Inhibiting poly(ADP-ribose) polymerase: a potential therapy against oligodendrocyte death

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Oligodendrocyte loss and demyelination are major pathological hallmarks of multiple sclerosis. In pattern III lesions, inflammation is minor in the early stages, and oligodendrocyte apoptosis prevails, which appears to be mediated at least in part through mitochondrial injury. Here, we demonstrate poly(ADP-ribose) polymerase activation and apoptosis inducing factor nuclear translocation within apoptotic oligodendrocytes in such multiple sclerosis lesions. The same morphological and molecular pathology was observed in an experimental model of primary demyelination, induced by the mitochondrial toxin cuprizone. Inhibition of poly(ADP-ribose) polymerase in this model attenuated oligodendrocyte depletion and decreased demyelination. Poly(ADP-ribose) polymerase inhibition suppressed c-Jun N-terminal kinase and p38 mitogen-activated protein kinase phosphorylation, increased the activation of the cytoprotective phosphatidylinositol-3 kinase-Akt pathway and prevented caspase-independent apoptosis inducing factor-mediated apoptosis. Our data indicate that poly(ADP-ribose) polymerase activation plays a crucial role in the pathogenesis of pattern III multiple sclerosis lesions. Since poly(ADP-ribose) polymerase inhibition was also effective in the inflammatory model of multiple sclerosis, it may target all subtypes of multiple sclerosis, either by preventing oligodendrocyte death or attenuating inflammation.

Keywords: poly(ADP-ribose) polymerase; multiple sclerosis; cuprizone; demyelination; oligodendrocyte apoptosis; Akt; AIF; JNK

Abbreviations: 4HQ = 4-Hydroxyquinazoline; AIF = apoptosis-inducing factor; DAPI = 4',6'-diamidino-2-phenylindole; ERK1/2 = extracellular signal-regulated kinase 1/2; JNK = c-Jun N-terminal kinase; MAG = myelin-associated glycoprotein; MAPK = mitogen-activated protein kinase; MBP = myelin basic protein; PARP = poly(ADP-ribose) polymerase
Introduction

Multiple sclerosis is a chronic disease of the central nervous system that is characterized by a presumed autoimmune inflammation, demyelination and axonal degeneration (Noseworthy et al., 2000). Although immunomodulatory treatments are available to counteract the common inflammatory pathology, no treatments exist to prevent demyelination, which may contribute to axonal degeneration, the best pathological correlate of clinical disability in multiple sclerosis (Naismith and Cross, 2005).

While destruction of myelin develops in association with inflammation, in the earliest lesions of pathological subtypes for patterns III and IV, apoptosis-like depletion of oligodendrocytes has been described, suggesting degenerative processes (Lucchinetti et al., 2000). An alternative hypothesis to the heterogeneous pathogenesis of multiple sclerosis even proposes that oligodendrocyte apoptosis represents the first and earliest stage of all lesions, resulting in primary demyelination that unmasks tissue antigens and secondary autoimmune inflammation (Barnett and Prineas, 2004). Depletion of oligodendrocytes then occurs progressively during lesion evolution (Frohman et al., 2006).

Recently, mitochondrial dysfunction has been suggested to play a role in the loss of oligodendrocytes and axons in multiple sclerosis (Kalman et al., 2007). Fulminate multiple sclerosis lesions with profound oligodendrocyte apoptosis (pattern III) reveal a pattern of hypoxia-like tissue injury, which seems to be induced by a dysfunction in complex IV of the respiratory chain (Lucchinetti et al., 2000; Aboul-Enein et al., 2003; Mahad et al., 2008). In such multiple sclerosis lesions, oligodendrocyte apoptosis follows a caspase-independent pathway (Aboul-Enein et al., 2003; Barnett and Prineas, 2004).

A non-inflammatory experimental primary demyelination, induced by a copper chelator cuprizone in weanling mice, results in multi-focal demyelination and loss of oligodendrocytes in particular brain areas, mainly the corpus callosum and superior cerebellar peduncle (Matsushima and Morell, 2001). A mitochondrial aetiology was assumed since giant mitochondria have been observed in the liver of cuprizone-treated mice (Suzuki, 1969). Supporting this notion, increased production of reactive oxygen species and decreased activity of various complexes of the respiratory chain were found in the mitochondria of cuprizone-treated oligodendroglia cells (Pasquini et al., 2007). However, in contrast to experimental autoimmunity encephalomyelitis, the number of T cells is negligible in the demyelinated corpus callosum and T cell activation has not been observed in the cuprizone model (Remington et al., 2007).

Impaired functioning of the mitochondrial respiratory chain results in excessive production of reactive oxygen species, which cause damage to various cellular components including DNA (Turrens, 2003). The nuclear enzyme poly(ADP-ribose) polymerase (PARP) functions as a DNA damage sensor and signalling molecule, which forms long branches of ADP-ribose polymers on a number of nuclear target proteins, including itself (Alano et al., 2004). Extensive DNA damage triggers overactivation of PARP, eventually resulting in cell dysfunction and death (Alano et al., 2004).

Additionally, PARP activity appears to be essential for the mitochondria-to-nucleus translocation of apoptosis-inducing factor (AIF), supporting the hypothesis that nuclear mitochondrial crosstalk dependent on poly(ADP-ribose)ation is critical in determining the fate of injured cells (Yu et al., 2002). This crosstalk is supposed to involve a PARP-dependent activation of c-Jun N-terminal kinase (JNK) and the cytoprotective phosphinositol-3 kinase-Akt pathway (Tapodi et al., 2005; Xu et al., 2006). Furthermore, PARP has been shown to function as a co-activator in the nuclear factor-κB-mediated transcription, regulating the expression of various pro-inflammatory proteins (Oliver et al., 1999).

PARP-mediated cell death and inflammation has been implicated in the pathogenesis of several central nervous system diseases (Kauppinen and Swanson, 2007). Inhibition of PARP activity reduced brain injury in ischaemia reperfusion and excito-toxicity (Endres et al., 1997; Mandir et al., 2000). It was also able to ameliorate inflammation in experimental autoimmune encephalomyelitis, the autoimmune model of multiple sclerosis (Scott et al., 2004).

Considering similar observations suggesting mitochondrial pathology and sparse inflammation in both the cuprizone model and pattern III multiple sclerosis (Lucchinetti et al., 2000; Aboul-Enein et al., 2003; Pasquini et al., 2007; Mahad et al., 2008), the focus of this study was (i) to reveal PARP activation in multiple sclerosis plaques and the cuprizone-mediated non-inflammatory primary demyelination model of multiple sclerosis; (ii) to determine whether the inhibition of PARP could exert a protective effect against experimental demyelination; and (iii) to determine the underlying mechanisms.

Materials and methods

Cuprizone model and the administration of PARP inhibitor

C57BL/6 male mice were purchased from Charles River Laboratories Magyarország Kft (Isaszeg, Hungary) and kept under standardized, specific pathogen-free circumstances. Starting at 8 weeks of age, mice received a diet of powdered rodent chow containing 0.2% cuprizone (bis-cyclohexanone oxalidihydrazone) (Sigma, Steinheim, Germany) by weight for 3, 5 and 6 weeks ad libitum to induce demyelination, as described previously (Hiremath et al., 1998). The PARP inhibitor 4-hydroxyquinazoline (4HQ, Sigma-Aldrich, Steinheim, Germany) (Banasik et al., 1992) was administered i.p. at a dose of 100 mg/kg body mass and a volume of 10 μl/g body mass every day (Veres et al., 2004), starting on the same day as the cuprizone treatment. Control mice received the same volume (10 μl/g) of saline solution instead of 4HQ. In order to follow the systemic effect of cuprizone, the weights of the mice were measured every week (Hiremath et al., 1998).

All animal experiments were carried out under legislation [1998/ XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration and Decree in Scientific Procedures of Animal Experiments (243/1998)] in laboratories in the University of Pecs. Licensing of procedures was controlled by The Committee on Animal
MRI and quantitative neuroimaging

At the beginning of treatment, and from the third week, mice were anaesthetized weekly by intraperitoneal injection of diazepam (5 mg/kg) and ketamine (80 mg/kg) (both purchased from Gedeon Richter Plc, Budapest, Hungary). The animals were then secured in an epoxy resin animal holder tube (Doty Scientific Inc., Columbia, SC, USA) custom modified to accommodate the tip of teeth and position the eyes of each animal in the same location −5.0 ± 0.5 mm above the isocentre of the magnet. A glass capillary filled with water:glycerol = 9:1 mixture was placed near the head of the animal, serving as an external signal intensity reference. Magnetic resonance images were obtained exactly as described before (Veres et al., 2003, 2004). The extent of demyelination in the corpus callosum was determined by calculating the mean signal intensity of the corpus callosum divided by the mean signal intensity of the reference capillary. The mean signal intensities were determined by freehand delineation of regions of interest in the corpus callosum or the reference capillary on coronal cross-sectional images exactly 1 mm posterior from the bregma by an investigator blind to the experiment.

Histopathology

After 5 weeks of treatment mice were terminally anaesthetized with intraperitoneally administered diazepam and ketamine and perfused via the left ventricle with 4% paraformaldehyde in a phosphate buffer containing picric acid. After overnight postfixation in the same fixative, brains were dissected. Brains were embedded in paraffin before histological analysis, and then 8 µm coronal sections were obtained at the level of 161, 181, 209 and 221 (Sidman, 1971). Demyelination was evaluated using luxol fast blue staining with cresyl violet. Scoring on a scale of 0–3 was performed by three independent investigators. A score of 0–3 was observed for each myelin status of a mouse not treated with cuprizone, whereas 3 indicated complete loss of myelin. The mean scores of coronal sections of the corpus callosum from four different regions stained with luxol fast blue-cresyl violet were calculated and the averages scores were used for statistical analysis.

Immunocytochemistry and confocal laser fluorescence microscopy for poly(ADP-ribose) and apoptosis-inducing factor in multiple sclerosis lesions

Poly(ADP-ribose) and AIF expression was studied in the lesions of 13 patients with multiple sclerosis and 5 control cases without neurological disease or brain lesions. The multiple sclerosis sample contained six cases with acute multiple sclerosis (Marburg, 1906), one case with relapsing remitting multiple sclerosis and six cases with chronic progressive multiple sclerosis (Table 1).

Lesion areas within the sections were defined according to activity: early pattern III lesions showed loss of myelin-associated glycoprotein (MAG), oligodendrocyte apoptosis and predominant infiltration by activated microglia; late active/inactive lesions in pattern III multiple sclerosis cases were densely infiltrated by macrophages with a variable content of myelin degradation products; normal appearing white matter areas were at least 1 cm apart from the active lesions. In pattern II lesions, early stages revealed scattered infiltration of the tissue with macrophages and activated microglia; myelin sheaths were still present, but showed signs of acute dissolution. In late active/inactive pattern II lesions, myelin was completely lost and macrophages contained myelin degradation products at various stages of chemical myelin disintegration. The lesions in patients with progressive multiple sclerosis were slowly expanding lesions with a small rim of active demyelination (early lesions) with microglia activation and some macrophages containing the earliest stages of myelin degradation. The late active/inactive lesion centres were completely demyelinated and contained a variable, but generally low amount of macrophages with myelin degradation products.

Active lesions following pattern III (Lucchinetti et al., 2000) were seen in four cases with acute multiple sclerosis, pattern II lesions were analysed in two cases of acute multiple sclerosis and one case with relapsing-remitting multiple sclerosis, and slowly expanding active lesions were present in six cases with progressive multiple sclerosis (Kutzelnigg et al., 2005).

Immunocytochemistry was performed on paraffin sections as described before (Marik et al., 2007) without antigen retrieval. Poly(ADP-ribose) antibody was purchased from Alexis Biotechnology, London, UK, and the AIF antibody from Chemikon International. For lesion characterization we used immunocytochemistry with antibodies against MAG, myelin oligodendrocyte glycoprotein, proteolipid protein, cyclic nucleotide phosphodiesterase and CD68, as described previously (Marik et al., 2007).

Fluorescence immunohistochemistry was performed on paraffin sections as described earlier (Bauer et al., 2007) with some modifications. Staining with primary antibody poly(ADP-ribose) or AIF was done overnight. As a second step, sections were incubated with a secondary biotinylated-anti-mouse antibody (Amersham Pharmacia Biotech; 1:200). This was followed by antigen retrieval by 60 min incubation in a plastic coplin jar filled with citrate buffer (0.01 M, pH 6.0) in a household food steamer device (MultiGourmet FS 20, Braun, Kronberg/Taunus, Germany). This first staining was finished with application of streptavidin-Cy2 (Jackson ImmunoResearch, West Grove, PA; 1:75) for 1 h at room temperature. After washing in tris-buffered saline, the sections were incubated overnight with anti-carbonic anhydrase II (The Binding Site Ltd, Birmingham, UK, for detection of oligodendrocytes). This was followed by washing and incubation with secondary Cy3-conjugated antibodies (Jackson ImmunoResearch). The staining was finished with 4’,6-diamidino-2-phenylindole (DAPI) (Sigma) counterstain. Sections were examined using a confocal laser scan microscope (Leica SP5, Mannheim, Germany). Recordings for Cy2 (excited with the 488 nm laser) and Cy3 (excited with the 543 nm laser) were done simultaneously and followed by recording for DAPI with a 405 nm laser.

Quantification of cells with poly(ADP-ribose) and AIF immunoreactivity

Poly(ADP-ribose)-positive cells were defined as cells with strong poly(ADP-ribose) immunoreactivity within the nuclei as well as in the cytoplasm, including cell processes; in the majority of these cells,
Confocal images were collected using an Olympus Fluoview FV-1000 laser scanning confocal imaging system and an Olympus HardSet Mounting Medium with DAPI, Vector Laboratories). Alexa 488 goat anti-rabbit secondary antibody (1:200, Molecular Probes) was applied for visualization. For immunofluorescent labelling, Alexa 488 goat anti-rabbit secondary antibody (1:200, Molecular Probes) and 3,3'-diaminobenzidine reaction were used for visualization. For quantification of AIF expression only those cells were counted that showed unequivocal immunoreactivity within their nuclei (Fig. 1P, Q and W).

Cells were counted manually (HL) in each of the above-defined areas in seven microscopic fields of 0.27 mm² each. The values given in Table 2 represent cells/mm².

### Immunohistochemistry, immunofluorescence and confocal microscopy of cuprizone lesions

Formation of poly(ADP-ribose)-positive nuclei appeared condensed and, in part, fragmented, suggesting apoptosis. In addition cytoplasmic poly(ADP-ribose) reactivity revealed signs of cell degeneration consistent with in part fragmented cell processes and cytoplasmic vacuolization (Fig. 1K, L, V, X and Y). For quantification of AIF expression only those cells were counted that showed unequivocal immunoreactivity within their nuclei (Fig. 1P, Q and W).

Cells were counted manually (HL) in each of the above-defined areas in seven microscopic fields of 0.27 mm² each. The values given in Table 2 represent cells/mm².

### Immunoblot analysis

Tissue samples were taken from animals killed after 3 or 5 weeks of treatment. Corpus callosum of the mice were carefully dissected and 25 mg of the tissue was homogenized in ice-cold 10 mM tris buffer, pH 7.4 [containing 0.5 mM sodium metavanadate, 1 mM ethylenedia-

minetetraacetic acid and protease inhibitor cocktail (1:200); all purchased from Sigma-Aldrich, Steinheim, Germany]. Homogenates (10 μg each) were loaded onto 10 and 12% sodium dodecyl sulphate polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes. The following antibodies were used: anti-MBP (1:1000) (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), anti-poly(ADP-ribose) (1:1000) (Alexis Biotechnology, London, UK), anti-AIF (1:330) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-Akt (Ser473) (1:1000) (R&D Systems, Minneapolis, MN, USA), anti-caspase-3 (1:1000), anti-nonphosphorylated Akt/protein kinase B (1:1000), anti-extracellular signal-regulated kinase 1/2 (ERK1/2) (Thr183/Tyr185) (1:1000), anti-phospho JNK (Thr183/Tyr185) (1:1000), anti-caspase-3 (1:1000) (all from Cell Signalling Technology,
Beverly, MA, USA), anti-phospho-p38-mitogen-activated protein kinase (MAPK) (Thr^{180}/Tyr^{182}) (1:1000) and anti-actin (1:10 000) (both from Sigma-Aldrich, Steinhein, Germany). Appropriate horseradish peroxidase-conjugated secondary antibodies were used at a 1:5000 dilution (anti-mouse and anti-rabbit IgGs; Sigma-Aldrich, Steinheim, Germany) and visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). Films were scanned and the pixel volumes of the bands were determined using National Institutes of Health Image J software (Bethesda, MD, USA).

**Caspase-3 activity assay**

Carefully dissected corpus callosum samples (20 mg) from animals treated for 3 weeks were homogenized in the lysis buffer (50 mM

**Figure 1** Activation of poly(ADP-ribose) polymerase (PAR) and nuclear translocation of AIF in different types of multiple sclerosis lesions. (A–I) Neuropathological characterization of multiple sclerosis pattern III lesions. (A) Hemispheric brain section of patient P III C, stained by immunocytochemistry for macrophages/microglia (CD68), shows multiple active lesions within the brain; the asterisk labels the lesion shown in Figs B–L (×0.3). Active pattern III lesion stained for myelin/oligodendrocyte glycoprotein (B) and MAG (C) showing loss of both myelin proteins in the centre of the lesion; in the very early lesion stages (+) MAG is completely lost from the lesion, while myelin/oligodendrocyte glycoprotein expression is partly preserved (×1.2). (D) Very early stage of pattern III lesion shows partial loss of myelin (stained for proteolipid protein), however, the inflamed vessels are surrounded by rim of preserved myelin (×40). (E) Within the active lesion oligodendrocytes that are stained for cyclic nucleotide phosphodiesterase show condensed nuclei reminiscent of apoptosis (×1200). Staining for CD68 shows only few activated microglia in the normal appearing white matter (F), a profound increase of activated microglia in early lesions showing selective loss of MAG (G); profound infiltration of the tissue with macrophages in late active portions of the lesion (H) and mainly perivascular accumulation of macrophages in the inactive lesion centre (I) (×200). Poly(ADP-ribose) expression in different lesion stages from the case shown in (A–I); in the normal appearing white matter (J) there is faint brown nuclear staining of glial cells; the nuclei are counterstained with haematoxylin (blue); in the area of MAG loss (the ‘+’ indicates the location of the area in (B), numerous cells are seen with intense nuclear and cytoplasmic reactivity for poly(ADP-ribose) (K); higher magnification of the cells in (L), shows different examples of poly(ADP-ribose) positive glial cells with dark condensed nuclei and partial cytoplasmic or cell process dissolution; the lesion centre (M) shows weak brown immunoreactivity in some nuclei, similar to that seen in the normal-appearing white matter (×200; inserts ×1200). AIF expression in similar lesion areas of the same case shown for poly(ADP-ribose) before; (N, O) purely mitochondrial AIF expression in the normal appearing white matter; (P, Q) in the early active (MAG loss) lesions AIF is seen not only in mitochondria, but also in nuclei; (R) in the inactive lesion centre AIF is only present in mitochondria (×200; inserts ×1200). Poly(ADP-ribose) and AIF expression in a slowly expanding lesion in progressive multiple sclerosis (ChMS D); (S) shows the location of the lesion in the subcortical white matter (×4) and (T) documents the hypercellular margin of the lesions with some macrophages with recent myelin degradation products (×100); no poly(ADP-ribose) expression was seen in the normal appearing white matter (U); however, there is a moderate number of small oligodendrocyte like glia cells with strong poly(ADP-ribose) reactivity within condensed nuclei and cell processes (V); the + labels the active lesion area in (S), (V) and (W). (W) AIF expression is enriched in the area of active lesion expansion (+); in the majority of the cells AIF is seen as cytoplasmic granules, representing mitochondria (upper insert), but there is also AIF reactivity in nuclei of cells, resembling oligodendrocytes (lower insert) (×200).
we demonstrated accumulation of the enzyme’s product by using lesions and control brains using Scheffe’s respective values found in normal white matter of control brains.

Statistics

The density of poly(ADP-ribose) and AIF-positive cells (Table 2) in each group of multiple sclerosis patients was compared with the respective values found in normal white matter of control brains using Scheffe’s post hoc ANOVA test; heteroscedasticity was minimized with the logarithmic transformation. The repeated body weight measurements were analysed using a random intercept fixed slope linear model considering a common distribution of initial weights but separate slopes for the treatment groups. Relative corpus callosum MRI signal intensities in the treatment groups were compared with a mixed effect analysis of variance where individuals were modelled as random effects. The histological degrees of corpus callosum myelination were compared using the non-parametric Mann–Whitney test. The immunoblot band intensities in the four treatment groups were normalized to the loading control and compared pairwise using Scheffe’s post hoc ANOVA test; heteroscedasticity was minimized with the logarithmic transformation. Differences were considered significant at values of $P<0.05$ or lower.

Results

PARP activation in multiple sclerosis lesions and control brains

In order to determine PARP activation in multiple sclerosis lesions, we demonstrated accumulation of the enzyme’s product by using anti-poly(ADP-ribose) immunofluorescence or immunohistochemistry. We observed very strong poly(ADP-ribose) reactivity in the nucleus and cytoplasm of single cells. This was most pronounced in patients with acute multiple sclerosis, in active lesions showing the characteristic pathological hallmarks of pattern III demyelination and containing high numbers of apoptotic oligodendrocytes, (Fig. 1A–I). The expression was seen in cells that, by the anatomy of their processes, mainly resembled oligodendrocytes (Fig. 1J–M and Fig. 2A and B). They contained a condensed, sometimes fragmented nucleus and their cytoplasm revealed, in part, fragmented cell processes or swelling and focal vacuoles (Fig. 1L). In addition, a few cells with astrocyte or macrophage morphology also showed strong poly(ADP-ribose) immunoreactivity. On the other hand, both multiple sclerosis and control tissue demonstrated weak-to-moderate labelling of nuclei for poly(ADP-ribose) (Fig. 1J and M). This was highly variable between cases, and independent of lesions in multiple sclerosis tissue. The observation of variable and moderate PARP activation in post-mortem tissues may reflect agonal events.

Quantitative analysis confirmed that poly(ADP-ribose) reactive glia cells were enriched in areas of initial and active myelin breakdown of pattern III lesions, as defined before (Marik et al., 2007) (Table 2). Similar poly(ADP-ribose) reactive oligodendrocytes, although in lower numbers, were also seen at the active edge of slowly expanding lesions in progressive multiple sclerosis (Fig. 1S–V) and in lowest numbers in patients with pattern II lesions (Table 2). Double staining and confocal laser-scanning microscopy confirmed that the majority of cells with strong poly(ADP-ribose) immunoreactivity also expressed the oligodendrocyte marker carbonic anhydrase II (Fig. 2C, E–H), but that scattered cells also co-expressed poly(ADP-ribose) with either glial fibrillary acidic protein (Fig. 2D) or CD68 (data not shown). Poly(ADP-ribose) reactivity in oligodendrocytes exceeded that of astrocytes both in number of positive cells and intensity of the staining (Fig. 1K, L, V and Fig. 2A, B).

Nuclear translocation of AIF in pattern III multiple sclerosis lesions

Since AIF is essential in mediating PARP-dependent cell death (Yu et al., 2002), we examined its expression in multiple sclerosis lesions. AIF reactivity in the normal brain, and with some exceptions in the normal appearing white matter of multiple sclerosis patients (Table 2), was confined to the mitochondria of neurons and glia cells (Fig. 1N, O). In multiple sclerosis lesions, AIF reactivity in mitochondria was enhanced (Fig. 1P–R, W) and seen not only in neurons and glia but also in macrophages. Within initial and active areas of multiple sclerosis pattern III lesions and much less in other active multiple sclerosis lesions (Table 2), we found a variable number of glia cells with nuclear AIF reactivity (Fig. 1P, Q and W) co-localized with increased anti-poly(ADP-ribose) staining in condensed nuclei, showing features of apoptosis (Fig. 2I–L). These data suggested that activation of PARP may result in AIF-mediated oligodendrocyte death in pattern III multiple sclerosis lesions.

Table 2 Poly(ADP-ribose) and nuclear AIF expression in multiple sclerosis

<table>
<thead>
<tr>
<th>Samples</th>
<th>MS III</th>
<th>MS II</th>
<th>Progressive</th>
<th>Controls</th>
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<tr>
<td>Cases</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>PAR early</td>
<td>71.4 ± 27.0***</td>
<td>1.6 ± 1.7</td>
<td>15.3 ± 9.2**</td>
<td>n.a.</td>
</tr>
<tr>
<td>PAR IA</td>
<td>22.2 ± 17.9**</td>
<td>0.2 ± 0.4</td>
<td>4.6 ± 7.3</td>
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<tr>
<td>PAR NWMP</td>
<td>5.0 ± 1.4</td>
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<td>1.4 ± 1.4</td>
<td>0.6 ± 0.7</td>
</tr>
<tr>
<td>AIF early</td>
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<td>11.9 ± 5.0***</td>
<td>n.a.</td>
</tr>
<tr>
<td>AIF IA</td>
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<td>3.3 ± 3.8</td>
<td>n.a.</td>
</tr>
<tr>
<td>AIF NWMP</td>
<td>4.9 ± 2.5***</td>
<td>1.0 ± 1.7</td>
<td>2.2 ± 2.6</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Quantitative analysis of cells with poly(ADP-ribose) immunoreactivity and nuclear AIF expression in glial cells in different types of multiple sclerosis lesions. The cases are identical to those described in Table 1; MS III = multiple sclerosis patients with pattern III lesions; MS II = multiple sclerosis cases with pattern II lesions; progressive = multiple sclerosis cases with slowly expanding lesions of progressive multiple sclerosis; PAR = poly(ADP-ribose); NWMP = normal white matter. The numbers represent cells with positive immunoreactivity/mm². Mean ± SD in normal white matter, in early lesions of multiple sclerosis pattern III cases or at sites of initial myelin destruction in pattern II lesions or slowly expanding lesions in progressive multiple sclerosis (early), and in the centre of the lesions that still contained myelin with poly(adenosine diphosphate) degradation products at different stages of digestion (IA). Significant difference from respective control normal white matter values was indicated *P<0.05, **P<0.01 and ***P<0.001.

Tris, pH 8) containing protease inhibitor cocktail (Sigma-Aldrich, Steinheim, Germany). Fluorometric assays were performed using fluorescent-labelled peptide substrate for caspase-3 (Ac-DEVD-AFC, Sigma-Aldrich, St Louis, MO, USA) and a fluorescence plate reader set at 360 nm excitation and 460 nm emission, as recommended by the manufacturer.
Cuprizone enhances PARP activation in the corpus callosum

In order to investigate the effect of PARP inhibition on experimental demyelination, we first examined the activation of PARP on cuprizone treatment. Cuprizone induced auto-poly(ADP-ribosyl)ation, i.e. activation of PARP in corpus callosum of mice after 3 weeks of treatment (\(P < 0.05\)) (Fig. 3A). Expression of poly(ADP-ribose) immunoreactivity in the apoptotic nuclei of oligodendrocytes was confirmed by confocal laser microscopy (Fig. 3B and C). In addition, 4HQ—a potent inhibitor of the enzyme (Banasik et al., 1992)—blocked both cuprizone induced and basal auto-poly(ADP-ribose)ylation at a dose of 100 mg/kg used throughout this study (\(P < 0.05\)) (Fig. 3A). This dose of 4HQ was previously found to be effective and devoid of any apparent toxic effect (Veres et al., 2004).

PARP inhibitor prevents weight loss, the systemic effect of cuprizone

Cuprizone caused weight loss in comparison to the control group (\(P < 0.001\)), which was effectively prevented by simultaneous administration of the PARP inhibitor (\(P < 0.001\)). 4HQ alone did not affect the growth rate (\(P = 0.28\)) (Fig. 4).

PARP inhibition protects against cuprizone-induced demyelination in the brain

Examination of the brain was performed by non-invasive in vivo MRI. In untreated mice, corpus callosum appeared hypointense on \(T_2\)-weighted images. Upon cuprizone feeding, \(T_2\)-weighted images of corpus callosum showed hyperintensity corresponding to demyelination (Merkler et al., 2005), which was most pronounced after 4 weeks. PARP inhibitor prevented cuprizone-induced hyperintensities in the corpus callosum (Fig. 5A).

Serial, quantitative neuroimaging indicated significant demyelination of the corpus callosum with cuprizone feeding after 3 weeks up to 6 weeks, which was most pronounced after 4 weeks of treatment and decreased thereafter. Inhibition of PARP prevented demyelination at all time points. When applied alone, 4HQ did not cause any changes in signal intensities (Fig. 5A).

Pathological analysis with luxol fast blue-cresyl violet staining revealed a profound demyelination in the corpus callosum of cuprizone-fed mice (Fig. 5B). According to a semi-quantitative histological analysis, 4HQ reduced the cuprizone-induced demyelination (\(P < 0.001\)) (data not shown). 4HQ alone did not affect myelination.

Quantitative MBP immunoblotting revealed decreased MBP expression after 5 weeks of cuprizone feeding (\(P < 0.01\)), which was reversed by the PARP inhibitor 4HQ (\(P < 0.05\)). The administration of the PARP inhibitor alone did not affect the MBP level (Fig. 5C). Similar results were found by MBP immunohistochemistry (data not shown).

Cuprizone induces caspase-independent AIF-mediated cell death, which is diminished by PARP-inhibition

Parallel to demyelination, we observed elevated expression of AIF in the corpus callosum of mice treated with cuprizone for 3 weeks,
an effect that was attenuated by 4HQ (Fig. 6A). Besides elevating its expression, cuprizone induced nuclear translocation of AIF. In cuprizone-treated mice, numerous cells showing typical shape and arrangement of oligodendrocytes gave strong nuclear anti-AIF immunostaining in the midline and cingular part of the corpus callosum, which were prevented by the PARP inhibitor (Fig. 6B–D). In contrast, cuprizone did not induce caspase-dependent cell death, as revealed by the absence of procaspase-3 cleavage determined by immunoblotting and a fluorescent caspase-3 assay (data not shown). Taken together, these data indicate caspase-independent AIF-mediated cell death in the corpus callosum of cuprizone-fed mice, similar to that in multiple sclerosis, which could be attenuated by inhibition of PARP.

Cuprizone treatment activates Akt and mitogen-activated protein kinases in the corpus callosum, and is modulated by PARP inhibition

Three weeks of cuprizone feeding induced activation of the MAPKs, i.e. JNK, p38-MAPK and ERK ½ (P < 0.01, respectively) and Akt (P < 0.05) indicated by immunoblotting utilizing phosphorylation-specific primary antibodies (Fig. 7). 4HQ treatment attenuated cuprizone-induced phosphorylation of JNK and p38-MAPK (P < 0.01 and P < 0.05, respectively) but not of ERK1/2 (Fig. 7A and B). 4HQ alone did not affect phosphorylation of the MAPKs.

In contrast to the effect on MAPKs, 4HQ enhanced cuprizone-induced phosphorylation of Akt (P < 0.05). In addition, PARP inhibition alone also resulted in increased phosphorylation of Akt (P < 0.05) (Fig. 7C and D).

Discussion

In this article, we used cuprizone-induced demyelination as an animal model for oligodendrocyte depletion, as observed in multiple sclerosis, and its prevention by PARP inhibition. Demyelination and oligodendrocyte death are two of the general features of multiple sclerosis, which have even been suggested to be the primary events in lesion evolution, and may contribute to chronic inflammation through epitope spreading and axonal degeneration, which correlates with clinical disability (Naismith and Cross, 2005). Alternatively, oligodendrocyte injury and tissue destruction may be the consequence of the inflammatory process of multiple sclerosis (Smith and Lassmann, 2002; Lassmann et al., 2007). Irrespective of the primary trigger for oligodendrocyte death in multiple sclerosis, mitochondrial dysfunction with subsequent apoptotic cell death is a cardinal feature in at least a subset of acute and chronic multiple sclerosis lesions (Aboul-Enein et al., 2003; Mahad et al., 2008) and this feature is shared between the cuprizone model and multiple sclerosis.

Mitochondrial dysfunction with excessive reactive oxygen species production suggested by previous studies (Suzuki, 1969; Hemm et al., 1971; Ludwin, 1978; Pasquini et al., 2007; Turrens, 2003) could cause the PARP activation observed by us.
of degenerating oligodendrocytes in the cuprizone model and active pattern III multiple sclerosis lesions. Overactivation of PARP promotes cell death by ATP depletion in the cell and regulating the release of AIF from mitochondria (Yu et al., 2002; Alano et al., 2004). AIF then translocates to the nucleus, leading to chromatin condensation, large-scale DNA fragmentation (>50 kb) and cell death in a caspase-independent manner (Lorenzo and Susin, 2004; Jurewicz et al., 2005). In fact, we observed nuclear translocation of AIF co-localized with poly(ADP-ribose) in several oligodendrocytes in both pattern III multiple sclerosis lesions and the cuprizone model. However, we were unable to detect caspase-3 activation in the corpus callosum of cuprizone-treated mice in agreement with previous findings (Copray et al., 2005; Pasquini et al., 2007).

Specificity and possible side-effects of a pharmacological agent are always an issue. However, 4HQ was reported to have a high potency for PARP-1 and no effects on enzymes other than PARP have been documented (Banasik et al., 1992). Therefore, it seems likely that prevention of the weight loss, diminished demyelination and oligodendrocyte loss induced by cuprizone can be assigned to the PARP inhibitory effect of 4HQ.

JNK and p38-MAPK activation are considered to promote cell death (Xia et al., 1995; Stariha and Kim, 2001; Ha et al., 2002; Jurewicz et al., 2003). Indeed, we observed that cuprizone increased phosphorylation of JNK and p38-MAPK in the corpus callosum, which was attenuated upon PARP inhibition. Cuprizone also induced ERK1/2 activation in the corpus callosum but it was not affected by the PARP inhibitor 4HQ, which can be explained by the notion that the MAPK/ERK kinase-ERK1/2 pathway is upstream to PARP activation (Tang et al., 2002; Kauppinen et al., 2006). Since ERK activation was found to promote oligodendrocyte survival (Cohen et al., 1996; Yoon et al., 1998), cuprizone-induced ERK activation may represent a protective mechanism against oligodendrocyte death. In conclusion, all effects of PARP inhibition on the MAPK pathways, i.e. suppressing JNK and p38 activation while not affecting ERK, could promote oligodendrocyte survival.

Cuprizone intoxication also resulted in Akt phosphorylation, which was further enhanced by co-administration of 4HQ. Furthermore, PARP inhibition alone caused phosphorylation of Akt in accordance with previous findings (Veres et al., 2003; Tapodi et al., 2005). Activation of Akt prevented neuronal apoptosis by inhibiting translocation of AIF to the nucleus (Kim et al., 2007), protected oligodendrocytes against tumour necrosis factor-induced apoptosis (Pang et al., 2007); and, by phosphorylating their respective upstream kinases, decreased activity of JNK and p38-MAPK (Park et al., 2002; Barthwal et al., 2003). Based on these data, our results may suggest that in response to cuprizone, the cytoprotective phosphatidylinositol-3 kinase/Akt pathway became activated, although it was insufficient to prevent oligodendrocyte death. Additional activation by PARP inhibition could be sufficient to protect oligodendrocytes against apoptosis, mediated partially by reduced activation of JNK and p38-MAPK and maintaining the integrity of the mitochondrial membrane systems preventing nuclear translocation of AIF.

In the cuprizone model of demyelination, the pathological changes are similar to pattern III lesions or lesions defined by Barnett and Prineas (Lucchinetti et al., 2000; Barnett and Prineas, 2004). The earliest change in these lesions is wide-spread oligodendrocyte apoptosis associated with microglia activation in the proximity of dying oligodendrocytes, while signs of humoral and cellular immune responses are minor. Besides the pathological similarities, we observed identical patterns of at least two key molecular mechanisms, i.e. PARP activation and caspase-independent AIF-mediated apoptosis of oligodendrocytes in both pattern III multiple sclerosis lesions and cuprizone-induced demyelination. Based on these pathological and molecular observations, it could be assumed that the apoptosis of oligodendrocytes, at least in a subgroup of multiple sclerosis patients and in the cuprizone model, happens via similar pathways. Thus, inhibition of PARP may be similarly effective in multiple sclerosis and may have several important aspects. By blocking demyelination, PARP inhibition may reduce inflammation through preventing epitope spreading. Besides, it also has a direct effect on inflammation...
indicated by a reduction in the clinical signs of experimental autoimmune encephalomyelitis (Scott et al., 2004). Inhibiting PARP may thus influence degenerative and autoimmune inflammatory processes in multiple sclerosis and provide an effective therapy targeting two basic mechanisms at the same time. Recently, plasma exchange has been shown to be highly efficient in patients with antibody-mediated pattern II lesions indicating the importance of mechanism-specific treatment strategies (Keegan et al., 2005). Similarly, PARP-inhibitors may be effective in patients with pattern III lesions characterized by primary oligodendrocyte death.

In summary, our data indicate that oligodendrocyte death occurs via very similar mitochondrial pathomechanisms in the cuprizone model and pattern III multiple sclerosis lesions.

Figure 5 Effect of cuprizone and 4HQ treatment on demyelination in corpus callosum. (A) Representative T2-weighted spin echo magnetic resonance images of brain coronal sections (upper panels) and quantification of T2 intensity changes in the corpus callosum (lower panels) of mice treated for 4 weeks. Arrows indicate hyperintensities (suggesting demyelination) or hypointensity (intact myelin status) in corpus callosum. Data are expressed as normalized mean signal intensities ± SD. *P < 0.001 compared with the cuprizone (CPZ) group. Experiments were repeated three times and at least five mice were included in each group. (B) Representative histopathology images and quantification of myelin status in the corpus callosum (arrows) on brain coronal sections of mice treated for 5 weeks. Blue staining by luxol fast blue indicates intact myelin sheath. Experiments were repeated at least three times and at least five mice were included in each group. (C) MBP expression in the dissected corpus callosum of mice treated for 5 weeks was detected by immunoblotting utilizing an anti-MBP antibody. Even protein loadings were confirmed by an anti-Akt antibody and immunoblotting. Representative immunoblots (left panel) from three experiments with similar results and densitometric evaluation (right panel) are shown. At least five mice were included in each group. Lane 1 = control; lane 2 = cuprizone (CPZ) treatment; lane 3 = cuprizone+4HQ (CPZ+4HQ) treatment; lane 4 = 4HQ only. Results on the diagram are expressed as the mean pixel densities ± SD; *P < 0.01 compared with control; ‡P < 0.05 compared with the cuprizone group.
Inhibition of PARP effectively attenuated oligodendrocyte depletion and protected against experimental demyelination mediated through a caspase-independent pathway involving nuclear translocation of AIF. Considering that PARP inhibition was also highly effective in experimental autoimmune encephalomyelitis (Scott et al., 2004), it may provide a therapeutic approach protecting against two basic processes in multiple sclerosis, inflammation and demyelination. Moreover, it may target all subtypes of multiple sclerosis either by preventing oligodendrocyte death, a key event in the formation of all new lesions or additionally, by targeting inflammation.
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