Meningeal inflammation plays a role in the pathology of primary progressive multiple sclerosis

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The primary progressive form of multiple sclerosis is characterized by accrual of neurological dysfunction from disease onset without remission and it is still a matter of debate whether this disease course results from different pathogenetic mechanisms compared with secondary progressive multiple sclerosis. Inflammation in the leptomeninges has been identified as a key feature of secondary progressive multiple sclerosis and may contribute to the extensive cortical pathology that accompanies progressive disease. Our aim was to investigate the extent of perivascular and meningeal inflammation in primary progressive multiple sclerosis in order to understand their contribution to the pathogenetic mechanisms associated with cortical pathology. A comprehensive immunohistochemical analysis was performed on post-mortem brain tissue from 26 cases with primary progressive multiple sclerosis. A variable extent of meningeal immune cell infiltration was detected and more extensive demyelination and neurite loss in the cortical grey matter was found in cases exhibiting an increased level of meningeal inflammation. However, no tertiary lymphoid-like structures were found. Profound microglial activation and reduction in neuronal density was observed in both the lesions and normal appearing grey matter compared with control cortex. Furthermore, cases with primary progressive multiple sclerosis with extensive meningeal immune cell infiltration exhibited a more severe clinical course, including a shorter disease duration and younger age at death. Our data suggest that generalized diffuse meningeal inflammation and the associated inflammatory milieu in the subarachnoid compartment plays a role in the pathogenesis of cortical grey matter lesions and an increased rate of clinical progression in primary progressive multiple sclerosis.

Keywords: B cells; clinical disability; grey matter lesion; microglia; neuropathology

Abbreviations: MOG = myelin oligodendrocyte glycoprotein
Introduction

In 10–15% of patients with multiple sclerosis, symptomatic onset is marked by progressive development of unremitting neurological dysfunction (primary progressive multiple sclerosis) and many questions remain as to the immunological and neurodegenerative changes that underlie this sub-type and how they might differ from those seen in secondary progressive multiple sclerosis. The age at onset of progressive disease and the rate of clinical progression are similar in secondary progressive and primary progressive multiple sclerosis (Confavreux and Vukusic, 2006; Scalfari et al., 2011) and grey matter atrophy in primary progressive multiple sclerosis is greater than that observed in relapsing–remitting multiple sclerosis, but reduced compared with secondary progressive multiple sclerosis (Ceccarelli et al., 2008).

The pathological hallmark of multiple sclerosis is the presence of inflammatory foci in the white matter of the CNS, accompanied by destruction of myelin and oligodendrocytes together with a variable degree of axon loss. Whereas early lesion formation involves the influx of peripheral immune cells, as multiple sclerosis progresses it is thought that inflammation becomes increasingly compartmentalized within the CNS (Lassmann et al., 2007; Reynolds et al., 2011). New gadolinium enhancing lesions on MRI become fewer (Thompson et al., 1991) and lesions generally display less prominent inflammation, become remyelinated or persist as chronic demyelinated plaques (Kutzelnigg et al., 2005; Frischer et al., 2009). Focal regions of demyelination are also observed in the grey matter of patients with multiple sclerosis, in both the neocortex (Bo et al., 2003; Kutzelnigg et al., 2005) and the deeper areas including the thalamus, hypothalamus, hippocampus, cerebellum and the spinal cord (Gilmore et al., 2009; Papadopoulos et al., 2009; Vercellino et al., 2009). Few comparable studies have been carried out on primary progressive multiple sclerosis brains, but generally differences between secondary progressive and primary progressive multiple sclerosis appear to be of a quantitative nature rather than fundamentally qualitative (Antel et al., 2012). There is little doubt that the pathology of primary progressive multiple sclerosis involves perivascular infiltration of peripheral immune cells (Revesz et al., 1994), which is accompanied by a similar level of axonal loss to that seen in secondary progressive multiple sclerosis (Tallantyre et al., 2009).

Numerous neuropathology and neuroimaging studies have highlighted the growing importance of cortical grey matter pathology in our understanding of the mechanisms underlying the cumulative increase in motor, sensory and cognitive symptoms that occurs as multiple sclerosis progresses. The pathological features of grey matter lesions substantially differ from those seen in white matter; blood–brain barrier disruption is moderate, and peripheral immune cell infiltration and complement deposition are reduced or absent (Bo et al., 2003; Brink et al., 2005; Leech et al., 2007). The primary feature of inflammatory activity in grey matter lesions is the prominent activation of microglia and association with overlying meningeal infiltrates (Kutzelnigg et al., 2005; Magliozzi et al., 2007, 2010; Dal Bianco et al., 2008). In support of this, damage to the glia limitans and more prominent cortical pathology, including a gradient of neuronal loss, extensive neurite injury and subpial demyelination, has been observed in cases with secondary progressive multiple sclerosis with more severe meningeal inflammation (Magliozzi et al., 2010). Furthermore, organized structures composed of T cells, B cells and plasma cells with a network of follicular dendritic cells resembling tertiary lymphoid follicles have been identified in the leptomeninges of a substantial proportion of patients with secondary progressive multiple sclerosis (Serafini et al., 2004; Magliozzi et al., 2007; Howell et al., 2011). Although the extent of cortical demyelination has been shown to be similar in cases with primary progressive multiple sclerosis compared with secondary progressive multiple sclerosis (Kutzelnigg et al., 2005), little is known about the relationship between inflammation and demyelination in primary progressive multiple sclerosis.

It is suggested that the steady development of permanent clinical disability in the progressive phase of multiple sclerosis may be attributed to the neuronal pathology in the grey matter (Reynolds et al., 2011), which includes prominent axonal degeneration, loss of synaptic density and reduction in glial and neuronal cell populations (Peterson et al., 2001; Wegner et al., 2006; Magliozzi et al., 2007, 2010). Despite the extensive investigation of the pathology associated with secondary progressive multiple sclerosis, few studies specifically examining post-mortem primary progressive multiple sclerosis tissues have been carried out. Our previous studies on secondary progressive multiple sclerosis pathology have demonstrated a good correlation between cortical demyelination, meningeal inflammation, neuronal loss and the rate of clinical progression (Howell et al., 2011; Reynolds et al., 2011). For this reason we have examined the relationship between meningeal inflammation and cortical pathology in primary progressive multiple sclerosis. The lack of acute relapses and reduction in contrast enhancing MRI activity in patients with primary progressive multiple sclerosis has led to the suggestion that neurodegeneration may be the primary pathology driving clinical progression, but there is little substantive evidence supporting this. An appreciation of the relationship between generalized inflammation, grey matter tissue damage and the clinical course of primary progressive multiple sclerosis will help our understanding of the potential mechanisms of the CNS injury that underlies the gradual progression of clinical disability in both secondary progressive and primary progressive multiple sclerosis.

Materials and methods

Post-mortem material

Human brain tissues examined in this study were provided by the UK Multiple Sclerosis Society Tissue Bank (UKMSTB) at Imperial College, London, UK and were obtained at autopsy with fully informed consent under ethical approval by the National Research Ethics Committee (08/MRE09/31). Eleven per cent of cases in the UKMSTB have a diagnosis of primary progressive multiple sclerosis (Reynolds et al., 2011). Twenty-six cases with primary progressive multiple sclerosis (10 male and 16 female), selected based on the availability of
sufficient snap and fixed frozen tissue blocks, were used in this study with a wide range of age at death (median = 66, age range 42–88 years) and disease duration (median = 25, range 3–53 years). Extensive general practitioner’s notes, including detailed neurologist reports, were available for all the cases (Reynolds et al., 2011). Summaries of the lifetime clinical histories (Table 1) were prepared for each patient by clinical neurologists with a specialist interest in multiple sclerosis (R.N., P.M.). Brain tissue samples from six age-matched control cases without neurological deficits were used (six males; median age at death = 68, range 35–84 years).

**Table 1 Clinical demographic details of the cases included in this study**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age at onset (years)</th>
<th>Age at wheelchair use (years)</th>
<th>Age at death (years)</th>
<th>Inflammation index</th>
<th>Post-mortem delay (h)</th>
<th>Cause of death</th>
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<tr>
<td>MS 369</td>
<td>F</td>
<td>45</td>
<td>49</td>
<td>67</td>
<td>++</td>
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<td>F</td>
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<td>37</td>
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<td>++</td>
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<tr>
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<td>M</td>
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<td>30</td>
<td>42</td>
<td>++</td>
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<tr>
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<td>M</td>
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<td>34</td>
<td>43</td>
<td>++</td>
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<tr>
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<td>66</td>
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<td>43</td>
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<td>88</td>
<td>+</td>
<td>13</td>
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</tr>
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<td>58</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>17</td>
<td>Bronchopneumonia</td>
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<tr>
<td>MS 313</td>
<td>M</td>
<td>37</td>
<td>58</td>
<td>66</td>
<td>+</td>
<td>54</td>
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<tr>
<td>MS 383</td>
<td>M</td>
<td>34</td>
<td>35</td>
<td>42</td>
<td>+</td>
<td>19</td>
<td>Aspiration pneumonia</td>
</tr>
<tr>
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<td>F</td>
<td>21</td>
<td>38</td>
<td>48</td>
<td>+</td>
<td>87</td>
<td>Pneumonia</td>
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<tr>
<td>MS 70</td>
<td>F</td>
<td>56</td>
<td>77</td>
<td>77</td>
<td>+</td>
<td>28</td>
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<td>F</td>
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<td>57</td>
<td>73</td>
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<td>78</td>
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<td>23</td>
<td>28</td>
<td>56</td>
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<td>8</td>
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<td>NA</td>
<td>58</td>
<td>0+</td>
<td>28</td>
<td>Breast cancer</td>
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<tr>
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<td>M</td>
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<td>62</td>
<td>70</td>
<td>0+</td>
<td>24</td>
<td>Pneumonia</td>
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<tr>
<td>MS 129</td>
<td>F</td>
<td>42</td>
<td>46</td>
<td>66</td>
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<td>20</td>
<td>Lung carcinoma</td>
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<tr>
<td>MS 188</td>
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<td>37</td>
<td>78</td>
<td>0+</td>
<td>14</td>
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<td>MS 315</td>
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<td>72</td>
<td>88</td>
<td>0+</td>
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<td>Aspiration pneumonia</td>
</tr>
<tr>
<td>MS 102</td>
<td>M</td>
<td>20</td>
<td>49</td>
<td>73</td>
<td>0+</td>
<td>11</td>
<td>Heart failure</td>
</tr>
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<td>Control cases</td>
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<td>0+</td>
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<td>Tongue carcinoma</td>
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<tr>
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<td>N/A</td>
<td>84</td>
<td>0+</td>
<td>5</td>
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<tr>
<td>C 41</td>
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<td>N/A</td>
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<td>0+</td>
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<td>Lung cancer</td>
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<tr>
<td>C 45</td>
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<td>N/A</td>
<td>77</td>
<td>0+</td>
<td>22</td>
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<td>C46</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>75</td>
<td>0+</td>
<td>24</td>
<td>Renal failure, multiple myeloma</td>
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<td>C48</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>68</td>
<td>0+</td>
<td>10</td>
<td>Metastatic colon cancer</td>
</tr>
</tbody>
</table>

The age at onset, age at wheelchair use, age at death and disease duration were calculated from retrospective analysis of clinical records. The inflammation index represents a semi-quantitative measure of meningeal and perivascular inflammation.

Tissue preparation

Post-mortem brains were cut into blocks (2 × 2 × 1 cm) and fixed in 4% paraformaldehyde for >12 h before being preserved in paraffin wax or cryoprotected using 30% sucrose and frozen in isopentane in dry ice. In addition, some blocks of unfixed tissue were snap frozen in cold isopentane prior to storage at −75°C. In total, 326 frozen blocks (50 fixed and 276 snap frozen) and 416 paraffin-embedded blocks from 26 cases with primary progressive multiple sclerosis and 24 paraffin-embedded blocks from control cases were examined.

Assessment of cellular infiltrates and the presence of tertiary lymphoid-like structures

The presence of immune cell infiltrates and screening for tertiary lymphoid-like structures was initially investigated using wax embedded brain blocks sampled as part of the routine neuropathological assessment of all cases processed by the UKMSTB, following the guidelines for brain dissection and processing proposed by BrainNet Europe (www.brainnet-europe.org). Sixteen tissue blocks, from anatomically defined brain areas, were assessed from each case. An index of general inflammation was assigned to each case based on the maximum inflammatory infiltrate noted in the meninges and perivascular loci of all blocks examined, as described previously (Howell et al., 2011). Cases were rated as exhibiting substantial (+ + grade) inflammation.
if one or more dense clusters (>50 cells) of small, round lymphocytic cells were noted. Moderate (+) infiltrates presented as small, more diffuse clusters of 5–50 cells in the meningeal or perivascular compartment and cases with few, scattered infiltrates containing fewer than five cells were rated as mild (0+). Cases classified as containing at least a single example of + + rated meningeal or perivascular inflammation were further sampled as these were the most likely to harbour significant immune cell aggregates and lymphoid-like structures. Double immunostaining for CD3-positive (blue) T cells and CD20-positive (brown) B cells (C and D) was used to screen for the presence of immune cell aggregates. A dense aggregate of CD3-positive T cells partially separated from an aggregate of CD20-positive B cells is shown in the meninges (C) together with a large mixed perivascular infiltrate of T and B cells in the white matter (D). Scale bars = 50 μm.

Figure 1 Meningeal and perivascular inflammation in primary progressive multiple sclerosis. Haematoxylin and eosin stained sections (14 blocks per case) were examined and the maximum degree of meningeal (A) and white matter perivascular (B) inflammation was used to determine the inflammatory rating of the case as mild (0+), moderate (+) or severe (++). Cases classified as containing at least a single example of ++ rated meningeal or perivascular inflammation were further sampled as these were the most likely to harbour significant immune cell aggregates and lymphoid-like structures. Double immunostaining for CD3-positive (blue) T cells and CD20-positive (brown) B cells (C and D) was used to screen for the presence of immune cell aggregates. A dense aggregate of CD3-positive T cells partially separated from an aggregate of CD20-positive B cells is shown in the meninges (C) together with a large mixed perivascular infiltrate of T and B cells in the white matter (D). Scale bars = 50 μm.

Immunohistochemistry
Snap-frozen sections were immediately fixed and endogenous peroxidase activity blocked by incubating with cold methanol/0.3% H₂O₂ prior to rehydration. Fixed sections were air dried and rehydrated in PBS before incubating with 0.3% H₂O₂ in methanol. Sections from paraffin embedded blocks were de-waxed and rehydrated. In preparation for immunostaining, all sections were subjected to heat-induced epitope retrieval in citrate buffer (10 mM; pH 6) or Dako epitope retrieval reagent (Dako Inc.), where appropriate, prior to overnight

B cells, CD35-positive follicular dendritic cells and Ig-A, G, and M-positive plasma cells to determine their status as aggregates or bona fide B cell follicle-like structures, as previously described (Howell et al., 2011).
incubation at room temperature in the primary antibody solution (Supplementary Table 1) and immunohistochemical staining was performed as described previously (Howell et al., 2010, 2011). Sections were counterstained with haematoxylin and mounted using Depex Polystyrene (DPX) or VectaMount™ AQ (Vector Labs).

Quantitative analysis of grey and white matter demyelination

Paraffin embedded tissue blocks were region-matched from superior frontal gyrus (sampled 1 cm rostral to the temporal pole), the superior temporal gyrus, hippocampus including parahippocampal gyrus (both at the level of the lateral geniculate body) and the primary visual cortex, all considered to be predilection sites for meningeal inflammation owing to their large sulci and meningeal compartments. Cortical tissue blocks were analysed for the presence of demyelination by immunostaining one section from each block, four blocks per case, for myelin oligodendrocyte glycoprotein (MOG). Images of entire MOG stained sections were generated by scanning stained slides at 1200dpi using a flat bed scanner, and areas of demyelinated pathology were manually outlined on the digitized images following microscopic examination of the stained slides (at ×100 magnification). Labelled areas of demyelination were subsequently measured using the area measures tool in ImageJ (http://imagej.nih.gov/ij/) and areas of total grey matter and white matter matter area per block, and the mean per cent grey matter and white matter demyelination, calculated per case.

Quantitative analysis of meningeal and parenchymal inflammation

The density of infiltrating cells was quantified using images of meninges overlying grey matter lesions or normal appearing grey matter regions (as identified microscopically in the MOG stained serial sections) in CD68, CD20 and CD3 stained sections. For each of the four brain regions analysed per case, four non-overlapping sample fields of meninges were captured at ×200 magnification (eight fields per section; total area 1.5 mm²) for quantification of positively stained cells overlying normal appearing grey matter and grey matter lesions. All grey matter lesions and normal appearing grey matter areas captured for analysis of the different cell phenotypic markers were region-matched within the same block from serial sections. Cell density was calculated as the mean number of cells per millimetre length of intact meninges as measured using ImageJ. For the evaluation of grey matter parenchymal inflammation, sections labelled with anti-CD68 antibody were used to quantify CD68-positive microglia by capturing four ×200 images from grey matter lesions and normal appearing grey matter parenchyma directly underlying sampled fields of meninges described above. Care was taken to capture the pial surface at the edge of every image so that the depth of cortex sampled remained constant throughout. Cell density was calculated as the mean number of CD68-positive cells per mm² of grey matter parenchyma with the exclusion of CD68-positive cells of the vasculature. The mean density of T or B lymphocytes per mm² of measured white matter perivascular space (n = 8 vessels) was also calculated. Infiltrates were quantified from four vessels (veins and/or arteries) from previously characterized normal appearing white matter and white matter lesion centres (active or chronic active lesion status determined by CD68 and MOG immunohistochemistry) per block, four blocks per case. Only vessels in cross-section presenting with a thin tunica media and with a total area (vessel and perivascular space) >0.003 mm² on microscopic examination were considered and the mean values per case compared.

Quantifying neuronal loss

Quantitative analysis of neurons was performed from cortical layers III and V. NeuN/SMI32 double-positive neurons were counted, and the numerical cell density calculated, from ×200 magnification images captured of layers III and V from both grey matter lesions and normal appearing grey matter regions (four images per layer, 16 fields per section). A small section of layer IV was present in all fields to ensure even depth of cortex was captured in each instance. Only cells with a visible nucleus (haematoxylin counterstain) were included in the count. Counts were expressed as total neurons per mm² per cortical layer, per block (four blocks), per case.

Neurite density

The density of neurofilament-H-positive neurites per block was calculated by manually counting the number of immunostained structures present in four grids (total area per field 0.0036 mm²) per ×200 image using Image ProPlus software (Media cybernetics Inc.) to ensure that the same neurofilament-H-positive profiles were not counted more than once within each captured field (Magliozzi et al., 2007). Four such sample images were taken from cortical layer III of grey matter lesions and normal appearing grey matter regions for each block (eight fields per block section) and the density of neurofilament-H-positive neurites per mm² calculated.

Image analysis

All quantification and analysis was performed with the researcher blinded to the case identity. Slides were examined using a Nikon E1000M or Leica DM2500 microscope and images were captured using a QICAM digital camera (QImaging Inc.). Image ProPlus, ImageJ and Photoshop CS2 (Adobe) software was used for analysis and generation of figures.

Statistical analysis

Data were analysed and expressed as mean ± standard error of mean (SEM), or median ± half the interquartile range, using Excel (Microsoft Office 2007, Microsoft Corp.) and plotted using PRISM 5.0 software (GraphPad). Data were compared by non-parametric Mann–Whitney test or the one-way analysis of variance (ANOVA) with Dunn’s multiple comparisons post-test, to calculate P-values. A non-parametric correlation test (Spearman’s rank correlation) was used to analyse the interdependence between the pathological and clinical markers and Kaplan–Meier survival curves were plotted for the analysis of the clinical course of the assigned sub-groups. A P < 0.05 was regarded as significant.

Results

Patient population

The 26 cases with primary progressive multiple sclerosis used in this study had a median age of disease onset (± half interquartile range in years) of 37.0 ± 10.5 and median disease duration of 25.0 ± 6.5
years, entirely in keeping with previous natural history studies of primary progressive multiple sclerosis (Confavreux et al., 1980; Cottrell et al., 1999; Scaffari et al., 2011). Males experienced an earlier age of onset, age at progression and age at first wheelchair use, when compared with females (Table 1). These clinical demographics demonstrate our study cohort to be accurately representative of the primary progressive multiple sclerosis population.

Meningeal and perivascular infiltrates, but an absence of tertiary lymphoid-like structures, characterizes inflammation in the primary progressive multiple sclerosis brain

Examination of post-mortem brain tissue of cases with primary progressive multiple sclerosis revealed varying degrees of inflammation in the leptomeninges and around white matter vessels (Fig. 1). The incidence of cases containing at least a single large meningeal and perivascular immune cell aggregate (rated substantial +++; Fig. 1A and B) was 8/26 (30% of examined cases with primary progressive multiple sclerosis; Table 1), which is reduced in comparison to that reported in secondary progressive multiple sclerosis (64/123, 52%; Fisher’s exact test, comparing primary progressive and secondary progressive multiple sclerosis, P = 0.055; Howell et al., 2011). Control brains showed minimal meningeal and perivascular inflammation (Table 1). Post-mortem interval and cause of death, whether infection-related or not, did not differ between the groups and did not differ compared with controls (data not shown). We selected additional frozen brain tissue samples from the eight cases with large immune cell aggregates (++) in order to investigate if ectopic tertiary lymphoid-like structures were present in our primary progressive multiple sclerosis cohort. Significant clusters of CD3-positive T cells and CD20 positive B cells were detected (Fig. 1C and D), but no Ki67- and CD20 double positive dividing B cells or CD35-positive follicular dendritic cells were noted. Immunostaining protocols were validated using human spleen as positive control material. Thus, significant aggregates of T and B lymphocytes were detected in the primary progressive multiple sclerosis, albeit at a lower frequency in comparison to cases with secondary progressive multiple sclerosis, but no ectopic lymphoid-like structures (rated +++) in Howell et al., 2011) were identified in the 742 cortical tissue blocks examined.

Increased cortical demyelination is associated with greater meningeal inflammation

MOG immunostaining of four region-matched cortical blocks per case revealed a wide variation in demyelinating lesion pathology in grey matter and subcortical white matter (Fig. 2A). Of the 26 cases with primary progressive multiple sclerosis, 25 exhibited cortical grey matter demyelination, including subpial lesions (Fig. 2B), with activated microglia at the lesion border (Fig. 2C) and in the outermost cortical layers (Fig. 2D). Lesions were frequently topographically associated with foci of inflammation in the meninges (Fig. 2E). Similar to secondary progressive multiple sclerosis, cortical grey matter lesions were predominantly detected at the depth of the cerebral sulci and tended to be larger in samples taken from the temporal gyrus, although the mean percentage of cortical demyelination was not significantly greater compared with other regions (mean percentage demyelination: superior frontal gyrus = 18.2%, hippocampus = 21.2%, temporal gyrus = 24.8%, occipital gyrus = 16.0%). The total grey matter lesion load varied between 0 and 87.8% in individual brain blocks and the mean area of demyelinated grey matter per case was 19.47 ± 3.32% (±SEM). The proportion of demyelinated white matter per block ranged from 0 to 96.3% of total white matter and the mean white matter lesion load was 12.12 ± 2.13% of total white matter sampled per block per case. The percentage area of demyelinated grey matter was significantly greater than the area of demyelinated white matter (P = 0.0487, non-parametric Mann–Whitney test, Fig. 2L).

In order to clarify the relationship between inflammation and demyelination, we quantified the immune cell infiltrates of the forebrain meninges (Fig. 2F) and white matter perivascular spaces (Fig. 2G), in region matched blocks of primary progressive multiple sclerosis and controls. Although no significant difference in the density of CD20-positive or CD3-positive infiltrates was found when comparing the meningeal inflammation overlying grey matter lesions and normal appearing grey matter regions, the mean density of total meningeal T cells (control 0.8 ± 0.3 and primary progressive multiple sclerosis 22.2 ± 3.1 cells/mm ± SEM, P = 0.0002) and B cells (control 0.3 ± 0.2 and primary progressive multiple sclerosis 4.6 ± 1.0 cells/mm ± SEM, P = 0.0006) was 28-fold and 15-fold higher, respectively, in primary progressive multiple sclerosis samples compared with controls (Fig. 2H). The density of perivascular CD3-positive T cells in normal appearing and demyelinated white matter was 6-fold higher in primary progressive multiple sclerosis compared with controls (P = 0.0004), but no difference in the number of perivascular white matter B cells was observed (P = 0.54; Fig. 2I). Numbers of activated CD68-positive macrophages in the meninges of cases with primary progressive multiple sclerosis showed no significant difference compared with controls (Fig. 2J) or between the meninges directly overlying grey matter lesions and normal appearing grey matter (data not shown). A significantly greater number of activated CD68-positive microglia were observed in primary progressive multiple sclerosis cortex (Fig. 2K) and both the grey matter lesions and normal appearing grey matter CD68-positive microglia differed quantitatively from controls (mean cells/mm² ± SEM: grey matter lesions = 100.0 ± 5.6, normal appearing grey matter = 96.2 ± 4.5, controls = 51.2 ± 7.2; P < 0.05 for controls versus grey matter lesions and controls versus normal appearing grey matter).

Although white matter lesion area did not correlate significantly with the sum of T and B cell numbers in white matter cuffs (Spearman r = 0.17 and 0.14, respectively), there was a significant moderate correlation between total meningeal lymphocytes (sum of CD3-positive and CD20-positive infiltrates) and the mean percentage cortical demyelination for each case (Fig. 2M, r = 0.47, P = 0.02).
Figure 2  Inflammation and demyelination in primary progressive multiple sclerosis cerebral cortex. Four regions of interest per case were assessed for white and grey matter demyelination and inflammation. MOG immunostaining revealed the presence of substantial cortical demyelination, often present as a ribbon-like subpial lesion (arrowheads) extending around the margins of a block from a (+ +)

(continued)
Greater neurite loss was associated with greater meningeal inflammation and cortical demyelination

Neurite pathology in cortical layer III was examined using a monoclonal antibody directed against the 200 kDa neurofilament protein (neurofilament-H) to investigate whether the extent of axon/dendrite loss differed between grey matter lesions and normal appearing grey matter regions, or between cases with primary progressive multiple sclerosis rated according to meningeal and perivascular inflammation (Fig. 3A–F). A significant decrease in the mean density of neurofilament-H-positive neurites was observed in grey matter lesions, being reduced by 45% compared with control grey matter and by 30% compared with normal appearing grey matter values (mean value ± SEM: grey matter lesions = 1986 ± 74, normal appearing grey matter = 2851 ± 91, controls = 3622 ± 83; $P < 0.0001$ for both grey matter lesions versus control and grey matter lesions versus normal appearing grey matter), which was supported by a significant inverse correlation between cortical lesion area and neurite density in the grey matter lesions (Fig. 3G; $r = -0.54; P = 0.007$). A trend towards greater neurite loss was seen with an increasing index of meningeal inflammation (Fig. 3H), which was consistent with the correlation noted between grey matter demyelination and meningeal lymphocyte density (Fig. 2). Interestingly, the level of total infiltrating B cells and T cells in the meninges, correlated modestly with density of neurofilament-H-positive neurites ($r = -0.45; P = 0.017$).

Neuronal loss in layers III and V of primary progressive multiple sclerosis cortex

Double immunostaining of neuronal nuclei and non-phosphorylated neurofilament epitopes (NeuN and SMI32 double positive) was used for the identification and quantitative analysis of neurons in cortical layers III and V (Fig. 4A–C). We were able to identify a significant reduction in the numerical density of neurons in primary progressive multiple sclerosis grey matter lesions compared with control cortex (Fig. 4D and E). In layer III grey matter lesions, a mean reduction of 19% was observed in total neuron density compared with control grey matter, while a reduction of 10% was seen when compared with the corresponding normal appearing grey matter (Fig. 4D; $P < 0.001$ when comparing grey matter lesions neuron density to controls). Layer V grey matter lesion neuron counts showed a similar reduction in total neuronal density (decreased by 18%) compared with controls and adjacent normal appearing grey matter (decreased by 8%); (Fig. 4E; $P < 0.05$ when comparing grey matter lesions neuron density with controls).

Cases with increased meningeal inflammation had more severe disease and died at a younger age

To investigate the relationship between inflammation and clinical activity, the time taken for the subject to reach retrospectively determined clinical milestones was compared with neuropathological measures of inflammation for each case (Fig. 5). A significantly earlier age at death was observed for the high (+ +) inflammation group compared with the low group (0 +) ($P < 0.05$) (Fig. 5A). In addition, cases rated as substantial for inflammation (+ +), had a significantly shorter disease duration in comparison with those cases with primary progressive multiple sclerosis with mild (0 +) meningeal/perivascular inflammation (Fig. 5B; $P < 0.01$). Interestingly, the mean time taken from which the patient became a wheelchair user and their eventual death was significantly different between the (+ +) and (0 +) rated groups [mean ± SEM, time from wheelchair use to death (years): + + = 7.9 ± 1.8, 0 + = 20.1 ± 3.9, $P = 0.017$]. In support of these clinicopathological findings, disease duration significantly correlated with total meningeal lymphocyte density ($r = -0.55; P = 0.0042$) and CD3-positive T cell density ($r = -0.55; P = 0.0043$). In contrast to the associations noted between meningeal inflammation, cortical grey matter pathology and clinical course, quantitative measures of demyelination and lymphocyte infiltration of the white matter did not correlate with any of the clinical milestones discussed above (data not shown).

Discussion

In this study we have shown that a greater degree of meningeal inflammation in cases with primary progressive multiple sclerosis was associated with more extensive cortical demyelination and neurite loss. Increased meningeal inflammation, in the absence of tertiary lymphoid-like structures, was associated with younger age at death and a shorter duration of disease, suggesting that diffuse meningeal inflammation and cortical neuronal pathology may be significant contributors to the pathological mechanisms driving the clinical
Figure 3 Reduced density of cortical neurites in cases with greater demyelination and meningeal inflammation. In demyelinated areas of the cortex (A), neurofilament H-positive neurite density was visibly decreased (C) in comparison with normal appearing grey matter (B and D). Arrowheads indicate the layer II/III border. A reduction in the number of neurofilament H-positive neurites can be clearly seen at ×200 magnification (E, grey matter lesion; F, normal appearing grey matter). The extent of cortical demyelination was inversely correlated with neurite density in grey matter lesions (G). Neurite density in both normal appearing grey matter and grey matter lesions was reduced with increased meningeal inflammation (H). Spearman’s non-parametric comparison (G), ANOVA and Dunn’s multiple comparison post-test *P < 0.05, **P < 0.01, ***P < 0.0001, in comparison with controls. Scale bars: A–D = 100 μm; E and F = 50 μm. GML = grey matter lesion; NAGM = normal appearing grey matter; NFil = neurofilament H-positive neurites.
progression of primary progressive multiple sclerosis. These findings are in keeping with our observations in secondary progressive multiple sclerosis (Magliozzi et al., 2007, 2010; Howell et al., 2011) and suggest that similar pathogenetic mechanisms prevail in the progressive phase of multiple sclerosis, irrespective of a preceding relapsing-remitting course or the presence of tertiary lymphoid-like structures in the meninges, although the severity of the pathological changes may differ.

**Neurodegeneration and progression of disease**

Substantial evidence now supports the view that cortical pathology is an important substrate for the progression and irreversible clinical disability in multiple sclerosis (Calabrese et al., 2010), and that it correlates better with measures of physical disability than MRI measures of white matter pathology (Pirko et al., 2007).

Figure 4 Neuronal loss in primary progressive multiple sclerosis. Images captured from cortical layers III and V of neuronal nuclei (NeuN) and SMI32-positive double-stained sections were used to quantify the density of neurons within normal appearing and demyelinated grey matter (A). Pyramidal cells were identified by the long apical dendrite and distinct morphology (B and C) and only NeuN- and SMI32 double positive neurons with visible haematoxylin stained nuclei were counted. Neuronal densities in layers III and V in grey matter lesions were significantly reduced in comparison with controls (D and E). ANOVA and Dunns multiple comparison post-test; *P < 0.05, **P < 0.01. Scale bars: A = 100 μm, C = 25 μm. GML = grey matter lesion; NAGM = normal appearing grey matter.
Neurites (Magliozzi et al., 2006; Wegner et al., 2010) in the thickness of the cortical ribbon (Magliozzi et al., 2007, 2010). This quantitative difference may reflect the overall shorter duration of the progressive phase in our secondary progressive multiple sclerosis cohort compared with primary progressive multiple sclerosis (primary progressive and secondary progressive multiple sclerosis clinical milestones are shown in Supplementary Table 2). However, determination of the onset of progressive disease is less accurate for secondary progressive multiple sclerosis and, therefore, one must be cautious about such interpretations.

**The extent of cortical demyelination is related to the severity of overlying meningeal inflammation**

Cortical demyelination, predominantly subpial and often extending around multiple gyri, exceeded the extent of white matter demyelination in our post-mortem primary progressive multiple sclerosis cohort, which is supported by previous observations in the forebrain (Kutzelnigg et al., 2005) and spinal cord (Tallantyre et al., 2009). Although previously described as exhibiting reduced overall inflammation and demyelination, primary progressive multiple sclerosis, like secondary progressive multiple sclerosis, encompasses significant heterogeneity, with cases displaying mild to substantial inflammation, demyelination, neuronal and axonal pathology. This is likely to reflect the overall course of disease, which can be highly variable between individuals (Revesz et al., 1994; Ingle et al., 2003). In this post-mortem study, our analysis of 742 cortical blocks from 26 cases of confirmed primary progressive multiple sclerosis, revealed diffuse meningeal inflammation, cortical demyelination and neuronal and axonal pathology to be interlinked. Cases displaying the most substantial qualitative and quantitative measures of inflammation and cortical demyelination had a shorter, more aggressive disease course. A positive correlation between the extent of cortical grey matter demyelination and meningeal lymphocyte density implies that the increased concentration of myeloid- and neurotoxic substances of the meningeal space, directly, or indirectly via the activation of cortical microglia, may play a major role in the induction of subpial pathology, as has been suggested for secondary progressive multiple sclerosis (Peterson et al., 2001; Bo et al., 2003; Serafini et al., 2004; Dal Bianco et al., 2008; Frischer et al., 2009; Magliozzi et al., 2010, Howell et al., 2011). In secondary progressive multiple sclerosis, a subset of cases defined by elevated, diffuse inflammation and tertiary lymphoid-like structures, exhibit a gradient of cortical damage, with neuronal losses greatest in superficial layers nearest the pial surface (Magliozzi et al., 2010). Our present findings strongly imply that inflammation, and the presence of a cytotoxic inflammatory milieu in the forebrain meninges, may also drive subpial pathology and contribute to a more severe disease in primary progressive multiple sclerosis, which is supported by previous descriptions of profound cortical demyelinating pathology, activated microglia and infiltrated meninges in primary progressive multiple sclerosis.

![Figure 5](https://example.com/figure5.png)

**Figure 5** Clinico-pathological comparisons revealed that a more severe clinical course was associated with greater inflammation at death in primary progressive multiple sclerosis. (A) Kaplan-Meier survival curves of age at death plotted against inflammatory rating (mild 0+, moderate +, substantial ++) revealed that cases rated as ++ for meningeal and perivascular inflammation had a younger age at death, in comparison with cases rated 0+ (mean age ± SEM years): ++ = 51.8 ± 3.8, + = 63.8 ± 4.9, 0 = 71.1 ± 3.4; one-way ANOVA *P* < 0.05 for ++ versus 0+]. (B) Cases characterized by substantial (+ +) meningeal/perivascular inflammation had a significantly reduced mean disease length in comparison to cases rated as mild (0+) (mean duration ± SEM + + = 14.6 ± 3.0, + = 24.8 ± 2.4, 0+ = 32.7 ± 4.0; one-way ANOVA *P* < 0.01 for ++ versus 0+).

Cortical grey matter atrophy, rather than inflammation or demyelination, is a powerful in vivo measure of pathology (De Stefano et al., 2003; Fisher et al., 2008; Fisniku et al., 2008). In patients with PPMS, brain atrophy over a 2-year period has been shown to be one of the best prognostic indicators of disease progression, validated by follow-up a decade later (Khaleeli et al., 2008). Imaging alterations in cortical volume will reflect processes of demyelination, inflammation and neurodegeneration, as well as subcortical white matter damage. Neurodegeneration in cortical grey matter manifests as quantified losses of inhibitory interneurons (Dutta et al., 2006), pyramidal neurons of layers III and V (Magliozzi et al., 2010), a marked reduction in the density of neurites (Magliozzi et al., 2007) and loss of neuropil (Dutta et al., 2006; Wegner et al., 2006), all of which result in a reduction in the thickness of the cortical ribbon (Magliozzi et al., 2010). Our analysis of cases with primary progressive multiple sclerosis has demonstrated significant reductions in neurite and neuronal number, but to a lesser degree compared with our previous study of cases with secondary progressive multiple sclerosis (Magliozzi et al., 2007, 2010).
multiple sclerosis tissues (Kutzelnigg et al., 2005, Frischer et al., 2009). The finding of an inverse relationship between neurite density and the extent of cortical demyelination suggests that a higher level of cytoxins in that part of the cortical grey matter, either endogenous or exogenous from the subarachnoid space, leads to both a greater degree of demyelination and increased neurite loss via the same or similar mechanisms. Demyelination does not appear to be dependent on neurite loss as neurite loss is also seen, although to a lesser extent, in the normal appearing grey matter, implying a differential sensitivity to the cytoxins. However, it remains possible that demyelination is due to antibody-dependent mechanisms, although there is little evidence for the presence of antibody and complement in cortical lesions (Brink et al., 2005).

Diffuse meningeal inflammation is prevalent in primary progressive multiple sclerosis but lymphoid structures are absent

Substantial evidence now supports the presence of tertiary lymphoid structures in the subarachnoid space of a considerable proportion of multiple sclerosis brains (Serafini et al., 2004; Magliozzi et al., 2007; Howell et al., 2011). The failure to identify any such structures in this study suggests that tertiary lymphoid tissue formation is an uncommon event in primary progressive multiple sclerosis when compared with secondary progressive multiple sclerosis, where such inflammatory aggregates were found in 40% of cases examined (Howell et al., 2011), although diffuse meningeal inflammation is still a feature of the pathology. However, the presence of immune cell aggregates containing a significant B cell component in the present study is in agreement with previous observations (Frischer et al., 2009), and suggests that the oligoclonal bands seen in primary progressive multiple sclerosis (Thompson et al., 1997) result from the meningeal infiltrates but are not dependent on the presence of tertiary lymphoid structures. It is possible that more extensive sampling may have revealed such structures, but our observation of a reduced frequency of ++ rated cases with primary progressive multiple sclerosis, predicts these structures, if present, would be found at an extremely low frequency in our primary progressive multiple sclerosis cohort. The development of tertiary lymphoid tissues in the meninges represents one end of the spectrum of meningeal inflammation that may further exacerbate pathology in addition to the diffuse, non-organized inflammation found throughout the forebrain and cord meninges. The absence of detectable follicle-like structures in this primary progressive multiple sclerosis study implies that their formation may occur early in the disease process, during the relapsing-remitting phase, as a result of the repeated acute inflammatory activity that is not a typical feature of primary progressive multiple sclerosis. This is in keeping with recent observations of meningeal inflammatory cell aggregates overlying subpial demyelinated lesions in cortical biopsies of early multiple sclerosis cases (Lucchinetti et al., 2011).

Conclusion

We have shown that there is a widespread demyelinating pathology in the primary progressive multiple sclerosis brain, that is greater in the cortical grey matter than underlying white matter, is predominantly subpial and is associated with inflammation of the overlying meninges. Inflammation, consisting of diffuse and clustered infiltrates but without ectopic lymphoidogenesis, associates with pathological changes that are similar but less extensive when compared with those reported in secondary progressive multiple sclerosis. This study also clearly illustrates that during the progressive stages of multiple sclerosis, neuronal pathology is accompanied by inflammation in both the perivascular and meningeal spaces in both primary progressive and secondary progressive multiple sclerosis. There is no indication that primary progressive multiple sclerosis is generally less inflammatory but encompasses a wide range of inflammatory activity similar to secondary progressive multiple sclerosis. This work further defines the nature of the partitioned immune response in progressive disease and suggests that effective immunotherapy of long-standing multiple sclerosis will need to target this inflammation.

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Supplementary material

Supplementary material is available at Brain online.

References


