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脱髄性ニューロパチーの病態解明と神経保護分子の解析

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研究代表者　馬場　広子

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総合研究報告書

脱髄性ニューロパチーの病態解明と神経保護分子の解析

研究代表者　馬場　広子　東京薬科大学教授

研究要旨　急性・慢性に経過する免疫性脱髄性ニューロパチーの症例の多くは未だに抗原の同定がなされず、病態も明らかではない。そこで本研究では、1）脱髄原因分子の探索と病態機序の解明、2）脱髄に伴う2次性軸索障害の病態解明、3）脱髄保護分子の探索を行い、脱髄性ニューロパチーの病態解明と予後改善を目指すことを目的とした。1）に関して末梢神経障害患者血清中の抗神経抗体を解析した結果、CIDP患者血清と高率に反応する分子量36Kの末梢髄鞘特異的分子を見出した。解析の結果P0遺伝子から発現し、髄鞘コンパクト部分に存在する接着分子であるとわかりL-MPZと命名した。この抗原ペプチドを用いたELISAの系を確立し、CIDP患者51例を含む113例の神経障害患者血清中の抗L-MPZ抗体の出現率および抗体価の検討を行い、特にCIDP患者血清に多く（23%）、高い抗体価が認められた。また、in vivo及びin vitro解析の結果病態との関連性が示された。2）髄鞘異常に伴う2次性軸索障害には軸索輸送関連分子等の異常を伴い、局所のCa^{2+}変化との関連性が示唆された。3）末梢神経髄鞘に多く存在するAX2は、siRNA実験などにより脱髄保護作用があることが見出された。AX2はヒト末梢神経髄鞘にも存在し、病態に関連することが示唆された。また、本研究を通じて脱髄保護作用を持つAX2の他にマクロファージの貪食作用を介して病態修飾に関わる分子を同定した。以上、本研究において、末梢神経障害の病態修飾に関わる新たな分子を見出すと共に、脱髄保護や組織修復に関わる複数の分子を明らかにした。

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（患者血清提供および臨床情報の提供）

A．研究目的

軸索障害が主体の免疫性ニューロパチーでは、抗神経抗体の関与が知られている。しかし、急性・慢性に経過する脱髄性ニューロパチーの多くの症例で、未だ抗原は同定されず、病態も明らかではない。また、予後や重症度、治療の反応性などの臨床的個人差に対する有効なマーカーも存在しない。そこで本研究では、1）脱髄原因分子の探索と病態機序の解明、2）脱髄に伴う2次性軸索障害の病態解明、3）脱髄保護分子の探索を行うことによって、脱髄性ニューロパチーの病態解明と予後の改善を目指すことを目的とした。

B．研究方法

1）患者血清を用いてSDS-PAGE?Westernを行い、血清中の抗神経抗体の有無および抗原の分画を同定した。抗原となる膜タンパク質に着目し、陽イオンを用いた2次元電気泳動/Western でそれぞれの患者血清が反応する抗原スポットを同定した。複数の血清が反応するスポットのMASS解析を行った。特に、その中でも反応血清数が多い36K末梢神経髄鞘特異的タンパク質を解析し、L-MPZと命名した。岐阜大抗L-MPZ陽性症例すべてがL-MPZの同じ部分のペプチドと反応したため、これを用いてELISA系を確立した。岐阜大および九大から提供を受けた113例(CIDP51例)の末梢神経障害患者血清および23例の健常人23例の血清の抗体価を測定した。L-MPZの病態への関与を調べる目的でL-MPZ抗原ペプチドをウサギ、ラット、マウスに投与し、抗体の産生と神経症状の有無を解析した。脱髄モデルマウスにL-MPZ抗体を尾静脈注入し、抗体の動向を調べた。マウス腹腔マクロファー

ジを用いて抗 L-MPZ 抗体の作用を調べた。また、九大の８０例に関して SDS-PAGE/Western を行い、L-MPZ 以外の抗神経抗体の有無を確認した。

２）髄鞘異常を伴うマウスの末梢神経および小脳における軸索異常を免疫組織、電顕、生化学的解析し、髄鞘異常との関連性を調べた。

３）リゾレシチン脱髄および髄鞘主要構成糖脂質であるスルファチド欠損マウスを用いて AX2 の作用機序を解析した。siRNA を用いて in vivo における AX2 の脱髄保護作用を調べた。また、末梢神経障害からの回復には髄鞘再生が重要である。そこで、マクロファージの貪食に機能する分子を調べると共に、スルファチド欠損マウスを用いて髄鞘再生の鍵を握る髄鞘形成細胞の分化に関与する分子を調べた。さらにヒトでスルファチド量の減少が報告されているため、スルファチド量と末梢神経の機能との関連性に関しても調べた。

（臨床面への配慮）

患者血清はすべて匿名化した上で、東京薬科大で解析した。解析結果を元に犬塚が臨床型との関連性を検討した。本研究は東京薬科大学ヒト組織等を研究活用するための倫理規程に則って倫理審査を受け承認されている。動物実験は、東京薬科大学動物実験指針に則って審査の上承認されている。

C．研究結果

１）岐阜大患者血清中の抗神経抗体を解析した結果、多くの症例で膜構成タンパク質に対する抗体がみられた。CIDP 症例と多く反応する分子、多くの Sjoegren 症候群症例と反応する分子などが見出された。すでに報告のある Periaxin や P0 に対する抗体も存在した。これらの中で、特に CIDP 血清 IgG と反応性が高い 36k 末梢神経髄鞘特異的タンパク質に着目して解析を実施した。その結果、この分子は P0 と共に P0 mRNA から産生されるこれまで未知の分子であることが明らかになり、これを L-MPZ と命名した。岐阜大の陽性例はすべて同じペプチドと反応し、動物でも全く同一部分の抗体ができることから、L-MPZ の抗原部位はきわめて反応性が高いことが示唆された。このペプチドによる ELISA 法を用いた抗 L一-MPZ 抗体価の検討では、CIDP23%（12 例）に抗体が陽性であった。その他 GBS や健常人でも陽性例はあったが抗体価は CIDP 患者に比して明らかに低値であった。糖尿病神経障害例で高い例が１例見られた。また、すべての患者血清中の抗体は L-MPZ の同一ペプチドと反応することがわかった。強制発現系および発達期動物の解析により、L-MPZ はミエリン形成に伴って P0 mRNA から P0 と共に産生され、細胞接着部位に集積することがわかった。L-MPZ を免疫した動物では抗体は出現するが明らかな神経障害は見られなかった。しかし、実験的脱髄を生じたマウスの尾静脈に抗体を注入すると脱髄部分に注入抗体の沈着が見られた。In vitro で抗 L-MPZ 抗体を作用したミエリンはマクロファージへの貪食が亢進し分泌するサイトカインの変化が見られた。２）髄鞘異常を伴うマウスの末梢神経および小脳の軸索では、加齢と共に蓄積物の増加による腫脹が見られた。これには局所の Ca^{2+}濃度変化が関与することがわかった。髄鞘異常と軸索局所での Ca^{2+}濃度変化の発生機序に関して今後も解析を継続する予定である。３）リゾレシチン脱髄モデルに AX2 siENA を投与した結果、AX2 が脱髄巣において cPLA2 活性化を抑制し、脱髄の抑制に関わることを示した。さらに、髄鞘の部分的形態異常を生じるスルファチド完全欠損マウスでも AX2 が過剰に発現することにより保護効果を示すことが示唆された。また、末梢神経髄鞘中のスルファチド量に応じて髄鞘の形態変化、伝導速度の低下が生じることが明らかになった。ヒトで血清スルファチドの減少例が報告されていることから、末梢神経障害のリスクファクターの可能性があることが示された。一方、視神経では含硫糖脂質の完全欠損により髄鞘形成細胞前駆細胞の増殖が変化することがわかった。脱髄時の髄鞘形成細胞の増加は髄鞘再生に重要であり、今後末梢神経における作用を調べる予定である。また、中枢神経系脱髄時のミクログリアと同様に、脱随巣

に出現するマクロファージの貪食に関わる分子を見いだし、組織修復との関連性が示唆された。

D．考察

脱髄性ニューロパチー患者血清中の抗体の多くは神経組織に存在する膜画分の蛋白分子を抗原とする。本研究では特に慢性脱髄性ニューロパチーで出現率の高い抗 L-MPZ 抗体に着目した。この抗体がニューロパチー発症の直接的原因になるかは現時点で不明であるが、脱髄巣に沈着することやマクロファージの貪食作用を亢進し、サイトカイン分泌パターンに影響することから、病態修飾作用は明らかにあると考える。昨年度脱髄保護の役割が示唆されたAX2はヒトでも髄鞘に存在するが、脱髄以外に髄鞘形態異常の場合にもAX2の保護作用が認められた。髄鞘の部分的形態異常はヒトにも存在する可能性があり、伝導速度低下など subclinical な危険因子となる可能性があることがわかった。

E．結論

本研究によって、脱髄性ニューロパチー患者血清中には神経組織中の膜蛋白を抗原とする抗体が高率に存在することが示された。徳にこの中から、複数の患者血清と反応する髄鞘糖タンパク質の抗原を新たに同定し、簡易測定系を開発し、脱髄病態修飾に関与する可能性を明らかにした。他にも複数の患者血清と病型特異的に反応する膜抗原があるため、これらの同定を今後引き続き実施する予定である。また、ヒト髄鞘にも存在する脱髄保護分子の候補を見出した。また、視神経における髄鞘形成細胞の増殖に関わる分子を見いだし、これがシュワン細胞にも適応するか今後検討する予定である。脱髄の修飾や修復には局所におけるマクロファージが重要である。本研究を通して、L-MPZおよび貪食特異的に出現する分子を新たに見出した。

以上、本研究を通じてニューロパチーの病態修飾や重症化の予防に関与しうる分子が複数同定された。これらを今後も検討していく予定である。

F．健康危険情報　該当なし

G．研究発表

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木村他：Role of Annexin 2 in the sciatic nerves of the shiverer and cerebroside sulfotransferase-deficient mice. 第５３回日本神経化学会（Neuro2010）

H．知的財産権の出願・登録状況（予定を含む）

１．特許取得　　該当なし

２．実用新案登録　該当なし

研究成果の刊行

Kajigaya et al., Increased numbers of oligodendrocyte lineage cells in the optic nerves of cerebroside sulfotransferase knockout mice. PJA Ser.B (in press).

Original Article

Title: 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 proligodendroblast 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*.

KEY WORDS: 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[1-4] and the maintenance of myelin structure[5,6]. In addition, several reports suggested that sulfatide may act as a negative regulator of oligodendrocyte terminal differentiation[7,8], as terminal differentiation and morphological maturation of oligodendrocytes were enhanced in cultures of CGT-[7] and CST-deficient[8] mice. Furthermore, elevated numbers of differentiated oligodendrocytes were found in the spinal cord and brain of CGT-[9] and CST-deficient mice[8,10]. 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[10], 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[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[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[14,15] for OPCs in both the developing and adult optic nerves, and proteolipid protein (PLP) for mature oligodendrocytes in the adult[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[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[17] (ssDNA, used at 1:200) was purchased from DakoCytomation (Kyoto, Japan).

Immunofluorescence. Immunohistochemistry was performed as previously described[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[18], glutamate/aspartate transporter (*GLAST*) cDNA[19], and macrophage colony-stimulating factor receptor (*c-fms*) cDNA[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[20] with minor modifications 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 previously[21] 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). Down-regulation of PLP mRNA expression occurs during normal

development[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 relatively lower in CST-deficient mice (Fig. 2D), and likely due to the increase in PLP mRNA-positive mature oligodendrocytes shown in Fig. 1. Thus, CST-deficiency causes an increase in oligodendrocyte lineage cells - primarily mature oligodendrocytes - in the optic nerve, which is consistent with data previously reported in the adult spinal cord[10].

To determine if other cell types exhibited increased numbers, we counted the numbers of astrocytes and microglia after *in situ* hybridization with two RNA probes: GLAST was used for astrocyte identification and *c-fms* for microglia[20]. No significant changes were observed in GLAST-positive (Fig. 3A) or *c-fms*-positive (Fig. 3B) cell numbers between wild-type and CST-deficient mice at both ages examined, indicating that although low amount of sulfatide was detected in astrocytes[24], its loss did not affect the cell numbers. Thus, the increase in cell numbers was restricted to oligodendrocyte lineage cells.

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[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[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 α on their surface. These markers are also present in early and late OPCs. Sulfated glycolipids produced by CST[8] appear in late OPC stage[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 G), 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), 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[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[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.*[8] 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 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[7,9], 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[25]). Our previous study[4] 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.*[10] 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[8]. Sulfatide expression is initiated in pre-myelinating oligodendrocytes when CST expression surges[16]. Loss of the CST enzyme resulted in the absence of proligodendroblast antigen (POA)[8], which is expressed in late OPCs prior to the appearance of sulfatide[26]. 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*[7,8] and *in vivo*[8]. 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[27)-33)]. 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[34)], tenascin-R[35)], and tenascin-C[36)]. 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[37)]. It has been reported that sulfatide mediates intracellular signaling pathways, including increased cytoplasmic calcium concentration, by

acting as a receptor of extracellular signals[38]. 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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