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Immunomodulatory effects of the ether phospholipid edelfosine in experimental autoimmune encephalomyelitis

Pierre Abramowski [a,b], Karin Steinbach [a,c], Axel R. Zander [d], Roland Martin [a,e,*]

[a] Institute for Neuroimmunology and Clinical MS Research (inims), Center for Molecular Neurobiology (ZMNH), University Medical Center Hamburg-Eppendorf, Falkenried 94, 20251 Hamburg, Germany
[b] Research Department Cell and Gene Therapy, Clinic for Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany
[c] Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, 1 Rue Michel Servet, 1211 Geneva, Switzerland
[d] Department for Stem Cell Transplantation, University Cancer Center Hamburg (UCCH), Martinistr. 52, 20246 Hamburg, Germany
[e] Neuroimmunology and MS Research (nims), Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, 8091 Zurich, Switzerland

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ABSTRACT

The 2-lysophosphatidylcholine analog edelfosine induces apoptosis in highly proliferating cells, e.g. activated immune cells. We examined mechanisms of action of edelfosine on immune functions in experimental autoimmune encephalomyelitis, a well-accepted animal model for multiple sclerosis. We observed activated caspase-3 expression in lymphoid organs and the central nervous system; however, edelfosine did not induce global apoptosis. Edelfosine improved the disease course and led to reduced frequencies of $CD4^+$ T cells infiltrating into the central nervous system. Our data suggest edelfosine as an interesting treatment candidate for multiple sclerosis.

1. Introduction

Apoptotic cell death is an elementary cellular response and involves the sequential activation of caspases. The stress-inducible, intrinsic apoptotic pathway involves cytochrome c release from mitochondria (Liu et al., 1996). The extrinsic pathway is induced by pro-apoptotic and pro-inflammatory cytokines, e.g. FasL, TRAIL and TNF-α, respectively, which induce the intracellular formation of specific death-inducing signaling complexes (DISCs) after binding to death domain receptors (Scaffidi et al., 1999; Micheau and Tschopp, 2003). Downstream caspases, e.g. caspase-3, are cleaved to execute cell death. Importantly, elimination of cells via apoptosis does not lead to inflammation or immune activation. Therefore, approaches to induce apoptosis in deregulated, e.g. self-reactive immune cells have been considered to treat autoimmune diseases, for instance multiple sclerosis (MS).

Many potentially pathogenic factors and disease mechanisms have been examined in animal models, particularly in experimental autoimmune encephalomyelitis (EAE) (Raine et al., 1980), and both animal and human studies point to a central role for autoreactive $CD4^+$ T cells in MS pathology (Sospedra and Martin, 2005). $CD4^+$ T cells are present in central nervous system (CNS) and cerebrospinal fluid (CSF) cellular infiltrates in both MS and EAE (Pettinelli and McFarlin, 1981; Richert et al., 1983; Martin et al., 1990; Ota et al., 1990). Further, albeit indirect evidence for their role stems from the observation that a large fraction of the genetic risk of MS is conferred by HLA-class II alleles of the HLA-DR15 haplotype (Jersild et al., 1973; Hillert and Olerup, 1993). Recently published large genome-wide association studies show over 130 single nucleotide polymorphisms besides HLA-DR as risk alleles for MS (Sawcer et al., 2011; Beecham et al., 2013), and interestingly many of these are involved in T cell activation and function (Lundmark et al., 2007; McElroy and Oksenberg, 2008).

Following priming with (auto)antigens to potentially autoreactive $CD4^+$ T cells in the periphery, activated $CD4^+$ T cells transmigrate across the blood brain barrier (BBB) and the blood CSF barrier (Cannella and Raine, 1995). Next, T cells are locally reactivated by antigen presenting cells within the CNS (Flügel et al., 2001). Subsequently, a number of immune cells including those of the innate (neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells) and adaptive ($CD4^+$ and $CD8^+$ T cells, B cells) immune system (Lucchinetti et al., 1996; Franciotta et al., 2008; Hauser et al., 2008) are recruited and form

* Corresponding author at: Neuroimmunology and MS Research, Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, 8091 Zurich, Switzerland. Tel.: +41 44 255 11 25; fax: +41 44 255 8064.
E-mail address: roland.martin@usz.ch (R. Martin).

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focal inflammatory lesions (Simpson et al., 1998). Immunodominant peptides of myelin proteins, e.g. myelin oligodendrocyte glycoprotein (MOG) peptide $MOG_{(35–55)}$, are used to induce chronic EAE in C57BL/6 mice, whereas relapsing-remitting EAE (RR-EAE) in SJL mice follows the injection of proteolipid protein (PLP) peptide $PLP_{(139–154)}$.

The synthetic 2-lysophosphatidylcholine (LPC) analog edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, ET-18-OCH_3) was found to induce selective apoptosis in leukemic cells (Diomede et al., 1993; Mollinedo et al., 1993). In contrast to other cytotoxic drugs edelfosine does not target the DNA and it does not directly interfere with the formation and function of the cellular replication machinery. Edelfosine binds to Fas/CD95 in a lipid raft-mediated process thereby exerting its cytotoxic activity (Gajate and Mollinedo, 2001; van der Luit et al., 2002). Detailed mechanistic studies have demonstrated that edelfosine-treated Jurkat leukemic T cells undergo apoptosis following recruitment of DISCs into lipid rafts (Gajate et al., 2009). Edelfosine accumulates in the inner leaflet of the plasma membrane in lipid rafts, which in turn induces the clustering of rafts and the recruitment of Fas into rafts with subsequent translocation of Fas, FADD and caspase-8 into rafts, DISC formation and activation of caspase-8 to induce apoptosis in the absence of FasL. Normal, resting cells are not able to take up significant amounts of edelfosine (Gajate et al., 2000). Additionally, edelfosine may accumulate in lipid rafts within the plasma membrane followed by endocytosis and translocation to the intracellular location of the CTP:phosphocholine cytidylyltransferase (CCT), the endoplasmic reticulum (ER) (Clement and Kent, 1999; Van der Luit et al., 2002, 2007). Here, edelfosine may inhibit the biosynthesis of phosphatidylcholine (PC) leading to mitotic arrest and apoptosis (Boggs et al., 1995; Van Der Luit et al., 2003). This effect was found in exponentially growing cells, which require high amounts of PC, while normal quiescent cells were not affected (van der Sanden et al., 2004; Zerp et al., 2008). The relative contribution of each mechanism may depend on the cell type and the concentration of edelfosine (Zoeller et al., 1995; Mollinedo et al., 1997; Tsutsumi et al., 1998). Thus, alkyl lysophospholipids like edelfosine may affect several cellular processes, probably with cell type-dependent emphasis but with the joint outcome of apoptosis induction.

Due to its immunomodulatory properties and its oral availability edelfosine had already been examined as a treatment for MS in the past (Munder and Westphal, 1990; Klein-Franke and Munder, 1992). Although not performed according to today's standards exploratory clinical trials demonstrated the drug's excellent safety profile. Parallel EAE studies, mostly with rats, but also with mice described a beneficial effect of edelfosine treatment on clinical outcome (Baker et al., 1991; Chabannes et al., 1992; Klein-Franke and Munder, 1992; Kovarik et al., 1995). So far, however, functional investigations on edelfosine-induced modifications of cellular responses in the context of EAE are limited. We therefore wanted to reassess and expand prior work in the EAE model to examine in more detail the mechanism/s of action of edelfosine as a potential treatment in autoimmune diseases, for instance MS.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in accordance with the guidelines of the local authorities (Behörde für Soziales, Gesundheit unf Verbraucherschutz Hamburg; G22/08).

2.2. Preparation of edelfosine

For in vivo applications, edelfosine (Medmark, Oberhaching, Germany) was dissolved in aqua ad injectabilia and diluted with PBS (PAA, Pasching, Austria). The diluted edelfosine was stored at −20 °C and thawed on the day of application. The injected edelfosine dose per mouse was 1, 10, 15 and 25 mg/kg edelfosine. Edelfosine was applied by intraperitoneal injection or by gavage.

2.3. Mice

C57BL/6J mice were purchased from the Jackson Laboratory and bred by the animal facility of the University Medical Center Hamburg Eppendorf. SJL/JHan™Hsd mice were purchased from Harlan Laboratories, Indianapolis, IN, USA.

2.4. Induction of EAE

For EAE experiments C57BL/6 mice or SJL mice were housed in "individually ventilated cages (IVC)"-racks at least 1 week prior to the active induction of EAE. On the day of immunization mice were injected subcutaneously at two sites of the flanks with 200 μg of $MOG_{(35–55)}$ (NeoMPS, San Diego, CA, USA) or 75 μg/ml of $PLP_{(139–151)}$ (NeoMPS) peptide in incomplete Freund's adjuvant (BD Difco Diagnostics, Sparks, MD, USA) supplemented with 4 mg/ml Mycobacterium tuberculosis H37 Ra (BD Difco). C57BL/6 mice were injected intravenously with 300 ng of pertussis toxin (Calbiochem Merck, Darmstadt, Germany) on the day of immunization and 48 h later. SJL mice received a single dose of 200 ng pertussis toxin on the day of immunization. Body weight and clinical score were monitored on a 0–5 scale with classifications of disease severity: 0 = healthy, 1 = limp tail, 2 = ataxia and/or paresis of hind limbs, 3 = paraplegia, 4 = paraplegia with forelimb weakness, 5 = moribund or dead.

2.5. Preparation of cells from secondary lymphoid organs and CNS of mice

For preparation of lymph nodes and spleens mice were sacrificed at day 9 after immunization. Single cell suspensions were prepared by mincing the tissue and pushing it through a 40 μm cell sieve. Cells derived from the spleen were resuspended in 5 ml red blood cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in ddH_2O) and incubated on ice for 7 min. Cells were resuspended in FACS buffer (0.1% BSA, 0.02% NaN_3 in PBS) or PBS + 1% FCS if the preparation was followed by recall experiments. At the acute phase of EAE mice were perfused transcardially with 50 ml PBS supplemented with 1% FCS to prepare the CNS. Dissected brain and spinal cord were digested for 60 min at 37 °C by addition of 1 mg/ml collagenase I and 0.1 mg/ml DNase I (both from Roche, Penzberg, Germany) in D-MEM (Gibco, Carlsbad, CA, USA). The cell suspension was passed through a 40 μm cell strainer and centrifuged (250 ×*g*, 10 min, 4 °C). Cells were separated from myelin and neurons by Percoll (GE Healthcare, Chalfont St. Giles, UK)-gradient centrifugation (30%/78%). CNS-infiltrating cells and microglia were collected from the interface. Cells were resuspended in FACS buffer, centrifuged (550 ×*g*, 10 min, 4 °C) and washed two additional times (250 ×*g*, 10 min, 4 °C).

2.6. Cell culture experiments

To determine T cell proliferation by [methyl-^{3}H]-thymidine incorporation, spleen- and lymph node-derived cells were seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) at 2×10^5 cells/well. Murine cells were cultured in 200 μl of complete mouse medium (50 μM β-mercaptoethanol, 10% FCS in RPMI1640 medium, GlutaMAX™ (Gibco)) supplemented with the disease-relevant peptide $PLP_{(139–151)}$, a polyclonal anti-CD3 antibody (145-2C11, from eBioscience, San Diego, CA, USA) or mitogenic Concanavalin A (Con A, from Sigma-Aldrich, St. Louis, MO, USA).

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