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Sialoadhesin deficiency ameliorates myelin degeneration and axonopathic changes in the CNS of PLP overexpressing mice

Chi Wang Ip,[a] Antje Kroner,[a] Paul R. Crocker,[b] Klaus-Armin Nave,[c] and Rudolf Martini[a,*]

[a]*Department of Neurology, Section of Developmental Neurobiology, University of Wuerzburg, Josef-Schneider Str. 11, D-97080 Wuerzburg, Germany*
[b]*Wellcome Trust Biocentre, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK*
[c]*Department of Neurogenetics, Max Planck Institute of Experimental Medicine, 37075 Goettingen, Germany*

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PLP overexpressing mice display demyelination and axonopathic changes, accompanied by an elevation of CD8+ T-lymphocytes and CD11b+ macrophages in the CNS. By crossbreeding these mutants with RAG-1-deficient mice lacking mature lymphocytes, we could recently demonstrate a pathogenetic impact of the CD8+ cells. In the present study, we investigated the pathogenetic impact of CD11b+ macrophages by crossbreeding the myelin mutants with knockout mice deficient for the macrophage-restricted adhesion molecule sialoadhesin (Sn). In the wild-type mice, Sn is barely detectable on CD11b+ cells, whereas in the myelin mutants, almost all CD11b+ cells express Sn. In the double mutants, upregulation of CD8+ T-cells and CD11b+ macrophages is reduced and pathological alterations are ameliorated. These data indicate that in a primarily genetically caused myelin disorder of the CNS macrophages expressing Sn partially mediate pathogenesis. These findings may have substantial impact on treatment strategies for leukodystrophic disorders and some forms of multiple sclerosis.

Keywords: Microglia; Macrophages; T-lymphocytes; Leukodystrophy; Multiple sclerosis; Animal model; Inflammation; Sialoadhesin; Siglec

Introduction

Myelin sheaths around larger caliber axons not only mediate the saltatory conduction of action potentials but are also necessary for the maintenance of axonal integrity and survival (Griffiths et al., 1998; Yin et al., 1998; Bjartmar et al., 1999; Frei et al., 1999; Wrabetz et al., 2000; Yin et al., 2000; Martini, 2001; Lappe-Siefke et al., 2003; Samsam et al., 2003; Edgar and Garbern, 2004; Edgar et al., 2004). This has crucial implications for several demyelinating disorders in humans that may be caused by distinct mechanisms including intoxication, diabetes autoimmunity and glial cell mutations. We have previously shown for the peripheral nervous system that mutations related to myelinating glial cells, the Schwann cells, cause a secondary activation of both T-lymphocytes and macrophage-like cells. By crossbreeding these mutants with either lymphocytic or macrophage mutants, we could show that both cell types significantly contribute to the primarily genetically caused demyelinating disorders (Schmid et al., 2000; Carenini et al., 2001; Mäurer et al., 2001, 2002; Kobsar et al., 2002, 2003, 2005; Ip et al., 2006b). Recently, we investigated a CNS myelin mutant, the PLP overexpressing mouse with regard of an elevation of immune-related cells and found a substantial increase in CD8+ T-lymphocytes and CD11b+ macrophage-like cells. Both by crossbreeding experiments with RAG-1-deficient mice and reconstitution with either CD8+ or CD4+ bone marrow stem cells, we could unequivocally identify the CD8+ T-lymphocytes as pathogenetically relevant (Ip et al., 2006a). The present study was designed to investigate the pathogenetic role of macrophage-like cells. For this purpose, we crossbred the PLP mutants with mice deficient in the macrophage-restricted adhesion molecule Sialoadhesin (Sn), a sialic acid binding protein involved in macrophage–lymphocyte interactions (Muerkoster et al., 1999; van den Berg et al., 2001). The absence of Sn leads to an amelioration of experimental autoimmune uveoretinitis (Jiang et al., 2006) and of the primarily genetically mediated demyelinating neuropathy in mice heterozygously deficient for the myelin component P0 (Kobsar et al., 2006). In the present study, we found that Sn is expressed by nearly all macrophage-like cells in the CNS of the PLP mutants, but only by a low percentage of macrophage-like cells in the CNS of wild-type mice. When Sn was knocked-out in PLP mutants, the number of CD8+ T-lymphocytes and macrophage-like cells was blocked or less elevated, respectively, and the pathological alterations, such as demyelination and axonopathy, were significantly reduced. Thus, the macrophage-restricted molecule Sn plays a pathogenic role in a primarily genetically caused neuropathy of the CNS.

Materials and methods

Animals and determination of genotypes

PLP-transgenic mice of the line 66 (Readhead et al., 1994) have been backcrossed to a C57/Bl6 genetic background and mated with

* Corresponding author. Fax: +49 931 20123697.
E-mail address: rudolf.martini@mail.uni-wuerzburg.de (R. Martini).
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C57/Bl6 wild-type mice to obtain heterozygous offsprings. Additionally, transgenic PLP mutants were crossbred with Sialoadhesin (Sn)-deficient mice (Oetke et al., 2006) in analogy to previous experiments (Kobsar et al., 2006). The animals were examined at the age of 12 months.

Presence of the autosomal *plp* transgene was displayed by a PCR amplification product obtained with a primer at the 3′ end of the PLP-gene (5′-CAGGTGTTGAGTCTGATCTACACAAG-3′) and a primer T7cos (5′-GCATAATACGACTCACTATAGG-GATC-3′) directed against the T7 promoter of the PLP cosmid cos901. The PCR was performed in a volume of 25 μl following a standard protocol with an annealing temperature of 55°C and an elongation time of 45 s at 72°C.

Determination of the Sn genotype was performed by PCR using the following primers: forward: CACCACGGTCACTGTGACAA; reverse: GGCCATATGTAGGGTCGTCT. This resulted in a 468-bp product for wt allele and a 1729-bp product for the mutated allele. Sn-deficient mice were characterized as true nulls, based on complete absence of detectable protein using antibodies to the N- and C-termini of Sn (Oetke et al., 2006).

Tissue preparation and immunohistochemistry

For the identification of macrophage-like cells, mice were transcardially perfused with 0.1 M cacodylate buffer containing 4% paraformaldehyde. The optic nerves were dissected and postfixed in the same fixative for 2 h and cryoprotected in 30% sucrose overnight. For T-lymphocyte staining, the animals were perfused with 0.1 M phosphate-buffered saline (PBS) only. After freezing in liquid nitrogen-cooled isopentane, 10-μm-thick longitudinal sections of the optic nerve were cut. For myelin basic protein (MBP) staining cross sections of the optic nerve at the region of 1200–1400 μm caudal to the pigment epithelium were used. Sections were preincubated for 30 min in 5% normal bovine serum in 0.1 M phosphate-buffered saline (PBS) and then incubated in one of the following primary antibodies diluted in 1% normal bovine serum (overnight, 4 °C). Rat anti mouse CD11b (Serotec, Oxford, UK) was used for detection of activated macrophages; rat anti mouse CD4 (Serotec, Oxford, UK) and rat anti mouse CD8 (Chemicon, Temecula, CA, USA) antibodies were used for the identification of T-lymphocytes; rabbit anti mouse MBP (MBL, Woburn, MA, USA) was applied to quantify myelin in the CNS of wild-type and PLP-tg mutants. To optimize immunolabeling for CD11b, sections were permeabilized with 0.3% Triton-X 100. To visualize primary antibodies, a biotinylated secondary antibody to rat (CD4, CD8, CD11b) or rabbit (MBP) Igs was applied for 1 h, followed by avidin/biotin reagent (Dako, Carpinteria, CA, USA) before incubation and staining with diaminobenzidine–HCl (DAB) and H_2O_2. To facilitate quantification of CD11b+ macrophage-like cells, sections were counterstained with hemalaun and only those immunoreactive profiles were scored that could be identified as cell bodies containing a nucleus.

For Sn immunoreactivity on macrophages, double immunofluorescence was applied on fresh frozen nerve sections using a rat anti-mouse CD169 antibody to Sn (Serotec, Eching, Germany) and a biotinylated rat antibody to CD11b (Serotec, Eching, Germany). CD169 antibody was detected with Alexa Fluor 488-labeled goat anti-rat antibody (Invitrogen/Molecular probes, Karlsruhe, Germany), followed by avidin–biotin blockade (Vector Laboratories, Burlingame, CA, USA) and application of the biotinylated CD11b antibody. This was detected by Streptavidin Cy3 (Biozol, Eching, Germany). The specificity of the immunoreaction was assessed by omission of the primary antibody.

Tissue preservation for light and electron microscopy

Optic nerves were processed for light and electron microscopy as previously described (Ip et al., 2006a). The mice were transcardially perfused using 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer. The nerves stayed in the same fixative overnight, followed by osmification and embedding in Spurr's medium. For light microscopic analysis, 0.5-μm-thick semithin sections were stained with alkaline methylene blue and investigated with an Axiophot 2 (Zeiss, Oberkochen, Germany). For electron microscopy, ultrathin sections of 70-nm thickness were counterstained with lead citrate and investigated using a Zeiss EM 10CR (Zeiss, Oberkochen, Germany).

Quantification of immune cells in the CNS

Quantification of the number of macrophage-like cells was performed as described (Ip et al., 2006a) with a light microscope (Olympus BH2, Olympus, Hamburg, Germany) using an ocular grid covering a defined area (0.0256 mm^2) at a final magnification of 600×. Only those profiles that showed nucleic/cell soma staining by hemalaun (see above) were counted, whereas immunoreactive structures consisting of processes only were not considered. The rostral region, covering the rostral 800–1500 μm of the optic nerve, was chosen as the area of interest. Investigation was performed on 4–6 optic nerve sections per animal. The cell number of macrophage-like cells was then calculated per mm^2.

T-cell staining as well as CD11b/Sn double immunofluorescence was analyzed on a Zeiss Axiophot 2 microscope using digital images acquired via a CCD camera and ImagePro 4.0 software at a final magnification of 300× measuring 4–6 consecutive sections. The entire region of the optic nerve was examined and the cell density was then calculated per mm^2.

Statistical analysis was performed by using the unpaired two-tailed Student's *t*-test for comparison of quantified profiles. Semiquantitative MBP scoring were analyzed by using the non-parametric Mann–Whitney *U* test.

Results

Sn is expressed on CNS macrophages of PLP overexpressing mutant mice

In order to investigate whether Sn is expressed on macrophage-like cells comprising macrophages and/or microglia of wild-type and PLP overexpressing mutant mice, we performed double immunohistochemistry using antibodies to Sn and CD11b. In wild-type mice only approximately 3% of the macrophage-like cells were Sn positive. In the PLP mutants, however, approximately 90% of the CD11b+ macrophages expressed Sn (Fig. 1).

Sn deficiency leads to a reduced upregulation of CD11b+ macrophages and CD8+ T-cells as well as an amelioration of myelin degeneration and axonopathic changes in PLP overexpressing mice

An upregulation of immune related cells (macrophages and CD8+ T-lymphocytes) has been observed in PLP overexpressing