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.

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 (Inglese *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 an 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

Table I Demographic and clinical characteristics of AHSCT cohort

Case	Kind of transplantation	Induction therapy/therapy following tx	Age at tx/gender	Yr first multiple sclerosis symptoms to tx	Clinical disease course	EDSS at tx	EDSS last f/u	Time of death after tx/cause of death
I	T cell depleted autologous peripheral blood stem cell transplantation	Busulphan, cyclophosphamide, ATG/G-CSF	40/m	9	sp	6	6	2 months/venoocclusive disease
II	T cell depleted autologous bone marrow graft	Plasmapheresis, IvIg, cyclophosphamide, ATG, TBI, methylprednisolone/G-CSF, prednisone	49/f	7	pp	8	8	7 months/pneumonia and thrombembolism
III	CD34 selected autologous peripheral blood stem cell transplantation	Busulphan, cyclophosphamide, ATG/G-CSF	44/f	19	sp	7	7	20 days/pneumonia and ARDS
IV	CD34 selected autologous peripheral blood stem cell transplantation	Busulphan, cyclophosphamide, ATG/G-CSF	47/f	24	sp	5.5	5.5	1.5 years/pneumonia and sepsis
V	Double selected (CD34+,CD3−) autologous peripheral blood stem cell transplantation	Busulphan, cyclophosphamide, ATG/G-CSF, lymphglobulin	29/m	6–7	pr	6	6	1 month/venoocclusive disease

tx = transplantation; yr = year; sp = secondary progressive multiple sclerosis; pr = progressive relapsing; ATG = anti-thymocyte globulin; G-CSF = granulocyte-colony stimulating factor; IvIg = intravenous immunoglobulins; TBI = total body irradiation; f/u = follow-up; pp = primary progressive multiple sclerosis.

collected by Dr Kolar/Dr Azzarelli (Indianapolis, USA), Dr Freedman/Dr Atkins (Ottawa, Canada), Dr Lucchinetti/Dr Openshaw (Rochester, USA, two cases) and Dr Lassmann/Dr Garcia-Merino (Vienna, Austria and Madrid, Spain). General neuropathology did not show evidence of other confounding CNS pathologies such as viral or bacterial infections.

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-KiM1P (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 (Bö *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 $62\,500\,\mu m^2$ each, defined by an ocular morphometric grid. The values in the tables represent the median number of cells or APP-positive axons per mm^2. 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, Inco., 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.
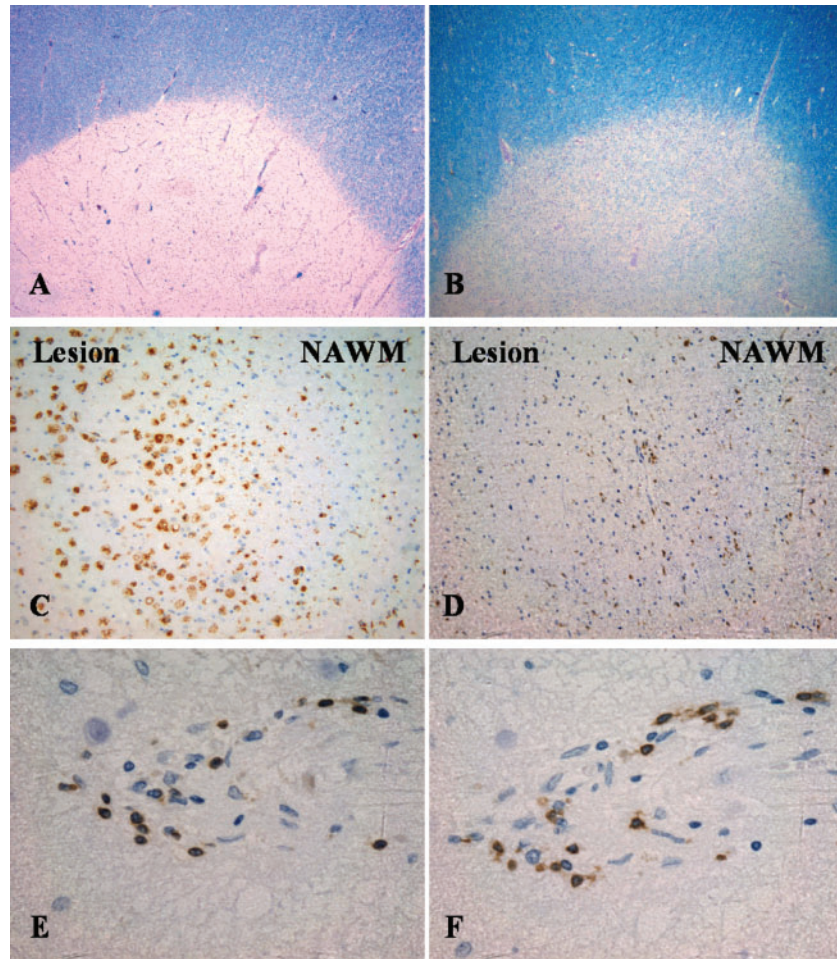
Fig. 1 Lesional pathology: In **A** a demyelinated and in **B** a remyelinated lesion is shown (LFB/PAS staining). High numbers of macrophages are seen at the plaque edge of chronic active lesions (**C**), whereas chronic inactive lesions lack such a macrophage rim (**D**) (KiMIP-staining). CD3-positive T cells (**E**) are dominated by CD8-positive cytotoxic T cells (**F**) and are predominantly located perivascularly. NAWM is normal appearing white matter. Original magnifications: **A** and **B**: ×4; **C** and **D**: ×20; **E** and **F**: ×40.

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/mm^2) compared with inactive lesion areas (median of 19 macrophages per mm^2; $P < 0.0001$). 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/mm^2 in
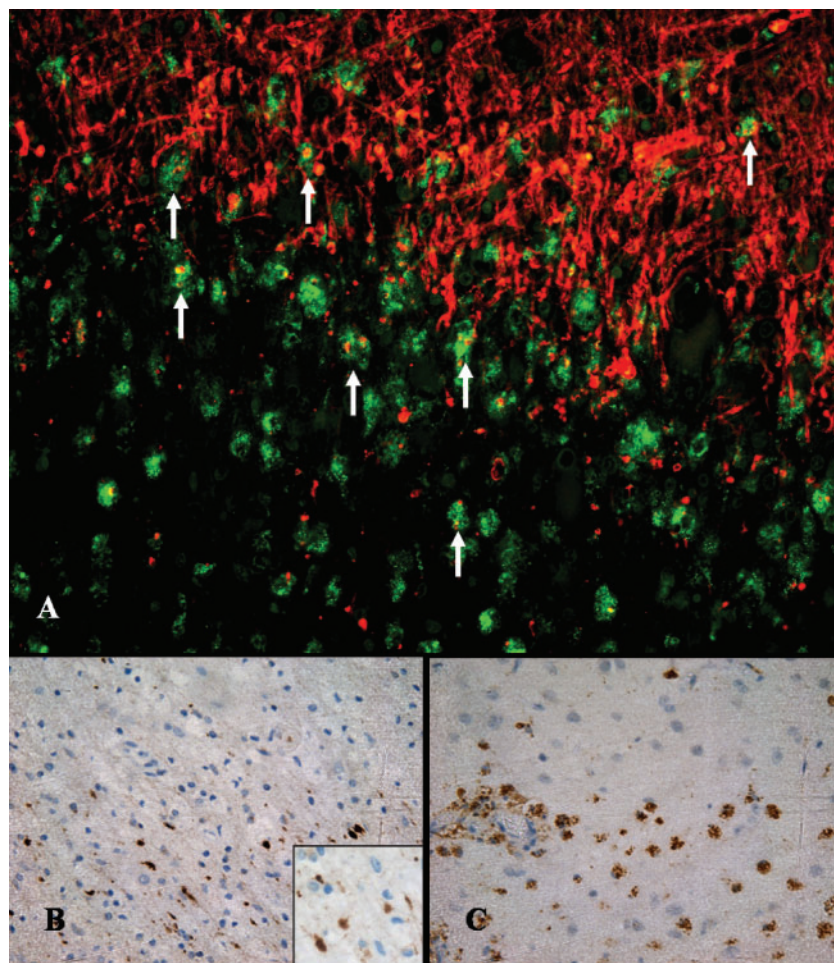
Fig. 2 Active demyelination and acute axonal damage. Double immunofluorescence for the myelin protein MBP (red) and the macrophage marker Ki-MIP (green) shows numerous macrophages with MBP-positive myelin degradation products (arrows), indicating an actively demyelinating lesion. High numbers of APP+ axons can be found at the lesion edge of active lesions (**B**). A acutely damaged axons are shown in higher magnification in the insert (**B**: APP staining). High numbers of macrophages as shown in **C** correlate with the acute axonal damage (KiMIP). Original magnifications: **A**: ×100; **B** and **C**: ×20.

Table 2 Staging of demyelinating and inflammatory activity in multiple sclerosis lesions

Case		I	II	III	IV	V	Sum
n Lesions		13	21	6	5	8	53
Demyelinating activity (lesion areas)	DM	7	16	5	5	8	41
	RM	4	10	2	2	2	20
Active		4	4	1	3	4	16
Inflammatory activity (lesions)	Chronic active	5	9	5	3	7	29
	Chronic inactive	8	12	1	2	1	24

DM = demyelinated lesion area; RM = remyelinated lesion area.

single lesions. Case 1 showed a median of 28 APP+ axons/mm^2 in the lesion centre (13 lesions examined) compared with the other cases with lower numbers (median of one to three APP+ axons/mm^2). APP positive axons were found in significantly higher numbers in active demyelinating lesion areas (median of 80 APP+ axons/mm^2) compared with inactive lesion areas (median of four APP+ axons/mm^2;

$P < 0.0001$). 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+

Table 3 Inflammatory cells per mm^2 or per vessel in the white matter plaques of the five investigated patients

Case (no. of investigated white matter plaques)	I (13)	II (21)	III (6)	IV (5)	V (8)	Median
T cells (CD3+) parenchyma	2.00 (0.32–16.73)	0.00 (0.00–2.29)	0.00 (0.00)	0.00 (0.00–0.32)	2.00 (1.60–17.60)	0.00
Cytotoxic T cells (CD8+) parenchyma	2.18 (0.00–8.32)	0.00 (0.00–0.89)	0.00 (0.00)	0.32 (0.00–0.80)	4.40 (0.80–12.48)	0.32
CD8/CD3 ratio parenchyma	1.00			1.00	1.00	1.00
Perivascular T cells	1.33 (0.00–8.86)	0.35 (0.00–1.42)	0.26 (0.05–0.41)	1.65 (0.75–2.00)	0.56 (0.17–1.44)	0.56
Perivascular cytotoxic T cells	1.00 (0.00–5.04)	0.30 (0.00–1.15)	0.28 (0.00–0.67)	0.44 (0.15–0.87)	0.77 (0.15–1.09)	0.44
CD8/CD3 ratio perivascular	0.75	0.86	1.00	0.27	1.00	0.86
B cells (CD20+ or CD79A+) parenchyma	0.00 (0.00–2.67)	0.00 (0.00–0.40)	0.00 (0.00–0.32)	0.00 (0.00)	n.a.	0.00
Perivascular B cells	0.05 (0.00–0.33)	0.00 (0.00–0.07)	0.00 (0.00–0.04)	0.00 (0.00)	n.a.	0.00
Plasma cells (CD138+) parenchyma	0.00 (0.00–1.07)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
Perivascular plasma cells	0.00 (0.00–0.07)	0.00 (0.00–0.20)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00

n.a. = not available.

Table 4 Macrophages/microglial cells and APP+ axons per mm^2 in active and inactive lesion areas

Case	I	II	III	IV	V	Median
n Active areas	4	4	1	3	4	
Macrophages/ active areas	265.60 (181.14–503.11)	215.82 (117.33–524.80)	209.60	240.00 (113.60–257.60)	575.20 (1.60–729.60)	240.00
Microglial cells/ active areas	29.59 (24.00–70.40)	124.45 (45.60–150.40)	136.00	91.20 (91.20–120.00)	31.20 (16.00–708.80)	91.20
APP+ axons/ active areas	211.23 (152.80–294.52)	80.34 (20.80–745.60)	62.40	3.20 (0.00–15.20)	217.60 (0.80–561.60)	80.34
n Inactive areas	11	26	7	7	10	
Macrophages/ inactive areas	14.93 (0.00–239.14)	23.60 (3.20–104.62)	33.40 (14.40–83.20)	19.20 (9.60–36.00)	16.00 (5.60–524.80)	19.20
Microglial cells/ inactive areas	78.40 (9.60–374.40)	68.00 (10.67–360.00)	105.40 (40.00–217.60)	105.60 (83.20–147.20)	92.80 (24.00–360.80)	92.80
APP+ axons/inactive areas	25.14 (8.35–35.66)	3.68 (0.00–31.20)	7.55 (1.07–10.29)	1.60 (0.00–3.20)	1.20 (0.00–28.80)	3.68

axons ranged from 15 to 745 APP+ axons/mm^2. 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/mm^2. 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
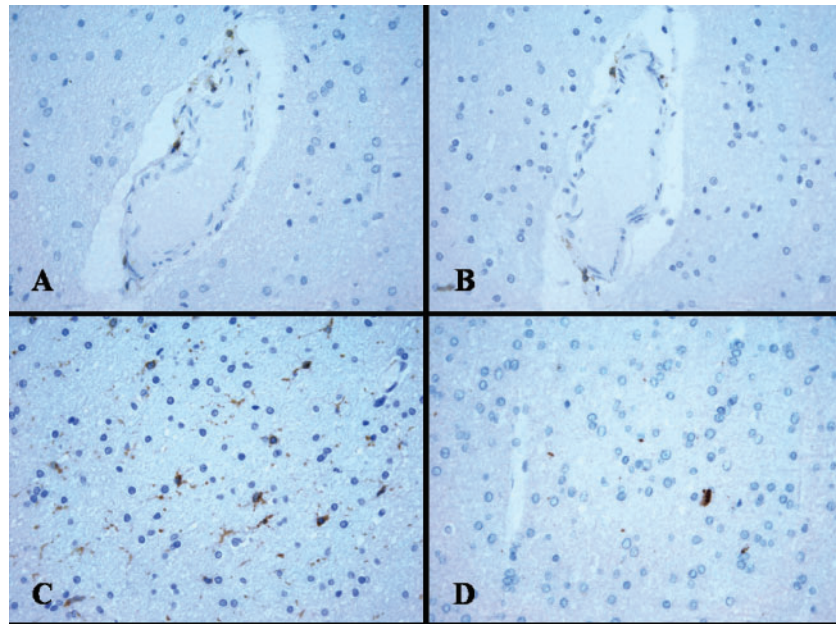
Fig. 3 Normal appearing white matter. The CD3-staining shows some T lymphocytes in the perivascular space (**A**). T lymphocytes are mainly cytotoxic T lymphocytes as shown with the CD8-staining (**B**). Activated microglial cells, positive for CR3/43, are found (**C**). Some axons are acutely damaged as seen in the APP -staining (**D**). Original magnifications: **A**–**D**: ×40.

Table 5 Inflammatory infiltrates and APP+ axons per mm^2 or per vessel in the NAWM

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

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.*,

2002). Surprisingly, we found no B cells, plasma cells or lymphoid follicle-like structures in our cases, although it is suggested that pretransplant plasma cells and, accordingly, possible pathogenetic antibodies persist in the CNS after extremely lymphoablative conditioning (Storek et al., 2004). Thus, the pathogenic role of plasma cells or antibody-mediated damage remains uncertain. With respect to quantitative data on inflammatory cells, the present study is clearly limited by the lack of adequate controls. It is impossible to find matching chronic archival multiple sclerosis autopsy cases fulfilling the same demographic, pathological and treatment (immunosuppressed versus non-immunosuppressed patients) variables. A comparison with existing data in the literature was therefore in our opinion the best possible procedure.

The histopathological correlate of the progressive brain atrophy and subsequent clinical progression seen after HSCT or in the Campath-1H trial may be ongoing demyelination and/or the acute axonal damage as reported in our study. Clinical progression after HSCT has not been documented in the patients studied here; this may be due to the fact that survival times were 2 months or less in three of the five cases presented here. Ongoing demyelination was proven in the present cases by the detection of LFB- and MBP-positive myelin degradation products in macrophages/microglia. These strict criteria for defining recent demyelinating activity are based on the sequence of myelin degradation in macrophages/microglia in EAE and multiple sclerosis lesions (Lassmann and Wisniewski, 1979; Brück et al., 1995). Recent in vitro data confirmed this suggestion that intracellular degradation of myelin proteins allows an estimation of the sequence of myelin degradation in multiple sclerosis lesions (van der Goes et al., 2005). Thus, the active demyelination observed in the present five cases has most probably occurred after AHSCT and is not a remnant of inflammatory attacks prior to transplantation. The same is true for ongoing acute axonal damage, although we cannot exclude ultimately that the lesions described here may have been initiated prior to AHSCT and have proceeded despite intense immunosuppression, especially in those cases with a short survival time after AHSCT. We observed a median number of 80 APP-positive axons/mm^2 in active demyelinating lesion areas. This compares to a mean of about 90 APP-positive axons/mm^2 in active demyelinating lesions from multiple sclerosis patients with a disease duration of 5–10 years (Kuhlmann et al., 2002). In experimental models, APP has been shown to be an early marker of axonal injury and APP immunoreactivity was positive up to 30 days after injury (Bramlett et al., 1997). Similar findings were reported in human tissues in which APP was used as a reliable marker of early axonal injury (Gentleman et al., 1993; McKenzie et al., 1996). APP immunoreactivity was present in adult trauma cases with a survival period of about 1 month but only rarely in patients with longer survival, indicating that APP-positive axons most likely represent recent damage (Ahlgren et al., 1996).

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 by T cells. The question is how long after T cell withdrawal (as here after AHSCT) do these cells lose their activation state or, on the contrary, possibly even adapt a 'memory' phenotype (Butovsky et al., 2005). It is possible that microglial cells/macrophages are committed earlier in the disease to a 'harmful' phenotype that persists over time and leads to continuing myelin and axonal damage even in the absence of T cells. Alternatively, microglia/macrophages could be 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

References

Ahlgren S, Li GL, Olsson Y. Accumulation of beta-amyloid precursor protein and ubiquitin in axons after spinal cord trauma in humans: immunohistochemical observations on autopsy material. Acta Neuropathol (Berl) 1996; 92: 49–55.

Bitsch A, Kuhlmann T, Stadelmann C, Lassmann H, Lucchinetti C, Brück W. A longitudinal MRI study of histopathologically defined hypointense multiple sclerosis lesions. Ann Neurol 2001; 49: 793–6.

Bitsch A, Schuchardt J, Bunkowski S, Kuhlmann T, Brück W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain 2000; 123: 1174–83.

Blanco Y, Saiz A, Carreras E, Graus F. Autologous haematopoietic-stem-cell transplantation for multiple sclerosis. Lancet Neurol 2005; 4: 54–63.

Bö L, Mörk S, Kong PA, Nyland H, Pardo CA, Trapp BD. Detection of MHC class II-antigens on macrophages and microglia, but not on astrocytes and endothelia in active multiple sclerosis lesions. J Neuroimmunol 1994; 51: 135–46.

Bramlett HM, Kraydieh S, Green EJ, Dietrich WD. Temporal and regional patterns of axonal damage following traumatic brain injury: a beta-amyloid precursor protein immunocytochemical study in rats. J Neuropathol Exp Neurol 1997; 56: 1132–41.

Brück W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA et al. Monocyte/macrophage differentiation in early multiple sclerosis lesions. Ann Neurol 1995; 38: 788–96.

Butovsky O, Talpalar AE, Ben-Yaakov K, Schwart M. Activation of microglia by aggregated ß-amyloid or lipopolysaccharide impairs MHC-II expression and renders them cytotoxic whereas IFN-gamma and IL-4 render them protective. Mol Cell Neurosci 2005; 29: 381–93.

Chen JT, Collins DL, Atkins HL et al. Brain atrophy after immunoablation and stem cell transplantation in multiple sclerosis. Neurology 2006; 66: 1935–7.

Coles AJ, Cox A, Le Page E, Jones J, Trip SA, Deans J et al. The window of therapeutic opportunity in multiple sclerosis. Evidence from monoclonal antibody therapy. J Neurol 2005; 253: 98–108.

Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. Brain 1997; 120: 393–9.

Gentleman SM, Nash MJ, Sweeting CJ, Graham DI, Roberts GW. b-Amyloid precursor protein (bAPP) as a marker for axonal injury after head injury. Neurosci Lett 1993; 160: 139–44.

Inglese M, Mancardi GL, Pagani E, Rocca MA, Murialdo A, Saccardi R et al. Brain tissue loss occurs after suppression of enhancement in patients with multiple sclerosis treated with autologous haematopoietic stem cell transplantation. J Neurol Neurosurg Psychiatry 2004; 75: 643–4.

Kerschensteiner M, Stadelmann C, Dechant G, Wekerle H, Hohlfeld R. Neurotrophic cross-talk between the nervous and immune systems: Implications for neurological diseases. Ann Neurol 2003; 53: 292–304.

Kornek B, Storch MK, Weissert R, Wallstroem E, Stefferl A, Olsson T et al. Multiple sclerosis and chronic autoimmune encephalomyelitis. A comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. Am J Pathol 2000; 157: 267–76.

Kuhlmann T, Lingfeld G, Bitsch A, Schuchardt J, Brück W. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. Brain 2002; 125: 2202–12.

Kutzelnigg A, Lucchinetti CF, Stadelmann C, Brück W, Rauschka H, Bergmann M et al. Cortical demyelination and diffuse white matter injury in multiple sclerosis. Brain 2005; 128: 2705–12.

Lassmann H, Wisniewski HM. Chronic relapsing experimental allergic encephalomyelitis: morphological sequence of myelin degradation. Brain Res 1979; 169: 357–68.

McKenzie KJ, McLellan DR, Gentleman SM, Maxwell WL, Gennarelli TA, Graham DI. Is beta-APP a marker of axonal damage in short-surviving head injury? Acta Neuropathol (Berl) 1996; 92: 608–13.

Medana I, Martinic MA, Wekerle H, Neumann H. Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes. Am J Pathol 2001; 159: 809–15.

Openshaw H, Lund BT, Kashyap A, Atkinson R, Sniecinski I, Weiner LP et al. Peripheral blood stem cell transplantation in multiple sclerosis with busulfan and cyclophosphamide conditioning: report of toxicity and immunological monitoring. Biol Blood Marrow Transplant 2000; 6: 563–75.

Pierce JE, Trojanowski JQ, Graham DI, Smith DH, McIntosh TK. Immunohistochemical characterization of alterations in the distribution of amyloid precursoe proteins and beta-amyloid peptide after experimental brain injury in the rat. J Neurosci 1996; 16: 1083–90.

Prineas JW, Kwon EE, Cho E-S, Sharer LR, Barnett MH, Oleszak EL et al. Immunopathology of secondary-progressive multiple sclerosis. Ann Neurol 2001; 50: 646–57.

Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. Brain Pathol 2004; 14: 164–74.

Storek J, Zhao Z, Lin E, Berger T, McSweeney PA, Nash RA et al. Recovery from and consequences of severe iatrogenic lymphopenia (induced to treat autoimmune diseases). Clin Immunol 2004; 113: 285–98.

Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. New Engl J Med 1998; 338: 278–85.

Traugott U, Reinherz EL, Raine CS. Multiple sclerosis. Distribution of T cells, T cell subsets and Ia-positive macrophages in lesions of different ages. J Neuroimmunol 1983; 4: 201–21.

Ulvestad E, Williams K, Mork S, Antel J, Nyland H. Phenotypic differences between human monocytes/macrophages and microglial cells studied in situ and in vitro. J Neuropathol Exp Neurol 1994; 53: 492–01.

van Bekkum DW. Preclinical experiments. Best Pract Res Clin Haematol 2004; 17: 201–22.

van der Goes A, Boorsma W, Hoekstra K, Montagne L, De Groot CJ, Dijkstra CD. Determination of the sequential degradation of myelin proteins by macrophages. J Neuroimmunol 2005; 161: 12–20.