white matter (WM) disease, but gray matter (GM) has been increasingly recognized to be also heavily involved (1). Cortical pathology, identified using magnetic resonance imaging (MRI), correlates with specific cognitive deficits, such as memory impairment and attention deficits (2, 3), and is a better predictor of cognitive decline than is WM pathology (4); this indicates the clinical relevance of GM pathology to patients with MS.

Based on their location within the cortex, demyelinated brain GM lesions have been classified into 3 different types (5). Type I lesions are mixed WM/GM lesions that can show inflammation to some degree. Type II lesions are small, purely intracortical lesions, mostly located around blood vessels. Type III cortical lesions reach from the pial surface downward into the cortex and may cover multiple gyri. A fourth cortical lesion type, spanning the cortex but not entering the subcortical WM, is sometimes defined (6) but will be disregarded here.

There is virtually no GM area within the CNS that is not affected by some degree of demyelination in patients with MS (7–9), and GM lesions may be quite extensive. Approximately 13% of the forebrain GM (10) and approximately 40% of the cerebellar cortex (11) were found to be demyelinated in patients with secondary progressive MS. Of all GM lesions, subpial Type III lesions are the most common in (chronic) MS (5), and these lesions may or may not be spatially associated with meningeal inflammation (12, 13). Unfortunately, Type III lesions are the most difficult lesion type to visualize with standard MRI techniques (14).

Although Type III lesions are the most common lesions in MS, it remains unclear whether these lesions, which have relatively minor inflammatory pathology (15), contribute to overall neurodegeneration in MS. So far, neurodegeneration in the MS neocortex has been investigated by a small number of groups. Some studies reported significant neurodegeneration in neocortical lesions but no detectable differences in neurodegeneration between normal-appearing GM (NAGM) and control cortex (16, 17). For example, Type I lesions had substantial glial (−36%) and neuronal (−10%) loss and a 47% loss of synapses, whereas in NAGM, the only evidence of neurodegeneration was rounded neurons, which are assumed to indicate axonal and/or dendrite loss (17). By contrast, another study reported significant neuronal loss in NAGM and neocortical GM lesions compared with controls (18). Thus, the effects of local GM demyelination on neurodegeneration remain unclear.

We investigated Type III lesions to understand the relationship between demyelination and neurodegeneration using immunohistochemistry for pan-neuronal, axonal, and myelin protein markers (NeuN, SMI312, and myelin proteolipid protein [PLP], respectively). Neurons and axons were characterized and quantified. We observed significant neuronal loss,
neuronal atrophy, and axonal loss in Type III lesions and NAGM compared with control cortex samples. Interestingly, we found no significant difference in neuronal loss, neuronal atrophy, or axonal loss between Type III lesions and NAGM in samples from patients with MS.

MATERIALS AND METHODS

Twenty-five tissue blocks from the brains of 11 patients with clinically and neuropathologically confirmed MS and 6 tissue blocks from the brains of 6 nondemented controls were obtained from the Netherlands Brain Bank (Amsterdam, the Netherlands; http://www.brainbank.nl/). All subjects had given written informed consent for autopsy and for the use of their brain and spinal cord tissue for scientific research. The postmortem protocol had been previously described in detail (19). Cortical lesions were identified and characterized using anti-PLP immunohistochemical staining, as previously described (14).

Tissue blocks were used to investigate neurodegenerative changes in Type III lesions compared with NAGM in each anatomic region. Blocks from the cingulate cortex were removed from 10 of 11 patients; 4 blocks contained GM lesions, 6 blocks contained NAGM, and 1 tissue block contained both GM lesions and NAGM. Blocks from the frontal cortex were obtained from 11 patients; 5 blocks contained GM lesions, 5 blocks contained NAGM, and 1 contained both. Finally, blocks from the temporal cortex were investigated in 8 patients; 2 tissue blocks contained GM lesions, 4 tissue blocks contained NAGM, and 2 had both NAGM and lesions.

Subsequently, MS frontal cortex tissue blocks and 6 anatomically matched tissue blocks from 6 control subjects were used to compare neuron density, neuron size, and axon density in patients with MS and controls. The control tissue did not contain Type III lesions. This analysis was limited to the frontal cortex because of the limited availability of high-quality control tissue.

Immunohistochemistry

Immunohistochemistry and digitization of staining were performed as previously described (20). Briefly, formalin-fixed paraffin-embedded tissue blocks were cut into 10-μm-thick sections. Sections were deparaffinized and rehydrated in a graded series of xylene and ethanol. Consecutive sections were stained for neuron cell bodies using Nissl (thionin; Thermo Fisher Scientific Inc, Waltham, MA) and for neurons (anti-NeuN, 1:1000; Millipore, Billerica, MA), myelin (anti-PLP, 1:1000; Serotec, Oxford, United Kingdom), activated microglia/macrophages (human leukocyte antigen, HLA-DR, 1:500; gift from Dr J. Hilgers, VU University Medical Center, Amsterdam, the Netherlands), and phosphorylated and nonphosphorylated heavy chains of neurofilaments (SMI312, 1:200; Covance, Princeton, NJ) using immunohistochemistry. Sections were rinsed after primary antibody incubation and subsequently incubated with biotin-labeled secondary antibodies. Sections were then incubated with streptavidin-biotin-peroxidase complex (Vectorstain, 1:200; Vector Laboratories Inc, Burlingame, CA). Finally, sections were rinsed, and peroxidase reaction was developed with 3,3′ diaminobenzidine-tetrahydrochloride dihydrate (DAKO, Glostrup, Denmark) as chromogen. Staining conditions were kept constant during the study.

Digitization and Morphometric Analysis

Morphometric analyses were undertaken using a Leica DM/RBE photomicroscope with a Xillix MicroImager digital camera (1,280 × 1,024 pixels) attached to a Microcomputer Imaging Device Elite image analysis system (Imaging Research Inc, St Catharines, Ontario, Canada). Images were taken as vertical stacks of 6 μm at 100 × magnification. Based on systematic random sampling and depending on the size of the section, 2 to 8 regions of interest (ROI) were selected. During digitization, care was taken to keep exposure conditions constant.

Within every ROI, morphometric analyses were performed where the distance between the pial surface and the WM/GM border was shortest to standardize measurements. The cortex was measured at 6 different points within every ROI, starting at the pial surface and ending at the leuкоcortical border. Three measurements in the superficial layers (I–III) and 3 measurements in the deeper layers (IV–VI) of the cortex were performed. The average scan area of each measurement was 41,991 μm². The 6 measurements were averaged, and average values were used in statistical analysis.

Number of neurons and average neuronal profile were measured using Microcomputer Imaging Device in-house segmentation scripts. These segmentation scripts were used to construct measuring templates based on local thresholding and clustering. Subsequently, these measuring templates were used to measure neurons in the original nonmodified images. Neuronal cell counts were Abercrombie-corrected (21), accounting for overestimation of cells caused by aberrant quantification of cells residing outside the imaged plane. Average neuron size was determined by measuring the proportional surface area stained for NeuN divided by the number of NeuN-positive objects identified using the segmentation scripts.

Axon density analysis was based on staining density (relative optical density [ROD]). Relative optical density was interpreted as a gray value ranging from 0 (no axons and therefore no staining and a white image) to 1 (a completely saturated black image caused by a very high density of axons). Subsequently, we corrected for interindividual staining intensity differences, which are largely caused by postmortem effects such as changes in the pH of tissue. Within every section, we measured ROD at 3 places within normal-appearing WM and used the average of these measurements to calculate relative ROD in GM (GM ROD measurement/average normal-appearing WM ROD measurement). Normal-appearing WM measurements were taken from locations without large blood vessels and without pathologic alterations. Microglia/macrophages positive for HLA-DR were visually quantified on a scale from 0 (no activated microglia/macrophages) to 4 (numerous activated microglia/macrophages), as previously described (20).

Statistical Analysis

All statistical analyses were performed using SPSS version 20.0. Mann-Whitney U tests were conducted to evaluate pairwise differences among groups (NAGM, Type III lesions, and controls), controlling for Type I error across tests by applying Bonferroni correction. After Bonferroni correction,
p < 0.017 was considered significant. Comparisons between Type III lesions and MS NAGM were not Bonferroni-corrected.

**RESULTS**

**Cases**

Thirty-one tissue blocks were obtained from 11 patients with MS and 6 controls. Ten of the patients had secondary progressive MS, and 1 had primary progressive MS. The ages of patients with MS and controls were similar (mean ± SD, 67.3 ± 13.1 vs 64.5 ± 4.3 years, respectively; p = 0.531). The mean ± SD disease duration among patients with MS was 34.7 ± 14.8 years. The postmortem delays among patients with MS and controls were also similar (mean ± SD, 9.2 ± 1.2 vs 7.7 ± 2.6 hours, respectively; p = 0.122). Clinical data are presented in Table 1.

**Cortical GM Lesions**

One hundred seventeen ROI were investigated in MS samples; 75 ROI contained NAGM, and 42 contained a lesion (1 lesion per ROI). Eighteen ROI were investigated in control samples. All except 2 cortical lesions extended from the pial surface into the cortex to cortical layer 3 or 4 and were defined as subpial Type III lesions. Two lesions extended from the pial surface to the WM border.

Immune activity within lesions was classified according to the distribution and density of HLA-DR–positive cells. In line with the literature (15) and based on semiquantitative analysis, there was minor to moderate microglial activity in Type III lesions and no significant difference in microglial activation between NAGM and Type III lesions (mean ± SD, 2.00 ± 0.97 vs 1.81 ± 1.05 microglia score, respectively; p = 0.358). There was no significant microglial activity in control cortex.

**Neuronal and Axonal Densities**

There was no significant difference in neuron density between Type III lesions and NAGM in the cingulate cortex (p = 0.593), frontal cortex (p = 0.104), or temporal cortex (p = 0.592). The same held true for axon density: There was no difference between Type III lesions and NAGM in the cingulate cortex (p = 0.156), frontal cortex (p = 0.989), or temporal cortex (p = 0.275). In the cingulate cortex, neurons were smaller in Type III lesions than in NAGM (−11.2%; p = 0.040). However, there was no difference in neuron sizes between Type III lesions and NAGM in the frontal cortex (p = 0.791) and temporal cortex (p = 0.086).

**Neuronal and Axonal Loss in MS Cortex Compared With Controls**

The frontal cortex was selected to investigate possible differences in neuron density and axon density between MS (NAGM and Type III lesions) and control cortex. After Bonferroni correction, neuron density (−25.4%; p = 0.001) and axon density (31.4%; p = 0.001) were significantly reduced in Type III lesions compared with control cortex (Fig.; Table 2). There was no significant difference in neuron sizes between Type III lesions and matched control cortex (−10.0%; p = 0.040). Subtle neurodegenerative changes were also found in NAGM. Neurons in NAGM were significantly smaller than those in controls (−13.1%; p = 0.005), and axon density was reduced by 33.0% (p < 0.001). Neuron density, however, was not significantly different in NAGM compared with controls. These results are summarized in Table 2.

**DISCUSSION**

Subpial cortical demyelination is extensive in chronic MS (11). We investigated this commonest of cortical lesion types and report significant neuronal loss, reduced neuron size, and axonal loss in MS cortical samples compared with control cortical samples. However, no significant differences in neuron density or axon density were found between Type III lesions and NAGM within MS cases. The only borderline significant difference was found for neuron size in Type III

**TABLE 1. Clinical Data**

<table>
<thead>
<tr>
<th>Subject</th>
<th>MS Type</th>
<th>Sex</th>
<th>Age at Death (years)</th>
<th>Postmortem Delay (hours:minutes)</th>
<th>MS Duration (years)</th>
<th>Cause of Death</th>
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</thead>
<tbody>
<tr>
<td>MS 1</td>
<td>SPMS</td>
<td>M</td>
<td>51</td>
<td>11:00</td>
<td>20</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>MS 2</td>
<td>SPMS</td>
<td>F</td>
<td>57</td>
<td>8:40</td>
<td>25</td>
<td>Euthanasia</td>
</tr>
<tr>
<td>MS 3</td>
<td>SPMS</td>
<td>F</td>
<td>84</td>
<td>7:35</td>
<td>50</td>
<td>Euthanasia</td>
</tr>
<tr>
<td>MS 4</td>
<td>SPMS</td>
<td>M</td>
<td>86</td>
<td>10:10</td>
<td>61</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>MS 5</td>
<td>SPMS</td>
<td>M</td>
<td>56</td>
<td>9:50</td>
<td>14</td>
<td>MODS</td>
</tr>
<tr>
<td>MS 6</td>
<td>SPMS</td>
<td>M</td>
<td>75</td>
<td>10:10</td>
<td>50</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>MS 7</td>
<td>SPMS</td>
<td>F</td>
<td>71</td>
<td>7:05</td>
<td>34</td>
<td>MODS</td>
</tr>
<tr>
<td>MS 8</td>
<td>SPMS</td>
<td>M</td>
<td>53</td>
<td>10:00</td>
<td>25</td>
<td>Euthanasia</td>
</tr>
<tr>
<td>MS 9</td>
<td>SPMS</td>
<td>F</td>
<td>56</td>
<td>8:25</td>
<td>32</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>MS 10</td>
<td>SPMS</td>
<td>M</td>
<td>80</td>
<td>9:45</td>
<td>45</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>MS 11</td>
<td>PPMS</td>
<td>M</td>
<td>71</td>
<td>8:45</td>
<td>26</td>
<td>MODS</td>
</tr>
<tr>
<td>Ctrl 1</td>
<td>—</td>
<td>M</td>
<td>68</td>
<td>7:17</td>
<td>—</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Ctrl 2</td>
<td>—</td>
<td>M</td>
<td>62</td>
<td>6:35</td>
<td>—</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Ctrl 3</td>
<td>—</td>
<td>M</td>
<td>70</td>
<td>7:30</td>
<td>—</td>
<td>Pancreatic carcinoma</td>
</tr>
<tr>
<td>Ctrl 4</td>
<td>—</td>
<td>F</td>
<td>65</td>
<td>12:50</td>
<td>—</td>
<td>Cardiac arrest</td>
</tr>
<tr>
<td>Ctrl 5</td>
<td>—</td>
<td>F</td>
<td>58</td>
<td>6:15</td>
<td>—</td>
<td>MODS</td>
</tr>
<tr>
<td>Ctrl 6</td>
<td>—</td>
<td>F</td>
<td>64</td>
<td>6:00</td>
<td>—</td>
<td>Cachexia</td>
</tr>
</tbody>
</table>

Ctrl, control; F, female; M, male; MODS, multiple organ dysfunction syndrome; PPMS, primary progressive MS; SPMS, secondary progressive MS.
lesions of the cingulate cortex, compared with NAGM of the same area (11.2% reduction). Compared with control cortex, both Type III lesions and NAGM showed features of neurodegeneration. On average, Type III lesions contained 25.4% fewer neurons and 31.4% reduced axon density compared with control cortex. We also found reduction in neuron size (13.6% on average) and significant axonal loss (−33.0%) in NAGM compared with controls. These changes are more subtle than

FIGURE. Neuronal loss in Type III cerebral cortical lesions in MS. Control (Ctrl) (A) and MS (B) sections immunostained for NeuN demonstrating overall neuronal loss in MS cortex. (C) Antimyelin PLP–immunostained section from a patient with MS (B) showing subpial demyelination (original magnification: 100 ×). High-magnification pictures from cortical layer II (D, E), with matching pictures demonstrating the quality of segmentation scripts (F, G) (digital zoom from original magnification: 100 ×).

TABLE 2. Cell Densities in the Frontal Cortex of Patients With MS and Controls

<table>
<thead>
<tr>
<th></th>
<th>NAGM</th>
<th>Type III Lesions</th>
<th>Control</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuron density, cells/mm²</td>
<td>106 ± 33.71</td>
<td>92 ± 25.74</td>
<td>123 ± 14.91</td>
<td>&lt;0.017*</td>
</tr>
<tr>
<td>Neuron size, μm²</td>
<td>103.80 ± 18.40</td>
<td>107.49 ± 24.75</td>
<td>119.49 ± 10.91</td>
<td>&lt;0.017†</td>
</tr>
<tr>
<td>Axons, ROD</td>
<td>0.45 ± 0.14</td>
<td>0.46 ± 0.19</td>
<td>0.67 ± 0.11</td>
<td>&lt;0.017†</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.
p = 0.017, Mann-Whitney U test after Bonferroni correction.
*Significant difference between Type III lesions and controls.
†Significant difference between NAGM and controls.
previously reported in the thalamus in MS (22) but are in line with other studies investigating neocortical neurodegeneration in MS. Those studies reported cortical neuronal loss of up to 20% in Type I GM lesions and Type III lesions compared with controls and approximately 10% neuronal loss in GM lesions as container category when compared with NAGM (16–18).

We previously showed that cortical atrophy, as measured by MRI, is predominantly explained by neuronal loss, neuron atrophy, and axonal loss, and, to a lesser extent, by demyelination (20).

Our current study confirms that neurodegeneration is, at least to a large extent, independent of demyelination because Type III lesions and NAGM showed significant signs of neurodegeneration compared with controls. Our results are consistent with another study that investigated cortical thickening and neurodegeneration in formalin-fixed brains in which there was approximately 10% cortical thinning independent of demyelination, with significant neurodegeneration in MS cortex compared with control cortex (17).

Axonal loss was a prominent neurodegenerative feature of Type III lesions and NAGM. Interestingly, in rat axotomy models, axonal transection was found to explain the 50% reduction in the number and size of neurons (23, 24). It is possible that the observed neuronal loss and smaller neuron size in subpial GM lesions and NAGM resulted predominantly from deafferentation after axonal degeneration in WM or GM. Local factors, such as meningeal inflammation (18), might have also affected neurodegeneration. In our sample, meningeal tissue was not specifically selected and was insufficiently present, but patients with aggressive disease course (i.e. a strong inflammatory phenotype, including the appearance of B-cell follicle-like structures in the meninges) may have lost up to approximately 65% of their neurons in the upper cortical layers (18). Microscopic evaluation showed that the patients included in the current study had mostly inactive WM lesions with minor microglial activation, with some sporadic mononuclear cell infiltrates. These findings suggest that the patients included in the present study had a relatively mild inflammatory phenotype, as is typical of patients with relatively long disease course at autopsy.

Other noninflammatory mechanisms, including mitochondrial dysfunction (25), glutamate excitotoxicity (26), and iron deposition (27), may play a role in MS neurodegeneration. Glutamate excitotoxicity, impaired iron homeostasis, and mitochondrial dysfunction were suggested to contribute to a state termed "virtual hypoxia" (28). During virtual hypoxia, neurons consume more energy than they can produce, leading to intracellular Ca$^{2+}$ overload, activation of Ca$^{2+}$-dependent apoptotic signaling pathways, and, eventually, neurodegeneration. Examination of these mechanisms was beyond the scope of the present study.

This study has some limitations. As indicated, patients with MS of relatively long duration (chronic) were selected, and this limits generalization to the entire population of patients with MS. Furthermore, control tissue that matched the quality of our MS tissue was only available for the frontal cortex, limiting any inference about regional differences in neurodegeneration. Finally, the current study had slightly more male than female patients with MS. Male patients with MS have been shown to develop more severe neurodegeneration (29). This effect is especially prominent in subcortical GM structures such as the thalamus (30). Whether sex affected the histopathologic measures presented here cannot be established because of the limited sample size. This might be the subject of a future study.

In conclusion, this study shows significant neurodegenerative changes in Type III lesions and NAGM of patients with chronic MS compared with nondemented controls. Interestingly, however, there are no significant differences in neuron density and axon density between NAGM and Type III lesions, suggesting that neurodegeneration may be largely independent of cortical demyelination.

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REFERENCES