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Macrophage colony stimulating factor is a crucial factor for the intrinsic macrophage response in mice heterozygously deficient for the myelin protein P0

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Abstract

Mouse mutants heterozygously deficient for the myelin protein P0 (P0+/−) resemble certain forms of human hereditary neuropathies. Endoneurial macrophages of intrinsic origin are intimately involved in the pathogenesis of the demyelinating neuropathy in these mutants. We have previously shown that deficiency for macrophage colony stimulating factor (M-CSF) prevents an increase of the number of endoneurial macrophages and alleviates the mutants' demyelinating phenotype. The aim of this study was to investigate which population of endoneurial macrophages – long-term resident macrophages or recently infiltrated macrophages – is affected by M-CSF deficiency. For this purpose, we generated bone marrow chimeric mice by transplanting GFP+ bone marrow into P0 mutants (P0+/−) and P0 mutants that lack M-CSF (P0+/− mcsf-op). This enabled us to discriminate recently infiltrated short-term resident GFP+ macrophages from long-term resident GFP− macrophages. Three months after bone marrow transplantation, P0+/− mice expressing M-CSF showed a substantial upregulation and activation of both GFP− and GFP+ macrophages in femoral nerves when compared to P0+/+ mice. In contrast, in P0+/− mcsf-op mutants, both GFP− and GFP+ macrophages did not substantially increase. Only small numbers of GFP+ but no GFP− macrophages were activated and phagocytosed myelin in chimeric P0+/− mcsf-op mutants, possibly reflecting recent activation outside the endoneurium before entering the nerve. Our findings demonstrate that M-CSF is crucial for the activation, in situ increase and myelin phagocytosis of both long-term and short-term resident endoneurial macrophages in P0+/− myelin mutants. M-CSF is, therefore, considered as a target candidate for therapeutic strategies to treat human demyelinating neuropathies.

Keywords: Myelin; Demyelination; Myelin phagocytosis; Macrophages; Activation; Macrophage colony stimulating factor; Peripheral neuropathy; Animal model

Introduction

Macrophages are important pathogenetic mediators in many degenerative disorders of the nervous system (Hendriks et al., 2005). In mice heterozygously deficient for the peripheral myelin protein P0 (P0+/−) mimicking some forms of inherited peripheral neuropathies in humans (Charcot-Marie-Tooth neuropathies), macrophages and T-lymphocytes have been identified to mediate the genetically determined demyelination (Carenini et al., 2001; Ip et al., 2006). The impact of macrophages became evident due to their numerical increase and due to their apparently active role in the phagocytosis of morphologically intact myelin. Furthermore, in P0+/− mutants additionally deficient in the cytokine macrophage colony stimulating factor (M-CSF), macrophage numbers remained as low as in normal P0+/+ wild-type controls, and the extent of demyelination was largely reduced (Carenini et al., 2001).

Resident endoneurial macrophages are the local macrophage population of the PNS and are scattered throughout the endoneurium in the normal PNS (Griffin and George, 1993; Monaco et al., 1992; Mueller et al., 2003), comprising approximately 5% of all endoneurial cells (Arvidson, 1977; Mueller et al., 2001, 2003; Oldfors, 1980). They undergo a physiological turnover, which exchanges around 40% of resident endoneurial macrophages in 3 months with hematogenous macrophages (Mueller et al., 2003; Vass et al., 1993). In recent studies using bone

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marrow chimeric mice, it was found that resident endoneurial macrophages are rapidly activated and able to generate an early intrinsic macrophage response by proliferation in traumatic (sciatic nerve crush) (Mueller et al., 2001, 2003) and acute inflammatory (experimental autoimmune neuritis) disease models (Müller et al., 2006). A similar study in P0+/− mutants demonstrated that the elevation of macrophage numbers in the mutants is mainly due to an intrinsic proliferation, affecting both 'long-term' resident macrophages and 'short-term' resident macrophages, the latter of which are defined as macrophages that are resident but have entered the nerve recently due to physiological turnover (Mäurer et al., 2003).

The objective of the present study was to determine which of the endoneurial macrophage populations is dependent on M-CSF.

Materials and methods

Animals and generation of P0+/− mcsf-op double mutants

Osteopetrotic mice carrying a homozygous spontaneous mutation in the *csF1* gene (mcsf-op) (Yoshida et al., 1990) are characterized by a reduced life span, lack of incisivi and their inability to breed. Thus, to generate mice heterozygously deficient for P0 (P0+/−) and homozygously deficient for M-CSF, P0+/− mice were first intercrossed with mice carrying a heterozygous *csF1* mutation. P0+/− mcsf-op mice were obtained in the F2 generation. Genotyping of the individuals has been performed as described (Carenini et al., 2001; Kalaydjieva et al., 2000). Age matched neuropathic P0+/− mcsf-wt and wild-type P0+/+ mcsf-wt mice served as controls. All animals were taken from the animal facility of the Department of Neurology, University of Würzburg and were kept under pathogen-free conditions.

Bone marrow transplantation

Bone marrow transplantation experiments were approved by the local authorities at the Bezirksregierung Muenster, Germany. At the age of 2 months, animals were bone marrow-transplanted with GFP+ bone marrow as described previously (Hickey et al., 1992; Mueller et al., 2003). In brief, bone marrow was taken from 8-week-old GFP-transgenic donor and injected (2×10^7 cells) into 2-month-old P0+/− mcsf-op ($n=4$), P0+/− mcsf-wt ($n=3$) and P0+/+ mcsf-wt ($n=2$) mice after a lethal irradiation with 5 Gy. Determining the percentage of GFP+ leukocytes from a blood smear validated the chimerism. Only animals with >95% GFP+ leukocytes were used for further experiments. In addition, splenic tissue from bone marrow chimeric mice was assessed for the distribution of GFP+, hematogenous cells. Ten micrometer cryosections were counterstained with DAPI (Vector Labs, Burlingame, California) and examined with a fluorescence microscope. Splenic follicles of bone marrow chimeric mice consisted nearly exclusively of GFP+ cells, whereas stromal cells, like blood vessel, were GFP− (Mueller et al., 2003). Three months later, at the age of 5 months, animals were sacrificed and examined.

Tissue preservation for immunohistochemistry and ultrastructural analysis

For immunohistochemistry, the mice were transcardially perfused using 4% paraformaldehyde in 0.1 M phosphate buffer. For ultrastructural analysis, ventral spinal roots were additionally immersed in 2% glutaraldehyde in 0.1 M cacodylate buffer.

For the immunohistochemical analysis, tissue was immersed in 4% paraformaldehyde at 4°C for additional 3 h, washed in phosphate buffer, placed in 10% sucrose overnight and then snap frozen. For ultrastructural analysis, ventral roots were placed in fixative overnight followed by osmification and embedding in Spurr's medium as described (Carenini et al., 2001).

Immunohistochemistry

Endoneurial macrophages of the femoral nerve were detected by immunohistochemistry for the macrophage specific marker CD68 (clone FA11, Serotec, Oxford, UK) or rat antibodies to mouse F4/80 (1:300; Serotec, Eching, Germany) on 10 μm thin cryosections. T-lymphocytes were detected by rabbit anti-mouse CD3 (Abcam AB16044, Cambridge, UK).

The primary antibodies CD68 and F4/80 were detected by Alexa 594-coupled streptavidin (molecular probes, Leiden, Netherlands) after incubation with a biotinylated secondary antibody (Vector Labs, Burlingame, California). All sections were counterstained with DAPI nuclear labeling (Vector Labs). Phagocytosis of myelin by macrophages was detected by colocalization of myelin basic protein (polyclonal MBP, Dako, Hamburg, Germany) with CD68+ macrophages (Mueller et al., 2003). For the colocalization, an Alexa 594-coupled secondary antibody visualized MBP, whereas CD68 was visualized by Alexa 350-coupled streptavidin. The same double-staining protocol was applied to colocalize CD3 and CD68.

Image and macrophage form factor analysis

Sections were examined under a Leica DM fluorescence microscope, and images were digitized and transferred to a PC using a Diagnostic Instruments SPOT II camera system (Visitron, München, Germany). The SPOT II software was used to merge fluorescent signals for GFP (green), CD68 (red) and DAPI (blue). GFP+ and GFP− endoneurial macrophages were counted using Analysis 3.1 Software (SIS, Münster, Germany). Analysis 3.1 was also used to determine the endoneurial area under study and a macrophage form factor (Shen, 2000; Soltys et al., 2001). The form factor is defined as $4\pi\times$ cell area/perimeter^2 and reflects a morphological grade of macrophage activation.

Ultrastructural analysis

Ultrathin sections were investigated with a Zeiss electron microscope 10CR (ZEISS, Oberkochen, Germany), equipped with a Proscan (Lagerlechfeld, Germany) slow scan camera and the corresponding software analysis system (Vario Vision 3.0 Doku; Soft Imaging System, Münster, Germany).