Novel modification of ceramide: rat glioma ganglioside GM3 having 3-*O*-acetylated sphingenine

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Abstract A novel *O*-acetylated GM3 containing 3-*O*-acetyl 4-sphingenine was isolated with one having a non-acetylated base from transplanted rat glioma tissue. The presence and position of the acetyl group were estimated by one- and two-dimensional proton nuclear magnetic resonance, and fast atom bombardment-mass spectrometries. In addition, the *O*-acetyl GM3 showed higher immunological activity toward anti-melanoma antibody in the presence of non-acetylated GM3 in complement-dependent liposome lysis than did non-acetylated or acetylated GM3 alone in the liposome, suggesting enhancement of immunological reactivity of the intact tumor cells by a small amount of *O*-acetyl GM3.

Key words: Ganglioside; Glioma; *O*-Acetyl sphingenine; *O*-Acetyl ceramide; *O*-Acetyl GM3; Rat

1. Introduction

Gangliosides (Ggls) and their *O*-acetylated derivatives have been minutely investigated as potential tumor-associated antigens. Many *O*-acetylated Ggls have been isolated from normal and tumor tissues or cells and their immunological activities toward monoclonal antibodies raised against tumor cells have been characterized. For instance, 9-*O*-acetyl (Ac) GD3 (9-*O*-Ac,*N*-Ac neuraminyl,*N*-Ac neuraminyllactosylceramide) was detected with an antibody as human melanoma-associated Ggl in melanoma tissue [1,2] and in fluids from patients [3], as well as being purified from melanoma cells [4] and human buttermilk [5], followed by characterization of the structure mainly by means of NMR spectroscopy. Other *O*-acetylated Ggls on the sialic acid moiety have been obtained from mammalian tissues and fluids [6–9] and from fish [10,11]. All these Ggls could be biochemically modified species from respective parent Ggls in normal or tumor cells by enzymatic *O*-acetylation [12] or intramolecular migration [13] after *O*-acetylation at the sialic acid, and not at the lipid moiety. Modification with the *O*-Ac residue at the latter has not yet been reported.

With respect to glycosphingolipids (GSLs) in glioma, human tumor tissue was found, in general, to contain a low concentration of Ggl, increased proportions of GM3, GM2 and GD3, and decreased proportions of the major normal brain Ggls [14,15]. In addition, we also isolated a human blood group B-active GSL, belonging to the ganglio series, from rat glioma cell line RG2 subcutaneous isografts [16]. We have continued examination of the acidic GSL component of rat glioma isografts, finding a novel *O*-acetylated GM3 containing 3-*O*-Ac 4-sphingenine. Furthermore, the immunological reactivity of the isolated *O*-Ac GM3 using liposomes is also demonstrated in this report.

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Abbreviations: GSL, glycosphingolipid; Ggl, ganglioside; Ac, acetyl; Cer, ceramide containing 4-sphingenine; GM3, *N*-acetylneuraminyllactosylceramide; 2D COSY, two-dimensional chemical shift-correlated spectroscopy; FAB-MS, fast atom bombardment-mass spectrometry; TLC, thin-layer chromatography; CMW, chloroform/methanol/water.

2. Materials and methods

2.1. Materials

Monoclonal antibody M2590 against GM3 from murine melanoma cells was obtained from COSMO Bio Co. (Tokyo). Guinea pig complement was a generous gift from Dr. Hiroki Mori, Department of Microbiology, Faculty of Pharmaceutical Science, Health Science University of Hokkaido. High performance precoated thin-layer chromatography (TLC) plates (silica gel) and deuterized dimethylsulfoxide (Me_2SO-d_6) were purchased from Merck (Germany). Authentic GM3 was isolated from rat liver in this laboratory. Other reagents were of analytical grade.

2.2. Purification of ganglioside from rat glioma tissue

Rat glioma tissue was obtained from normal Fisher rats after transplantation of cell line RG2, as reported previously [16]. The acidic lipid fraction of chloroform/methanol/water (CMW) extracts from the tissue (1,235 g wet wt.) was isolated by anion-exchange column chromatography using DEAE-Sephadex A-25 (Pharmacia-LKB, Uppsala, Sweden), and was repeatedly chromatographed on a silica gel column (Iatron, Tokyo) without saponification until the sugar moiety of the isolated Ggl appeared to be homogeneous under proton NMR analysis. GSL from normal Fisher rat brain was also purified without alkaline treatment as described above. Each purified GSL was analyzed for chemical structure as below.

2.3. Saponification of purified O-Ac gangliosides

The purified *O*-Ac Ggl (0.1 mg) was treated with 1 ml of 1% sodium methoxide in methanol for 2 h at room temperature, followed by neutralizing with acetic acid. The saponified Ggl was obtained after removal of salt from the mixture by gel-filtration chromatography using a Sephadex LH-20 (Pharmacia-LKB, Uppsala, Sweden) column (1 × 20 cm) by running with CMW (60:30:4.5).

2.4. NMR and mass spectrometries

The one-dimensional (1D) or 2D NMR spectrum of purified GSL (1–5 mg) was measured in a solution of Me_2SO-d_6/D_2O (98:2) at 90°C by 500 MHz, in Hokkaido University High-Resolution NMR Laboratory, as reported earlier [17]. The fast atom bombardment-mass spectrum (FAB-MS) of intact Ggl was obtained on a JEOL HX-500 apparatus at Analytical Center, Hokkaido University, using triethanol amine as a matrix, as reported previously [17].

2.5. Immunological studies

Immunostaining of Ggl on a TLC aluminum plate was performed as

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previously described [17] by a modification of the procedure of Magnani et al. [18]. Immunological reactivity of purified *O*-Ac GM3 toward anti-melanoma antibody was examined in a liposome lysis system depending on complement according to the method of Nores et al. [19], with some modification. Briefly, 500 nmol of both cholesterol and dipalmitoyl phosphatidylcholine, 20 nmol of dicetylphosphate and variable amounts of *O*-Ac GM3, GM3 or a mixture of both (*O*-Ac GM3/GM3, 65:35) were dried and dispersed in 0.1 ml of marker solution containing 0.1 M 4-methyl-umberiferyl phosphate (Wako Pure Chemicals, Tokyo). The resultant liposomes were thoroughly dialyzed against 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl to remove untrapped fluorescence marker. Aliquots of the liposome suspension were incubated with 1 mg per ml of M2590 antibody and guinea pig complement. The liberation of 4-methylumberiferone from the liposomes was measured with a spectrofluorophotometer (RE-510; Shimadzu) after incubation with alkaline phosphatase (*E. coli*, type III; Sigma) for 10 min at room temperature. Each assay was performed in triplicate and plotted as mean values in Fig. 4C.

3. Results and discussion

3.1. Isolation of O-Ac GM3

A GM3 that migrated slightly faster than authentic GM3 was purified together with the ordinary GM3 from the glioma acidic fraction as demonstrated in Fig. 1. The oligosaccharide structures of these GM3s were independently confirmed by gas chromatography-mass spectrometry of partially methylated alditol acetates (data not shown), and by 1D NMR and FAB-MS spectra. The faster-migrating GM3 was saponified to give an R_f spot similar to that of standard GM3 (Fig. 1), demonstrating an alkaline-labile group bound to the Ggl molecule responsible for the faster migration. The yield of whole GM3 containing the faster-migrating form was 28 mg from 1.235 g of wet wt. tissue.

3.2. FAB-MS study

As demonstrated in Fig. 2, the main pseudo molecular ion of the GM3 at m/z 1305 was due to a GM3 molecule (M_r = 1264) including an additional Ac residue, composed of sphingenine as a long chain base, 24:0 as a fatty acyl and an *N*-Ac-neuraminic acid moiety. The other molecular ions at m/z 1333, 1277, 1249, 1221 and 1193 were consequently assigned to

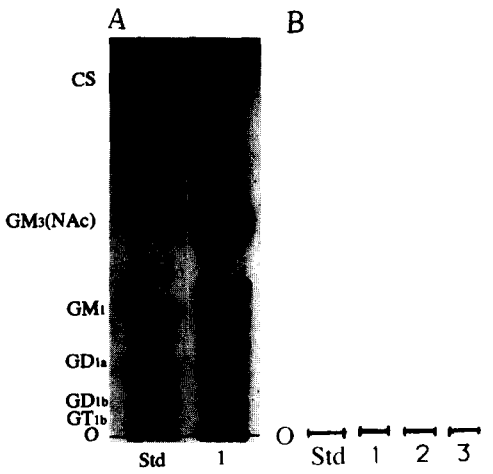

Fig. 1. Thin layer chromatography of GM3 from rat glioma subcutaneous isografts. The crude acidic fraction (A) and isolated GM3 (B) from rat glioma were chromatographed on TLC, developed with CMW (60:35:8) and stained by orcinol-sulfuric acid. (A) Std, acidic fraction of rat whole brain; lane 1, acidic fraction from rat glioma; CS, sulfated GalCer. (B) Std, standard GM3; lane 1, glioma *O*-Ac GM3; 2, glioma GM3; 3, saponified *O*-Ac GM3. O, origin.

monoAc GM3 with 26:0, 22:0, 20:0, 18:0 and 16:0 as the fatty acyls. The fragment ions due to mono-Ac-dihexosylceramide (*O*-Ac LacCer in the figure), -monohexosylCer (*O*-Ac GlcCer) and -Cer were also observed with the same lipid species as those of the Ac GM3. These fragments indicate the presence of monoAc group on Cer of the GM3 molecule, not on the sugar moiety. The Ac group presumably attaches to hydroxyl oxygen at C-3 of sphingenine, since the Cer moiety of the GM3 was composed of sphingenine (98%) and non-hydroxy fatty acids (26:0, 9%; 24:0, 35; 22:0, 12; 20:0, 16; 18:0, 23; 16:0, 3; and others) as analyzed by gas-layer chromatography.

3.3. NMR study

The 1D NMR spectrum of the faster-migrating GM3 is

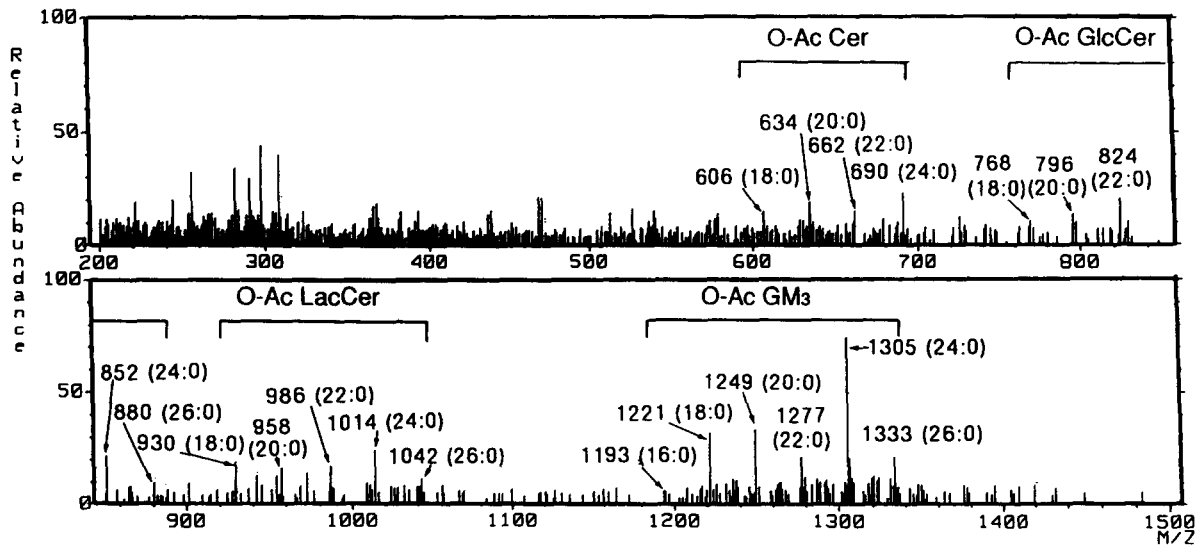

Fig. 2. Negative FAB-MS spectrum of *O*-Ac GM3. The numbers in parentheses at the fragment indicate carbon number and unsaturated grade of fatty acid.

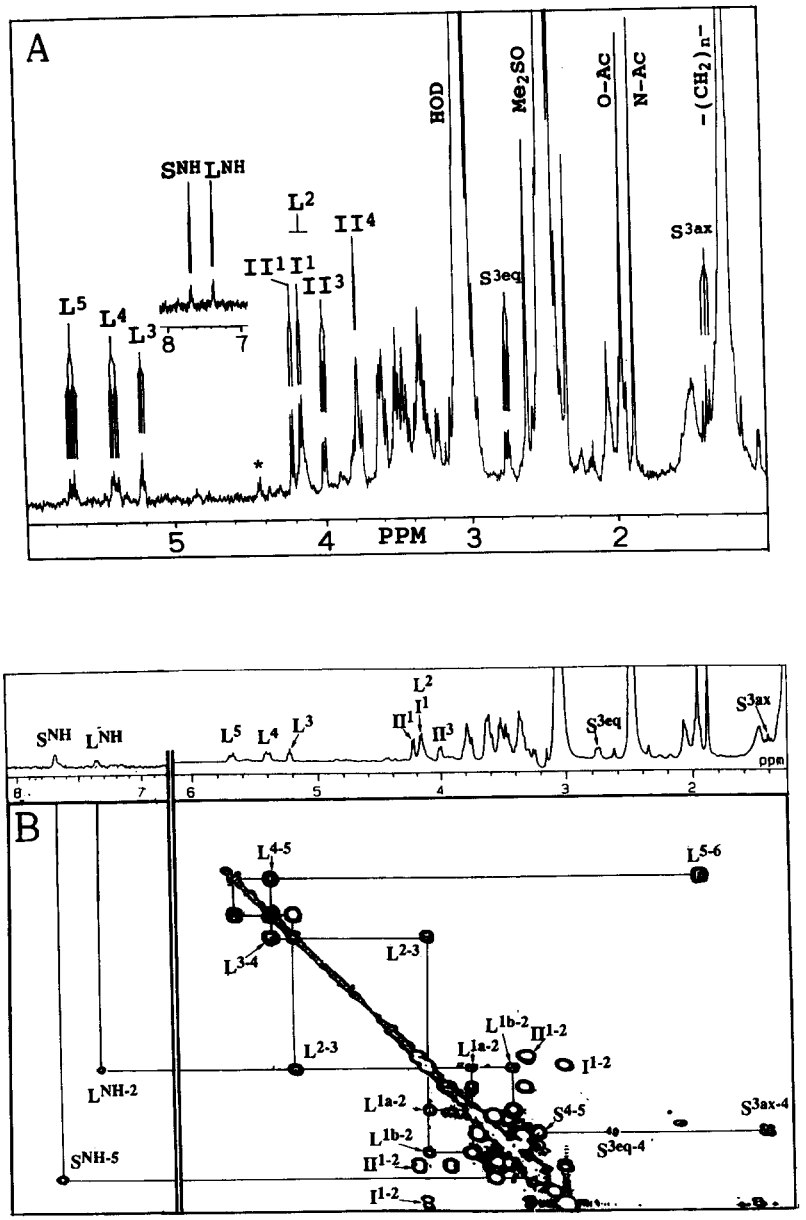

Fig. 3. 1D (A) and 2D (B) NMR spectra of *O*-Ac GM3. L, long chain base; S, sialic acid, I^1, H-1 on Glc; II^1, H-1 on Gal; L^{2-3}, connectivity between H-2 and H-3 on the long chain base. The peak marked with an asterisk is a contaminant.

shown in Fig. 3A, demonstrating a typical singlet ^{1}H at δ1.959 due to *O*-Ac-methyl ^{1}Hs, a lower field-shifted triplet 1H at 5.224 (abbreviated as L^3), probably due to 1H attached to the carbon with an acetoxy group, and olefinic ^{1}Hs at 5.402 and 5.679 due to ^{1}Hs attached to the C-4 and -5 on sphingenine, respectively. These ^{1}Hs as well as other ^{1}Hs were assigned by 2D chemical shift-correlated spectroscopy (COSY) as shown in Fig. 3B, and summarized in Table 1. The partial 2D spectrum of the Ac GM3 showed that L^3 coupled both with a broad L^2, overlapped with the anomeric ^{1}H (I^1) on Glc, and olefinic L^4 judged from the presence of cross-peaks L^{2-3} and L^{3-4}, respectively, and that the L^2 further coupled with L^{1a} and L^{1b} from L^{1a-2} and L^{1b-2} and with amide ^{1}H (L^{NH}), which was previously assigned to the ^{1}H bearing on the Cer moiety [16,17], from L^{NH-2}. Based on these couplings, the ^{1}Hs from L^1 to L^5 were assigned to H-1 to H-5, respectively, on sphingenine, and therefore, an Ac group was confirmed to exist at C-3-*O* on sphingenine of the GM3. Thus, the assigned C-3-H on sphingenine was detected neither in any neutral GSLs from glioma, nor in any GSLs from normal rat brain, indicating the occurrence of non-acetylation on these GSLs, and further suggesting that expression of *O*-Ac Cer on GM3 might be a tumor-associated phenomenon.

The *O*-acetylated sialylparagloboside at the Cer was also obtained from the tissue as a minor Ggl (data not shown). The presence of an *O*-acetyltransferase acting on the long chain base has been reported earlier only in yeast [20]. The present report might be, therefore, the first evidence of this in mammalian tissue, if Ggls or the precursor lipids were acetylated by *O*-acetyltransferase which would exist in glioma. The extent of the lipid *O*-acetylation in glioma GM3 was estimated with inte-

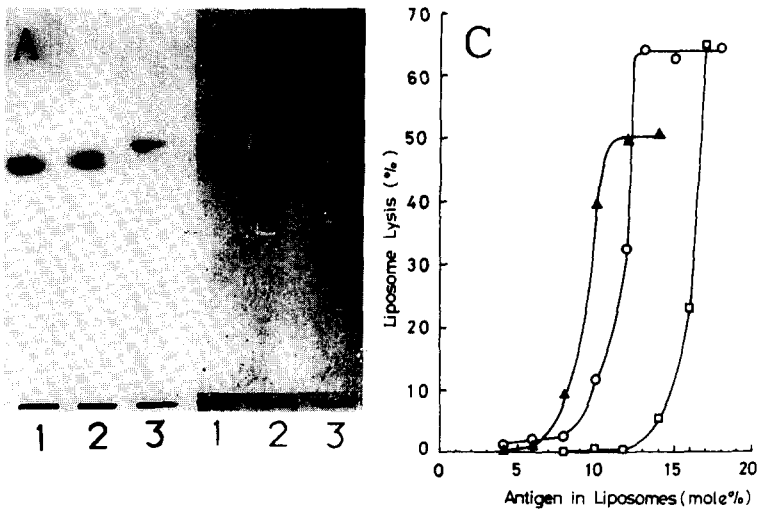

Fig. 4. Immunological activity of *O*-Ac GM3: (A) stained with orcinol-sulfuric acid; (B) immunostained on TLC with M2590 using 1/100 of the respective lipids in panel A. Lane 1, authentic GM3; 2, glioma GM3; 3, *O*-Ac GM3. (C) Result from antibody complement-dependent liposome lysis. Abscissa indicates the amount of antigen GM3s (mol%) added when the liposomes were prepared. ○, liposome with GM3; □, *O*-Ac GM3; ▲, a mixture of GM3 and *O*-Ac GM3 in a molar ratio of 65:35.

gration of H-5 (L^5), the chemical shift of which was distinct between *O*-acetylated and non-acetylated Ggls (see Table 1), to 35%.

3.4. Immunological study of O-Ac GM3

The antigenicity of the purified *O*-Ac GM3 from glioma was estimated using an antibody against murine melanoma, M2590, which reacted with GM3 and GM3 lactone containing *N*-Ac-neuraminic acid [21], revealing that *O*-Ac GM3 was strongly stained by the antibody together with non-acetylated GM3, as demonstrated in Fig. 4B. Furthermore, in the study using complement-dependent liposome lysis, at 10 mol% of antigen, liposomes independently with GM3 and with *O*-Ac GM3 released 12% and 0%, respectively, of trapped fluorescence, whereas that with mixed antigen with GM3 and *O*-Ac GM3 liberated 42% of the marker, as shown in Fig. 4C. These data indicate that liposomes with mixed antigen, in which the ratio of the composition might be close to that of intact glioma cells regardless of other lipid components, were significantly more sensitive to the melanoma antibody than liposomes with GM3 or *O*-Ac GM3 only. A threshold density with recognition by the antibody in an all-or-none pattern was observed, in agreement with a previous result [19], but the mol% extent at the threshold was slightly different in the GM3 liposomes. Though it is not clear why liposomes with *O*-Ac GM3 had much weaker reactivity toward antigen than liposomes with GM3, or why the existence of the inferior reactive *O*-Ac GM3 on liposomes enhanced the activity, evidence that GSLs form a cluster in the cell membranes [22] led us to consider preferential forming of the cluster to react with the antibody through the presence of the *O*-Ac GM3, and/or extended exposure of the carbohydrate moiety above the membranes by the introduction of a hydrophobic and sterically hindered Ac residue in the lipid portion, as compared to those from non-acetylated GM3. Further investigation is required for clarification.

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Table 1
NMR chemical shift (δ, ppm) of *O*-Ac GM3 and reference ceramide (Cer) contained in GM3

	H-1[a]	H-1[b]	H-2	H-3	H-4	H-5	H-6[a]	H-6[b]	H-7	H-8	H-9[a]	H-9[b]	-CONH-	CH_3CO-
O-Ac GM3[a]														
Cer	3.450	3.808	4.16	5.224	5.402	5.679	1.963	–	–	–	–	–	7.356	1.956
β-Glc	4.159	–	3.17	3.32	3.3	3.3	3.620	3.77	–	–	–	–	–	–
β-Gal	4.220	–	3.321	4.007	3.780	3.35	3.483	3.610	–	–	–	–	–	–
NeuAC	–	–	–	1.386[b] 2.757[c]	3.610	3.515	3.518	–	3.239	3.39	3.39	3.594	7.678	1.881
GM3														
Cer[d]	3.513	3.945	3.79	3.951	5.391	5.562	1.950	–	–	–	–	–	7.285	–
Cer[e]	3.432	3.993	3.769	3.876	5.343	5.533	1.931	–	–	–	–	–	–[f]	–

[a] Measured at 90°C.
[b] Ax ^{1}H.
[c] Eq ^{1}H.
[e] Measured at 30°C [23].
[f] Not reported.

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