Association of GM4 ganglioside with the membrane surrounding lipid droplets in shark liver

Yu-Teh Li,[1],* Eiko Sugiyama,* Toshio Ariga,[†] Jun Nakayama,[§] Masayoshi Hayama,[§] Yoichiro Hama, Hiroki Nakagawa,** Tadashi Tai,[‡] Karol Maskos,[§§] Su-Chen Li,* and Takeshi Ksama****

Department of Biochemistry,* Tulane University Health Sciences Center School of Medicine, New Orleans, LA 70112; Clinical Research Center,[†] Eisai Co. Ltd., Koishikawa, Tokyo 112-8088, Japan; Central Clinical Laboratories,[§] Shinshu University Hospital, Matsumoto 390-8621, Japan; Department of Applied Biological Sciences,** Faculty of Agriculture, Saga University, Saga 840-8502, Japan; Department of Tumor Immunology,[‡] The Metropolitan Institute of Medical Science, Honkomagome, Tokyo 113-8613, Japan; Coordinated Instrumentation Facility,[§§] Tulane University, New Orleans, LA 70118; and Instrumental Analysis Research Center for Life Science,*** Tokyo Medical and Dental University, Tokyo 113-8510, Japan

Abstract   By TLC, GM4 was found to be the major ganglioside in the liver of six shark species examined: *Odontaspis taurus, Negaprion brevirostris, Sphyrna lewini, Mustelus griseus, Mustelus manazo,* **and** *Prionace glauca.* **A detailed analysis of the glycosphingolipids (GSLs) in the liver of** *O. taurus* **(sand tiger shark) showed that it contained approximately 110 nmol of lipid-bound sialic acid per gram of wet tissue, of which 80% was GM4. By extracting the liver of** *O. taurus* **with chloroform/methanol, followed by chromatographic separation of GSLs using DEAE-Sephadex A-25 and Iatrobeads columns, we have isolated GM4 in pure form with a yield of approximately 5 mg per 100 g of wet tissue. The structures of both the sugar chain and the ceramide moiety of this GM4 were analyzed by chemical analysis, mass spectrometry, and NMR spectroscopy. Similar to GM4 isolated from other sources, 92% of fatty acids in the ceramide of this GM4 were 2-hydroxylated. However, unlike the long-chain bases found in other GSLs, the total long-chain bases in this GM4 were found to contain 43% octadecasphingenine and 50% nonadecasphingenine.** Immunohistochemical analysis using a monoclonal antibody against GM4 revealed that the hepatocytes of both *M. griseus* (spotless smooth hound) and *M. manazo* (smooth hound) were filled with lipid droplets and GM4 was primarily associated with the membrane structure surrounding lipid droplets.—Li, Y.-T., E. Sugiyama, T. Ariga, J. Nakayama, M. Hayama, Y. Hama, H. Nakagawa, T. Tai, K. Maskos, S-C. Li, and T. Ksama. **Association of GM4 ganglioside with the membrane surrounding lipid droplets in shark liver.** *J. Lipid Res.* 2002. **43:** **1019–1025.**

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Ganglioside GM4 (NeuAcα2$\rightarrow$3GalCer) was first revealed as a minor ganglioside of human brain by Kuhn and Wiegandt (2). After a detailed examination of the regional distribution of GM4 in human brain, Ledeen et al. concluded that this ganglioside was a major component of myelin gangliosides (3). Subsequently, Yu and Iqbal identified GM4 as a specific marker for human myelin and oligodendroglial perikarya (4). Kim et al. examined the immunocytochemical localization of several classes of gangliosides and detected GM4 in all oligodendrocytes (5). Using a monoclonal antibody specific to GM4, Ozawa et al. reported that the expression of GM4 in chicken cerebellum was associated with astrocytes, and not with myelin (6). The occurrence of GM4 in extraneural tissues was first reported in chicken egg yolk (7) and chicken thymus (8). This ganglioside has also been detected in mouse erythrocytes (9, 10), rat kidney (11), chicken embryonic liver (12), chicken liver (13), and frog liver (14). Among these tissues, GM4 was found to be the major ganglioside in chicken egg yolk (7), chicken embryonic liver (12), and frog liver (14). While studying the distribution of glycosphingolipids (GSLs) in marine organisms, we found that the liver samples from six shark species that we examined were unusually rich in ganglioside GM4 when analyzed by TLC. We have subsequently performed a detailed structural characterization of GM4 isolated from the liver of *Odontaspis taurus* (sand tiger shark). We have also used immunohistochemical analysis to study the lo-

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calization of GM4 in the liver of *Mustelus griseus* (spotless smooth hound) and *Mustelus manazo* (smooth hound).

MATERIALS AND METHODS

Materials

O. taurus, *Negaprion brevirostris* (lemon shark), and *Sphyrna lewini* (scalloped hammerhead) were caught off the coast of the Gulf of Mexico. *M. griseus*, *M. manazo*, and *Prionace glauca* (blue shark) were caught near the coast of Southern Japan. Ceramide glycanase was isolated from leech (*Macrobdella decora*) (15). The monoclonal antibody against GM4 (MAb AMR 10) was prepared as described previously (6). The followings were from commercial sources: galactosylceramide, GM3, GM1, ceramide, octadecasphingenine, NeuAc, clostridial sialidase (type X), D_2O, CD_3OD, and DMSO-d_6 (Sigma-Aldrich, St. Louis, MO); DEAE-Sephadex A-25 (Amersham Pharmacia Biotech,Uppsala, Sweden); Iatrobeads (6RS 8060) (Iatron Laboratory Tokyo, Japan); precoated silica gel 60 TLC plates and HPTLC plates (Merck, Darmstadt, Germany). All other chemicals used were of analytical grade.

TLC analysis

The solvent systems used for the analysis of GSLs were: *A*) chloroform-methanol-water (60:35:8, v/v/v); *B*) chloroform-methanol-2 N NH_4OH (60:35:8, v/v/v); and *C*) 1-propanol-NH_4OH-water (75:5:25, v/v/v). GSLs were visualized by spraying the plates with the diphenylamine-aniline-phosphoric acid reagent (16, 17) followed by heating at 110–120°C for 15–20 min. To detect gangliosides, the plates were sprayed with the resorcinol-HCl reagent (18) and also heated at 110–120°C for 15–20 min. The quantitative estimation of gangliosides on a TLC plate was accomplished by using a Scan Jet 2c/ADF scanner (Hewlett Packard, Palo Alto, CA) and analyzed by the National Institutes of Health Image 1.61 program.

Analysis of gangliosides in the liver of different shark species

For the analysis of gangliosides in the liver of different shark species, 20 g of the liver sample from *O. taurus, N. brevirostris, S. lewini, M. griseus, M. manazo, and P. glauca* were separately extracted with 20 volumes of chloroform-methanol (1:1, v/v), subsequently passed through a DEAE-Sephadex column, and analyzed by TLC according to the procedure described by Ledeen et al. (3).

Isolation of the major ganglioside from the liver of *O. taurus*

For a typical isolation, 166 g of the fresh liver of *O. taurus* were extracted twice with 20 volumes of acetone and filtered through a Buchner funnel. GSLs were subsequently extracted from the acetone treated residue successively with 20 volumes each of chloroform-methanol (2:1, v/v), and chloroform-methanol (1:1, v/v). The combined extracts were evaporated to dryness, treated with 40 ml of 0.6 N NaOH in methanol at 40°C for 2 h, and neutralized with acetic acid. After removing methanol using a flush evaporator, the mixture was resuspended in water, exhaustively dialyzed against water in a cold room, and the retentate was lyophilized to obtain a crude GSL fraction. This fraction was dissolved in 25 ml of chloroform-methanol-water (30:60:8, v/v/v, solvent D) and applied onto a DEAE-Sephadex A-25 column (1.5 × 30 cm, acetate form) that had been equilibrated with solvent D. The column was washed with 300 ml of the same solvent to remove neutral lipids. Mono-sialogangliosides were eluted with 300 ml of 0.01 M of sodium acetate in solvent D. Di- and tri-sialogangliosides were successively eluted with 300 ml each of 0.02 M and 0.08 M of sodium acetate in solvent D, respectively. The eluates were separately evaporated to dryness, suspended in water, dialyzed exhaustively against water, and lyophilized.

Over 90% of the total lipid-bound sialic acids were recovered in the mono-sialoganglioside fraction. The major ganglioside in this fraction was initially designated as ganglioside S and further purified by Iatrobeads column chromatography. The mono-sialoganglioside fraction obtained from DEAE-Sephadex-A-25 chromatography (containing 5 mg of lipid-bound sialic acid) was dissolved in 1.5 ml of chloroform-methanol (9:1, v/v) and applied onto an Iatrobeads column (1.5 × 35 cm) that had been equilibrated with the same solvent. After washing with the same solvent, the column was eluted with a linear gradient generated from 1.8 liter of chloroform-methanol (40:60, v/v) in the reservoir and 1.6 liter of chloroform-methanol (85:15, v/v) in the mixing chamber. The flow rate was 20 ml/h and 10 ml fractions were collected. Gangliosides in each fraction were analyzed by TLC using solvent A. With this procedure, 8 mg of pure ganglioside S was isolated from 166 g of the fresh liver of *O. taurus*.

Analytical methods

The lipid-bound sialic acid was analyzed by the resorcinol-HCl method (18) using NeuAc as standard. Periodate oxidation of GSLs was carried out in chloroform-methanol (1:2, v/v) and 0.08 M of sodium metaperiodate at room temperature for 24 h. The neutral sugar in ganglioside S was determined by gas chromatography (GC) after methanolysis and trimethylsilylation as described by Bhatti et al. (19). To determine the long-chain base composition, 50 μg of ganglioside S dissolved in 0.5 ml of 2 N HCl in CH_3OH was subjected to methanolysis for 5 h according to the procedure described by Sweeley and Moscatelli (20). After methanolysis, fatty acid methyl esters were removed by extraction twice with N-hexane. The lower methanol phase was evaporated to dryness, dissolved in a small volume of $CHCl_3$, and passed through a small silica gel column to remove traces of fatty acid methyl esters (20). The long-chain bases were then converted to trimethylsilyl derivatives by reacting with a mixture of hexamethyldisilazane-trimethylchlorosilane-pyridine (1:1:5, v/v/v). An aliquot of the reaction mixture was analyzed by a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan) fitted with a cross-linked 5% phenyl methyl silicone capillary column (DB-5, 0.25 mm id × 30 m, J&W Scientific, Folsom, CA). GC/mass spectrometry (MS) was carried out under the same condition using a Finnigan TSQ 70 quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a Varian 4300 GC (Palo Alto, CA). For the analysis of fatty acids, another 50 μg of ganglioside S were subjected to methanolysis for 3 h at 100°C with 0.2 ml of 1 N HCl in anhydrous methanol. After adding an equal volume of water to the hydrolyzate, the fatty acid methyl esters were extracted with N-hexane and analyzed by GC and GC/MS.

Secondary ion mass spectrometry and collision-induced dissociation mass spectrometry

Secondary ion mass spectrometry (SIMS) spectra of ganglioside S were recorded on a Finnigan TSQ 70 quadrupole mass spectrometer in the negative and positive ion mode equipped with a cesium ion gun. Approximately 1 μg of the GSL sample dissolved in chloroform-methanol (2:1, v/v) was loaded on the SIMS target tip with 0.5 μl of triethanolamine for the negative ion mode or 3-nitrobenzyl alcohol for the positive ion mode. The SIMS primary beam was Cs^+ accelerating at 20 kV. The SIMS spectrum was obtained by acquisition of 16 scans at a scan rate of 200 U/sec. Collision-induced dissociation (CID) was induced by Argon gas at 3 m Torr under 15 eV of CID energy and the spectrum was obtained by acquisition of 32 scans at a scan rate of 200 U/sec.

NMR spectroscopy

For NMR analyses, ganglioside S (1.5 mg) was exchanged with D_2O, lyophilized, exchanged again with CD_3OD, and evaporated to dryness. The exchanged sample was then dissolved in 600 μl of DMSO-d_6. The NMR spectra were recorded at 25°C on a Bruker DRX 500 MHz spectrometer. Bruker probes (5 mm, inverse with XYZ gradients, and broad band with Z gradients) were used for all measurements. The ^{13}C signal assignments rely on the initial delineation of the individual proton spin systems within each molecule using a variety of two-dimensional techniques, including gradient versions of the double-quantum filtered COSY (DQF-COSY) (21, 22), total correlation spectroscopy (TOCSY) (23, 24), nuclear Overhauser enhancement in rotating frame (ROESY) (25, 26), indirectly detected heteronuclear single-bond ^1H-^{13}C correlation (HSQC) (27–29), and ^1H-detected heteronuclear multiple bond connectivity (HMBC) (30, 31).

Enzymatic hydrolysis

Ganglioside S (2 to 5 nmol) was dissolved in 100 μl of 10 mM of sodium acetate buffer, pH 5.5, and incubated with 4 units of clostridial sialidase at 37°C overnight. The reaction mixture was first heated in a boiling water bath for 3 min to stop the reaction, and then evaporated to dryness using a centrifugal vacuum evaporator, and analyzed by TLC using solvent A. The same amount of the ganglioside was incubated overnight with 0.2 units of ceramide glycanase in 100 μl of 50 mM sodium acetate buffer, pH 5.0, at 37°C as described previously (15).

Immunohistochemical analysis

Shark livers from *M. griseus* and *M. manazo* were fixed with 20% formalin buffered with 45 mM phosphate buffer (pH 7.4) for 48 h, and then incubated with a 0.88 M hypertonic gum-sucrose solution overnight. The tissue specimens were immediately frozen in an OCT compound (Sakura Finetechnical Co., Tokyo, Japan) at –80°C. Frozen sections sliced at 15 μm thickness were placed on Matsunami-Adhesive-coated slides (Matsunami Glass, Osaka, Japan) and then air-dried. After blocking with 1% BSA in PBS, these tissue specimens were incubated overnight with the anti-GM4 antibody (MAb AMR-10) at 4°C. After washing with PBS, they were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (DAKO A/S, Glastrup, Denmark) for 1 h at room temperature. The peroxidase reaction was developed with a diaminobenzidine/H_2O_2 solution and counter staining was performed with hematoxylin. In a control experiment, the primary antibody was omitted from the staining procedure, and no specific staining was found. In addition, tissue sections treated with chloroform-methanol (2:1, v/v) for 24 h were also immunostained with the AMR-10 antibody. Hematoxylin and eosin staining were performed on liver sections for morphological examination.

RESULTS AND DISCUSSION

Composition of GSLs in the liver of *O. taurus*

By TLC, GM4 was found to be the major ganglioside in the liver of *O. taurus, N. brevirostris, S. lewini, M. griseus, M. manazo,* and *P. glauca* (result not shown). This finding prompted us to initiate the detailed analysis of GM4 in *O. taurus,* which is readily available from the Gulf of Mexico.

Figure 1 shows that the crude GSL fraction derived from the liver of *O. taurus* contained one major ganglioside. This ganglioside was initially named ganglioside S. The TLC-mobility of ganglioside S was found to be faster
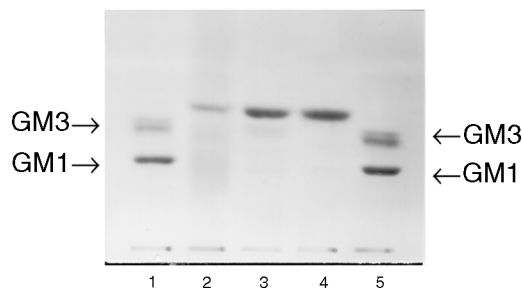

Fig. 1. Analysis of the crude lipid extract, the mono-sialoganglioside fraction, and ganglioside S isolated from the liver of *O. taurus* by TLC. Lane 1 and 5 are authentic gangliosides GM3 and GM1; lane 2, the crude lipid extract; lane 3: the mono-sialoganglioside fraction; lane 4, the purified ganglioside S. The plate was sprayed with the resorcinol-HCl reagent (18).

than that of GM3 using solvent A. The content of the total lipid-bound sialic acid in this liver sample was found to be approximately 110 nmol per gram of wet tissue. More than 98% of the total gangliosides were determined to be mono-sialogangliosides and about 80% of them was ganglioside S. The livers of *N. brevirostris, S. lewini, M. griseus, M. manazo,* and *P. glauca* were also found to contain comparable levels of ganglioside S. The yield of ganglioside S from the liver of *O. taurus* was approximately 5 mg per 100 gram of wet tissue. One of the minor mono-sialogangliosides was found to move as a doublet with a TLC-mobility similar to that of GM3. This slow moving ganglioside was converted to lactosylceramide by clostridial sialidase and to sialosyllactose by ceramide glycanase. Thus, this minor ganglioside was identified to be GM3.

Characterization of ganglioside S

The pure ganglioside S moved as a single band on TLC under solvent systems A, B, and C (results not shown). This ganglioside contained NeuAc and galactose in a molar ratio of 1:1 and was refractory to ceramide glycanase. This indicated the absence of a lactose core in ganglioside S (15). The galactose residue of this ganglioside was resistant to periodate oxidation and this ganglioside was converted to galactosylceramide by clostridial sialidase. These results indicated that the sugar chain of ganglioside S consisted of a sialic acid linked to the C3 position of the galactose residue.

Table 1 shows the composition of fatty acids and the long-chain bases found in ganglioside S. Similar to the GM4 isolated from other tissues (3, 13, 14), ganglioside S was found to be also rich in 2-hydroxy fatty acids. The major fatty acids in this ganglioside are C24:0h (31%), C23:0h (19%), C22:0h (24%), and C24:1h (14%), and 92% of total fatty acids are 2-hydroxylated. The long-chain bases of ganglioside S consist of octadecasphingenine (43%), nonadecasphingenine (54%), and icosasphingenine (3%). It is intriguing that ganglioside S is so rich in nonadecasphingenine that is only infrequently found in other GSLs (32). Unlike the sugar chains of GSLs, the biological importance of the ceramide moiety has not been

TABLE 1. Fatty acid and long-chain base composition of ganglioside S

Components[a]	%[b]
Fatty acids	
2-hydroxy fatty acids	
C16:0h	.64
C20:0h	2.03
C21:0h	1.34
C22:0h	23.67
C23:0h	18.58
C24:1h	14.46
C24:0h	31.02
subtotal	91.74
non-hydroxy fatty acids	
C16:0	2.52
C18:0	2.01
C22:0	1.23
C24:1	0.75
C24:0	1.75
subtotal	8.26
Long-chain bases	
d18:1	42.96
d19:1	54.32
d20:1	2.72

[a] The structures of fatty acids and long-chain bases were analyzed by GC/MS.

[b] These compositions were determined by gas chromatography equipped with FID.

fully appreciated. The unusual long-chain bases of ganglioside S may have special biological implications.

Structural analysis of ganglioside S by mass spectrometry

Figure 2 shows the negative ion SIMS spectrum of ganglioside S. The spectrum is consistent with the presence of the deprotonated molecule $[M-H]^-$ for a GSL with NeuAc-hexose (Hex) attached to ceramides containing sphingenines N-acylated with 2-hydroxy fatty acids. As shown in Fig. 2, the sphingenine and fatty acid compositions of major deprotonated molecules are: m/z 1089 (d18:1/C22:0h), m/z 1103 (d18:1/C23:0h and d19:1/C22:

0h), m/z 1117 (d18:1/C24:0h and d19:1/C23:0h), and m/z 1131 (d19:1/C24:0h). The spectrum also clearly shows the presence of fragment ions corresponding to ceramide (fragmentation point "a" shown in Fig. 2) and hexosyl ceramide (fragmentation point "b" shown in Fig. 2). The ions of m/z 308 and 290 are that of $[NeuAc-H]^-$ and $[NeuAc-H_2O-H]^-$, respectively (33), and the ion at m/z 468 is derived from $[(NeuAc-Hex)-H_2-H]^-$. The fragment ions at m/z 751 and 765 are assigned to the lyso-ganglioside corresponding to NeuAc-Hex-octadecasphingenine and NeuAc-Hex-nonadecasphingenine, respectively. These lyso-form ions are usually produced from the gangliosides containing 2-hydroxy fatty acids.

Figure 3 shows further characterization of the major deprotonated molecular ion of m/z 1,117 by CID-MS. The ion from the elimination of a NeuAc residue from the precursor ion was detected at m/z 826 that corresponds to a mono-hexosyl ceramide. Further elimination of a Hex from the ion at m/z 826 yielded the ion of ceramide at m/z 664. The CID also produced a major ion at m/z 290 that was assigned to $[NeuAc-H_2O-H]^-$. The SIMS and CID spectral analyses suggest that ganglioside S is a sialosylhexosylceramide. The mass spectral profile of deprotonated molecules and their resulted fragment ions from the cleavage of the glycosidic linkages of NeuAc and Hex agree well with fatty acid and long-chain base composition of this ganglioside shown in Table 1.

NMR analysis of ganglioside S

The ^{1}H- and ^{13}C-NMR assignments for ganglioside S are summarized in **Table 2**. The anomeric configurations of the two sugar residues in ganglioside S can be readily discerned from the one-dimensional ^{13}C spectrum of this ganglioside as shown in **Fig. 4**. The appearance of the resonance at 104.85 ppm (Fig. 4, G1) for the anomeric (C1) carbon of the Gal residue indicates that this sugar is in the pyranose form and has a β-anomeric linkage, since the anomeric carbon of methyl β-galactopyranoside resonates
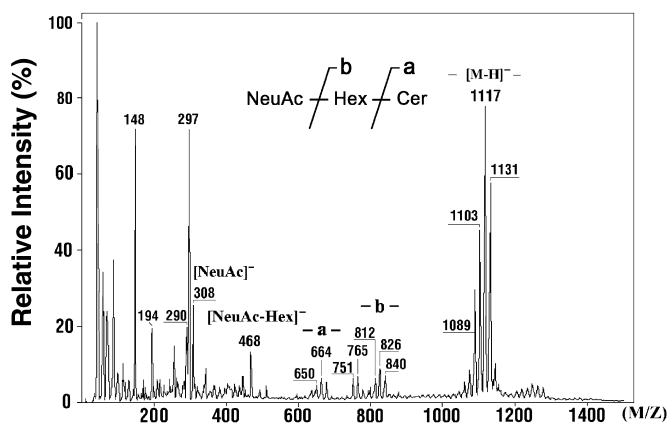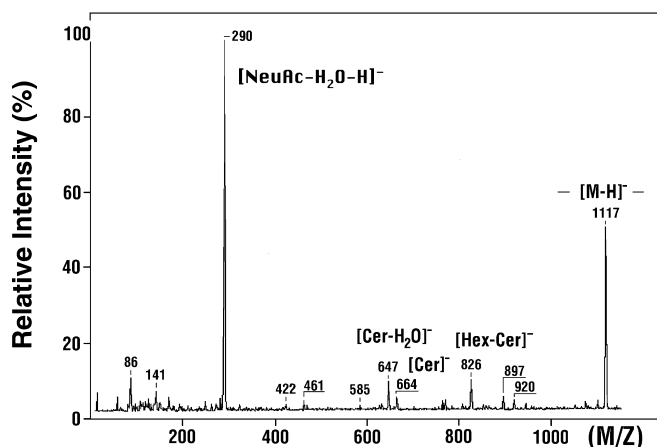

Fig. 2. Negative ion secondary ion mass spectrometry (SIMS) spectrum of ganglioside S isolated from the liver of *O. taurus*. The ions at m/z 148 and 297 are cluster ions of triethanolamine used as SIMS matrix. To save space, the labels for m/z 308 and m/z 468 are shortened. As indicated in the text, m/z 308 and m/z 468 represent $[NeuAc-H]^-$ and $[(NeuAc-Hex)-H_2-H]^-$, respectively.

Fig. 3. CID spectrum of the major deprotonated molecule $[M-H]^-$ of m/z 1,117 derived from the negative SIMS of ganglioside S shown in Fig. 2.

TABLE 2. ^{1}H and ^{13}C chemical shifts of ganglioside S, interglycosidic NOE, and heteronuclear multiple bond correlations observed in the ROESY and HMBC spectra

Position	^{1}H	^{13}C	Interglycosidic HMBC/ROESY
NeuAc			
1		170.93	
2		100.00	Gal-H3/NeuAc-C2
3	2.685[a]; 1.484[b]	42.19	
4	3.576	67.97	
5	3.437	53.52	
6	3.371	74.14	
7	3.215	69.30	
8	3.571	71.95	
9	3.581[a]; 3.337[b]	64.30	
NHCO		172.79	
NHAc	1.887	23.44	
Gal			
1	4.084[c]	104.85	Sph-H1[a,b]/Gal-C1; Gal-H1/Sph-H1[b]
2	3.308	69.30	
3	3.908	76.80	
4	3.703	67.89	
5	3.234	76.25	
6	3.469[a]; 3.469[b]	61.25	
Sph			
1	3.947[a]; 3.520[b]	68.98	Gal-H1/Sph-C1
2	3.796	53.52	
3	3.942	71.64	
4	5.353	131.87	
5	5.548	132.34	
6	1.926	32.66	
7	1.296	29.61	
FA			
1		174.35	
2	3.805	71.79	
3	1.545[a]; 1.406[b]	35.32	

NMR chemical shifts are given in ppm from the spectra obtained at 500 MHz in DMSO-d_6 at 25.0 +/− 0.1°C. The ^{1}H and ^{13}C chemical shifts are expressed relative to DMSO (2.49 and 39.50 ppm, respectively). Sph, sphingosine; FA, fatty acid.

[a,b] Denote two different protons attached to the same carbon.

[c] This proton has a spin-spin coupling ($^3J_{1,2}$) of 7.7 Hz with the H-2 proton.

Fig. 4. The structure of GM4 and one-dimensional ^{13}C-NMR spectrum of ganglioside S showing the assignments of the seven signals detected between 80 and 180 ppm. The complete assignments of ^{1}H and ^{13}C chemical shifts of NeuAc and Gal and part of ceramide are shown in Table 2.

at 104.5 ppm. In agreement with the α-NeuAc residues found in sialosyloligosaccharides (34), the anomeric (C2) carbon of the NeuAc in ganglioside S was found to resonate at 100.0 ppm (Fig. 4, N2). The ^{1}H-NMR analysis also supports the β-anomeric configuration of the Gal residue, since the anomeric proton (δ 4.084) of this sugar residue has a large vicinal coupling constant ($^3J_{1,2}$ = 7.7 Hz) as indicated in Table 2. As shown in Fig. 4, in addition to the two anomeric carbons, the five signals between 120 and 180 ppm can be readily assigned to that of the carbonyl carbon (C1) of fatty acyl moieties (174.35 ppm, Fig. 4, F1), the carbonyl carbon of the acetamido group of NeuAc (172.79 ppm, Fig. 4, N10), the carboxyl carbon of NeuAc (170.93 ppm, Fig. 4, N1), and the vinyl carbons (132.34 ppm, Fig. 4, S5; and 131.84 ppm, Fig. 4, S4) of sphingosine.

Interpretation of the ^{1}H-^{1}H DQF-COSY and TOCSY spectra led to the assignment of the proton relay signals corresponding to four spin systems. The HM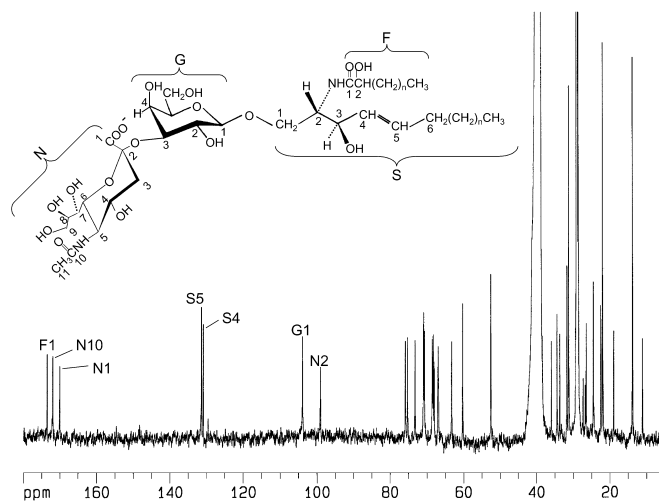BC and ROESY two-dimensional NMR experiments supplied definitive structural information regarding connections to the four spin systems and allowed a view of the overall structure. Detailed data from HSQC and HMBC spectra suggested that the ceramide unit consisted of two spin systems, a sphingenine, and an α-hydroxy fatty acyl ester. In the ^{1}H-NMR spectrum, a large coupling constant ($J_{4,5}$ = 15.4 Hz) between C4 and C5 protons of the sphingenine indicated the trans geometry of the double bond. The primary structure of the sugar moiety of ganglioside S was identified by NMR spectroscopy to be NeuAcα2→3Galβ1→1′Cer based on the following observations.

In this ganglioside, C3 of the β-Gal (76.80 ppm) residue has moved downfield by 3 ppm in respect to the corresponding methyl galactopyranoside, indicating that C3 of β-Gal is substituted. In the ROESY spectrum, the anomeric proton of β-Gal residue shows a cross peak with the more upfield H1 proton, H1[B], (Table 2) of the sphingenine moiety. The results of ROESY experiment as shown in Table 2 suggest that the linkage between β-Gal and the ceramide in ganglioside S is Galβ1→1′Cer.

After completely assigning the carbon spectrum (Table 2), we were able to make an unambiguous determination of the interresidue linkage from the long-range C-H correlation (HMBC). The interresidue correlations observed in the HMBC spectra of the studied molecule are also listed in Table 2. The presence of the strong cross peak, Gal-H3/NeuAc-C2 in the HMBC spectra, establishes the NeuAcα2→3Gal interglycosidic linkage in the disaccharide part of the molecule. The presence of the strong cross peaks, Gal-H1/sphingenine-C1 and sphingenine-H1[A,B]/Gal-C1 (Table 2), in the HMBC spectra shows that the β-Gal residue is directly connected with the C-1 ceramide hydroxy group by a glycosyl linkage (Galβ1→1′Cer). Based on chemical analysis, MS, and NMR spectroscopy, the structure of ganglioside S was established to be GM4 (NeuAcα2→3Galβ1→1'Cer).
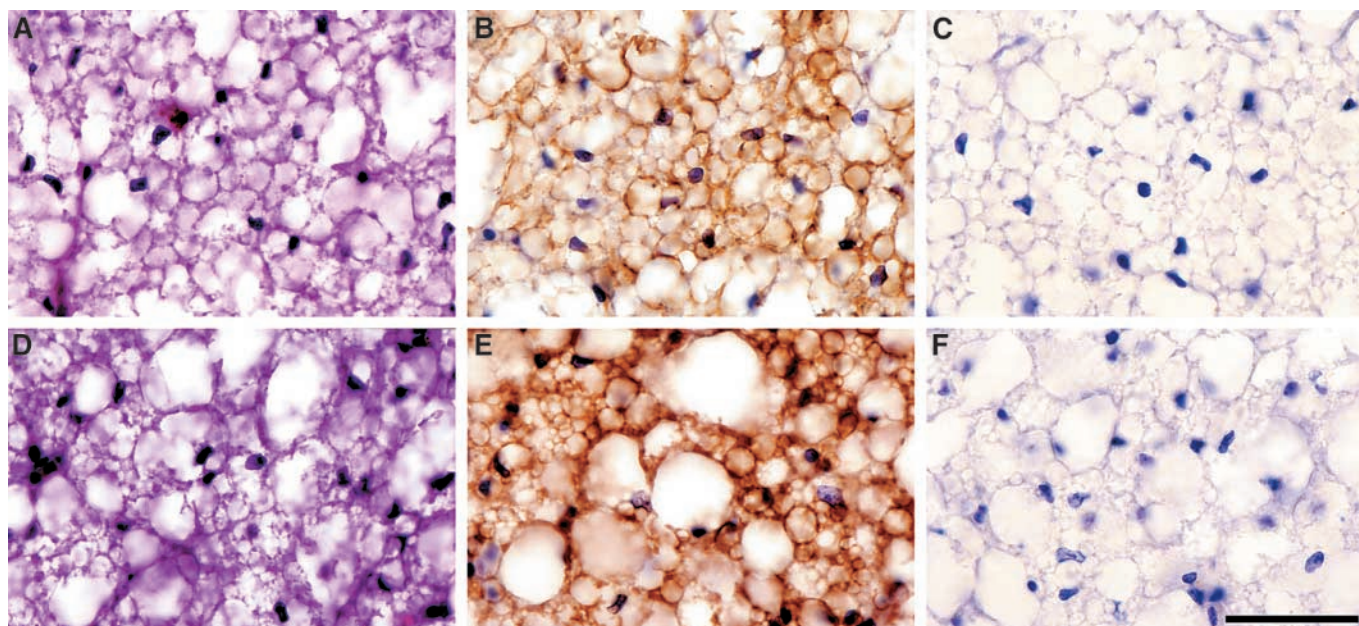
Fig. 5. Immunohistochemical analysis showing the expression of GM4 in the frozen section prepared from the liver of *M. griseus* and *M. manazo*. A and D: The hematoxylin and eosin stain showing the storage of lipid materials as droplets in the hepatocytes of *M. griseus* (A) and *M. manazo* (D). B and E: The immunostaining with the monoclonal antibody AMR-10 showing the expression of GM4 mainly in the limiting membrane surrounding lipid droplets found in the hepatocytes of *M. griseus* (B) and *M. manazo* (E). C and F: The negative control experiment for *M. griseus* (C) and *M. manazo* (F) omitting the AMR-10 antibody from the staining procedure. Bar = 50 μm.

Immunohistochemical analysis of the liver of *M. griseus* and *M. manazo*

The high GM4 content in the liver of six shark species prompted us to use the immunohistochemical analysis to study the localization of GM4 in the frozen sections of the liver of *M. griseus* and *M. manazo*. We realize that for histological analysis, paraffin-embedded sections are superior to frozen sections. However, paraffin-embedded sections are not suitable for preserving GSLs in tissue sections. As shown in **Fig. 5**, lipid materials were found to be stored in the hepatocytes as droplets in both *M. griseus* (Fig. 5A) and *M. manazo* (Fig. 5D). This observation confirmed the finding of Corner et al. (35). Immunohistochemical analysis using a monoclonal antibody against GM4 (MAb AMR-10) revealed that GM4 was primarily associated with the limiting membrane surrounding lipid droplets in the hepatocytes of *M. griseus* (Fig. 5B) and *M. manazo* (Fig. 5E). In addition, varying amounts of GM4 were also detected in the lipid droplets themselves. However, bile ducts located in the portal areas did not express GM4 (results not shown). To exclude the possibility that the AMR-10 antibody might have cross-reacted with glycoproteins containing the NeuAcα2→3GalNAc-epitope, immunohistochemical analysis was also performed on tissue sections of *M. griseus* liver pretreated by chloroform-methanol (2:1, v/v) to remove GSLs. No positive staining (data not shown) was detected after chloroform/methanol treatment, indicating that the positive immunological staining was due to the presence of GM4. Sharks have been known to have large livers that store large amounts of lipid materials (35, 36). Due to the high lipid content, it was difficult to make thin frozen sections of shark liver for histological studies. Among various shark liver samples processed, only the liver of *M. griseus* and *M. manazo* gave frozen sections suitable for histological analysis.

Beside to be closely associated with myelin (3–5) and astrocytes (6) in neural tissues, GM4 has been shown to exhibit immunosuppressive activity in inhibiting T-cell proliferative responses to tetanus toxoid (37). GM4 has also been shown to specifically interact with myelin basic protein and protect gangliosides against neuraminidase (38). Although GM4 has been found in a number of specialized tissues, the biological significance of this ganglioside is still not well understood. The liver of *O. taurus* contains the comparable amount of GM4 as that found in the frog liver (117 nmol/g wet tissue) (14). However, the GM4 isolated from frog liver contains both NeuAc and *N*-glycolylneuraminic acid, whereas the GM4 isolated from shark liver contains exclusively NeuAc. The presence of GM4 in shark liver has never been reported. It is intriguing that in the liver of *M. griseus* and *M. manazo*, lipid materials are stored as droplets that are encapsulated by a membrane and GM4 is part of this membrane structure. GSLs, primarily located on the plasma membrane of animal cells, have been shown to play a wide variety of biological functions and their associations with membrane components have been the subjects of intensive studies (39–42). It would be important to study the composition of the lipid droplets as well as the interaction of GM4 with proteins and membrane macromolecules surrounding the droplets in the future.

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ERRATA

In the article "Association of GM4 ganglioside with the membrane surrounding lipid droplets in shark liver" by Yu-Teh Li et al., published in the July 2002 issue of the *Journal of Lipid Research* (Volume **43**, pages 1019–1025), the correct spelling of the last author's name is as follows: Takeshi Kasama.