Autologous haematopoietic stem cell transplantation fails to stop demyelination and neurodegeneration in multiple sclerosis

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The present study analyses autopsy material from five multiple sclerosis patients who received autologous stem cell transplantation. A total of 53 white matter lesions were investigated using routine and immunohistochemical stainings to characterize the demyelinating activity, inflammatory infiltrates, acutely damaged axons and macrophages/microglial cells. We found evidence for ongoing active demyelination in all of the five patients. The inflammatory infiltrate within the lesions showed only very few T cells and CD8+ cytotoxic T cells dominated the T cell population. B cells and plasma cells were completely absent from the lesions. High numbers of acutely damaged axons were found in active lesion areas. Tissue injury was associated with activated macrophages/microglial cells. The present results indicate that ongoing demyelination and axonal degeneration exist despite pronounced immunosuppression. Our data parallel results from some of the clinical phase I/II studies showing continued clinical disease progression in multiple sclerosis patients with high expanded disability system scores despite autologous stem cell transplantation.

Keywords: multiple sclerosis; stem cell transplantation; demyelination; neurodegeneration

Abbreviations: APP = amyloid precursor protein; ASCT = autologous stem cell transplantation; BMT = bone marrow transplantation; EDSS = Expanded Disability Status Scale; EAE = experimental allergic encephalomyelitis; HSCT = haematopoietic stem cell transplantation

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Introduction

Autologous stem cell transplantation is considered a potential therapeutic option to treat severe multiple sclerosis refractory to conventional treatment. This therapeutical approach was prompted by transplant experiments in animals with experimental allergic encephalomyelitis (EAE), an experimental model of multiple sclerosis. Remissions or cure of EAE were achieved in animals after high-dose chemotherapy or high-dose total body irradiation and autologous bone marrow transplantation (BMT) (van Bekkum, 2004). Approximately 250 multiple sclerosis patients have received autologous stem cell transplantation (ASCT) as part of phase I and II open trials (Blanco et al., 2005). Haematopoietic stem cell transplantation (HSCT) appears to be ineffective for patients with progressive disease and high pretransplantation Expanded Disability Status Scale (EDSS) scores, whereas patients with less accumulated disability and more inflammatory disease...
activity might benefit from HSCT. A marked reduction in MRI activity is observed after HSCT. However, brain tissue loss is ongoing, independent of visible MRI activity, suggesting persisting demyelination or neurodegeneration (Inglessi et al., 2004).

The neuropathological hallmark of multiple sclerosis is the demyelinated plaque associated with inflammation, gliosis and axon loss. Accumulation of autologous stem cell transplantation amyloid precursor protein (APP) in damaged axons has been shown to be a reliable marker of acute axonal damage in experimental and human tissue (Gentleman et al., 1993; Pierce et al., 1996; Ferguson et al., 1997; Bitsch et al., 2000). Recent and active demyelination may be confirmed by the detection of myelin protein or Luxol fast blue (LFB)-positive degradation products in macrophages or microglia (Brück et al., 1995). Inflammation, especially CD8-positive T cells and macrophages/microglia have previously been shown to correlate with acute axonal damage (Trapp et al., 1998; Kuhlmann et al., 2002). In general, the inflammatory infiltrate is thought to be detrimental, but it may also be beneficial and may have neuroprotective effects (Kerschensteiner et al., 2003). Inflammation and demyelination are probably responsible for the early development of symptoms during a multiple sclerosis attack. During the progressive phase of the disease, inflammation is less prominent. New focal white matter lesions are rare, but radial expansion of pre-existing lesions is common, even when inflammation is sparse (Prineas et al., 2001). This is associated with progressive axonal damage within demyelinated plaques and in the normal appearing white matter (Ferguson et al., 1997; Kornek et al., 2000; Kuhlmann et al., 2002; Kutzelnigg et al., 2005). In this stage of the disease, the inflammatory process appears to be trapped behind a closed or repaired blood–brain barrier (Serafini et al., 2004; Kutzelnigg et al., 2005).

The aim of this study was to characterize the histopathology of multiple sclerosis lesions in patients who had received autologous stem cell transplantation. To our knowledge only a single case with limited histopathological data from a patient with autologous haematological stem cell transplantation has been published; this patient is included in our study (Openshaw et al., 2000). Our results demonstrate ongoing acute axonal damage and active demyelination following autologous stem cell transplantation in the absence of substantial lymphocyte infiltration of the lesions.

**Material and methods**

**Patients**

We investigated paraffin-embedded autopsy tissue from five multiple sclerosis patients who had received autologous stem cell transplantation. The postmortem study of the brain of these patients was approved by the Ethics Committee of the University of Göttingen. Different protocols for the autologous stem cell transplantation were applied. Median disease duration before transplantation was 9 years. Clinical characteristics and therapy-related data are summarized in Table 1. The autopsy cases were

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**Table 1** Demographic and clinical characteristics of AHSCT cohort

<table>
<thead>
<tr>
<th>Case</th>
<th>Kind of transplantation</th>
<th>Induction therapy/therapy following tx</th>
<th>Age at tx/ course</th>
<th>Gender</th>
<th>Yr first symptoms</th>
<th>Clinical disease to tx</th>
<th>EDSS at tx</th>
<th>EDSS last f/u</th>
<th>Time of death after tx/cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T cell depleted autologous peripheral blood stem cell transplantation</td>
<td>Busulphan, cyclophosphamide</td>
<td>40</td>
<td>m</td>
<td>9</td>
<td>6</td>
<td>2 months/venoocclusive disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>T cell depleted autologous bone marrow graft</td>
<td>Busulphan, cyclophosphamide, ATG, G-CSF, prednisone</td>
<td>49</td>
<td>f</td>
<td>7</td>
<td>6</td>
<td>7 months/pneumonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>CD34 selected autologous peripheral blood stem cell transplantation</td>
<td>Busulphan, cyclophosphamide, ATG, G-CSF</td>
<td>44</td>
<td>f</td>
<td>19</td>
<td>6</td>
<td>20 days/pneumonia and ARDS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>CD34 selected autologous peripheral blood stem cell transplantation</td>
<td>Busulphan, cyclophosphamide, ATG, G-CSF</td>
<td>47</td>
<td>f</td>
<td>24</td>
<td>5.5</td>
<td>15 years/spe\dski</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>CD34 selected autologous peripheral blood stem cell transplantation</td>
<td>Busulphan, cyclophosphamide, ATG, G-CSF</td>
<td>29</td>
<td>m</td>
<td>6-7</td>
<td>6</td>
<td>1 month/venoocclusive disease</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** G-CSF = granulocyte-colony stimulating factor; f/u = follow-up; pr = progressive relapsing; pp = primary progressive multiple sclerosis; pp = primary progressive multiple sclerosis; g = secondary progressive multiple sclerosis; IV = intravenous immunoglobulin; TBI = total body irradiation; tx = transplantation; yr = year; sp = secondary progressive multiple sclerosis; pp = primary progressive multiple sclerosis; f/u = follow-up.
Histopathology
Specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Slices of 4 μm thickness were stained with haematoxylin and eosin (HE), Luxol–fast blue (LFB) and periodic acid–Schiff (PAS) as well as Bielschowsky’s silver impregnation. Immunohistochemical staining was performed with a biotin–avidin or an alkaline phosphatase/anti-alkaline phosphatase technique. The primary antibodies used were anti-myelin basic protein (MBP, Boehringer Mannheim, Germany), anti-CD3 (T cells, Dako, Denmark), anti-CD8 (cytotoxic T cells, Dako, Denmark), anti-CD20 (B cells, Dako, Denmark), anti-CD79a (B cells, Dako, Denmark), anti-CD138 (plasma cells, Dako, Denmark), anti-KM1P (macrophages/microglial cells, Dr Radzun, University of Göttingen, Germany), anti-MRP 14 (early activated macrophages, BMA Biomedicals, Switzerland), anti-APP (acutely damaged axons, Boehringer Mannheim, Germany) and anti-CR3/43 (MHC class II—activated macrophages/microglia, Dako, Denmark). Double immunofluorescence staining was performed with primary antibodies directed against MBP and Ki-M1P to detect recent myelin degradation products in macrophages. Secondary antibodies were Cy-2 and Cy-3 labelled.

Histopathological classification of multiple sclerosis lesions
A total of 53 white matter lesions from five multiple sclerosis patients were analysed. They were classified according to both demyelinating and inflammatory activity. In addition, 18 areas of normal-appearing white matter (NAWM) were investigated. The NAWM was chosen to be as remote as possible from the lesions. As autopsy specimens were small, a certain proximity to the lesions was unavoidable.

The demyelinating activity was classified as described earlier (Bitsch et al., 2001). Active demyelinating lesions (active) were partially demyelinated and diffusely infiltrated by macrophages/microglia containing LFB-positive myelin degradation products that were immunoreactive for myelin proteins such as MBP. Inactive demyelinated lesions (DM) were also infiltrated by macrophages/microglia, but these cells contained only PAS-positive myelin degradation products and no myelin proteins that could be detected by immunocytochemistry. Demyelination was complete. In remyelinating lesions (RM), thin, sometimes irregularly arranged myelin sheaths indicative of remyelination were present. As white matter lesions might show more than one of these features, a total number of 77 different lesion areas were investigated.

Concerning the inflammatory activity, the lesions were divided into chronic active and chronic inactive lesions, with chronic active being defined as the presence of a macrophage/microglial rim at the plaque edge. Chronic inactive lesions lacked such a macrophage/microglial rim (Bo et al., 1994).

Morphometry
The number of T cells, B cells, plasma cells, macrophages/microglial cells and APP-positive axons stained with the corresponding antibodies was determined per square unit of tissue on serial sections. Macrophages and microglia were differentiated morphologically in Ki-M1P immunohistochemistry since markers distinguishing these cell types are not available. Macrophage-like cells were identified as large, round cells with foamy cytoplasm and eccentric nuclei. Microglial cells had a stellate morphology with distinct cell processes. Counting was done in the lesion centre or at the plaque edge. Additionally the density of T cells, B cells, plasma cells and APP-positive axons was determined in the NAWM. The number of cells or APP-positive axonal profiles was counted in at least 10 standardized microscopic fields of 62 500 μm² each, defined by an ocular morphometric grid. The values in the tables represent the median number of cells or APP-positive axons per mm². Moreover, we determined the number of perivascular T cells, B cells and plasma cells in the Virchow Robin space of at least 10 vessels in the lesion centre as well as in the NAWM. Quantitative data are given as median number of cells per vessel.

Statistics
For statistical analysis the Spearman rank correlation test and Mann–Whitney U-test were applied. Non-parametric quantitative data were expressed as median and range. We used the GraphPad PRISM© software (GraphPad Software, Inc., San Diego, CA, USA).

Results
Lesional activity
Demyelinating activity
Seventy-seven lesion areas from 53 white matter lesions were classified based on demyelinating activity. Of the 77 investigated lesion areas, 16 showed active demyelination (Fig. 2A), 41 were completely demyelinated (Fig. 1A) and 20 lesion areas showed remyelination (Fig. 1B). All five included multiple sclerosis cases contained plaques with active demyelination exhibiting LFB+, and MBP+ myelin remnants within macrophages.

Inflammatory activity
Of the 53 white matter lesions, 29 were classified as chronic active lesions, characterized by a macrophage/microglial rim at the plaque border (Fig. 1C), and 24 were chronic inactive lesions (Fig. 1D).

The numbers of lesion areas/lesions investigated are summarized according to their demyelinating and inflammatory activity in Table 2. Lesions showing active demyelination were all chronic active inflammatory lesions, except for two that were actively demyelinating throughout the entire extent of the lesion.
Inflammation

T cells, B cells and plasma cells

In general, low numbers of T cells were found within the parenchymal plaques and in the perivascular space. Most CD3-positive T cells were CD8-positive (Fig. 1E and F). The fact that the CD8 count is often higher than the CD3 count as shown in Table 3 is most probably due to the sampling of the tissue in consecutive sections especially when such low numbers of T cells are present. Both antibodies gave reliable staining patterns in appropriate control tissues. B cells were completely absent, with even fewer plasma cells evident. There was no accumulation of T cells, B cells or plasma cells at the lesion edge. Quantitative data are shown in Table 3. Lymph follicle-like structures were not detected in any of the five cases when carefully inspecting the CD20 or CD79a stainings.

Macrophages/microglia

Concerning the ‘demyelinating activity’, active demyelinating lesion areas showed significantly higher numbers of macrophages (median of 240 macrophages/mm²) compared with inactive lesion areas (median of 19 macrophages per mm²; P<0.0001). No significant differences were found between microglial cells in active and inactive lesion areas. Macrophages outnumbered microglial cells in active lesion areas, whereas microglial cells dominated in inactive lesion areas. The median numbers of macrophages and microglial cells in active and inactive lesion areas are shown in Table 4.

With respect to ‘inflammatory activity’, macrophages and microglial cells were present in the plaque centre, and in 29 of 53 lesions. They also accumulated at the plaque border (chronic active lesions). In general, microglial cells dominated the lesion centre, whereas the macrophage/microglial rim was either dominated by macrophages or microglial cells. Early activated MRP14+ macrophages were not present in any lesion.

Acute axonal damage

The density of APP-positive axons within the lesion was variable, ranging from 0 to 216 APP+ axons/mm² in
single lesions. Case 1 showed a median of 28 APP+ axons/mm² in the lesion centre (13 lesions examined) compared with the other cases with lower numbers (median of one to three APP+ axons/mm²). APP positive axons were found in significantly higher numbers in active demyelinating lesion areas (median of 80 APP+ axons/mm²) compared with inactive lesion areas (median of four APP+ axons/mm²; \( P<0.0001 \)). Data are shown in Table 4. Thirteen of the 53 investigated lesions showed pronounced acute axonal damage at the plaque border with high numbers of APP+ axons (Fig. 2B and C). These ‘rims’ of APP+ axons were identified in all included cases and typically co-localized with the macrophage rim at the chronic active plaque border (Fig. 2D). Within these regions the density of APP+...
axons ranged from 15 to 745 APP+ axons/mm². In general, the number of APP+ axons in the lesion centre and at the plaque border (‘rims’ of APP+ axons) correlated with the number of macrophages ($r = 0.4270$, $P = 0.0003$).

### Normal appearing white matter (NAWM)

Very few T cells were found in the NAWM parenchyma, but some perivascular cuffs of T cells were present (Fig. 3A and B). Almost no B cells were found and plasma cells were absent. Microglial cell activation, as revealed by CR3/43 staining (Fig. 3C) and by morphological evaluation, was evident. Acutely damaged axons, as shown by APP+ staining, were seen (Fig. 3D), with the median ranging from 0 to 18 APP+ axons/mm². Detailed quantitative data are shown in Table 5.

### Discussion

The present study describes the histopathological changes found in autopsies of multiple sclerosis patients who had received ASCT. Histopathological and immunocytochemical examinations revealed a marked suppression of inflammatory activity with very few T cells and no B cells or plasma cells within the lesions, either parenchymally or perivascularly. However, we found signs of ongoing active demyelination as well as evidence for recent acute axonal damage in areas of macrophage/microglial activation. These results indicate that microglia/macrophage-mediated acute axonal damage and active demyelination may occur after autologous stem cell transplantation or the immunosuppression associated with it. It is highly unlikely, although not completely excluded, that these observations are remnants of inflammatory attacks prior to the transplantation. The pathological data challenge the efficacy or even the harmfulness of intense immunosuppression associated with or without autologous stem cell transplantation in progressive multiple sclerosis, as well as raise questions regarding when, during disease evolution, this therapy should be considered.

Early clinical trials with ASCT were primarily performed in severely disabled patients with progressive disease since therapeutic options for the progressive stage of multiple sclerosis with high EDSS scores are particularly...
unsatisfactory. These trials showed an effective reduction of MRI activity, the neuropathological correlate being the intense suppression of inflammation as confirmed in the present study. Despite the profound suppression of MRI inflammatory activity, patients with high EDSS scores progressed clinically after HSCT, and ongoing tissue loss based on MRI atrophy parameters was observed (Inglese et al., 2004). Effective suppression of inflammation in the face of ongoing atrophy was also observed in the Campath-1H study (Coles et al., 2005).

When multiple sclerosis patients were treated with an anti-leucocyte monoclonal antibody (CD 52, Campath-1H), the number of new gadolinium-enhancing lesions was effectively suppressed. However, patients with SPMS showed sustained accumulation of disability due to uncontrolled progression marked by ongoing cerebral atrophy. In contrast, patients with RRMS had a reduction in disability 6 months after Campath-1H infusion. This study suggests that once the cascade of events leading to irreversible tissue injury is established, suppression of inflammation does little to slow or prevent ongoing brain atrophy and clinical progression (Coles et al., 2005).

The present study may provide important insight on the pathological basis of the observed MRI changes after HSCT, namely the suppressed MRI activity and the ongoing tissue loss. It is generally accepted that gadolinium enhancement indicates influx of inflammatory cells from the periphery into the central nervous system. This has been shown to be significantly suppressed by HSCT or the immunosuppression associated with it. The morphological correlate of this observation may be the significantly reduced number of T cells in the lesions as compared with the mean number of lymphocytes in chronic multiple sclerosis plaques reported in the literature (Traugott et al., 1983; Kuhlmann et al.,

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### Table 5 Inflammatory infiltrates and APP+ axons per mm2 or per vessel in the NAWM

<table>
<thead>
<tr>
<th>Case (n NAWM areas)</th>
<th>I (5)</th>
<th>II (5)</th>
<th>III (2)</th>
<th>IV (3)</th>
<th>V (3)</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells (CD 3+) parenchyma</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00−1.60)</td>
<td>3.20 (3.20−4.80)</td>
<td>0.00</td>
</tr>
<tr>
<td>Cytotoxic T cells (CD8+) parenchyma</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>2.40 (1.60−2.40)</td>
<td>0.00</td>
</tr>
<tr>
<td>Perivascular T cells (CD3+)</td>
<td>0.40 (0.35−0.65)</td>
<td>0.35 (0.10−0.55)</td>
<td>0.05 (0.00−0.10)</td>
<td>0.70 (0.15−0.70)</td>
<td>0.18 (0.05−0.30)</td>
<td>0.35</td>
</tr>
<tr>
<td>Perivascular cytotoxic T cells (CD8+)</td>
<td>0.50 (0.00−0.60)</td>
<td>0.15 (0.10−0.35)</td>
<td>0.13 (0.05−0.20)</td>
<td>0.20 (0.05−0.30)</td>
<td>0.25 (0.00−0.68)</td>
<td>0.20</td>
</tr>
<tr>
<td>B cells (CD20+ or CD79A+) parenchyma</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00−0.80)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>n.a</td>
<td>0.00</td>
</tr>
<tr>
<td>Perivascular B cells</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
<td>n.a</td>
<td>0.00</td>
</tr>
<tr>
<td>APP+ axons parenchyma</td>
<td>17.60 (6.40−20.00)</td>
<td>3.20 (0.80−17.60)</td>
<td>12.80 (1.60−24.00)</td>
<td>0.80 (0.80−1.60)</td>
<td>0.00 (0.00)</td>
<td>3.20</td>
</tr>
</tbody>
</table>

Fig. 3 Normal appearing white matter. The CD3-staining shows some T lymphocytes in the perivascular space (A). T lymphocytes are mainly cytotoxic T lymphocytes as shown with the CD8-staining (B). Activated microglial cells, positive for CR3/43, are found (C). Some axons are acutely damaged as seen in the APP-staining (D). Original magnifications: A–D: × 40.
Thus, the APP immunoreactivity observed in our patients must for the most part be attributed to the post-AHSCT/immunosuppression period, implying that this type of treatment does little to stop or prevent further development of axonal damage at that disease stage.

Microglia/macrophages are the main candidates for mediating myelin and axon injury in the absence of a prominent inflammatory component within the lesions. One of the key questions is therefore the cause of macrophage/microglial activation observed in the present study. A standard view is that macrophages/microglial cells are activated secondary to tissue damage meaning that the microglial activation seen here could simply be the consequence and not the cause of ongoing axonal loss. Nevertheless, the observations made in the present five cases raise the question of whether tissue damage progresses in multiple sclerosis, even when inflammation is almost completely abolished, or whether the immunosuppressive regime used for autologous stem cell transplantation is insufficient to block the inflammatory reaction within the brain. Active demyelination and acute axonal damage was clearly associated with areas of macrophage/microglial activation in the lesions and the NAWM in the present cases. Whether these developed from resident parenchymal microglia or originated from haematogenous monocytes (either as new immune cells maturing from the transplant or as ‘old’ cells from the host) can never be clarified. However, the lack of MRP-14 expression, a peripheral monocyte marker (Ulvestad et al., 1994), is evidence of CNS origin of these cells. Microglial cells/macrophages are good candidates for mediating axonal damage (Ferguson et al., 1997; Bitsch et al., 2000; Kuhlmann et al., 2002). Alternatively, ongoing tissue damage in multiple sclerosis patients after AHSCT may also be driven by T cells. Although the number of T cells is effectively reduced, it is notable that CD8+ T cells dominate, as shown by the CD8/CD3 ratio (Table 3). A correlation between acute axonal damage and CD8-positive T cells has been described (Bitsch et al., 2000; Kuhlmann et al., 2002), suggesting that cytotoxic T cells may be the main effector cells mediating this process (Medana et al., 2001). Whether CD8+ T cells survived the severe immunosuppression, or alternatively infiltrated the CNS after transplantation remains unanswered. Finally, we must consider the possibility that the chemotherapy regimen used for immunoablation is itself toxic to the CNS. Chen et al. (2006) have shown accelerated brain atrophy also in non-multiple sclerosis patients following HSCT.
Multiple sclerosis patients may therefore be even at higher risk for CNS chemotoxicity due to their pre-existing local disease.

In conclusion, our data show that axonal damage and demyelination are both active in patients with long-standing disease and high EDSS scores following autologous stem cell transplantation and the immunosuppression associated with it. It is therefore unlikely that AHSCT and/or potent immunosuppression halts disease progression, despite suppressing the inflammatory aspects of the disease. These data correlate with post-AHSCT MRI and clinical studies demonstrating continued atrophy and clinical progression in some patients at advanced disease stages. There is an urgent need for effective therapies which prevent ongoing demyelination and neurodegeneration in progressive multiple sclerosis.

Acknowledgements
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References