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ORIGINAL PAPER

Sphingosine Toxicity in EAE and MS: Evidence for Ceramide Generation via Serine-Palmitoyltransferase Activation

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Abstract Multiple sclerosis (MS) is a demyelinating disorder characterized by massive neurodegeneration and profound axonal loss. Since myelin is enriched with sphingolipids and some of them display toxicity, biological function of sphingolipids in demyelination has been investigated in MS brain tissues. An elevation of sphingosine with a decrease in monoglycosylceramide and psychosine (myelin markers) was observed in MS white matter and plaque compared to normal brain tissue. This indicated that sphingosine toxicity might mediate oligodendrocyte degeneration. To explain the source of sphingosine accumulation, total sphingolipid profile was investigated in Lewis rats after inducing experimental autoimmune encephalomyelitis (EAE) and also in human oligodendrocytes in culture. An intermittent increase in ceramide followed by sphingosine accumulation in EAE spinal cord along with a stimulation of serine-palmitoyltransferase (SPT) activity was observed. Apoptosis was identified in the lumbar spinal cord, the most prominent demyelinating area, in the EAE rats. TNFα and IFNγ stimulation of oligodendrocytes in culture also led to an accumulation of ceramide with an elevation of sphingosine. Ceramide elevation was drastically blocked by myriocin, an inhibitor of SPT, and also by FTY720. Myriocin treatment also protected oligodendrocytes from cytokine mediated apoptosis or programmed cell death. Hence, we propose that sphingosine toxicity may contribute to demyelination in both EAE and MS, and the intermittent ceramide accumulation in EAE may, at least partly, be mediated via SPT activation, which is a novel observation that has not been previously reported.

Keywords Ceramide · Demyelination · EAE · Multiple sclerosis · Normal appearing white matter · Serine-palmitoyltransferase

Abbreviations

EAE	Experimental autoimmune encephalomyelitis
FMCs	Fast migrating cerebrosides
GalCer	Galactosylceramide
GC-MS	Gas chromatography-mass spectrometry.
HPLC	High performance liquid chromatography
HPTLC	High performance thin-layer chromatography
MGC	Monoglycosylceramide
MS	Multiple sclerosis
NAWM	Normal appearing white matter
SC	Spinal cord
SPT	Serine palmitoyltransferase

Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune demyelinating disease of the central nervous system (CNS) leading to progressive demyelination and

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neurological deterioration, as manifested pathologically by axonal transection and also axonal loss [1]. There has been speculation that several factors contribute to MS pathogenesis, including toxic environment, viral infection, hereditary linkage, and autoimmune demyelination. Demyelination may occur by different mechanisms in different subpopulations of MS patients [2]. The inflammatory microenvironment contains a variety of substances such as proteolytic enzymes, cytokines, oxidative products, and free radicals that can injure neurons and their axons [3]. Animal and human studies have indicated that inflammatory cytokines play a critical role in pathogenesis in MS lesion [4] by fostering inflammation and demyelination [5], although the exact mechanism remains mostly unknown. The consensus now is that MS is a chronic inflammatory autoimmune demyelinating disease of the CNS, as evidenced by immune cell infiltration and accompanying inflammatory processes. However, the etiology of MS is still unknown [6]. This leaves us with many major questions, including the primary cause of the CNS inflammation, the primary target antigen in MS, the stimulation of autoimmunity, etc. [6, 7]. Since sphingolipids are critical components of myelin, one such unanswered question is: do toxic sphingolipids and lysosphingolipids participate in MS demyelination?

The CNS, more specifically myelin, is enriched with sphingolipids and lysosphingolipids such as galactosylceramide (GalCer) and ceramide, sphingoids (sphingosine or sphingenine and sphinganine), and psychosine [8]. An increasing concentration of these sphingolipids during brain development and myelinogenesis implies that (lyso) sphingolipids may participate in cell growth, differentiation, myelinogenesis, and maintenance of the structural and functional integrity of myelin [8, 9]. Although mounting evidence suggests that regulatory pro-inflammatory cytokines such as tumor necrosis factor (TNF)α, interferon (IFN)γ, transforming growth factor (TGF)-β, interleukin (IL)-6, etc., play essential roles in fostering inflammation and demyelination in MS [10, 11], the exact mechanism of cytokine mediated demyelination due to sphingolipid toxicity still remains elusive. Two pro-inflammatory cytokines, TNFα and IFNγ, act synergistically in mediating apoptosis in a wide variety of cells including cardiac myocytes, oligodendrocytes, astrocytes, etc. [12–14], and they are abundant in MS brain (and NAWM) [15] and EAE tissues [16]. Also, elevated levels of both TNFα and IFNγ were found in MS patients after 8 weeks of endurance training [17].

A malfunction of sphingolipid metabolism was reported in MS tissues [18] and a very early report suggested that sphingosine was elevated in MS brain [19]. This is also supported by our earlier observation [20] and by a more recent publication [21]. Employing a chromatographic methodology developed in our laboratory [8] that used as little as 30–50 mg of tissues, we reported an elevation of sphingosine in MS brain. Although a correlation between the MS demyelination and oligodendrocyte cell death exists [22, 23], the precise biochemical pathway of cytokine mediated accumulation of ceramide and sphingosine in MS has not been explored in detail.

In this investigation, we report that the elevation of sphingosine level in EAE spinal cord may be triggered by de novo generation of ceramide. A major portion of ceramide generation is contributed via serine-palmitoyltransferase (SPT) activation, which is a novel observation in this investigation. Employing human oligodendrocytes in cell culture, we provide further evidence that sphingosine toxicity may have a significant role in oligodendrocyte cell death that contributes to progressive demyelination in MS and EAE.

Materials and Methods

Materials

MS and normal brain tissues were obtained as a generous gift from the National Neurological Research Specimen Bank (Los Angeles, CA, USA). Male Lewis rats, purchased from Charles River Laboratories (Wilmington, MA, USA) were used in this study in accordance with the 'Guide for the Care and Use of Laboratory Animals' from the US Department of Health and Human Services (National Institutes of Health, Bethesda, MD, USA), and approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina (Charleston, SC, USA). Silicic acid was purchased from Sigma (St. Louis, MO, USA), and pre-coated TLC plates (E. Merck, Kenilworth, NJ, USA) and solvents of analytical grades were obtained from Fisher Scientific (Pittsburgh, PA, USA). A human oligodendroglioma (HOG) cell line was a generous gift from Prof. Robert K. Yu, IMMAG, (Augusta, GA, USA). Human TNFα and IFNγ were purchased from PeproTech (Rocky Hill, NJ, USA). We have chosen HOG cells, which is an oligodendrocyte cell line of human origin, for our study [24].

Human Tissue Analyses

We performed analysis of sphingolipid from human MS, NAWM, and control brain tissues. To evaluate the sphingolipid profile, we analysed six different sets of human tissues. For lipid analysis, approximately 30–50 mg of tissue from a plaque area and a NAWM from the MS tissue were dissected carefully, and an identical anatomical position (matching the NAWM) from the white matter from normal brains (of individuals died of automobile accident) was used as controls.

Animal Studies

We have carried out animal studies to address this question: Does lipid profile in an animal model of EAE match with the human analysis? To delineate the lipid profile, we have induced EAE in Lewis albino rats, isolated the spinal cord (lumbar and thoracic) and brains, analysed the sphingolipid profile as well as assayed the enzyme(s) relevant to our observation.

Induction of EAE in Lewis Rats

EAE was induced in Lewis albino adult rats (approximately 6–8 weeks old) by subcutaneous injection of a homogeneous emulsion (0.2 ml) of guinea pig myelin basic protein (MBP) in phosphate-buffered saline (PBS, 0.5 ml from 1 mg/ml), and complete Freund's adjuvant or CFA (0.5 ml) containing *Mycobacterium tuberculosis* (3 mg/ml). The animals were carefully monitored for onset of EAE and graded for decline in motor function based on following changes: limb tail paralysis (EAE1), hind limb weakness (EAE2), hind limb partial paralysis (EAE3), and finally bilateral hind limb paralysis (EAE4) that occurred at approximately 12 days post-inoculation [25]. At least four animals in each group were used for this study to obtain a statistical significance. Control (sham) animals were injected with CFA in PBS without guinea pig MBP. Control and EAE animals were sacrificed under anesthesia at different grades of EAE and then brains and spinal cords were collected for sphingolipid analysis. To ascertain the region-specific elevation of sphingolipids and induction of apoptosis, each brain was dissected into four regions such as fronto-temporal, parieto-occipital, cerebellum, and brainstem while each spinal cord was dissected into thoracic and lumbar regions. Dissected brain and spinal cord segments were preserved at −70 °C until use.

Purification and Assay of Tissue Sphingolipids (Ceramide, Monoglycosylceramides or MGCs, Sphingoids, and Sphingomyelin) From Brains and Spinal Cords

Lipids were extracted from 30 to 50 mg of brain or spinal cord tissues, and sphingolipids and phospholipids were quantitatively resolved and purified using a silicic acid column (0.4 cm x 14 cm), as reported previously [8]. Briefly, ceramide was eluted from the column using chloroformacetone (9:1, v/v) and quantified by high performance thin-layer chromatography (HPTLC) followed by gas chromatography-mass spectrometry (GC-MS). Ceramide bands in HPTLC were first resolved using chloroform:methanol:acetic acid (95:4.5:0.5, v/v/v) visualized by iodine absorption followed by spraying with copper sulfate-sulfuric acid mixture [8]. The bands were visualized after heating at 110 °C, scanned, and quantified using Image J program. Total sphingolipids and sphingoids were eluted using tetrahydrofuran:water (7:1, v/v) after removal of MGCs (using chloroform:methanol, 23:2, v/v). Sphingomyelin and phosphatidylcholine were eluted using methanol. Purified MGCs and sphingomyelin were assayed using HPTLC and quantified after scanning. Individual component was expressed in μg/g (ceramide) and ng/g (sphingoids) of tissue weight and ng/mg of protein using reference standards. Sphingoids, purified by Dowex chromatography from tetrahydrofuran:water elution were further assayed by high performance liquid chromatography (HPLC) after fluorescent tagging [8, 26]. Lipid analysis methodology used [8] here was highly reproducible with requisite precautions as determined by the coefficient of variation (CV) within and between the groups. CV values were averaged within each set to get an average value for better interpretation of the data.

Apoptosis in EAE Spinal Cord and Brain as Determined by Internucleosomal DNA Fragmentation

To determine the tissue degeneration in EAE, the CNS tissues from control and EAE animals were examined for programmed cell death (apoptosis) employing a modified internucleosomal DNA fragmentation method [27]. Briefly, 100 mg of the CNS tissue from the control and EAE animals was surgically removed, homogenized, and digested with proteinase K (250 ng/μl) in Tris-HCl buffer (pH 8.0) containing 10 mM EDTA and 0.5% SDS for 24 h. DNA from digested tissues was extracted with an equal volume of chloroform:phenol (1:1, v/v), which was thoroughly pre-equilibrated with 0.5 M Tris-HCl buffer (pH 8.0) to avoid any DNA loss [25, 27]. Total genomic DNA was precipitated with 2 volumes of absolute ethanol in presence of $MgCl_2$ to a final concentration of 10 mM. The DNA pellet was digested with RNase A (to remove any contaminating RNA) and subjected to resolution on 1.6% agarose gels. After staining with ethidium bromide (1 μg/ml), the gels were photographed using Polaroid film (positive/negative) Type 665 and the negative was used to examine the presence of internucleosomal DNA fragmentation in EAE samples.

Determination of Serine-Palmitoyltransferase (SPT) Activity in EAE Spinal Cords

The activity of the SPT for de novo ceramide synthesis [28] was assayed in both thoracic and lumbar regions of the EAE and normal spinal cord microsomes, as described previously [29]. Briefly, tissues were disrupted with five strokes in a Potter-Elvehjem tissue grinder in 50 mM HEPES (pH 7.4), 0.25 M sucrose, and 5 mM EDTA.

Following disruption, the cells were lysed. The lysates were centrifuged for 15 min at 18,000 g. The supernatant was retained and centrifuged for 1 h at 100,000 g. The resulting microsomal pellet was suspended in 50 mM HEPES (pH 7.4), 5 mM EDTA, 5 mM DTT, and 20% (w/v) glycerol. All assays were performed under linear conditions with 100 µg of microsomal protein in the presence of 100 mM HEPES (pH 8.3), 2.5 mM EDTA, 5 mM DTT, 0.05 mM pyridoxal phosphate, 0.2 mM palmitoyl-CoA, and 1.0 mM serine (with 2 µCi/assay of ^{3}H-serine, Vt = 100 l). Reactions were carried out for 30 min at 37 °C and terminated by the addition of 0.2 ml of 0.5 N NaOH. Labelled lipid product was extracted and quantified by liquid scintillation counting. The SPT activity was expressed as percent of control spinal cord sections and was inhibited by the SPT inhibitor, myriocin [29].

Cell Culture Studies

To mimic degeneration of the oligodendrocytes in MS, we employed the HOG cells [24] in our cell culture studies and exposed these cells to two major pro-inflammatory cytokines (TNFα and IFNγ), widely known to be secreted in MS and EAE to predominantly affect the sphingolipid metabolism.

Sphingolipid Analysis in HOG Cells

Cell culture studies were initiated to examine the lipid profile after exposure of HOG cells to the pro-inflammatory cytokines (TNFα and IFNγ), as these cytokines were found to be secreted in MS brain and EAE spinal cord [15, 16]. Briefly, HOG cells were grown in DMEM (high glucose) containing sodium bicarbonate, 10% FBS and penicillin-streptomycin (Cellgro, Manassas, VA, USA). Cells were grown to 80–85% confluent state before changing the FBS concentration to 0.2%. Cytokine solutions (TNFα, 100 ng/ml; IFNγ, 100 ng/ml; TNFα + IFNγ, 100 ng/ml each) were added to cell cultures and incubated overnight. Cells were collected after trypsinization, washed with cold PBS, and preserved at −40 °C for sphingolipid analysis. Purified ceramide fraction was analysed by HPTLC [8] and GC-MS while the sphingosine content was quantified by HPLC [8, 26].

Myriocin Treatment to Block SPT Regulation After Cytokine Exposure

We used myriocin (5 µM), an inhibitor of SPT, to block the ceramide generation in HOG cells after stimulation with cytokines, as described previously [30], and examined the sphingolipid profile. Cells were grown to 70–80% confluent state and divided into four groups [Group # 1: untreated cells or control; Group # 2: cells treated with TNFα (100 ng/ml); Group # 3: cells treated with myriocin along with TNFα stimulation; and Group # 4: cells treated with TNFα, IFNγ, and combination of both cytokines (100 ng/ml each) in presence and absence of myriocin]. After 24 h of incubation, cells were trypsinized, collected in cold PBS, and washed once with PBS. Lipids were extracted and ceramide purification was carried out, as described previously [8]. The protein content was measured by BCA (Pierce, Rockford, IL, USA) method [8]. Fifteen microlitre of the ceramide solution (adjusted to equal protein concentration) was applied and resolved by HPTLC along with standard ceramide. Individual ceramide bands were scanned and quantified from the standard ceramide concentrations and confirmed by GC-MS [8].

RT-PCR for Examination of mRNA Expression of the SPT Gene

HOG cells were cultured equally in 4 × 60 mm petri-dish and incubated for 48 h. The medium was changed to 0.2% FBS and the cells were grown for another 24 h before exposure to the defined amounts of cytokines (TNFα and IFNγ). After incubating overnight, total RNA from the cells was isolated using a standard protocol [31]. Briefly, total RNA was prepared from the lysed cells according to specifications and used for preparation of cDNA. We used cDNA for PCR amplification of SPT gene and also β-actin as the housekeeping gene. The annealing of primers was carried out at 58 °C. We performed PCR for 30 cycles for β-actin and SPT1, while PCR for 25 cycles for SPT2. We used following primer sequences for SPT1, SPT2, and β-actin genes. SPT1 gene, forward: 5′-GCT AAC ATG GAG AAT GCA CTC-3′ and reverse: 5′-CTT CCT CCG TCT GCT CCA C-3′; SPT2 gene, forward: 5′-AGA TTA TCA CCT CCA TGA AGT-3′ and reverse: 5′-ATC TAT CTC CTT CAA AGC AGT-3′; and β-actin gene, forward: 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ and reverse: 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′.

Western Blotting for Examination of Caspase-3 Activation

Cytokine mediated apoptotic cell death was demonstrated in caspase-3 activation using Western blotting. Briefly, HOG cells were incubated with TNFα only and TNFα plus IFNγ, as described earlier. Cells were collected after treatment with the cytokines and lysed in Laemmeli buffer. The protein extracts were resolved using SDS-PAGE. The resolved protein bands were transferred onto a PVDF membrane (GE Healthcare Biosciences, Marlborough, MA, USA) and probed using an active caspase-3 monoclonal antibody (Cell Signalling, Sacramento, CA, USA). Active

caspase-3 bands were identified in lanes representing treatments with both cytokines.

Determination of Cell Death by Flow Cytometry

Next, the efficacy of myriocin, a specific inhibitor of SPT, to protect from cell death was examined using flow cytometry. Briefly, HOG cells were cultured in a 12-well dish and incubated with cytokines in presence or absence of myriocin (5 μM), as described previously [31]. The following sets were prepared for this study: control cells (untreated, set # 1), TNFα (set # 2), TNFα + myriocin (set # 3), TNFα + IFNγ (set # 4), and TNFα + IFNγ + myriocin (set # 5). Cells from all treatments were collected and washed with cold PBS. Cells were labeled with propidium iodide, washed, and fixed using methanol. The fixed cells were then analyzed by flow cytometry using Cell Quest program. In each set, minimum five wells were used to obtain a statistical evaluation.

Immunocytochemical Staining for Ceramide and SPT1 in HOG Cells

To confirm our observation that TNFα upregulated ceramide concentration by stimulating SPT activity, the SPT1 and ceramide levels were examined by immunocytochemical staining using SPT1 and ceramide specific antibodies [32]. Briefly, HOG cells were grown and cultured on poly-D-lysine coated cover slips. After 48 h of incubation, cells were exposed to TNFα overnight (media containing 0.2% FBS) while the control sample was treated with the media containing 0.2% FBS only (without TNFα). Cells were then washed and fixed using 4% paraformaldehyde. After blocking, cells were stained with the primary antibody (rabbit IgG for ceramide and mouse IgG for SPT1, respectively) followed by the secondary antibody conjugated with fluorescein isothiocyanate (FITC, for detection of ceramide) and tetramethylrhodamine (TRITC, for detection of SPT1). The fluorescent staining was observed using confocal microscopy with identical imaging conditions.

Use of FTY720 to Determine Ceramide Upregulation in HOG Cells

The drug fingolimod or FTY720 (Novartis), approved for clinical MS therapy [33–35], is an immunomodulatory agent that inhibits ceramide synthesis by blocking ceramide synthase. We tested the inhibitory efficacy of FTY720 in HOG cell culture after stimulation with TNFα (100 ng/ml) and combination of TNFα and IFNγ (100 ng/ml each). Cells, cultured in 5 × 100 mm dishes for studying the inhibitory efficacy of FTY720, were grown to confluency before exposing to media containing 0.2% FBS. The following five sets were prepared for this study: control (untreated, set # 1), TNFα (100 ng/ml, set # 2), TNFα + FTY720 (300 nM, set # 3), TNFα + IFNγ (100 ng/ml, set # 4), and TNFα + IFNγ + FTY720 (set # 5). Cells were collected after overnight treatment, washed with PBS, and saved at −20 °C for ceramide analysis. Lipids from each set of cells were extracted and purified, as described previously [8].

Use of Sphingosine to Demonstrate Induction of Cell Death in Oligodendrocytes

Two 96-well plates were cultured with 10,000–12,000 cells/well and incubated for 48 h. Cells were treated with sphingosine at desired concentrations (as described below) in DMEM containing 0.2% FBS and 0.04% dimethyl sulfoxide (DMSO) and incubated for 24 h. Stock solutions of DMEM, 0.2% FBS, and 0.04% DMSO (Media A) were prepared and used for dilution. A 40 μM sphingosine stock solution was prepared by evaporating 20 μl of 10 mM sphingosine (in ethanol), adding 2 μl of 0.04% DMSO, and mixing with 5 ml of the media containing 0.2% FBS (stock). This stock solution was diluted to a range of 0.25–30 μM concentrations. Two sets of control were used, one set containing the regular media with 10% FBS and the second set cultured in Media A. Cells were incubated for another 24 h and the cell viability was measured using cell counting kit 8 (Dojindo Molecular Technologies, MD, USA).

Statistical Analysis

All animal experiments were repeated three times using four animals per group. Analysis of variance (ANOVA) with Tukey's post-hoc analysis was used to compare the treatment groups. All p values reported were two sided and a $p < 0.05$ was considered statistically significant. All statistical analysis was performed using MS Excel v2010 or R (V3) on a Windows XP platform.

Results

Summary of Results from MS Brain and EAE Spinal Cord

There were (i) an upregulation of sphingosine in MS brain and EAE spinal cord, (ii) an ultimate decrease in ceramide level in MS brain, and (iii) also intermittent increases in psychosine and ceramide levels in EAE spinal cord but ultimate decreases in both components in MS brain. However, no change in sphingolipid profile (including the composition of gangliosides) was observed in EAE brain. All of the

results from our human and animal studies are elaborately described below.

Human and Animal Studies

Sphingosine Level was Elevated in MS Brain

Sphingolipid content such as ceramide, monoglycosylceramide (MGC), and sphingoids were analysed in six control and six MS brain tissues. Sphingoid content, specifically sphingosine, was increased by 1.5-fold in MS plaques while psychosine was increased in NAWM but reduced to 0.5-fold of the control in plaque (Fig. 1a). A reduction in MGC (GalCer and fast migrating cerebrosides or FMCs [36]) content in NAWM (0.65-fold) and plaque (0.5-fold) (data not shown) is consistent with demyelination because MGC is a myelin component and known to be decreased in myelinolytic disorders [37]. However, total ceramide showed a decreasing trend in NAWM (0.75-fold) and also in MS plaque (0.65-fold, data not shown). To further understand the mechanism of sphingosine elevation during the course of MS demyelination, we performed animal studies and induced EAE in Lewis rats. The animals were graded (EAE1, EAE2, EAE3, and EAE4) according to the severity of neurological impairment, and the levels of the CNS ceramide and sphingoids were quantified during the progression of EAE.

Sphingolipid Concentration was not Altered in EAE Brains

Brains surgically collected from the control and EAE animals were dissected into the following four regions: frontotemporal hemisphere, parieto-occipital hemisphere, cerebellum, and brainstem. Sphingolipids such as ceramide and MGC, sphingomyelin, and sphingoid contents were measured in each sample. All components remained unaffected in each brain region (data not shown), indicating that induction of EAE in this model did not primarily affect brain. Furthermore, no induction of apoptosis in EAE brains was identified. Since the animals suffered severe neurological damage with paralysis, the disability might be caused by neurological impairment and dysfunction in spinal cord rather than in brain. Therefore, we decided to assay the sphingolipid profile in both thoracic and lumbar spinal cord from the rats at different stages of EAE.

Sphingosine and Ceramide Were Elevated in EAE Spinal Cord

Sphingolipid contents in spinal cord segments (thoracic and lumbar regions) were assayed, as described previously [8], and induction of apoptosis was determined by analysing internucleosomal DNA fragmentation, as reported previously [27]. Like MS brain, increases in sphingosine (2.0–2.5-fold) and psychosine (1.5–1.75-fold) in EAE grades 2 and 3 (lumbar cord) appeared to be noteworthy (Fig. 1b). Thoracic cord showed a similar profile with moderate elevations in sphingosine and psychosine (data not shown). Psychosine content diminished considerably with EAE progression, specifically in EAE grade 4, and an almost same phenomenon was observed in MS brain (Fig. 1a). Ceramide content showed an increasing trend in early EAE stage followed by a decreasing trend through EAE progression in thoracic as well as in lumbar cord (Fig. 1c). (a) Thoracic region: There was a moderate increase (1.2-fold) in ceramide content (grades 2 and 3) in the thoracic cord, but the levels were reduced to control in EAE grade 4 (Fig. 1c). An indication of onset of demyelination was reflected by a moderate reduction in MGCs (0.9–0.85-fold). (b) Lumbar region: Demyelination was indicated by a greater decrease (from 0.7 to 0.6-fold) in MGC content. Increased ceramide (1.4–1.5-fold) in early EAE followed by a decreasing trend in late EAE (grade 4) was observed (Fig. 1c), but sphingomyelin was unaltered (data not shown). Since both sphingosine and psychosine were toxic and caused apoptosis in vitro [38, 39], the internucleosomal DNA fragmentation indicating occurrence of apoptosis in both thoracic and lumbar cord was examined.

Induction of Apoptosis in EAE Spinal Cord

The genomic DNA profile was examined by DNA laddering in EAE spinal cord (lumbar and thoracic regions) at grades 1–4. Our findings (data not shown) agree with a previous report of absence of internucleosomal DNA fragmentation in thoracic spinal cord in EAE [25]. In contrast to the brain and thoracic spinal cord, induction of apoptosis was observed in lumbar spinal cord in EAE grades 3 and 4, as indicated by presence of internucleosomal DNA fragmentations (Fig. 2). Interestingly, a gradual increase in sphingosine in EAE grades 2 and 3 (Fig. 1b) supported our perception that sphingosine caused toxicity to foster demyelination and apoptosis in EAE spinal cord.

Increase in SPT Activity in EAE Spinal Cord

Elevation in ceramide levels (1.2–1.4-fold) occurred in lumbar spinal cord regions in EAE grades 2 and 3 (Fig. 1c) while sphingomyelin content showed no alteration. Since the activation of SPT is the alternative source of sphingoid via ceramide generation, the SPT activity was determined in control and EAE spinal cord, as reported previously [29]. A moderate increase in the SPT activity proportionately to EAE severity was observed in thoracic spinal cord while the SPT activity in lumbar spinal cord was markedly elevated in EAE grades 2 and 3 (Table 1). This enzyme

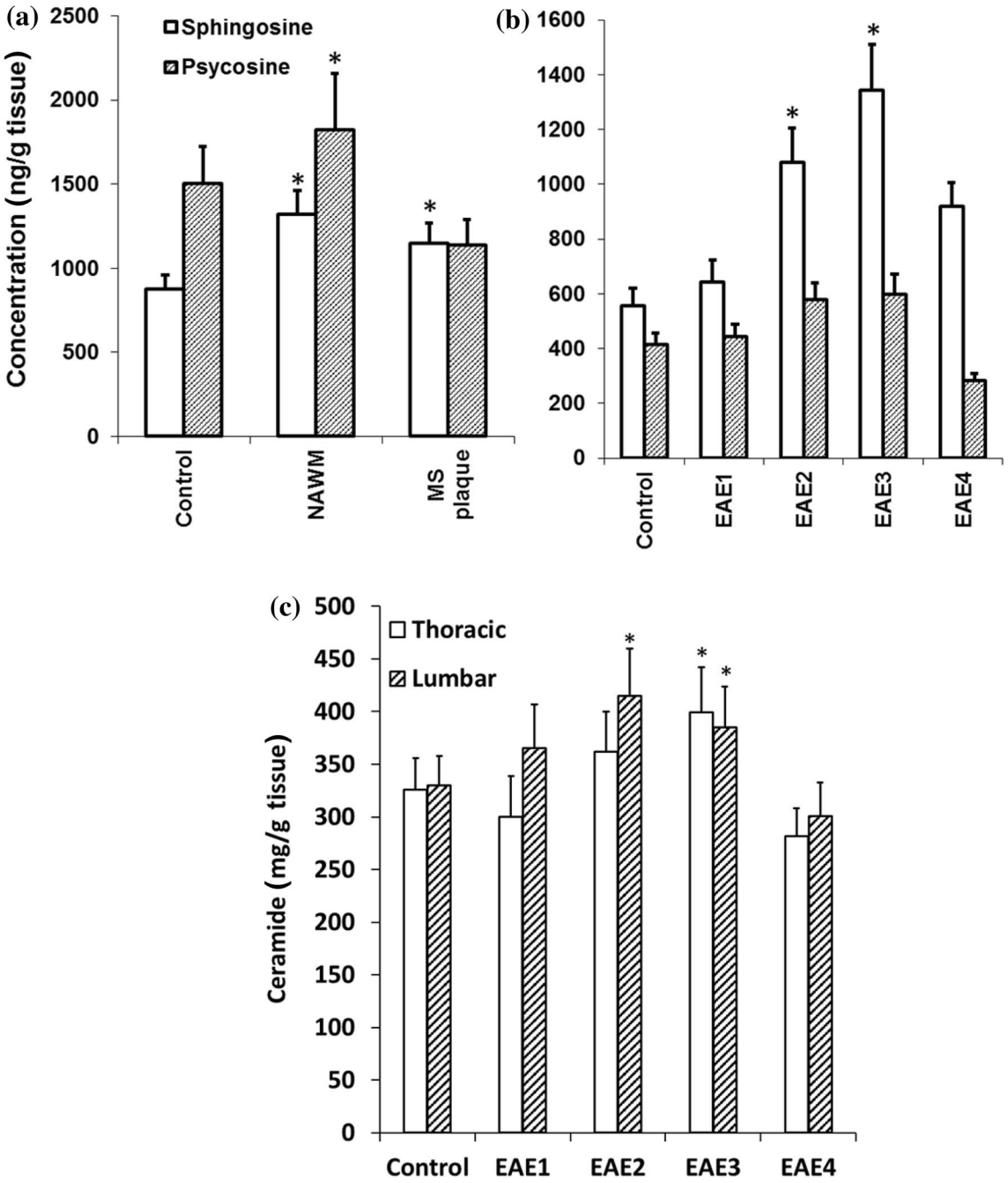

Fig. 1 Sphingosine and psychosine contents in MS brain (human) and EAE lumbar spinal cord and ceramide content in thoracic and lumbar spinal cord regions in EAE rats. Sphingosine (*bar with no line*) and psychosine (*bar with dashed line*) were purified and assayed by HPLC, as described in the text. **a** MS brain tissues ($n=6$). **b** Lumbar spinal cords from EAE rats ($n=4$). MS plaque and NAWM indicated a significant difference ($*p<0.05$) when compared with corresponding control tissues while data from EAE grade 2 (EAE2) and EAE grade 3 (EAE3) showed a significant variability ($*p<0.05$) in lumbar spinal cords. **c** Lipids were isolated from thoracic region (*empty bar with no line*) and lumbar spinal cord region (*bar with dashed line*) of EAE and control rats ($n=4$). Ceramide was purified by column chromatography and analysed by HPTLC and GC-MS, as described in the text. TLC bands were developed using chloroform:methanol:acetic acid (95:4.5:0.5, v/v). TLC bands were visualized by char spray and individual band was scanned and the concentration of each band was determined using Image J program. When compared with controls, significant difference was observed in thoracic spinal cord in EAE3 ($*p<0.05$) and lumbar spinal cord in EAE2 and EAE3 ($*p<0.05$)

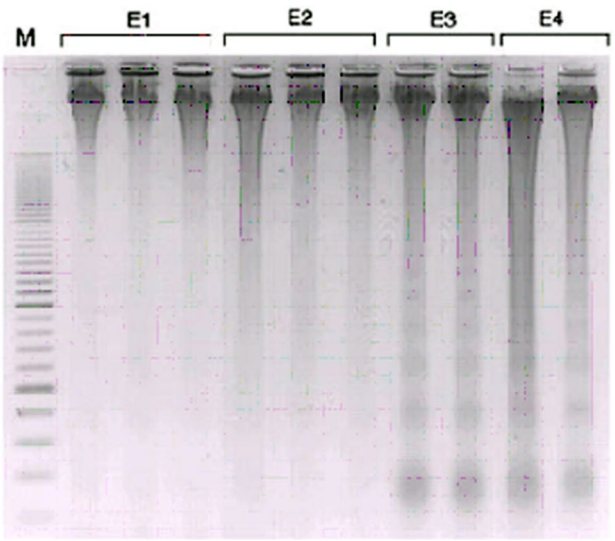

Fig. 2 Internucleosomal DNA fragmentation in lumbar spinal cord in different EAE grades. Genomic DNA samples were isolated from EAE lumbar spinal cord segments (1-cm long) and subjected to electrophoresis using 1.6% agarose gel. The DNA bands were visualized after staining with ethidium bromide. Internucleosomal DNA fragmentation (180 bp DNA ladder pattern), a well-recognized hallmark of apoptosis, indicated induction of apoptosis only in EAE3 and EAE4. E1, EAE1; E2, EAE2; E3, EAE3; E4, EAE4; and M, 100 bp DNA ladder markers

Table 1 SPT activity in EAE spinal cord

	SPT activity in percentage (%)	
	Spinal cord regions	
Tissue	Thoracic	Lumbar
Control	100	100
EAE1	103 ± 12	120 ± 15
EAE2	152 ± 18*	193 ± 20*
EAE3	131 ± 17	161 ± 18*
EAE4	98 ± 10	112 ± 12

SPT activity was measured in EAE spinal cord from thoracic and lumbar regions. SPT activity was determined using spinal cord homogenate and radioactive substrate and expressed in percent (%) compared to control ($p < 0.05$, between control and EAE2, thoracic cord; between control and EAE2 and also control and EAE3, lumbar cord; and $p = 4$)

*indicates $p < 0.05$ compared to control

activity corresponded well with our sphingoid and ceramide assays and indicated that the elevation in ceramide was partly mediated by stimulation in SPT activity. Our cell culture studies (described below) indicated that nearly 70% elevation in ceramide content might be due to increase in SPT activity.

Cell Culture Studies

Our cell culture studies produced some important results: (i) elevations in ceramide and sphingosine after cytokine stimulation led to HOG cell death, (ii) elevation in ceramide was blocked by myriocin and prevented HOG cell death, and (iii) a major portion of HOG cells died due to sphingosine production.

Pro-inflammatory Cytokines Stimulated Production of Ceramide and Sphingosine in HOG Cells in Culture

Although a large number of pro-inflammatory cytokines, which promote demyelination, have been identified in MS lesions, we have focused on examining the effect of two specific cytokines, TNFα and IFNγ on oligodendrocytes degeneration. Both cytokines are involved in demyelination in MS and perturbation of lipid metabolism [11, 40], indicating that TNFα and IFNγ act synergistically [41] and they together stimulate ceramide generation via sphingomyelinase activation [41, 42]. We have tested our hypothesis whether these two cytokines may also stimulate SPT activity in HOG cells in culture, based on an earlier report [24]. We found that these two cytokines, TNFα and IFNγ (combined effect), elevated ceramide level (1.3–1.4-fold) and sphingosine level (3.5-fold) in HOG cells (Table 2). We speculated that blocking the cytokine stimulation of ceramide generation (principal source for sphingosine generation) would protect the HOG cells from degeneration. Hence, HOG cells were exposed to these two cytokines in presence or absence of myriocin and FTY720 based on the concept that cytokine mediated de novo ceramide synthesis would be blocked by myriocin, a specific inhibitor of SPT, and also by FTY720. FTY720 is an immunomodulatory drug and a ceramide synthase inhibitor. It is a sphingosine-1-phosphate receptor modulator and an analog of myriocin

Table 2 Elevation in ceramide and sphingosine levels in HOG cells after cytokine stimulation

HOG cells treatment	Ceramide (ng/mg protein)	Sphingosine (ng/mg protein)
Control	910 ± 30	2.99 ± 0.2
+ TNFα	1050 ± 55	4.1 ± 0.35
+ IFNγ	975 ± 48	6.23 ± 0.55*
+ TNFα + IFNγ	1260 ± 63*	10.4 ± 0.8*

Ceramide and sphingosine (purified from HOG cells by column chromatography) were analysed by TLC and HPLC, respectively, as described in the text. Standard deviation (SD) value was obtained from a set of three independent experiments

*indicates $p < 0.05$ compared to control

[43]. A substantial inhibition of ceramide production by FTY720 and myriocin was observed in HOG cells (Fig. 3a, b). We would like to clarify that bands co-migrating with the standard ceramides (lanes 1–3, Fig. 3a) were scanned and their concentrations were determined (Fig. 3b) using the Image J software.

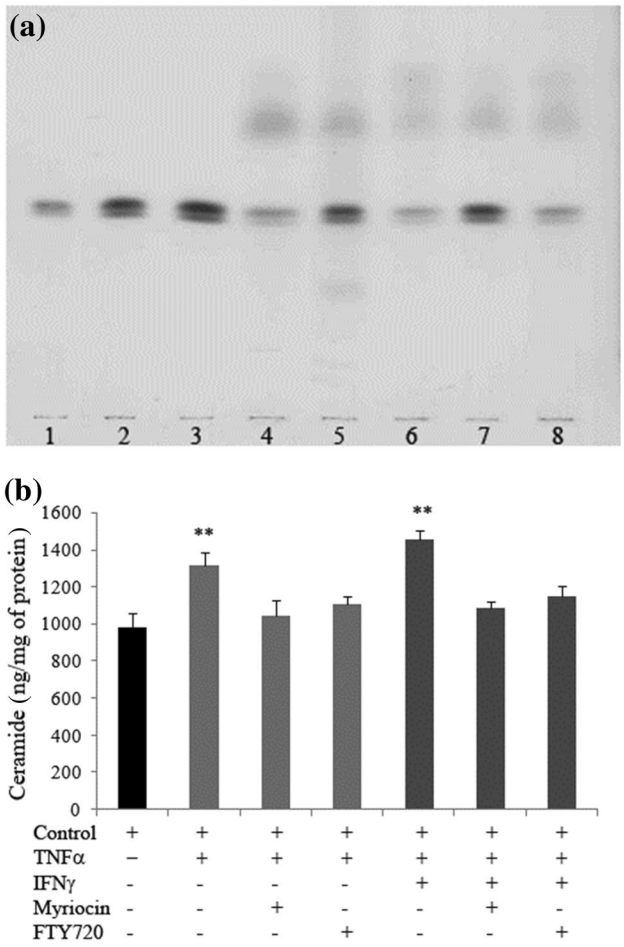

Fig. 3 Purification and estimation of ceramide contents in HOG cells. **a** Thin-layer chromatogram of purified ceramide fractions from HOG cells after cytokine stimulation followed by FTY720 treatment. The purified ceramide fractions from lipid extract was dissolved in a defined volume of chloroform:acetone according to the protein concentration and an equal volume of the solution was applied on an HPTLC and developed using chloroform:methanol:acetic acid (95:4.5:0.5, v/v/v). *Lanes 1–3*, standard ceramide at increasing concentrations; *Lane 4*, control; *Lane 5*, TNFα; *Lane 6*, TNFα + FTY720; *Lane 7*, TNFα + IFNγ; and *Lane 8*, TNFα + IFNγ + FTY720. HOG cells were incubated with cytokines with or without FTY720 for 24 h. **b** The individual ceramide bands visualized (co-migrating in *lanes 1–3*), were scanned, and presented as bar graph. Myriocin and FTY720 inhibited ceramide generation in HOG cells exposed to cytokines (TNFα and IFNγ alone or in combination). Myriocin appeared to be more effective than FTY720 in inhibiting ceramide synthesis. A significant difference was indicated by $*p < 0.05$ or $**p < 0.005$ when compared to control

TNFα Alone and in Combination with IFNγ Induced Apoptosis in HOG Cells

Western blotting was used to test our hypothesis that TNFα and IFNγ might induce apoptosis in HOG cells by activating caspase-3. A clearly sharp increase in active caspase-3 (17 kDa fragment) band was identified in two lanes that contained the protein extract from cells exposed to cytokines (Fig. 4a).

Upregulation of mRNA Expression of SPT Gene in HOG Cells After Cytokine Exposure

The stimulatory effect of TNFα and IFNγ was tested at the genetic level by examining the mRNA expression of SPT gene by employing RT-PCR. The levels of mRNA expression of both SPT1 and SPT2 genes were examined after cytokine stimulation in HOG cells. TNFα and IFNγ alone as well as in combination stimulated SPT expression but

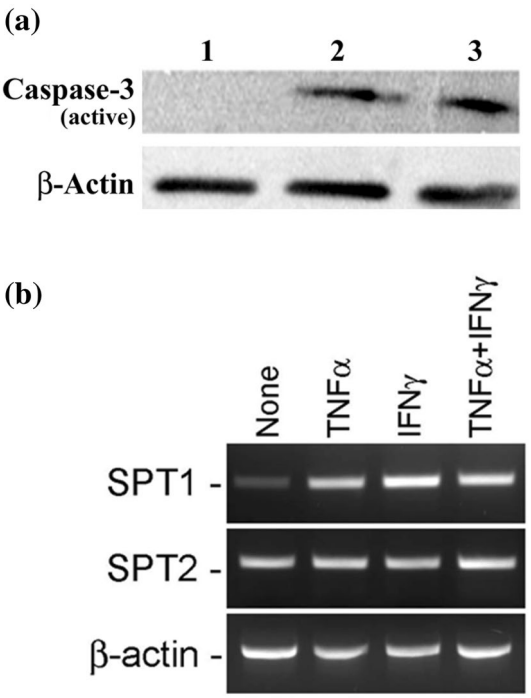

Fig. 4 Western blotting for demonstration of cytokine induced caspase-3 activation for apoptosis in HOG cells and RT-PCR experiments to examine mRNA expression of SPT1 and SPT2 genes in HOG cells after cytokine stimulation. **a** HOG cells were untreated (*Lane 1*) and treated with TNFα (*Lane 2*) or TNFα plus IFNγ (*Lane 3*). Proteins were extracted and subjected to Western blotting to examine caspase-3 activation. **b** HOG cells were exposed to TNFα and IFNγ alone or in combination. Total RNA was isolated and RT-PCR was used for observation of mRNA expression of SPT1 and SPT2 genes. An increase in SPT1 mRNA expression was observed after cytokine stimulation while a subtle increase in SPT2 was observed following stimulation with dual cytokines

the cytokine exposure stimulated SPT1 expression more than SPT2 expression (Fig. 4b). Although IFNγ itself showed a high stimulation, combination of both cytokines might produce some retrospective effect under experimental conditions [31]. Our current data strongly support the notion that ceramide upregulation by these cytokines was also mediated, at least partly, due to increase in SPT activity.

Immunocytochemical Identification of Upregulation of SPT1 and Ceramide and Their Co-localization in Oligodendrocyte Culture

In order to further confirm that TNFα upregulated SPT1 activity, we employed immunocytochemical staining of SPT1 (since SPT1 was more upregulated) and ceramide after TNFα stimulation in HOG cells. When compared with control cells, TNFα stimulation increased the expression of SPT1 along with upregulation of ceramide in HOG cells (Fig. 5a, b).

Myriocin Partially Rescued HOG Cells From Cytokine Stimulatory Effects

Both TNFα and IFNγ upregulated ceramide and sphingosine levels in HOG cells leading to cell death due to caspase-3 activation. Hence, we postulated that if ceramide/sphingosine elevation was mediated by SPT activity, cell death could be inhibited using myriocin. Our data indicated that, in fact, myriocin protected a large portion of the HOG cells from cell death after cytokine exposure for 24 and 48 h, and the efficacy of myriocin was prominant after incubation for 24 h rather than 48 h (Fig. 6). It should be noted that no difference was found between the two sets of controls.

Sphingosine Caused Oligodendrocyte Cell Death

To test whether sphingosine could mediate apoptosis in oligodendrocytes, HOG cells were exposed to different concentrations of sphingosine. A gradual decrease in cell growth was observed with sphingosine at concentrations between 0.5 and 30 μM (Fig. 7). A mild increase in cell growth due 0.25 μM spingosine supports the concept that sphingosine at a lower concentration mediates oligodendrocyte cell growth via activation of transient receptor

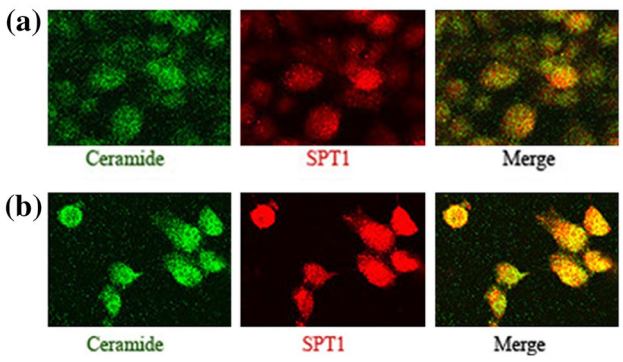

Fig. 5 Immunocytochemical localization of SPT1 and ceramide levels in HOG cells after cytokine stimulation. HOG cells were incubated with or without TNFα for 24 h, fixed, and stained with ceramide and SPT1 antibody. **a** Control cells. **b** TNFα treated cells showed an overexpression of ceramide (*green*) and SPT1 (*red*). (Color figure online)

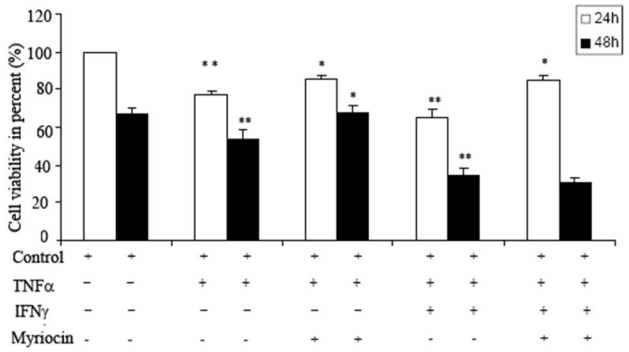

Fig. 6 Myriocin prevented oligodendrocyte cell death after cytokine stimulation. HOG cells were incubated with cytokines in presence or absence of myriocin for 24 and 48 h. Cells were collected and stained with propidium iodide and cell death was assayed by flow cytometry. TNFα induced cell death was prevented by myriocin. Myriocin inhibited cell death largely at 24 h. Control was measured as 100% viable (*$p < 0.05$ and **$p < 0.005$)

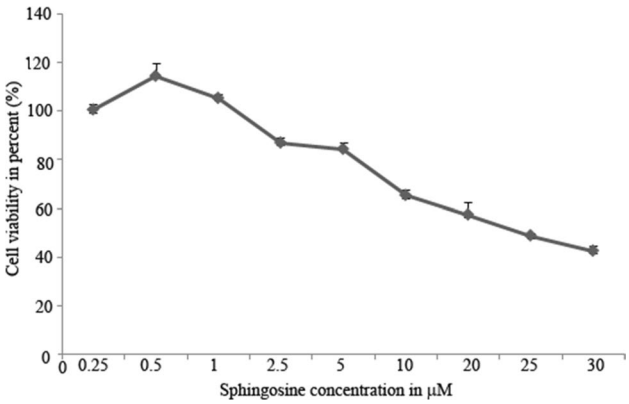

Fig. 7 Sphingosine induced cell death in oligodendrocytes. Cell viability was measured after addition of sphingosine at an increasing concentration to HOG cells and incubating for 24 h. Cell death was assayed using propidium iodide employing flow cytometry. A gradual decrease in cell viability was observed with increasing sphingosine concentration ($n = 4$). No difference was noted between two sets of controls

potential melastatin-3 (TRPM3), as reported in an earlier study [44].

Discussion

We earlier reported an elevation in sphingosine level in MS brain [20] and this observation was subsequently substantiated by the results from other studies, as reported in two earlier publications [19, 21]. While searching for the source of sphingosine elevation, we used an EAE rat model and observed an intermittent ceramide elevation in EAE grades 2 and 3, but ceramide level was reduced to normal level in EAE grade 4. Our novel findings from the current investigation showed that the increase in SPT activity (for ceramide synthesis) might play a significant role in EAE spinal cord triggering ceramide accumulation leading to in vivo sphingosine generation. Degeneration of oligodendrocytes is a well-known hallmark in MS as well as in EAE and we report that accumulation of sphingosine appears to be a causative agent for oligodendrocyte death. Oligodendrocytes may also die in different pathological scenarios such as adrenoleukodystrophy [45] and other neurodegenerative diseases [46, 47].

Our sphingolipid analysis in MS brain tissues reveals a decrease in monoglycosylceramide (MGC, such as GalCer and FMCs) [37, 47] in NAWM (35%) and in MS lesion (60%) reflecting the demyelination, because MGC is a myelin component [37, 45]. A decrease in ceramide (0.75-fold) along with MGC in NAWM and MS plaque was observed. Psychosine was elevated in NAWM but reduced in MS plaque. The reductions of ceramide and psychosine along with MGC in MS plaque are consistent with the notion that psychosine and ceramide are plausible myelin components [36, 37]. Although a previous report [46] suggested that ceramide was upregulated in plaque area, our current data indicated a decrease in ceramide content and agreed with recent reports [18, 40].

Although NAWM once was considered to be a normal appearance area, recent brain imaging indicated the pathogenic alternations in NAWM [48]. Moreover, significant abnormality including demyelination has been identified in NAWM of MS patients [49, 50]. Hence, biochemical changes in NAWM reported in our current investigation correspond well with the pathogenesis in demyelinating disease [51]. In addition, an increase in sphingosine content in NAWM and plaque in MS tissues, as we have shown here, is in agreement with the previous studies [19, 21]. However, the mechanism of ceramide/sphingosine accumulation and their effects on oligodendroglia remained unresolved in previous studies.

To evaluate the mechanism of sphingosine accumulation, EAE was induced in adult Lewis rats, and the animals were graded on the clinical basis, as described in an earlier report [25]. Since lesions in EAE rats are mostly associated with lumbar spinal cord leading to paralysis, sphingolipid contents in EAE spinal cord (thoracic and lumbar regions) were examined. As in MS, a reduction in MGC content was also recognized in EAE spinal cord indicating demyelination.

A transient elevation in ceramide (1.2-fold) in the thoracic spinal cord was observed in EAE grades 2 and 3, while ceramide accumulation was more in the lumbar region (1.1–1.4-fold) in EAE grades 1 and 2. The upregulation (in EAE grades 1 and 2) and down regulation (in EAE grade 3) of SPT activity in thoracic spinal cord suggested that ceramide elevation occurred by de novo synthesis in EAE [41, 52]. Although our data in EAE animals on ceramide do not show a complete compatibility with that in MS tissues, we think that a similar phenomenon of ceramide elevation may also occur in early MS onset followed by a gradual reduction with the disease progression. In fact, a moderate increase in C16:0 and C18:0 ceramide was observed in MS and NAWM when compared with corresponding control tissues [52]; moreover, a large increase in ceramide (1.9-fold) in MS cerebrospinal fluid and at the onset of EAE due to ceramide synthase (CerS6) activation was reported [53]. A trend in reduction of ceramide in late EAE (EAE grade 4) with increasing sphingosine concentration justified the decline of ceramide concentration due to demyelination, a condition that was also observed in MS samples. In lumbar spinal cord, a 2.5-fold increase in sphingosine (ceramide breakdown by ceramidase) and 1.5–1.75-fold increase in psychosine content were observed in EAE grades 2 and 3. This accumulation of sphingosine (and psychosine) could possibly be associated with elevation in ceramide concentration by stimulation in SPT activity [29, 44, 54], a condition also known to occur in ceramide induced apoptosis [29, 55].

A normal level of SPT activity in thoracic spinal cord in EAE grade 3 after initial elevation (EAE grade 2) may reflect down regulation of SPT by accumulated sphingosine as observed in cultured neurons [55]. Although our observation has established that SPT activation is a source of ceramide generation in MS and EAE, we do not rule out other alternative ceramide sources such as hydrolysis of sphingomyelin or breakdown of glycosphingolipid although exploring these possibilities are beyond the scope of our present investigation. The role of SPT activation in neurological diseases is of increasing interest because a mutation in one subunit of SPT has been reported in human hereditary motor and sensory neuropathy type 1 [56, 57].

The reduction in psychosine (and ceramide) in MS plaque may be attributed to severe demyelination since both GalCer and psychosine are potential substrates for β-galactosidase [58]. An increased sphingosine

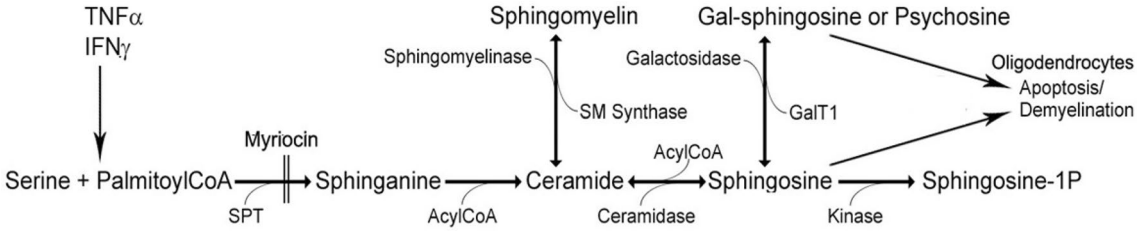

Fig. 8 Schematic presentation of the pathways for cytokine mediated synthesis of ceramide leading to sphingosine/psychosine generation and oligodendrocyte degeneration in MS and EAE

concentration in EAE grade 2 may have been produced by breakdown of ceramide by ceramidase [43]. Our observation of a region-specific elevation in sphingosine in spinal cord (EAE grade 2) agrees with a previous immunohistological study that indicated EAE gradually progressed from lumbosacral to thoracic spinal cord [59]. Apoptosis, as found by DNA laddering, occurred concurrently in the lumbar spinal cord in EAE grade 3 and 4, and this has also been reported by previous studies [27, 60]. Although elevation in ceramide and sphingosine was observed in thoracic spinal cord, no apoptosis was detected, employing DNA laddering, in this spinal cord region. This indicates that either ceramide/sphingosine at this low concentration does not initiate apoptosis and/or that is beyond the level of our detection. The elevation in psychosine in the lumbar region indicates that psychosine mediated myelinolysis may also occur.

A unique observation in this study is that a normal tissue maintains constant amounts of the free and ceramide-bound sphingosine/sphinganine ratio and these have been determined in EAE and MS tissues. It is worth mentioning that the ratio of free sphingosine/sphinganine and ceramide-bound sphingosine/sphinganine is increased in EAE tissues to an average 2–2.5 (1–1.25 in control) and 5 (3.0 in control), respectively, indicating that these ratios may be used as markers for tissue degeneration (apoptosis) since both sphingosine (not sphinganine) and ceramide (not dihydroceramide) are potent mediators of apoptosis in vitro. A similar variation in their molar ratio is also observed in NAWM and MS plaque when compared with corresponding normal brain tissues. Our results suggested that demyelination in EAE/MS did proceed via a sphingosine mediated apoptotic pathway through SPT activation in addition to sphingomyelin-ceramide mediated apoptosis [28, 42, 61]. However, further experiments are necessary to ascertain the precise mechanism of in vivo generation of ceramide and sphingosine for demyelination in MS.

The concept that TNFα signalling in MS demyelination also proceeds via de novo synthesis of ceramide was further supported by our in vitro studies using HOG cell line. TNFα mediated elevation in de novo ceramide synthesis and sphingosine accumulation (breaking down of ceramide by ceramidase) has been reported in other cells and its importance has also been discussed in a previous report [62]. Exposure of HOG cells to TNFα and IFNγ led to elevation in ceramide and was blocked by myriocin, a potent inhibitor of SPT. Apparently, the efficacy of inhibition of ceramide generation by myriocin (≥90%) indicated that cytokine mediated SPT activation could be a major source of ceramide in human MS (as HOG is a human cell line). Based on our studies, we depict the schematic pathways (Fig. 8) for cytokine mediated ceramide synthesis leading to sphingosine/psychosine generation and oligodendrocyte cell death in MS and EAE.

Fingolimod or FTY720, an immunomodulatory agent and a ceramide synthase inhibitor [34, 35], has been introduced as a potent therapeutic agent for MS. Our cell culture data using FTY720 indicated that ceramide/sphingosine synthesis was inevitably inhibited by FTY720 to prevent cell death in oligodendrocytes. However, inhibition by both inhibitors strongly suggests that a major portion of ceramide generation (70%) by TNFα and IFNγ is mediated via SPT activation because both myriocin and FTY720 are inhibitors of ceramide synthesis. Although activation of ceramidase is a potent cause of sphingosine generation, the precise mechanism of such myriocin/FTY720 inhibition is not clear. Further work will be needed to delineate the mechanism of myriocin and FTY720 mediated antagonism of cell death in oligodendrocytes.

Sphingosine has been shown to induce HOG cell death in a dose dependent manner our current investigation and this is in agreement with other studies showing that sphingosine can trigger apoptotic cell death in human gastric cancer cells [63], hepatoma cells [64], and rhabdomyosarcoma cells [39], possibly by inhibiting protein kinase C, and down regulating the ERK activation [65], and this process is also dependent on lysosomal proteases [66]. Thus, our data strongly suggest that sphingosine, in addition to ceramide, is a plausible secondary mediator of signal transduction for causing apoptotic death in oligodendrocytes in MS and EAE. However, a detail signaling mechanism for

cell death in oligodendrocytes was not pursued, as this was beyond the scope of our study.

Conclusion and Future Direction

Our study for the first time demonstrated that the elevation of ceramide and sphingosine occurred in EAE via in vivo ceramide generation (SPT activation). Two major cytokines, TNFα and IFNγ, acted synergistically in cultured oligodendrocytes leading sphingosine/psychosine accumulation for triggering cell death. Our study agreed well with previous studies that reported cytokine mediated de novo synthesis of ceramide [41] leading to sphingosine elevation in other cells [14, 64]. Hence, this study may help in the future for development of novel and potent therapeutic strategies for the treatment of demyelinating disease by targeting the ceramide biosynthetic pathway.

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