The role of sulfoglucuronosyl glycosphingolipids in the pathogenesis of monoclonal IgM paraproteinemia and peripheral neuropathy

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Abstract: In IgM paraproteinemia and peripheral neuropathy, IgM M-protein secretion by B cells leads to a T helper cell response, suggesting that it is antibody-mediated autoimmune disease involving carbohydrate epitopes in myelin sheaths. An immune response against sulfoglucuronosyl glycosphingolipids (SGGLs) is presumed to participate in demyelination or axonal degeneration in the peripheral nervous system (PNS). SGGLs contain a 3-sulfoglucuronic acid residue that interacts with anti-myelin-associated glycoprotein (MAG) and the monoclonal antibody anti-HNK-1. Immunization of animals with sulfoglucuronosyl paragloboside (SGPG) induced anti-SGPG antibodies and sensory neuropathy, which closely resembles the human disease. These animal models might help to understand the disease mechanism and lead to more specific therapeutic strategies. In an in vitro study, destruction or malfunction of the blood-nerve barrier (BNB) was found, resulting in the leakage of circulating antibodies into the PNS parenchyma, which may be considered as the initial key step for development of disease.

Keywords: IgM paraproteinemia and peripheral neuropathy, HNK-1/SGlcA epitope, sulfoglucuronosyl glycosphingolipids (SGGLs), anti-MAG/SGGL antibody, blood-nerve barrier (BNB)

Introduction

Glycosphingolipids (GSLs) are located primarily on plasma membranes and are particularly abundant in the nervous system. They are known to play important roles in biological functions, such as cellular differentiation, modulation of signal transduction, and immune reactions. Recent studies have focused on the immunological properties of GSLs and their role in pathogenic mechanisms of several immune-mediated peripheral neuropathies, such as Guillain–Barré syndrome.1,2 This field has recently undergone remarkable expansion.3,4

In plasma cell dyscrasias, one clone of plasma cells developed from B lymphocytes multiplies excessively and produces a large quantity of a single type of monoclonal antibody (immunoglobulin), known as the IgM M-protein. Monoclonal gammopathy of undetermined significance is the most common plasma cell dyscrasia, accounting for more than 60% of these diseases. It is sometimes closely associated with malignant disorders such as multiple myeloma, Waldenstrom’s macroglobulinemia, primary amyloidosis, and heavy-chain disease. These diseases are more common among older people. IgM paraproteins accumulate in serum or urine of approximately 1% of patients above 50 years of age, and its incidence increases to approximately 3% in patients above 70 years of age. The incidence of plasma cell dyscrasia is increased in African-American male populations.5–7 In this review, I will describe the pathological role of certain GSLs that

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are specific for the PNS on IgM paraproteinemia and peripheral neuropathy.

**IgM paraproteinemia and peripheral neuropathy**

Approximately 10% of patients with plasma cell dyscrasia have associated peripheral neuropathies that occur 6–10 times more frequently in these patients than in age-matched controls. This disease is referred to IgM paraproteinemia and peripheral neuropathy. Clinical manifestations are distal symmetric sensory symptoms followed by motor weakness with progressive proximal involvement. The disease often shows slow progression of a demyelinating neuropathy, which can be demonstrated by performing nerve conduction studies or nerve biopsies, in addition to a variable degree of axonal loss associated with sensory ataxia and impaired gait. Most patients have chronic, slowly progressive demyelinating neuropathy, predominantly in sensory ataxia. Neurophysiological examination typically shows a widespread slowing of sensorimotor nerve conduction velocity with marked delay of distal latencies, suggesting that nerve fiber endings are particularly affected.

Although the pathogenesis of IgM paraproteine mia and peripheral neuropathy is unknown, there is evidence that deposits of excessive amounts of abnormal IgM M-proteins are found in patient’s nerve biopsies, especially in the PNS myelin. The intraneural injection of IgM M-proteins into animals reveals demyelination in the sciatic nerve, suggesting that the IgM M-protein may play a causative role in the disease.

Latov et al. first reported that IgM M-protein in a patient with sensorimotor neuropathy and IgM gammopathy reacted with an antigen in the PNS. In about 50–60% of the patients with neuropathy, the monoclonal IgM M-protein possesses an antibody activity against myelin components in the PNS. The myelin antigen was subsequently characterized to be MAG. The epitope was later shown to be on the oligosaccharide moiety of MAG. It also reacted with the mouse monoclonal antibody HNK-1, which recognized a surface antigen of natural killer cells.

Although about half of the IgM M-protein reacted with MAG, the pathogenic role was debatable since MAG is distributed in both the PNS and central nervous system (CNS); however, only peripheral nerve injury occurred in the disease. In this regard, much attention has been focused on the reactivity of the monoclonal IgM M-protein from patients with peripheral neuropathy and PNS-specific glycosphingolipids (GSLs). Indeed, at least two GSLs strongly reacted with IgM M-protein from patient sera, suggesting that they may be the true target antigens for potentially harmful IgM M-protein in the disease.

**Characterization of PNS-specific glycosphingolipids recognizing monoclonal IgM M-protein in patients with IgM paraproteinemia and peripheral neuropathy**

Ilyas et al. first reported that monoclonal IgM M-protein of patients with IgM paraproteinemia and peripheral neuropathy cross-reacted to acidic GSLs in the ganglioside fraction from human sciatic nerve. Freddo et al. found that the reactive PNS-specific GSLs lacked sialic acid; therefore, they were not gangliosides. We isolated two acidic GSLs that bound to the IgM M-protein from human cauda equina by DEAE-Sephadex A-25, Iatrobeads, and high performance liquid column chromatographies. The major acidic GSL migrated between GM1 and GD1a and the minor acidic GSL migrated between GD1a and GD1b (Fig. 1). Their structures were elucidated by sugar analysis, enzymatic digestion, mild acid hydrolysis, permethylation, fast atom bombardment mass spectrometry, and nuclear magnetic resonance studies.

Gas–liquid chromatography analysis revealed that the acidic GSLs contained glucose, galactose, N-acetylgalactosamine, glucuronic acid (GlcA), and long-chain base with molar ratio of 1:2.01:1.07:0.73 and 0.96 for the major GSL, and 1:2.97:1.88:0.93, and 0.82 for the minor GSL, respectively. The major fatty acids of both GSLs were C16:0, C18:0, C18:1, C20:0, C22:0, and C24:0, and C24:1. The long chain base was mainly C18:1 sphingosine.

Negative fast atom bombardment mass spectrometry gave the sugar sequence information in addition to the molecular weights. Their core structure was confirmed to be paragloboside by high-performance thin-layer chromatography-immunostaining using anti-paragloboside monoclonal antibody (mAb).

Both acidic GSLs lacked sialic acid but contained sulfated GlcA as their acidic moiety. The presence of GlcA in mammalian GSLs is very rare. We confirmed its presence in GSLs by capillary gas–liquid chromatography–chemical ionization mass spectrometry using authentic 3-D-GlcA. The sulfate group in GlcA was attached to the 3 position by periodate oxidation and permethylation–reduction studies. In this procedure the terminal sulfated
GlcA yielded a sulfated glucose residue. Subsequent acetolysis, reduction, and acetylation of the permethylated GSLs produced 2,4-di-O-methyl-1,3,5,6-tetra-O-acetyl-glucitol, 2,3,6-tri-O-methyl-1,4,5-tri-O-acetylglucitol, 2,4,6-tri-O-methyl-1,3,5-tri-O-acetyl-galactitol, and 3,6-di-O-methyl-1,4,5-tri-O-acetyl-1,3,5-tetra-O-acetyl-2-deoxy-2-N-methyl-acetoamido-glucitol. The structure of 2,4-di-O-methyl-1,3,5,6-tetra-O-acetyl-glucitol was characterized by fragment ions at m/z 43, 117, 129, 159, 189, and 233 as detected by gas–liquid chromatography–electron ionization mass spectrometry. These results indicate that the sulfated group is attached to the 3 position of GlcA. Nuclear magnetic resonance studies confirmed that the paragloboside core structure and the further established sulfated GlcA were terminally linked and that all the sugars are O-linked. These results indicate that the structures of the two acidic GSLs, named as sulfoglucuronosyl glycosphingolipids (SGGLs) are identified as follows: (a) GlcA(3-sulfate)(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)-Glc(β1′)ceramide or IV3GlcA(3-sulfate)nLcOse4-Cer (SGPG) and (b) GlcA(3-sulfate)(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)-Glc(β1′)ceramide or VPGlcA(3-sulfate)nLcOse6Cer (SGLPG) as shown in Fig. 2. The structures of these SGGLs were independently confirmed by Chou et al. as well as by total chemical synthesis.

The carbohydrate epitope recognizing in patients with IgM M-paraproteinemia and peripheral neuropathy and its expression in glycoproteins and neural adhesion molecules

The mouse monoclonal antibody anti-HNK-1 (Leu-7; VC1.1), which recognizes the surface antigen of natural killer cells, is known to cross-react with MAG; therefore the antigenic determinant for HNK-1 is presumably present on MAG. We have reported that HNK-1 and IgM M-protein both bind to acidic GSLs of the PNS. Therefore, HNK-1/SGGL and human IgM M-protein from patients with neuropathy must share an affinity for a common epitope. SGPG was found to be extremely labile to acid. After treatment with 0.05 M hydrochloric acid in methanol at room temperature, SGPG was converted to the lactone form of desulfated SGPG. After base treatment, the lactone form was converted to desulfated SGPG. Although desulfated SGPG was immunostained with patient’s serum, neither the lactone form of desulfated SGPG nor the methyl ester of desulfated SGPG were reactive, suggesting that the free carboxyl group, but not the sulfate group, in the terminal GlcA residue is necessary to bind IgM M-protein from neuropathy patients, whereas both the sulfate and carboxyl groups are required for reactivity with HNK-1.
To further define the precise structural requirement for this carbohydrate epitope, we tested 14 chemically synthesized SGGLs and their nonsulfated derivatives with defined carbohydrate chain lengths and aglycone structures, including ceramide, 2-(tetradecyl)hexadecyl residue, and 2-(trimethylsilyl)ethyl residue. These synthetic SGGLs were tested for immunoreactivity with anti-HNK-1 antibody, VC1.1, mouse mAb NGR50, which was prepared using naturally occurring SGPG as the immunogen as described later, and sera (LT and YT) from two patients with IgM paraproteinemia and demyelinating neuropathy using high-performance thin-layer chromatography-immunostaining and enzyme-linked immunoabsorbent assay. The mAb VC1.1 reacted with SGGL analogs containing a minimum of the two terminal sugars of 3-sulfoglucuronic acid (SGlcA)-Gal-, but not with the nonsulfated derivatives of SGGLs nor with SGGLs having a modified ceramide structure. On the other hand, mAb NGR50 reacted only with SGPG and SGLPG. This suggests that mAb NGR50 reacts with an epitope that includes ceramide structure. The human patient serum LT reacted with all synthetic SGGLs except those with SE aglycone structure. On the other hand, another human patient serum YT, similar to the VC1.1, reacted with SGPG, SGLPG, and SGGL analogs containing a minimum of two terminal sugars of SGlcA-Gal-. All antibodies reacted more strongly with synthetic SGGLs with longer carbohydrate chains than those with shorter chains. These results indicate that, with some exceptions, anti-SGGL antibodies recognize a minimum of two sugars bearing the following structures, 3-sulfoglucuronosyl \(\beta\)-3-galactosyl; SGlcA-Gal- and the aglycone ("ceramide") structure appear to play an important role for antibody–antigen interaction. In this regard, Schmitz et al. reported that both SGlcA and the neolactosyl core structures in synthetic SGGLs are essential for recognition of the L2/HNK-1 antibody and these structures may be responsible for the biological function of certain glycoconjugates.

Some of the carbohydrates on the cell surface have been shown to be associated with cell–cell recognition and adhesion. The HNK-1 carbohydrate epitope is known to be recognized as the surface antigen on a number of human lymphocytes, including natural killer cells and are characteristically expressed on a series of cell adhesion molecules in MAG, including P0, PMP-22, NCAM, L1, J1, and P012),15),23),30),47)–49) and also on some GSLs in the PNS. Variability in the relative strength of the binding of the IgM M-proteins to these glycoconjugates as MAG, SGPG, P0 and PMP-22 has been described. Shiina et al. reported that serum IgM M-proteins from 4 of the 12 patients with IgM paraproteinemia and peripheral neuropathy strongly immunostained the entire myelin, whereas serum IgM M-proteins from the remaining eight patients bound to a region surrounding the myelin sheath in the cross-section, suggesting that there are variabilities in the fine specificity of IgM M-proteins with anti-SGPG reactivities. They also reported that the reactivities against MAG and SGPG may play more important roles in the pathogenesis of IgM paraproteinemia and peripheral neuropathy than those against P0 and PMP-22. The HNK-1 or SGlcA epitope expressed on glycoconjugates modulate...
synaptic plasticity by affecting adhesive and anti-adhesive properties and cell adhesion, migration, and neurite outgrowth.

In addition to HNK-1 (Leu-7 or CD57), specific antibodies against the SGlcA epitope, including Elec-39, NC-1,50 BM-89,57 and VC1.1,58 have been reported. We43 have produced the mouse mAb NGR50 by immunizing C3H/HeN inbred strain female mice with purified SGPG absorbed to acid-treated Salmonella minnesota mutant R595 and studied its antigenic specificity. Thin-layer chromatography immunostaining revealed that mAb NGR50 reacted specifically with SGPG and SGLPG, but not with the desulfated derivatives of SGGLs and other GSLs. Western blot analysis showed cross-reactivity with human MAG and several glycoproteins in the 20–30 kDa range, but not with rat MAG. Failure to react with rat MAG implies that the occurrence of the SGlcA epitope on glycoproteins is dependent upon the animal species. An immunocytochemical study of rat sciatic nerve using mAb NGR50 revealed positive staining in the outer surface of the myelin sheath and Schwann cells, as well as in the intervening connective tissues.

Jungalwala and co-investigators reported the presence of its binding protein, SBP-1, in the rat cerebellum and that its expression was developmentally regulated.59,60 During development of the rat cerebral cortex, the level of SBP-1 decreased after embryonic (E) day 18 to an almost undetectable level by postnatal (P) day 10; whereas in the cerebellum, the expression of SBP-1 was maximal at P7.

**Biosynthesis of SGGLs and cloning of key enzymes in the biosynthesis of HNK-1 epitope**

Biosynthetically, at least four glycosyltransferases are required: lactosyl ceramide (LacCer; Galβ1-4Glcβ1-1′Cer)N-acetyllactosaminyl transferase (LacCer-GlcNAcT) to form lactotriaosyl ceramide (LcOse3Cer); LcOse3Cer-galactosyl transferase (LcOse3Cer-GalT) to form neolactotetraosyl ceramide (IV3GlcA-nLcOse4Cer); and IV3GlcA-nLcOse4Cer-sulfotransferase (SuT) to form SGPG (IV3GlcA(3-sulfate)nLcOse4Cer). Activities of these enzymes have been demonstrated in the brains of chickens and rodents;62–66 the key enzymes in the biosynthesis of HNK-1 epitope are β1,3-GlcAT, which transfers a GlcA to a terminal galactose, and SuT, which adds a sulfate group to the GlcA.

Das et al. first reported that glucuronyltransferase (GlcAT-1) in embryonic chicken brains catalyzed the in vitro biosynthesis in GlcA containing GSLs starting from neolactotetraosylceramide (nLcOse3Cer) and neolactohexaosylceramide (nLcOse6Cer). Chou et al. also reported that the enzyme in the adult rat cerebral cortex catalyzed the transfer of GlcA from UDP-GlcA to the terminal galactose of the neolacto (type 2) series of GSLs to form β 1-3-linked glucuronosyl neolacto-GSLs. The enzyme was highly specific for the neolacto series of acceptor GSLs, nLcOse3Cer, nLcOse5-Cer, and neolactoctaoysylceramide (nLcOse6-Cer). Considerable activity of GlcAT was present in the adult rat cerebral cortex, even though SGGLs almost completely disappeared from the cortex by P15. In the cerebellum, although the levels of SGGLs increased with development, the specific activity of GlcAT declined.

Oka et al. found in rat brain extract two different GlcATs that were separated by UDP-GlcA-Sepharose CL-6B column chromatography, designated as GlcAT-L and GlcAT-P. GlcAT-L was recovered predominantly in the effluent fraction and catalyzed the transfer of GlcA from UDP-GlcA to GSL acceptors. GlcAT-P was recovered in the eluting fraction and catalyzed the transfer of GlcA to glycoprotein acceptors, suggesting that GlcAT-P is associated with the biosynthesis of HNK-1 epitope on glycoproteins such as N-CAM and other cell adhesion molecules. GlcAT-P specifically recognized the N-acetyllactosamine (Galβ1-4GlcNAc) structure at the nonreducing terminals of glycoproteins, whereas GlcAT-L had a similar activity to that reported by Das et al. and Chou et al. Further studies have demonstrated that GlcAT-L or GlcAT-D is active on both glycoproteins and GSLs in vitro. The GlcAT-P was highly specific to the terminal type II structure, Galβ1-4GlcNAc, while the GlcAT-S recognized not only the type II structure, Galβ1-4GlcNAc, but also the type I structure, Galβ1-3GlcNAc. These acceptor specificities were similar to those of the native enzymes. The GlcAT-D transferred a GlcA to not only type 2 (Galβ1-4GlcNAc) but also type 1 (Galβ1-3GlcNAc) glycan chains, suggesting that the HNK-1 epitope expressed on type 1 glycan chain may occur, although the existence of such glycans has not previously been reported. The enzyme was localized specifically in the brain, and was barely detectable in other tissues, including sciatic nerve fibers, thymus, and liver. Yavuz
et al.\textsuperscript{70} reported that GlcAT-P expressed on mouse brain was also expressed in \textit{E. coli} cells along with other glycosyltransferases and showed activity for transfer of GlcA to neolactotetraose and neolactohexaose.

Some phospholipids were reported to stimulate the activities of glycosyltransferases, such as β1-4GalT\textsuperscript{77} and α2-3sialyltransferase.\textsuperscript{78} GlcAT-P was activated dramatically in the presence of sphingomyelin.\textsuperscript{70} In GlcAT-D, phosphatidylinositol and phosphatidylcholine increased the enzymatic reaction by 4.4- and 2–3-fold, respectively, whereas phosphatidylcholine slightly decreased the rate.\textsuperscript{73} Phosphatidyl inositol is specifically required for expression of the activity of the recombinant enzymes toward the GSL acceptor, paragloboside.\textsuperscript{79}

Terayama \textit{et al.}\textsuperscript{80} first isolated a cDNA clone encoding GlcAT-P from a rat brain by a PCR-based cloning method. The primary structure deduced from the cDNA sequence predicted a type II transmembrane protein with 347 amino acids. Mitsumoto \textit{et al.}\textsuperscript{83} also cloned a cDNA encoding GlcAT-P from human brain that was 98.2% identical to rat GlcAT-P in amino acid sequence and demonstrated that the human GlcAT-P gene was located on chromosome 11q25. Transfection of the GlcAT-P cDNA into COS-1 cells not only induced expression of the HNK-1 epitope on the cell surface but also marked morphological changes of the cells, suggesting that the HNK-1 epitope is associated with a cell–substratum interaction. Further study of the gene indicated that the predicted amino acid sequence of mouse GlcAT-P was 96.2% and 98.2% identical to those of the rat and human enzymes, respectively.\textsuperscript{85} Alternatively spliced isoforms of mouse GlcAT-P are present in the brain and encode two proteins that are identical throughout their length except for an additional 13 amino acids in the N-terminal cytoplasmic domain of the major form. The coding region of GlcAT-P was composed of 5 exons spanning approximately 6 kb, and the GlcAT-P gene was mapped to the A4 region of mouse chromosome 9.

GlcAT might catalyze the transfer of GlcA to the Gal residue of proteoglycans. Kitagawa \textit{et al.}\textsuperscript{85} cloned a cDNA from human placenta, which encoded GlcAT-1 responsible for the biosynthesis of the glycosaminoglycan–protein linkage region, GlcA/β1-3Gal/β1-3Gal/β1-4xyl/β1-O-Ser, of the proteoglycan by a PCR strategy based on motifs conserved GlcAT-P into putative proteins. The amino acid sequence showed 43% identity to the rat GlcAT-P, and the highest sequence identity was found in the COOH-terminal catalytic domain.

During a screening of a rat brain cDNA library using GlcT-P cDNA corresponding to the catalytic region of GlcAT-P as a probe, Seiki \textit{et al.}\textsuperscript{72} obtained a novel cDNA clone encoding a GlcAT-D, later named as GlcAT-S. The cDNA sequence contained an open reading frame encoding 324 amino acids, with type II transmembrane topology. The amino acid sequence revealed 49% homology to rat GlcAT-P. In this regard, using the RNA of rat E13 brain as a template of reverse transcription-PCR and designing primers to the highly conserved regions found in the alignment of amino acid sequence of GlcAT-P and GlcAT-1, Shimoda \textit{et al.}\textsuperscript{71} independently isolated a cDNA clone encoding a GlcAT-D, which was involved in the biosynthesis of the HNK-1 epitope on the neuronal cells. Imiya \textit{et al.}\textsuperscript{84} isolated a cDNA and genomic clones encoding the mouse GlcAT-S. The amino acid sequence of mouse GlcAT-S was 98.1% identical to that of rat GlcAT-S. Northern blot analysis revealed that the mouse GlcAT-S transcript was specifically expressed in the nervous system. Moreover, the mouse GlcAT-S gene was composed of four exons spanning over more than 25 kilobase pairs. Southern blot analysis and chromosomal mapping indicated that the mouse GlcAT-S gene was a single copy gene and mapped to the A4-B region of mouse chromosome 1. The GlcAT-S transcript was specifically expressed in the nervous system. The crystal structures of these GlcATs have been reported.\textsuperscript{85,86}

Sulfortransferase (SulT) is also considered to be a key enzyme in the biosynthesis of the HNK-1 epitope. Subsequently, Bakker \textit{et al.}\textsuperscript{77} reported the isolation of a cDNA clone encoding SulT from rat brain by an expression cloning strategy that involved the cotransfection of GlcAT-P cDNA. The clone isolation predicts a protein of 356 amino acids, with characteristics of a type II transmembrane protein and with no sequence similarity to other known SulTs. The enzyme is expressed as a soluble fusion protein, and cell homogenate transfection with the full-length cDNA transfers a sulfate from a sulfate donor to acceptor substrates containing a terminal GlcA. The cloned cDNA was shown to induce HNK-1 reactivity in CHOP \textit{P} cells only in combination with a GlcAT, indicating that these two enzymes, together with common enzymes already present in the cells, are required and sufficient for the biosynthesis of the HNK-1 epitope on glycoproteins.
Expression of glucuronyltransferases and sulfotransferase in mouse brain and rat embryos

Inoue et al.\(^{52}\) and Nagase et al.\(^{58}\) reported different region-specific expression patterns of two GlcATs (GlcT-P and GlcAT-S) or GlcAT-D. In mouse brain, strong expression of GlcAT-P and moderate expression of GlcAT-S were observed in the neuronal cells of several nuclei of limbic-related regions and of the sensory system and the cerebellum. In rat day P8 cerebellum, GlcAT-S mRNA was expressed in the internal and external granule layers as well as the embryonic pallium and retina. On the other hand, the expression of GlcAT-P was mostly localized in the Purkinje cell layer.\(^{71}\)

The HNK-1 epitope was observed in a subpopulation of myotomal cells and migrating myoblasts in the limb bud. Nagase et al.\(^{58}\) investigated the expression patterns of genes encoding two GlcATs (GlcAT-P, GlcAT-D) and SuIT, which are required for biosynthesis of the HNK-1 epitope. In rat day E11.5, the GlcAT-P gene was expressed in the non-migrating longitudinal fibers, whereas the GlcAT-D gene was expressed in the migrating myoblasts in the limb bud. Thus, differential expression of GlcAT genes may relate to the epaxial/hypaxial or migrating/non-migrating myoblast lineages, whereas the expression of the SuIT gene was ubiquitously observed in the embryo, which was confirmed by complete absence of in situ hybridization signal when the SuIT sense probe was used. In our study, however, GlcAT-P expression did not show significant developmental regulation in mouse brains. In contrast, GlcAT-S showed a transient expression pattern from E14 to E18.\(^{59}\) Expression of GlcAT-S is presumed to be involved in the transient expression of SGPG in developing mouse embryonic brains.

Yamamoto et al.\(^{30}\) generated mice with targeted deletion of the GlcAT-P gene; the HNK-1 carbohydrate disappeared almost completely in GlcAT-P-deficient mice, but a trace of HNK-1 immunoreactivity remained on the surfaces of the soma and proximal dendrites of a subset of neurons in some limited regions. These remaining HNK-1 carbohydrates in GlcAT-P-deficient mice were localized predominantly in the perineuronal nets and assumed to be synthesized by GlcAT-S, suggesting the possibility that the two GlcATs synthesize structurally and functionally different HNK1 carbohydrates in vivo. From this point of view, GlcAT-P-deficient mice constituted a useful tool for clarifying the structural and functional roles of the remaining the HNK-1 carbohydrate epitope. Interestingly, the GlcAT-P \(-/-\) mice exhibited normal development of gross anatomical features; however, the adult mutant mice exhibited reduced long-term potentiation at hippocampal CA1 synapses and showed a defect in spatial memory formation. GlcAT-P-deficient mice showed impaired hippocampus-dependent spatial learning. Although HNK-1 plays an essential role in synaptic plasticity and memory formation, it remains unclear how HNK-1 regulates these functions.

Expression of SGGLs in tissues of the nervous system

SGGLs are present mainly in the PNS tissues, cauda equina, and sciatic nerve, of human, bovine, cat, dog, and monkey.\(^{39,40}\) They were present in high concentrations and seemed to express almost equal amounts in these species.\(^{30}\) They were also present in the sciatic nerve of rat, mouse, rabbit, guinea pig, and chicken, but in much lower concentrations. Therefore, the occurrence of SGGLs was species-dependent, and higher in non-rodent mammals than in rodents.\(^{40,68,91}\) SGGLs were absent in the CNS of adult brain from the same species. The concentration of SGLPG was about one-fifth of that for SGPG in most tissues.\(^{91}\) Although SGGLs did not express in the spinal cord,\(^{68,91}\) they were expressed in brain stem and dura mater.\(^{91}\) SGGLs were also expressed in human dorsal root ganglion,\(^{92}\) and motor and sensory neurons in the PNS,\(^{93}\) in comparable amounts as in the sciatic nerve. SGGLs were minor components in human sympathetic ganglion,\(^{92}\) inner ear,\(^{94}\) eighth nerve,\(^{94}\) and optic nerve.\(^{95}\) Interestingly, SGGLs were not detectable in the CNS myelin,\(^{92}\) but expressed as minor components in rat Schwann cells,\(^{91}\) Schwanoma,\(^{91}\) several tumor cells of neural origin,\(^{39,95}\) brain microvessels,\(^{41}\) human umbilical veins,\(^{41}\) bovine brain microvascular endothelial cells,\(^{97}\) human brain microvascular endothelial cells,\(^{98}\) peripheral nerve endothelial microvascular endothelial cells,\(^{99}\) as well as in the neural crest cells of fish and birds in low amounts.\(^{100}\) SGGLs were found in the adult goldfish brain. It is speculated from these observations that SGGLs have some role during neural cell differentiation and in regeneration. In the CNS of higher animals, neuronal differentiation is minimal after maturation. However, it is known that in fish brain and in the PNS, neural cell differentiation and regeneration is possible even in the adult.\(^{30}\) SGGLs were present in the purified myelin fraction from cat PNS nerve, but their level
was not enriched in whole homogenate.\textsuperscript{40,101} SGGLs were present in the adult rodent cerebellum. SGGLs were localized specifically in Purkinje cells and their arbors or dendrites in the molecular layer.\textsuperscript{102} However, they were not detected in the cerebellum mice mutants of the Purkinje cell degeneration model, in which Purkinje cell loss was the primary defect. The loss of SGGLs in Purkinje cell-deficient mutants was specific, since most of the major lipids were not significantly affected and only the percentage composition of other lipids, such as sulfatides and gangliosides, were altered in adult cerebellum of the mutants. Other GlcA-containing GSLs, which do not contain the sulfate group, have been reported in insects such as \textit{Calliphora vicina} and \textit{Tenebrio molitor}\textsuperscript{103,104} (Table 1).

Subcellular localization studies of bovine spinal accessory nerve tissue,\textsuperscript{91} human dorsal root ganglion,\textsuperscript{92} and human sensory nerve of the PNS\textsuperscript{93} have demonstrated that SGPG was relatively abundant in the axolemma-enriched fraction (1.71–3.33 µg/mg protein) and myelin (1.34–1.63 µg/mg protein).

**Developmental expression of SGGLs in rat and mouse brains**

SGGLs were expressed in selected regions of the CNS tissues of fetal, newborn, and adult rodents and their expression is developmentally regu-
In rat cerebral cortex, SGGLs were developmentally regulated and were maximally expressed at day E15, but undetectable between day of birth (P1) and P14 after birth. They were not present in the adult rat brain but expressed maximally in the subpopulation of differentiated neurons of embryonic brain during development, thus they may be considered as stage-specific antigens.\textsuperscript{60,68} Immunoreactivity in the cerebral cortex appeared first in the mantle layer of neural tube of day E10. Staining then appeared more strongly in the molecular layer (layer I) and subplate (layer III), and more faintly in the intermediate zone and cortical plate in E15. In postnatal age, the whole brain was almost uniformly stained. However, immunoreactivity gradually disappeared 3 to 4 weeks after birth, except in specific neuronal cell types.\textsuperscript{107}

SGGLs are also detected in the cerebellum even in adult rats.\textsuperscript{106} The levels of SGGLs increased during postnatal development of the cerebellum, contrary to their diminishing expression in the cerebral cortex. Interestingly, the developmental profile of SGGLs in cerebellum appeared to be biphasic. The first phase appears near birth, and then the level decreases at P7. This pattern may reflect developmental events such as cellular migration and differentiation. Based on immunocytochemical studies of SGGLs, Bergmann glial fibers, the granular cell layer, and nascent white matter were stained at P2. Immunoreactivity of the granular cells was diminished drastically at P7, but remained constant in the white matter. Thereafter, the second phase of SGGL expression occurred at P10 and reached a maximum at P20; this phase may reflect the massive growth of dendritic trees in Purkinje cells. By P15, the expression of SGGLs was greatly reduced except in the molecular layer, where the immunostaining persisted until adulthood. Therefore, SGGLs may serve as regulatory markers of cellular differentiation in the cerebellum. In the adult rat cerebellum, Purkinje cell dendritic trees of the molecular layer and synapses of the deep cerebral layer were strongly stained, but not in the granular cell layer or the white matter.\textsuperscript{108} The high level of SGGLs remaining in Purkinje cells in adulthood suggests that SGGLs may play an integral role in Purkinje cell function.\textsuperscript{106}

In mouse embryos, the SGlcA epitope, detected using HNK-1 antibody, appeared at the early stages of E8.5 and E10.5.\textsuperscript{101} Immunoreactivity against the HNK-1 epitope appeared in neuroepithelial cells prior to axonal outgrowth and along the walls of the early neural tube. This expression pattern suggests that the SGlcA epitope may be associated with the organization of early neuronal setting and axonal growth patterns,\textsuperscript{101} and a further study indicated that SGPG was expressed at E16.\textsuperscript{89}

In rat PNS, the concentration of SGGLs increases with age and then declines slightly. The rate of increase in the level of SGGLs between day 5 to 20 was similar to the rate of deposition of myelin in the nerve. The rate of accretion of SGGLs was rapid for the first P30 days and plateaus at P40–60. This developmental pattern appeared to coincide with the process of myelination.\textsuperscript{68,109} In the mouse sciatic nerve, immunoreactivity against the HNK-1 epitope appeared first in unmyelinated fibers until P21, and strongly in myelinated fibers at 8 weeks.\textsuperscript{110}

**Experimental animal models of IgM paraproteinemia and peripheral neuropathy by sensitization of animals with SGPG**

In IgM paraproteinemia and peripheral neuropathy, studies on nerve biopsy from patients have revealed IgM deposits only in the PNS myelin and moderate demyelination with widening of the myelin lamellae. The IgM M-protein deposits are found in myelin with wide spacing of lamellae, which is the hallmark of this form of neuropathy.\textsuperscript{12,15,111,112} Moreover, direct injection of anti-SGGL/MAG antibody into cat sciatic nerve resulted in demyelination, which was associated with complement deposition.\textsuperscript{19,113} Thus, an antibody-dependent complement-mediated mechanism is presumed to participate in the pathogenesis of this autoimmune disease. To further clarify the pathogenic role of SGGLs, we sensitized rabbits and Lewis rats with highly purified SGPG.\textsuperscript{20,114–116}

In rabbit, anti-SGPG antibody titers in sera were detected 2 to 4 weeks after the initial inoculation and reached a maximum 6 to 8 weeks postinoculation. The animals showed weight loss, sluggishness in righting response from the outstretched position, and a slight to mild weakness predominantly in their hind feet 2 to 5 weeks postinoculation. Electrophysiological studies revealed slightly diminished conduction velocity and conduction block in the sciatic nerves of the SGPG-inoculated rabbits. Immunization of rabbits with purified SGPG produced mild neurological symptoms and electrophysiological dysfunction.\textsuperscript{114} This finding strongly suggests the involvement of SGPG in the process of demyelination.
In Lewis rats, anti-SGPG antibody titers in the sera were detected 2 to 4 weeks after injection and reached a maximum 8 weeks post-inoculation. Interestingly, anti-SGLPG antibodies were detected at 6 weeks and reached a maximum 12 weeks post-inoculation. Electrophysiological examination of the sciatic nerves revealed nerve conduction abnormalities that consisted of a conduction block and mild decrease in conduction velocity. In addition, sensitization of rats with SGPG induced minor but clear clinical signs of neuropathy, consisting of mild tail muscle tone loss and walking disabilities. Morphological studies showed (a) axonal changes in the lateral aspects of the dorsal columns in the spinal cord; and (b) damage to the endothelial cells in the spinal cord, which suggested a breakdown of the blood-brain barrier (BBB). In this regard, we reported that SGPG was present in the microvessels of adult rat brain. Thus, the present finding supports our hypothesis that disruption to the capillaries, which may be induced by the circulating anti-SGPG antibodies, may contribute to subsequent nerve damage. Interestingly, intraneural injection of the rat anti-SGPG antibody with guinea pig complement into the sciatic nerve of Lewis rats revealed extensive demyelination and axonal degeneration, along with mild to moderate clinical symptoms. Morphologically, vesiculation and loosening of the myelin sheath were observed 8 h post-injection, followed by extensive demyelination and macrophage infiltration after 4 days (Fig. 3). Since rat MAG does not bear the SGlcA epitope, it supports the concept that SGGLs may serve as primary target antigens in experimental IgM paraproteinemia and demyelinating neuropathy.

Ilyas et al. reported that four cats immunized with SGPG developed clinical signs of sensory neuropathy within 11 months after initial immunization. All cats demonstrated characteristics of unsteadiness, falling, hind limb weakness and ataxia. In two cats, the ataxia and hind limb paralysis were so severe that the animals had to be euthanized. Pathological examination revealed sensory ganglionitis with inflammatory infiltrates in the dorsal root ganglia. No overt signs of pathology were noted in the examined roots or nerves. High-titer anti-SGPG antibodies were detected in all 4 cats immunized with SGPG but not in the three control cats. This study suggests that these anti-SGPG antibodies play a role in the pathogenesis of this neuropathy. It has suggested that immunization of cats with SGPG induces anti-SGPG antibodies along with a sensory neuropathy that closely resembles human disease.

These animal models might help us understand the disease mechanism and lead to more specific therapeutic strategies.

Pathological role of SGGLs on IgM paraproteinemia and peripheral neuropathy

1. Interaction between SGGLs and blood-nerve barrier. Although the primary causal role of humoral immunity has been postulated in IgM paraproteinemia and peripheral neuropathy, the mechanism by which large molecules such as immunoglobulins can traverse the BBB or BNB to enter the endoneurium is not fully understood. However, in this disease, the destruction or malfunction of the BBB or the BNB results in leaking of...
circulating antibodies into the PNS parenchyma. This has been considered as the initial key step for the development of disease process. Since endoneurial microvascular endothelial cells (MECs) are one of the cardinal components making up the structural basis of the BBB/BNB, knowledge on the cellular characteristic of MECs using cell culture techniques may provide new insights regarding the pathogenetic mechanism of this immune-mediated neuropathy.

To test the hypothesis that circulating antibodies trigger permeability changes in the microvascular structure, we\(^{37}\) established an *in vitro* model of the BNB by coculturing a bovine MEC monolayer and rat astrocytes in Transwell chambers. We analyzed the effect of anti-IgM SGPG antibody obtained from a patient with IgM paraproteinemia and demyelinating peripheral neuropathy against cultured bovine MECs. Permeability studies revealed that the antibody facilitated the leakage of \(^{14}\)C-inulin and \(^{125}\)I-labeled human IgM through bovine MEC monolayers. A direct cytotoxicity of this antibody against bovine MECs was also shown by a leakage study using \(^{51}\)Cr-incorporated bovine MECs. This cytotoxicity depended on the concentration of the IgM antibody, and was almost completely blocked by preincubation with the pure antigen, SGPG. This study strongly supports the hypothesis that immunological insults against bovine MEC-bound SGGLs induce the destruction or malfunction of the BNB, which results in penetration of the immunoglobulin molecule that attaches to the peripheral nerve parenchyma. It is also an intriguing possibility that SGGLs, which bear the same carbohydrate epitope as several cell-adhesion glycoproteins, may actually participate in the formation of the BBB/BNB and the maintenance of barrier function. This study showed that bovine MECs and the peripheral nervous tissues shared many GSLs, including SGPG, as common antigens; hence, the presumed cascade of pathological processes-immunological function of the BNB is: (a) increased permeability across the BNB, (b) leakage of immunoglobulins into the endoneurial space, and (c) subsequent immunological attack of peripheral myelin and axon, ultimately leading to their destruction, which can be logically explained in those patients with anti-GSL antibody. In SGPG sensitive rat study, damage in the capillaries was induced in the dorsal horn of the spinal cord lumbar region,\(^{20}\) suggesting the damage to endothelial cells increases the leakage of plasma proteins into the nerve parenchyma. However, it is not clear how immunological insults against bovine MECs actually occur in this disease. There have been few studies on the pathological changes of endothelial cells in IgM neuropathy. Meier *et al.*\(^{118}\) reported that the gap between the adjacent endoneurial cells are enlarged in IgM neuropathy, and Powell *et al.*\(^{119}\) showed microvascular changes, including endothelial cytoplasmic enlargement occasionally obliterating the vessel lumen, and intracytoplasmic actin-like filaments in dysglobulinemic neuropathies. Detailed characterization of the antibodies was not performed. In both reports, pathological changes in endothelial cells were completely different from those of acute inflammatory changes, but minimal presumably due to the deposition of circulating immune complexes typically observed in vessels in collagen disease with vasculitis such as periarteritis nodosa.\(^ {120} \)

**2. Inflammatory effect of SGGLs on microvascular endothelial cells that may be involved in the pathogenesis of IgM paraproteinemia and peripheral neuropathy** Although the functional roles of SGGLs are not clear, they are able to support cell adhesion of Schwann cells *in vitro*\(^ {121} \) and they may have implications in neural cell adhesion.\(^ {54} \)

The most interesting aspect of the regulated expression of SGGLs in bovine MECs is the induction of their synthesis by proinflammatory cytokines. Treatment of bovine MECs with interleukin (IL)-1\( \beta \), a proinflammatory cytokine, was shown to induce accumulation of SGGLs.\(^ {122} \) The SGPG content in bovine MECs increased 8 fold after stimulation for 4h; this resulted in an enhanced attachment of human lymphocytes on IL-1\( \beta \)-activated bovine MEC monolayer surface. Further studies indicate that inflammatory cytokines, such as tumor necrosis factor (TNF)-\( \alpha \) and IL-1\( \beta \) stimulate the GlcAT-P and GlcAT-S genes and elevate the SGPG concentration in cerebromicrovascular endothelial cells, SV-HCEC, promoting T cell adhesion.\(^ {123} \) In siRNA HNK-1\(^ {5} \) (siHNK-1)-transfected cells, SGPG expression was down-regulated after stimulation in addition to reducing T cell adhesion. In addition, up-regulation of GlcAT genes in the cells was observed after cytokine stimulation mediated by NF\( \kappa \)B signaling; controversially, inhibition of SGPG expression by siHNK-1 intercepted the NF\( \kappa \)B activity.\(^ {124} \)

Selectins are known to be involved in the interaction between leukocytes and vascular endothelial cell adhesion, leading to lymphocyte homing, platelet binding, and neutrophil extravasation.\(^ {125} \) Needham and Schnaar\(^ {26} \) have reported that L-
and P-selectins bind to a surface adsorbed with SGGLs in a Ca\textsuperscript{2+}-independent manner, but not with E-selectin. Interestingly, we\textsuperscript{122} have found that a significantly larger number of human lymphocytes attach to the IL-1\textbeta-activated bovine MECs than to the unstimulated bovine MECs. Interestingly, attachment of human lymphocytes to the IL-1\textbeta-activated bovine MEC cells can be blocked either by incubation of the human lymphocytes with an anti-L-selectin antibody or by application of an anti-SGPG antibody significantly reduced the attachment of human lymphocytes onto the bovine MEC monolayer (*P < 0.05; **P < 0.001). Error bars indicate the SD.

Fig. 4. Effect of anti-L-selectin and anti-SGPG antibodies on the adhesion of human lymphocytes. Filled bars represent IL-1\textbeta-activated bovine MECs and the hatched bar represents the nonactivated bovine MEC monolayer. Preincubation with anti-L-selectin and/or anti-SGPG antibody significantly reduced the attachment of human lymphocytes onto the bovine MEC monolayer (*P < 0.05; **P < 0.001). Error bars indicate the SD.

Therapeutic strategies in patients with IgM paraproteinemia and peripheral neuropathy

Based on evidence regarding the pathogenicity of anti-MAG/SGGL antibodies, therapeutic effects have been documented reducing circulating IgM protein or anti-MAG/SGGL antibodies by removal (plasmapheresis or plasma exchange), inhibition (intravenous immunoglobulin, IVIg), or reduction of synthesis (corticosteroid, immunosuppressive, cytotoxic agents or interferon-\alpha etc).\textsuperscript{128} In an earlier study, reducing the IgM M-protein concentration by plasmapheresis or plasma exchange has been reported to ameliorate the symptoms of patients.\textsuperscript{24} Plasma exchange was temporarily effective in approximately half of the patients both alone and in combination with other therapies.\textsuperscript{11} Corticosteroids alone had no effect, but approximately half of the patients responded to corticosteroids given in association with other therapies.\textsuperscript{11} IVIg was effective in half of the patients. Patients who had anti-MAG IgM antibodies, showed significant improvement at 4 weeks with IVIg compared with placebo.\textsuperscript{129} Some agents, such as interferon-\alpha,\textsuperscript{130} chlorambucil,\textsuperscript{11} cyclophosphamide,\textsuperscript{131} and Rituximab (monoclonal antibody against CD20 antigen) improved the sensory ataxia.\textsuperscript{132,133} A plausible possibility is that blockage of leukocyte/endothelial cell connections by applying excess amounts of polysaccharides or by administration of monoclonal antibodies to block these oligosaccharide epitopes might be an effective therapeutic strategy.\textsuperscript{97}
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Profile

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