A Novel Role of Lactosylceramide in the Regulation of Tumor Necrosis Factor α-mediated Proliferation of Rat Primary Astrocytes

IMPLICATIONS FOR ASTROGLIOSIS FOLLOWING NEUROTRAUMA*

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The present study describes the role of glycosphingolipids in neuroinflammatory disease and investigates tumor necrosis factor α (TNFα)-induced astrogliosis following spinal cord injury. Astrogliosis is the hallmark of neuroinflammation and is characterized by proliferation of astrocytes and increased glial fibrillary acidic protein (GFAP) gene expression. In primary astrocytes, TNFα stimulation increased the intracellular levels of lactosylceramide (LacCer) and induced GFAP expression and astrocyte proliferation. D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCl (PDMP), a glucosylceramide synthase and LacCer synthase (GalT-2) inhibitor, inhibited astrocyte proliferation and GFAP expression, which were reversed by exogenous supplementation of LacCer but not by other glycosphingolipids. TNFα caused a rapid increase in the activity of GalT-2 and synthesis of LacCer. Silencing of GalT-2 gene using antisense oligonucleotides also attenuated the proliferation of astrocytes and GFAP expression. The PDMP and antisense-mediated inhibition of proliferation and GFAP expression was well correlated with decreased Ras/ERK1/2 pathway activation. Furthermore, TNFα-mediated astrocyte proliferation and GFAP expression was also inhibited by LY294002, a phosphatidylinositol 3-kinase 3-kinase inhibitor, which was reversed by exogenous LacCer. LY294002 also inhibited TNFα-induced GalT-2 activation and LacCer synthesis, suggesting a phosphatidylinositol 3-kinase-mediated regulation of GalT-2. In vivo, PDMP treatment attenuated chronic ERK1/2 activation and spinal cord injury (SCI)-induced astrocyte proliferation with improved functional recovery post-SCI. Therefore, the in vivo studies support the conclusions drawn from cell culture studies and provide evidence for the role of LacCer in TNFα-induced astrogliosis in a rat model of SCI. To our knowledge, this is the first report demonstrating the role of LacCer in the regulation of TNFα-induced proliferation and reactivity of primary astrocytes.

Traumatic injury to the adult central nervous system (CNS)1 results in a rapid inflammatory response by the resident astrocytes, characterized mainly by hypertrophy, proliferation, and increased glial fibrillary acidic protein (GFAP) expression, resulting in astrogliosis (1–4). Tumor necrosis factor-α (TNFα) has been identified as one of the first cytokines to appear following CNS injury and has been implicated in exacerbation of CNS injury. TNFα induces proliferation of both primary astrocytes (5,6) and human astroglioma cell lines (7,8) as well as GFAP overexpression (9). Although many reasons have been put forward to explain the obvious lack of CNS regeneration following injury/neurotrauma, the robust formation of the glial scar, as a result of astrogliosis, is also known to interfere with subsequent neural repair or axonal regeneration (2,10). Thus, considerable effort is being directed toward understanding the mechanisms involved in astrocyte proliferation and reactivity in order to design therapeutic approaches to modulate gliosis, which is an impediment to neuronal recovery and axonal regeneration.

Studies from our laboratory and others have shown the involvement of sphingolipids such as ceramide and psychosine in the potentiation of cytokine-mediated inflammatory disease (11–14). In addition, we have recently reported the involvement of lactosylceramide in the regulation of inducible nitric-oxide synthase gene expression and the efficacy of the glycosphingolipid biosynthesis inhibitor (PDMP) in attenuating spinal cord injury (SCI)-induced inflammatory disease, demonstrating significantly improved functional outcome post-SCI (15). The activation of sphingomyelinases and the resulting sphingomyelin-ceramide pathway has been closely linked with TNFα-induced apoptosis in numerous cell types. However, TNFα is also known to cause sphingosine 1-phosphate and lactosylceramide generation through activation of sphingosine kinase and lactosylceramide synthase (GalT-2), respectively, which have been implicated in inducing cell proliferation, which is antagonistic to the effects observed with ceramide (16,17). Whereas sphingosine 1-phosphate is mitogenic for primary astrocytes (18), LacCer has been linked with hyperproliferation of aortic smooth muscle cells in atherosclerosis (17).
In the present study, we sought to delineate the role of glycosphingolipids in TNFα-induced astrocyte proliferation and GFAP expression. LacCer generated through TNFα stimulation was found to be the effector molecule that regulated TNFα-induced proliferation of astrocytes and GFAP expression through the Ras/MEK/ERK pathway. LacCer generation in response to TNFα stimulation was found to be regulated in a PI3K-dependent manner, since 2-(4-morpholinyl)-8-phenyl-4H-1-benzo pyran-4-one (LY294002 or LY; a PI3K inhibitor) attenuated TNFα-induced GaIT-2 activation and LacCer production.

**Materials and Methods**

**Reagents**—Recombinant rat TNFα was obtained from Calbiochem. Dulbecco’s modified Eagle’s medium and fetal bovine serum were from Invitrogen (Grand Island, NY). A 20-mer antisense oligonucleotide (5’-CCC TGG AGC GCA GTC TT-3’) was obtained from Integrated DNA Technologies (Coralville, IA). The medium was incubated at 37 °C in 5% CO2 for 24 h prior to transfection.

**BrdU Activity assay**—The activity of GaIT-2 was measured using [3H]UDP-galactose as the galactose donor and GlCer as the acceptor as described previously (15). Briefly, following stimulation, cells were harvested in PBS, and cell pellets were suspended in Triton X-100 lysis buffer. Cell lysates were sonicated, and protein quantification followed. LacCer generation in response to TNFα stimulation was found to be regulated in a PI3K-dependent manner, since 2-(4-morpholinyl)-8-phenyl-4H-1-benzo pyran-4-one (LY294002 or LY; a PI3K inhibitor) was used for binding with agaro se-conjugated Ras-binding domain (RBD) of Raf-1, which was expressed in BL21 (Invitrogen). Echerichia coli was transformed by pGEX-2T/GST-RBD in the presence of 0.1 mM isopropyl 1-thio-β-D-galactopyranoside as described previously (20).

**RSD**

1. Quantification of Ras Activation—After stimulation, primary astrocytes in 6-well plates were washed with ice-cold phosphate-buffered saline (PBS) and lysed in membrane lysis buffer (0.5 ml of 25 mM TRIS pH 7.5, 150 mM NaCl, 1% Igepal CA 630, 0.25% sodium deoxycholate, 10% glycerol, 1 mM EDTA, 25 mM NaF, 1 mM of sodium orthovanadate, and EDTA-free Complete™ protease inhibitor mixture). After centrifugation (5,000 × g) at 4 °C for 5 min, supernatant was used for the Ras activation assay. 100 µg of supernatant was used for binding with agaro se-conjugated Ras-binding domain (RBD) of Raf-1, which was expressed in BL21 (Invitrogen). Echerichia coli was transformed by pGEX-2T/GST-RBD in the presence of 0.1 mM isopropyl 1-thio-β-D-galactopyranoside as described previously (20).

2. Measurement of LacCer Synthesis—Cultured cells were incubated in growth medium containing [14C]galactose (5 Ci/mM) for 24 h as described previously. The medium was removed, and the cell monolayer was washed with sterile PBS. After stimulation with TNFα (1 nM) for various periods of time, cells were harvested and washed with ice-cold PBS and lysed by sonication. 200 µg of protein was used for extraction of lipids using chloroform/methanol/HCl (100:100:1). The organic phase was dried under nitrogen. Glycosphingolipids were resolved by high-performance thin layer chromatography using chloroform/methanol/0.25% KCl (70:30:4, v/v/v) as the developing solvent. The gel area corresponding to GalT-2 activity was measured on a scintillation counter. Assay without exogenous GlCer served as blank, and their radioactivity counts were subtracted from all respective data points.

3. GalT-2 Antisense Oligonucleotide—A 20-mer antisense oligonucleotide of the following sequence (5’-CCC TGG AGC GCA GTC TT-3’) was obtained from Invitrogen. A scrambled oligonucleotide (5’-CTG ATA TCG TCG ATA TCG AT-3’) was also synthesized and used as control. Cells were counted and plated a day before transfection, and the following day they were treated with Oligofectamine (Invitrogen)-oligonucleotide complexes (200 nM oligonucleotide) under serum-free conditions. 48 h following transfection, the protein levels of GaIT-2 were analyzed using polyclonal antibodies raised against rat GaIT-2 (Abgent Inc.). 48 h following transfection, the cells were stimulated with TNFα (1 ng/ml), and BrdU incorporation was assayed 18 h following stimulation.

**Plasmids, Transient Transfection, and FACS Analysis—Dominant Negative Ras expression vector (pCMVRasN17) was purchased from BD Biosciences. pEGFP expression plasmid was purchased from Clontech. p110Δkin, a kinase-deficient version of p110 (the catalytic subunit of PI3K) was obtained from Tanti et at. (21). 3 × 105 cells/well were cultured in 6-well plates for 1 day before the transfection. Transfection was performed with plasmid concentration constant (2.5 µg/transfection) and 8 µl of Fugene transfection reagent (Roche Applied Science). 24 h following transfection, the cells were placed in serum-free media overnight. Following stimulation for 18 h, the cells were trypanopinized and pelleted, the cell pellets were washed with PBS and finally resuspended in 100 µl of PBS. The cells were fixed in 70% ethanol at 4 °C for 1 h. Following fixation, cells were pelleted and the cell pellets were washed with PBS three times. The DNA was stained with 7-amino-actinomycin D (7-AAD). Cell cycle analysis was done. Events were acquired using a BD Biosciences FACSCalibur equipped with a 488-nm argon laser and CellQuest software. pEGFP was acquired using a 515–545-nm bandpass filter (FL1), and 7-AAD was acquired using a 515–545-nm bandpass filter (FL1).
For 60 min. 50–100 units of reverse transcriptase by incubating the tubes at 42 °C. The primer sequences for GFAP (forward, 5'-cag ata ctc ttc tgt ttc ttg-3') were synthesized in a 50-μl reaction containing 5 μg of total RNA and 50–100 units of reverse transcriptase by incubating the tubes at 42 °C for 60 min.

Real-time PCR—Total RNA isolation from rat spinal cord sections was performed using TRIzol (Invitrogen) according to the manufacturer's protocol. Real-time PCR was conducted using Bio-Rad iCycler (iCycler iQ Multi-Color Real Time PCR Detection System; Bio-Rad). Single-stranded cDNA was synthesized from total RNA as described. The primer sets for use were designed (Oligoperfect™ designer, Invitrogen) and synthesized from Integrated DNA Technologies (Coralville, IA). The primer sequences for GFAP (forward, 5'-cag ata ctc ttc tgt ttc ttg-3') were annealed to the RNA according to the manufacturer's protocol. cDNA was synthesized in a 50-μl reaction containing 5 μg of total RNA and 50–100 units of reverse transcriptase by incubating the tubes at 42 °C for 60 min.

Induction of SCI in Rats—Sprague-Dawley female rats (225–250 g in weight) were purchased (Harlan Laboratories, Durham, NC) for induction of SCI. All rats were given water and food pellets ad libitum and maintained in accordance with the Guide for the Care and Use of Laboratory Animals of the United States Department of Health and Human Services (National Institutes of Health, Bethesda, MD). We have used a clinically relevant weight drop device for the induction of SCI in rats as described earlier (22). Briefly, rats were anesthetized by intraperitoneal administration of ketamine (80 mg/kg) plus xylazine (10 mg/kg) followed by laminectomy at T12. While the spine was immobilized with a stereotactic device, injury (30 g/cm force) was induced by dropping a weight of 5 g from a height of 6 cm onto an impounder gently placed on the spinal cord. Sham-operated animals underwent laminectomy only. However, no prophylactic antibiotics or analogues were used in order to avoid their possible interactions with the experimental therapy of SCI.

Treatment of SCI—Within 30 min after induction of SCI, rats received the glycosphingolipid inhibitor, PDMP (Mentreya). PDMP was dissolved in 5% Tween 80 in saline and diluted with sterile saline (0.85% NaCl) at the time of intraperitoneal administration to SCI rats. Animals (six per group) were randomly selected to form four different groups: vehicle (VHC) (5% Tween 80 in saline)-treated sham (laminectomy only) and SCI and PDMP (20 mg/kg in 5% Tween 80)-treated sham and SCI. A single dose of PDMP was administered every 24 h after the first dose 72 h after injury. Animals were sacrificed under anesthesia 1 h, 4 h, 12 h, 24 h, 48 h, 72 h, and 1 week following treatment.

Preparation of Spinal Cord Sections—Rats were anesthetized and sacrificed by decapitation. Spinal cord sections with the site of injury as described earlier were placed on the spinal cord. Sham-operated animals underwent laminectomy. Sections of spinal cord to be used for histological examination and pEGFP-positive events. Following total RNA extraction using TRIzol (Invitrogen) per the manufacturer's protocol, single-stranded cDNA was synthesized from total RNA. 5 μg of total RNA was treated with 2 units of DNase I (bovine pancreas; Sigma) for 15 min at room temperature in an 18-ml volume containing 1X PCR buffer and 2 mM MgCl₂. It was then inactivated by incubation with 2 μl of 25 mM EDTA at 65 °C for 15 min. 2 μl of random primers were added and annealed to the RNA according to the manufacturer's protocol. cDNA was synthesized in a 50-μl reaction containing 5 μg of total RNA and 50–100 units of reverse transcriptase by incubating the tubes at 42 °C for 60 min.

Statistical Analysis—All values shown in the figures are expressed as the means ± S.E. of values obtained from at least three independent experiments. The results were examined by one- and two-way analysis of variance; then individual group means were compared with the Bonferroni test. p < 0.05 was considered significant.

RESULTS

TNFα-induced Proliferation of Rat Primary Astrocytes Is Mediated by GSL—TNFα stimulation of primary astrocytes, resulting in proliferation of astrocytes and their reactive transformation characterized by increased GFAP expression, is a complex multistep process. In the present study, we tested whether GSLs were somehow involved in astrocyte proliferation. Increasing concentrations of TNFα (0, 0.1, 1, and 5 ng/ml) induced proliferation of astrocytes, which was assayed by BrdUrd incorporation (Fig. 1A). To address the involvement of GSL in TNFα-mediated proliferation, primary astrocytes were pretreated for 0.5 h with several concentrations of the glycosphingolipid inhibitor PDMP (0, 10, 20, 30, and 50 μM) or its corresponding inactive enatiomer, InPDMP (0, 10, 20, 30, and, 50 μM), followed by stimulation with TNFα (1 ng/ml) for 18 h. PDMP dose-dependently inhibited cellular proliferation assayed by BrdUrd incorporation (Fig. 1B). While InPDMP has no effect (Fig. 1C). TNFα (at a concentration of 1 ng/ml) and PDMP (25 μM) were used for subsequent studies. Furthermore, increasing doses of lactosylceramide (LacCer) induced proliferation of astrocytes; however, GluCer did not have a similar effect (Fig. 1D). Additionally, exogenously supplemented LacCer but not GluCer was able to bypass PDMP-mediated inhibition of TNFα-induced proliferation (Fig. 1E). A similar trend was observed with regard to GFAP gene expression. Pretreatment of astrocytes with PDMP inhibited TNFα-induced GFAP mRNA and protein expression, which was reversed by exogenously supplemented LacCer (Fig. 1F). Furthermore, as shown in Fig. 2, exogenous supplementation of other GSL metabolites such as GalCer (Fig. 2A) and gangliosides GM1 (Fig. 2B), GM2 (Fig. 2C), and GD3 (Fig. 2D) neither induced proliferation themselves nor reversed the PDMP-mediated inhibition of TNFα-induced proliferation, thus indicating this to be a LacCer-specific effect. Therefore, a metabolite of the glycosphingolipid pathway, LacCer, may play a role in the regulation of TNFα-mediated proliferation of astrocytes and GFAP expression, two processes that accompany astrogliosis.

TNFα Stimulation Results in Altered Levels of LacCer—To understand the mechanism of TNFα-induced astrocyte proliferation mediated by LacCer, in situ levels of lactosylceramide were quantified. [3H]LacCer was released and characterized by Rf value using commercially available standard LacCer by HPTLC as described under “Materials and Methods.” As shown in Fig. 3A, a sharp increase in LacCer levels was observed within 2–5 min following stimulation with TNFα. Upon TNFα stimulation, LacCer levels increased to ~2.5-fold of those observed in unstimulated cells. Correspondingly, a rapid increase in GalT-2 enzyme activity was also observed upon TNFα stimulation (Fig. 3B). The role of GalT-2 and its product LacCer in...
cell proliferation was further confirmed by silencing GaIT-2 gene using antisense (AS) DNA oligomers against rat GaIT-2 mRNA and a sequence-scrambled oligomer as a control. As shown in Fig. 3C, diminished protein levels of GaIT-2 by AS oligonucleotides correlated with diminished synthesis of $[^{14}C]$LacCer upon TNF$\alpha$ stimulation. Silencing of GaIT-2 with AS oligomers decreased the TNF$\alpha$-induced astrocyte proliferation (Fig. 3D), whereas supplementing LacCer exogenously bypassed the inhibition, presumably because the signaling events downstream of LacCer can be triggered upon the addi-
Lactosylceramide-mediated Regulation of Astrogliaosi

Correlating with decreased astrocyte proliferation, diminished GFAP mRNA (Fig. 3E) and protein levels (Fig. 3F) were observed upon GaIT-2 silencing using AS oligomers. However, in the presence of exogenous LacCer, AS-mediated inhibition of GFAP expression was blunted, thus further establishing the involvement of LacCer in astrogliosis.

**Activation of Small GTPase Ras and ERK1/2 Is Involved in LacCer-mediated Regulation of TNFα-induced Proliferation**

Since we have previously established the redox-dependent regulation of small GTPase Ras by LacCer (15), the possible involvement of Ras in LacCer-mediated regulation of TNFα-induced astrocyte proliferation was investigated. Primary astrocytes were transiently co-transfected with dominant negative Ras, DN-Ras (H-Ras N17 mutant), and pEGFP as a transfection marker followed by cell cycle analysis of the GFP-gated cells by FACS. Upon TNFα and LacCer stimulation, the percentage of cells in S-phase was significantly increased in the mock-transfected group. However, the DN-Ras-transfected group showed a significantly decreased percentage of cells in S-phase (Fig. 4A). TNFα- and LacCer-induced GFAP mRNA and protein expression was also significantly attenuated in DN-Ras-transfected cells and GFAP expression (Fig. 4B). These results show that Ras is involved and necessary for cellular proliferation as well as for GFAP expression. The inability of exogenous LacCer to bypass the inhibition by DN-Ras demonstrated that Ras is necessary for LacCer-mediated proliferation and GFAP gene expression and suggests that Ras is downstream of LacCer in the signaling cascade that induces astrogliosis. The role of Ras was further confirmed by assaying Ras activity using the glutathione S-transferase-conjugated Raf-1 RBD (Ras binding domain). As expected, TNFα stimulation enhanced the activation of Ras, which was attenuated upon PDMP pretreatment. PDMP-mediated inhibition of TNFα-induced Ras activation was fully reversed by the addition of LacCer (Fig. 4C). Furthermore, TNFα-induced Ras activation was also attenuated upon silencing of GaIT-2 expression by AS oligonucleotides, thus further confirming LacCer-mediated regulation of Ras activation (Fig. 4D). To further examine the signaling events downstream of Ras that mediate proliferation and GFAP expression, we investigated the involvement of two well established downstream effectors of Ras, ERK1/2 (Fig. 4E) and PI3K (Fig. 5). In correlation with the effect on proliferation and regulation of GFAP expression reported earlier (9), inhibition of the ERK1/2 pathway by PD98059 (a MEK1/2 inhibitor) pretreatment attenuated TNFα-mediated astrocyte proliferation (Fig. 4E) and GFAP (Fig. 4F) expression, and this effect could not be reversed even by exogenous supplementation of LacCer, indicative of MEK-ERK1/2 being downstream of LacCer in the signaling cascade. Further, the effect of MEK1/2 inhibitor observed on cell proliferation was also confirmed by directly assaying ERK1/2 activation using antibodies specific for the phosphorylated (activated) form of ERK1/2. TNFα-induced phosphorylation of ERK1/2 was inhibited both by PDMP and MEK1/2 inhibitor PD98059. However, exogenous LacCer supplementation could only reverse PDMP-mediated inhibition of ERK1/2 activity and not PD98059-mediated inhibition (Fig. 4, G and H, respectively). In addition, AS-mediated silencing of GaIT-2 expression attenuated TNFα-induced ERK1/2 activation (Fig. 4F). These results establish LacCer-mediated regulation of astrocyte proliferation and GFAP expression to be through the small GTPase Ras/ERK1/2 pathway.

**The Role of PI3K in TNFα-mediated Regulation of Astrocyte Proliferation**

The involvement of the second effector of Ras, PI3K, in astrocyte proliferation was also examined. PI3K has been reported to be involved in cell survival pathways and pro-
FIG. 4. The involvement of small GTPase Ras and ERK1/2 in LacCer-mediated regulation of TNF-α-induced proliferation and GFAP gene expression in primary astrocytes. Dominant negative Ras (DN-Ras) was transiently co-transfected with pEGFP (transfection marker) in primary astrocytes followed by stimulation with TNFα and/or LacCer. The cell cycle status of the GFP-gated cell population was assayed by FACS analysis (A), and GFAP mRNA and protein expression (B) was assayed in DN-Ras- and mock-transfected primary astrocytes. The effect of inhibition of LacCer synthesis via PDMP (C) or through AS-mediated silencing of GalT-2 (D) was examined on Ras activation using a glutathione S-transferase-tagged Ras-1 Ras binding domain as described under “Materials and Methods.” Pretreatment with LacCer and/or PDMP (25 μM) for 0.5 h or transfection with AS oligomers for 48 h was followed by TNFα stimulation for 5 min, cell lysates were used to assay levels of activated Ras, which is represented as a graph following densitometric analysis of the autoradiograph, and the data are expressed as arbitrary density units (A.D.U.). To examine MEK/ERK pathway involvement in proliferation and GFAP expression, upon pretreatment for 0.5 h with PD98059 (30 μM), a MEK1/2 inhibitor, and/or LacCer (10 μM), followed by stimulation with TNFα for 18 h, cell proliferation (E) and GFAP expression was assayed (F). ERK1/2 activation was assayed upon LacCer depletion using PDMP (G) or GalT-2 silencing (I) or in response to the MEK inhibitor PD98059 (H). PDMP or PD98059 was pretreated for 0.5 h followed by stimulation with TNFα for 20 min and immunoblot using phosphorylated ERK1/2 as described under “Materials and Methods.” In the case of AS-mediated silencing of GalT-2, cells were transfected with the oligomers. 48 h following transfection, the cells were stimulated with TNFα for 20 min, following which cells lysates were prepared and assayed for ERK1/2 activation. *** p < 0.01 in A as compared with unstimulated pcDNA-transfected cells. #, p < 0.001 as compared with mock-transfected TNFα-stimulated cells in A.
signaling events such as activation of the Ras/ERK1/2 signaling cascade, which regulates proliferation and GFAP expression.

The Efficacy of PDMP in Attenuation of Astrogliosis in SCI—To test the physiological relevance of our observations and further investigate the role of LacCer in astrogliosis in vivo, we examined the effect of PDMP in the rat SCI model. Rapid and chronic activation of ERK1/2 has been proposed to be a mechanism that operates in astroglial activation following acute CNS injury (23, 24). Furthermore, astrogliosis triggered in response to secondary inflammatory disease has been widely reported to be detrimental for axonal regeneration and recovery in SCI (25–27). As shown in Fig. 6A, a robust activation of ERK1/2 is observed within 1 h post-SCI. Activated ERK1/2 levels steadily rise until 48 h and remain substantially elevated even until 1 week post-SCI. However, PDMP treatment 0.5 h post-SCI effectively attenuates chronic ERK1/2 activation (Fig. 6A). Additionally, PDMP treatment effectively attenuated GFAP mRNA (Fig. 6B) and protein expression (Fig. 6C), which was highly up-regulated in VHC-treated SCI. Furthermore, double immunofluorescence analysis of spinal cord sections from the lesion epicenter of VHC-treated SCI rats showed a significant increase in GFAP (Fig. 7D) and activated ERK1/2 (Fig. 7E) levels and their co-localization (Fig. 7F) 24 h following injury, whereas PDMP-treated SCI rats showed significantly attenuated GFAP expression (Fig. 7H) and ERK1/2 activation (Fig. 7K), as well as their co-localization (Fig. 7L), thus demonstrating the efficacy of PDMP in vivo in controlling chronic ERK1/2 activation and GFAP expression. To determine whether administration of PDMP suppressed astrogliosis after SCI, we counted the number of GFAP and BrdUrd double
positive cells in tissue sections. Double immunostaining with GFAP and BrdUrd revealed numerous proliferating astrocytes along the margins of the lesion in the VHC-treated SCI (Fig. 8B) as compared with VHC-treated sham (Fig. 8A), PDMP-treated sham (Fig. 8C), or PDMP-treated SCI (Fig. 8D). Correspondingly, PDMP-treated rats had a significantly better neurological outcome with a BBB score of 14 as compared with the VHC-treated rats with a score of 7, which could be due to attenuation of inflammatory events such as iNOS expression and proinflammatory cytokine expression as reported earlier (15) as well as astrogliosis as shown in this report (Fig. 8F).

Thus, these studies indicate the involvement of glycosphingolipids in astrogliosis at the site of lesion in an in vivo model of SCI. These observations find critical relevance in other neuroinflammatory diseases as well, since astrogliosis and its detrimental effects are common to a number of CNS disorders.

FIG. 6. ERK1/2 activation and GFAP mRNA and protein expression at the lesion epicenter following SCI. Phosphorylated ERK1/2 levels were assayed by immunoblot analysis of protein samples derived from spinal cord sections of vehicle (VHC) or PDMP-treated sham operated or SCI rats. The ratio of pERK/ERK is depicted as well (A). GFAP mRNA levels were quantified by real time PCR analysis (B), and protein levels were quantified by immunoblot analysis (C) from RNA and protein samples derived from spinal cord sections of vehicle (VHC)- or PDMP-treated sham-operated or SCI rats at various time points. Data are represented as mean ± S.D. *** p < 0.001 as compared with VHC-treated sham; # p < 0.001 as compared with VHC-treated at 12 h.

FIG. 7. Double immunofluorescence staining of spinal cord sections from the lesion epicenter for pERK/GFAP co-localization. Immunofluorescent microscopy images of spinal cord sections from sham and SCI rats, stained with antibodies to pERK (red) and GFAP (green) as described under "Materials and Methods." A–C, GFAP (A), pERK (B), and their co-localization (C) in VHC-treated sham. D–F, GFAP (D), pERK (E), and their co-localization (F) in VHC-treated SCI. G–I, GFAP (G), pERK (H), and their co-localization (I) in PDMP-treated sham. J–L, GFAP (J), pERK (K), and their co-localization (L) in PDMP-treated SCI rats.

DISCUSSION

Astrogliosis is a prominent and ubiquitous reaction characterized by proliferation of astrocytes with up-regulated expression of GFAP. Although the functional role of astrogliosis is not clearly defined, numerous studies have documented its pathological interference with the function of residing neuronal circuits, thus preventing axonal remyelination and inhibiting axonal regeneration (28, 29). We have previously reported the involvement of LacCer in inducible nitric-oxide synthase gene expression in primary astrocytes and the anti-inflammatory efficacy of PDMP treatment in protecting against white matter vacuolization, demyelination, and neuronal apoptosis resulting in a profoundly improved neurological outcome in a rat model of SCI (15). Since PDMP treatment profoundly attenuated the inflammatory disease process post-SCI, including GFAP expression, which is a characteristic feature of astrogliosis, in this study we sought to investigate the involvement of GSL in proliferation of astrocytes and GFAP expression, the two processes that culminate in astrogliosis. This study demonstrates a novel pathway of LacCer-mediated regulation of TNFα-induced astrocyte proliferation and GFAP expression through signaling events involving PI3K and the Ras/ERK1/2 pathway in primary astrocytes. These conclusions are based on the following findings (1). TNFα-stimulation induced the activity of GαT-2 and increased the production of LacCer (2). The inhibition of GSL synthesis by PDMP or antisense oligonucleotides to GαT-2 inhibited astrocyte proliferation and GFAP expression, which was reversed by LacCer but not by other GSLs (GlucCer, GaIcCer, GM1, GM3, and GD3) (3). Inhibition of LacCer synthesis also inhibited the activation of the Ras/ERK1/2 pathway (4). PI3K through an as yet unknown mechanism regulated GαT-2 enzyme activity and LacCer production (6). PDMP treatment effectively attenuated chronic ERK1/2 activation and GFAP expression in a rat model of SCI.

Fig. 9 shows a schematic representation of the possible mechanism of regulation of TNFα-induced astrocyte proliferation and GFAP expression by LacCer. Based on data presented in this report, TNFα through the activation of PI3K results in...
the activation of GalT-2, leading to LacCer biosynthesis. LacCer generation recruits and activates the small GTPase (Ras) that activates the downstream ERK1/2 pathway, thus resulting in astrocyte proliferation and GFAP expression and triggering astrogliosis. Studies from our laboratory and others have reported the mechanism for the LacCer-mediated regulation of Ras to be reactive oxygen species-dependent in primary astrocytes (15) and other cell types (17). TNFα-induced activation of the small GTPase Ras could be through the direct activation of Src kinases associated with the LacCer-enriched glycosphingolipid signaling domains (GSDs) present on the cell surface. A number of studies have shown that several transducer molecules such as Src kinase associate with these GSDs and form functional units known as lipid rafts, which mediate signal transduction and cellular functions (30). In particular, LacCer has been shown to play an important role in the stabilization of GSDs (31). The addition of exogenous LacCer to the plasma membrane also initiates a similar downstream signaling cascade following incorporation into the plasma membrane. The amphipathic properties of GSLs that make them capable of being incorporated into cellular membrane have been extensively exploited to identify the functions of specific GSLs (32, 33). LacCer addition to the outer leaflet of the plasma membrane bilayer could induce redistribution and reorganization of the lipids in both leaflets of the plasma membrane, possibly resulting in the sorting of LacCer into GSDs. Coupling of the inner and outer leaflets of the lipid bilayer has been documented in artificial membranes (34), and this phenomenon is also thought to occur at the plasma membrane of living cells, based on the localization of various lipid-anchored proteins at the plasma membrane (35, 36). If such a coupling occurs, changes in the inner leaflet protein localization could result in the activation of Src kinases that have been shown to directly associate with LacCer in GSDs (33). ROS generation and Src kinase activation may be followed by Grb/SOS-mediated Ras activation, which triggers the downstream MEK1/2-ERK1/2 pathway.

FIG. 8. Double immunofluorescence staining of spinal cord sections from the lesion epicenter for BrdUrd/GFAP co-localization. Immunofluorescent microscopy images of spinal cord sections from sham and SCI rats stained with antibodies to BrdUrd (red) and GFAP (green) as described under “Materials and Methods.” GFAP/BrdUrd double-positive cells are indicated with arrowheads, and GFAP/BrdUrd cells are indicated with arrows. Representative images for sections from VHC-treated sham (A), VHC-treated SCI (B), PDMP-treated sham (C), and PDMP-treated SCI (D) rats. Quantitative analysis of the average density of GFAP and BrdUrd double-positive cells (E). F. BBB Locomotor score of PDMP- and VHC-treated rats 10 days post-SCI. Data are represented as mean ± S.D. ***, p < 0.001 in (E) as compared with VHC-treated sham; #, p < 0.001 in E as compared with VHC-treated SCI.

FIG. 9. Schematic diagram showing the regulation of TNFα-induced GFAP expression and proliferation of primary astrocytes resulting in astrogliosis.
pathway. The data presented in this study identify a glyosphingolipid, LacCer, as a bioactive signaling molecule regulating astrogliosis by mediating astrocyte proliferation and GFAP expression. In addition, the blockade of trauma-mediated ERK activation and GFAP expression in the SCI model (as reported in this study) and the inflammatory process and neuronal apoptosis (15) by PDMP further establish LacCer, generated through Galt-2 stimulation, to be a potent signaling lipid molecule that triggers inflammation and astrogliosis and show that it may have relevance to various neuroinflammatory diseases.

Glial cells can secrete TNFα, which, in turn, can act on these cells in an autocrine manner. TNFα can induce the proliferation of astrocytes (5, 6) and overexpression of GFAP (9), both components of astrogliosis. Astrogliosis is a prominent and ubiquitous reaction of astrocytes to injury so as to gain from the potential neurotrophic effects while at the same time tempering their scarring effect. This report proposes GSL modulation as a potential target to attenuate astrogliosis and the inflammatory disease processes in neuroinflammatory diseases.

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REFERENCES

1. Mucke, L., and Ediddington, M. (1993) FASEB J. 7, 1226–1232
2. Bidet, J. L., Malhotra, S. R., Privat, A., and Gage, F. H. (1997) Trends Neurosci. 20, 570–577
3. Dhine, M., Block, F., Korr, H., and Topper, R. (2001) Brain Res. 902, 178–189
4. Barna, B. P., Estes, M. L., Jacobs, B. S., Hudson, S., and Ransohoff, R. M. (1990) J. Neuroimmunol. 30, 239–243
5. Barna, B. P., Estes, M. L., Jacobs, B. S., Hudson, S., and Ransohoff, R. M. (1990) J. Neuroimmunol. 30, 239–243
6. Selma, K. W., Farrow, M., Raine, C. S., and Brosnan, C. F. (1990) J. Immunol. 144, 129–135
7. Lachman, L. B., Brown, D. C., and Dinarello, C. A. (1987) J. Immunol. 138, 2911–2916
8. Bettea, J. R., Gillespie, G. Y., Chung, I. Y., and Benveniste, E. N. (1990) J. Neuroimmunol. 30, 1–13
9. Zhang, L., Zhao, W., Li, X., Alston, D. L., Barker, J. L., Chang, Y. H., Wu, M., and Rabinow, D. R. (2004) Neuroreport 11, 409–412
10. Steeves, J. D., and Tetzlaff, W. (1998) Ann. N. Y. Acad. Sci. 860, 412–424
11. Lachman, L. B., Brown, D. C., and Dinarello, C. A. (1987) J. Immunol. 138, 2911–2916
12. Belazs, E., and Schwab, M. E. (1997) Brain Pathol. 7, 33–41
13. Moon, L. D., Brecknell, J. E., Franklin, R. J., Dunnett, S. B., and Fawcett, J. W. (1999) J. Neurosci. Res. 66, 317–326
14. Pettus, B. J., Bielawski, J., Porcelli, A. M., Reames, D. L., Johnson, K. R., and Hannun, Y. A. (2003) J. Neurochem. 88, 583–593
15. Fawcett, J. W., and Asher, R. A. (1999) in The Brain Book. Oxford, England: Oxford University Press, pp. 13–41
16. Mandell, J. W., and VandenBerg, S. R. (1999) J. Neurosci. Res. 66, 611–672