Increased numbers of oligodendrocyte lineage cells in the optic nerves of cerebroside sulfotransferase knockout mice

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Abstract: Sulfatide is a myelin glycolipid that functions in the formation of paranodal axo-glial junctions in vivo and in the regulation of oligodendrocyte differentiation in vitro. Cerebroside sulfotransferase (CST) catalyzes the production of two sulfated glycolipids, sulfatide and prolodendroblast antigen, in oligodendrocyte lineage cells. Recent studies have demonstrated significant increases in oligodendrocytes from the myelination stage through adulthood in brain and spinal cord under CST-deficient conditions. However, whether these result from excess migration or in situ proliferation during development is undetermined. In the present study, CST-deficient optic nerves were used to examine migration and proliferation of oligodendrocyte precursor cells (OPCs) under sulfated glycolipid-deficient conditions. In adults, more NG2-positive OPCs and fully differentiated cells were observed. In developing optic nerves, the number of cells at the leading edge of migration was similar in CST-deficient and wild-type mice. However, BrdU proliferating OPCs were more abundant in CST-deficient mice. These results suggest that sulfated glycolipids may be involved in proliferation of OPCs in vivo.

Keywords: oligodendrocyte, sulfatide, cerebroside sulfotransferase, optic nerve, myelination

Introduction

Galactocerebroside and its sulfated analogue, sulfatide, are two major glycolipids, constituting 30% of the total myelin lipids. The roles of these glycolipids in oligodendrocyte development and myelination in vivo have been examined using mutant mice with disruptions in genes for either ceramide galactosyltransferase (CGT) or cerebroside sulfotransferase (CST). These studies demonstrated that galactolipids are important for the formation of paranodal axo-glial junctions and the maintenance of myelin structure. In addition, several reports suggested that sulfatide may act as a negative regulator of oligodendrocyte terminal differentiation, as terminal differentiation and morphological maturation of oligodendrocytes were enhanced in cultures of CGT- mice. Furthermore, elevated numbers of differentiated oligodendrocytes were found in the spinal cord and brain of CGT- and CST-deficient mice. Thus, sulfated glycolipids may be important for controlling oligodendrocyte-lineage cell numbers in addition to the timing of differentiation. During development, migration, proliferation and apoptosis of oligodendrocyte precursor cells (OPCs) influence the total number of oligodendrocytes. An increase in proliferation and decrease in apoptosis of oligodendrocyte-lineage cells was observed in 15-day-old CST-deficient spinal cords, suggesting that changes in these processes may, in part, be responsible for the increase in oligodendrocytes in the adult spinal cord. However, how the migration of OPCs is affected by conditions of sulfated glycolipid-deficiency is not known, nor is it understood if these potential changes influence the number of oligodendrocytes.

During optic nerve development, bipolar OPCs originate from the floor of the third ventricle and...
migrate to the optic nerves through chiasmal regions around postnatal day 0 (P0). Some of these migrating cells reach the region of the lamina cribrosa around P4, and can be widely seen throughout the nerves by P7.\cite{11} During this time, oligodendrocytes initiate terminal differentiation and myelination. The final number of oligodendrocytes is strictly regulated by the induction of apoptosis in excess cells.\cite{12,13} Thus, the developing optic nerve allows us to highlight the migration of OPCs and to address the timing of myelination under CST-null conditions.

In this study we focused on the numbers of oligodendrocyte-lineage cells from the early migrating stage through adulthood and the timing of myelination in CST-deficient mouse optic nerves to determine the role of sulfated glycolipids in the regulation of oligodendrocyte migration, proliferation and myelin formation. To identify oligodendrocyte-lineage cells, we used two markers: NG2 chondroitin sulfate proteoglycan\cite{14,15} for OPCs in both the developing and adult optic nerves, and proteolipid protein (PLP) for mature oligodendrocytes in the adult.\cite{16}

**Experimental procedures**

**CST-KO mice.** CST-deficient mice were kindly provided by Dr. Koichi Honke (Kochi University Medical School, Nankoku, Japan). Genotypes were determined by PCR as previously described.\cite{4} Mice were maintained in the animal facility of the Tokyo University of Pharmacy and Life Sciences under University Guidelines for Care and Use of Animals. The experiments were performed after securing the University Animal Use Committee Protocol Approval.

**Antibodies.** The polyclonal antibody against NG2 (used at 1:200) was purchased from Chemicon (Temecula, CA). The rat monoclonal antibody against bromodeoxyuridine (BrdU, used at 1:100) was purchased from Abcam (Cambridge, UK). The rabbit polyclonal antibody against single stranded DNA\cite{17} (ssDNA, used at 1:200) was purchased from DakoCytomation (Kyoto, Japan).

**Immunofluorescence.** Immunohistochemistry was performed as previously described\cite{4} with minor modifications. Briefly, CST-deficient mice and wild-type controls of various ages were fixed by transcardial perfusion with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. Ten-µm-thick cryosections of the optic nerves were permeabilized for one hour in 0.1 M PB, containing 0.3% Triton X-100 and 10% goat serum (PBTGS). Primary antibodies were diluted to appropriate concentrations in PBTGS. Alexa 488-conjugated anti-rabbit IgG or Alexa 594-conjugated anti-rat IgG (Molecular Probes, Eugene, OR) were used as secondary antibodies. Sections were counterstained with either diamidino-2-phenylindole (DAPI, Molecular Probes) or propidium iodide (PI, Vector Laboratories, Burlingame, CA). Images were captured with a Pascal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). Digitized images were transferred to a laboratory computer for analysis using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). Nerve areas were measured by AxioVision Rel.4.6 (Carl Zeiss).

To identify proliferating cells, mice were treated with an intraperitoneal injection of BrdU (Roche Diagnostics, 100 µg/g body weight) 24 hours before sacrifice. After fixation, sections were stained with DAPI then post-fixed with 4% PFA. The sections were denatured in 2 N HCl, neutralized with 0.1 M sodium borate buffer (pH 8.5) and extensively washed in phosphate buffered saline (PBS). They were incubated with appropriate primary and secondary antibodies for detecting BrdU. Images were captured by fluorescence microscopy with an AxioCam HRc CCD camera (Carl Zeiss).

For developmental studies using 1- and 2-day-old mice, each optic nerve with the attached eyeball was mounted in OCT compound after transcardial fixation. The blocks were cut transversely from the eyeball. Once the tip of the optic nerve appeared, 10-µm serial optic nerve sections were collected sequentially on a total of 25 glass slides. Each slide contained 6, 10-µm-thick sections that were a total of 250-µm apart from each other. In total, 1.5 mm (250 µm x 6) of optic nerve from each retina was serially sectioned. Slides containing sections from 6 different positions were prepared from two wild-type and two CST-deficient mice. This method was used to determine the leading edge of cell migration along the nerve. Data were collected from 6 sections at each position and mean values were calculated.

**In situ hybridization.** Riboprobes were generated from plasmids containing the following mouse cDNAs: PLP cDNA,\cite{18} glutamate/aspartate transporter (GLAST) cDNA,\cite{19} and macrophage colony-stimulating factor receptor (c-fms) cDNA.\cite{20} Digoxigenin (DIG)-labeled antisense and sense cRNA probes were generated using a DIG RNA labeling kit (Roche Applied Science).

In situ hybridization was performed using methods described elsewhere\cite{20} with minor modifica-
tions as follows: pre-hybridization was carried out for three to four hours at 65 °C in hybridization buffer containing 50% formamide, 5X SSC, 200 µg/mL yeast tRNA, 100 µg/mL heparin, 1X Denhardt’s solution, 0.1% Tween-20, 0.1% CHAPS, and 5 mM EDTA. Hybridization was performed overnight with 100 ng/mL riboprobe at 65 °C. After pre-incubation in the blocking buffer (20% heat-inactivated sheep serum, 0.1% Triton X-100, 2 mg/mL bovine serum albumin in PBS), the sections were incubated overnight with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) at 4 °C. Finally, the sections were treated with freshly prepared colorizing substrate, nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche Diagnostics), in the presence of 5 mM levamisole. Sense probes were used as negative controls. Sections were counterstained with nuclear fast red solution (Sigma).

Electron microscopic analysis. Electron microscopy was performed as described previously21) with minor modifications. CST-deficient mice and wild-type controls at 10 days of age were fixed by transcardial perfusion followed by overnight fixation with 2% PFA and 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Optic nerves were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for two hours, dehydrated in ethanol, and embedded in Epon 812 (TAAB Laboratories, Berks, UK). Transverse ultrathin sections were cut with an ultramicrotome (EM UCG, Leica) and stained with uranyl acetate and lead citrate. The sections were examined by electron microscopy (JEM-1011, JEOL, Japan).

Statistical analysis. Statistical analysis was performed using Microsoft Excel 2003 (Microsoft Corporation). Data were expressed as the mean ± standard error of the mean (S.E.M.). A Student’s t-test was performed for comparisons between CST-deficient and wild-type mice. A p value of less than 0.05 was regarded as statistically significant.

Results

The number of oligodendrocyte lineage cells was increased in adult CST-deficient optic nerves. To determine whether the loss of sulfated glycolipids influenced the number of oligodendrocyte lineage cells in adult optic nerves, we measured the numbers of mature oligodendrocytes and OPCs by means of PLP mRNA in situ hybridization and NG2 immunohistochemistry, respectively. In 4-week-old animals, in which myelination was still in progress, PLP mRNA was abundantly expressed in oligodendrocytes from CST-deficient as well as wild-type animals (Fig. 1A and B). In contrast, expression of PLP mRNA in 22-week-old mice was reduced in both animals (Fig. 1C and D).
mRNA expression occurs during normal development,\textsuperscript{22,23} and this was unchanged in the CST-deficient condition. However, more PLP mRNA-positive mature oligodendrocytes were found in CST-deficient optic nerves than in wild-type controls at both ages (Fig. 1E). In 4-week-old animals, OPCs were identified with an anti-NG2 antibody (Fig. 2A and B). The number of NG2-positive cells per area was also higher in CST-deficient mice than in wild-type mice, although only a few NG2-positive cells were found in the adult (Fig. 2C). The percentage of the total number of cells comprised of NG2-positive cells was calculated. Each value represents the mean ± SEM of the data obtained from four animals. *, P < 0.05; ***, P < 0.001 vs. control.

The increase in oligodendrocyte precursor cells occurs prior to the onset of myelination in the optic nerve. During normal development excess oligodendrocytes are eliminated by programmed cell death.\textsuperscript{12} However, our results from adult mice suggest that the regulation of cell numbers was affected in CST-deficient optic nerves. In order to examine the changes in OPC numbers during development, the optic nerves of 5-day-old CST-deficient and wild-type mice were stained with an anti-NG2 antibody. Total cell numbers were determined by nuclear staining using PI. As shown in Fig. 4, NG2-positive cells in 5-day-old animals appeared to have fine cell processes and were aligned between the nerve fibers (Fig. 4A–F), indicating that they were post-migratory OPCs. Total numbers of PI-positive cells (Fig. 4G), the number of NG2-positive cells (Fig. 4H), and the ratio of NG2-positive cells to the total number of cells (Fig. 4I) were significantly increased in mutant compared to wild-type optic nerves. In 5-day-old optic nerves, marker proteins for mature myelin, including PLP, myelin-
associated glycoprotein and myelin basic proteins, were not detected by immunohistological analysis, demonstrating that neither wild-type nor CST-deficient oligodendrocytes formed myelin at this age (data not shown). Thus, the number of OPCs was increased in CST-deficient mice prior to myelination.

To determine if cell death or proliferation was in part responsible for the increase in cell numbers at postnatal day 5, ssDNA- or BrdU-positive cell numbers were counted and compared between CST-deficient and wild-type optic nerves. Apoptotic cells positive for ssDNA\(^\text{17}\) were rarely found in either group of animals (Fig. 4J). No significant difference was observed in the numbers of BrdU-positive cells (Fig. 4K), suggesting that the increase in cell numbers occurred before BrdU incorporation at 4 days of age.

**CST-deficient optic nerves show an increase in proliferating NG2-positive cells during early stages of development.** During optic nerve development, bipolar-shaped OPCs migrate from the brain toward the retina. They express NG2 and platelet derived growth factor receptor \(\alpha\) on their surface. These markers are also present in early and late OPCs. Sulfated glycolipids produced by CST\(^\text{8}\) appear in late OPC stage.\(^\text{16}\) To detect migrating and proliferating cells, we administrated BrdU to 0- and 2-day-old wild-type and CST-deficient mice. After 24 hours, serial transverse sections were obtained by cutting the optic nerves from the retinal to the chiasmal sides (Fig. 5A). Size of each cross section was measured and no significant difference was observed between two groups in all positions. The average number of cells per nerve area was calculated at six different distances away from the retina as described in the materials and methods. This method allowed for a direct comparison of NG2-positive cells at each position between wild-type and CST-deficient animals.

In wild-type mice (white columns in the graphs in Fig. 5) NG2-positive cells were abundant at the chiasmal side (red cells in F), and these cells gradually decreased toward the retinal side (B compared to D). No NG2-positive OPCs were present at the migration endpoint (position 1) at one day of age (day 1; Fig. 5H), while two days later some OPCs had reached the endpoint (Fig. 5K). Also, the number of NG2-positive cells (Fig. 5H and K) and the ratio of NG2-positive cells to total cells (Fig. 5I and L) increased at all positions between day 1 and day 3. The ratios of proliferating NG2-positive cells to the total number of NG2-positive cells were relatively lower in the regions at the forefront of migration (position 2 at day 1 and position 1 at day 3) in both ages (Fig. 5J and M). Thus, OPCs actively migrate and proliferate during these two days in normal development.

In 1-day-old CST-deficient mice (blue columns), the number of NG2-positive cells and the ratio of NG2-positive cells to total cells at position 2 were similar to wild-type (Fig. 5H and I), suggesting that the number of OPCs at the migration forefront was not different in these two types of animals. In contrast, the ratio of NG2-positive cells to total cells (Fig. 5I) and the ratio of proliferating NG2-positive cells to total NG2-positive cells (Fig. 5J) in the mutant mice were significantly higher than those in wild-type at all other positions at day 1. In 3-day-old mice, the number of NG2-positive cells and ratios were higher in the mutants at all positions (Fig. 5K and L). These results suggest that during early development the increase in OPCs in mutant optic nerves is due to an increase in OPC proliferation rather than differences in migration.

**Influence of sulfatide loss on the onset of myelination.** Primary cultures of CST-deficient oligodendrocytes showed early expression of MBP.\(^\text{8}\) Additionally, an increase in the number of terminally differentiated oligodendrocytes, as determined by PLP mRNA expression, was found in various brain regions in 7-day-old CST-deficient mice.\(^\text{8}\) These studies using molecular markers for myelin suggest an earlier onset of myelination in CST-deficient mice. However, myelin formation itself was not directly examined. Therefore, to determine the influence of CST-deficiency on the timing of myelination in vivo, the number of myelinated axons was determined by electron microscopic analysis (Fig. 6A and B). In 10-day-old CST-deficient mice, a decreased percentage of axons covered with compact myelin was found (Fig. 6C), suggesting that myelination is delayed in mutant optic nerves.

**Discussion**

In the present study, we demonstrated that the number of mature oligodendrocytes was significantly increased in the optic nerves of adult CST-deficient animals. This increase in oligodendrocyte-lineage cells occurred early in development, indicated by the higher numbers of NG2-positive OPCs present in 5-day-old CST-deficient optic nerves. Given that this difference was not observed at the forefront of migration and the percentages of proliferating NG2-positive cells were higher in 1- and 3-day-old mutant...
mice, it can be concluded that CST-deficiency affects proliferation but not migration of OPCs. In spite of the increase in OPCs, the appearance of myelin sheaths was relatively delayed in development. Thus, during early development, sulfated glycolipids regulate the number of oligodendrocyte lineage cells by controlling proliferation after migration and the onset of myelination in the optic nerve.

A previous report by Hirahara et al. showed that the number of PLP mRNA-positive mature oligodendrocytes was increased in the brain of 7-day-old CST-deficient mice where myelination had just

Fig. 4. Number of OPCs and the proportion of dying or proliferating cells in 5-day-old optic nerves of wild-type and CST-deficient mice. (A–F) Double labeling of NG2 (green)/PI (red) was performed on optic nerves from 5-day-old wild-type (+/+; A–C) and CST-deficient (−/−; D–F) mice. Scale bar represent 25 µm. (G–I) Total cell numbers (G) or the number of NG2-positive cells (H) per area, and percentages of NG2-positive cells (I) in both wild-type (WT) and CST-deficient (CSTKO) optic nerves are shown. (J, K) One day after BrdU injection, the densities of dying cells (J) and proliferating cells (K) were examined using specific antibodies against ssDNA or BrdU, respectively. Each value represents the mean ± SEM of the data obtained from three individual animals of each genotype.

*, P < 0.05; ***, P < 0.001 vs. control.
begun. Moreover, a recent study using CST-deficient mice demonstrated that numerous CC1-positive oligodendrocytes were maintained in the spinal cord through at least 7 months of age.10) Our present study adds to this evidence by showing that increases in oligodendrocytes were also found in 4-week-old (at which point myelination has almost terminated) and 22-week-old optic nerves. Taken together,
current data suggest that excess numbers of oligodendrocytes can be observed during myelinating stages and greater numbers of oligodendrocytes are sustained into adulthood in various CNS regions. Similar increases in differentiated oligodendrocytes were also reported in CGT-deficient mice, suggesting that the loss of sulfatide is predominantly responsible for the changes in cell numbers observed in the CNS.

In normal animals, the number of oligodendrocytes is strictly regulated by axons during development. The number of myelinating cells that survive appears to be precisely matched to the number and length of axons requiring myelination; this is achieved by competition for limiting amounts of survival signals (for review). Our previous study showed that nodal numbers (as indicated by sodium channel clusters) were not significantly changed in 4-week-old CST-deficient optic nerves, indicating that the length of each myelin segment was not affected. Additionally, the study by Shroff et al. revealed that oligodendrocytes in 2-day-old CST-deficient spinal cords appeared to have fewer processes, indicating that axons may require a greater number of oligodendrocytes to compensate for the fewer number of oligodendrocytic processes. Although it is still unknown if oligodendrocytes with fewer processes are present in the adult, under conditions of CST-deficiency each oligodendrocyte may form less myelin rather than surviving without having formed any myelin at all.

The present results shown in Fig. 5 indicate that proliferation of NG2-positive cells primarily occurred after migration; an increase in proliferating cells may explain the difference in NG2-positive cell numbers between wild-type and CST-deficient neonates. Such dysregulation of OPC numbers in early development may be responsible, at least in part, for the increase in oligodendrocyte-lineage cells in adults. Thus, the loss of glycolipids results in changes in OPC proliferation but not migration during early development. Competitive PCR analysis detected a low level of CST mRNA at embryonic day 11.5 in brain which was followed by an up-regulation as development progressed, with the highest levels appearing in the adult brain. Sulphatide expression is initiated in pre-myelinating oligodendrocytes when CST expression surges. Loss of the CST enzyme resulted in the absence of proligodendroblast antigen (POA), which is expressed in late OPCs prior to the appearance of sulfatide. Thus, both POA and sulfatide are likely candidates for being responsible for the inhibition of post-migratory OPC proliferation in normal developing optic nerves.

Several reports have demonstrated that sulfatide inhibits terminal differentiation both in vitro and in vivo. However, our electron microscopic analysis did not show an earlier onset of myelination in 10-day-old CST-deficient mouse optic nerves. In vivo, the onset of CNS myelination is influenced by interactions with axons or other surrounding cells, including astrocytes. It is possible that these interactions may compensate for the loss of sulfatide in the mutant optic nerve.

The molecular mechanisms underlying these changes caused by loss of sulfated glycolipids are currently unknown. Previous studies have demonstrated the variety of sulfatide function. Negatively charged sulfatide is known to bind to positively charged proteins, including extracellular matrix proteins like laminin, tenascin-R, and tenascin-C. Sulfatide may also play a role in regulating the associations between proteins and lipid rafts, as suggested by the change in neurofascin 155 in cholesterol-enriched fractions in the CGT-deficient CNS. It has been reported that sulfatide mediates intracellular signaling pathways, including increased cytoplasmic calcium concentration, by acting as a receptor of extracellular signals. Without sulfated glycolipids, OPCs may fail to receive exogenous signals resulting in the observed changes in OPC proliferation and onset of myelination.
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