Disruption of GM2/GD2 synthase gene resulted in overt expression of 9-\(O\)-acetyl GD3 irrespective of Tis21

Keiko Furukawa,* Wei Aixinjueluo,* Takeshi Kasama,§ Yuki Ohkawa,* Michiko Yoshihara,* Yusuke Ohmi,* Orie Tajima,* Akio Suzumura,* Daiji Kittaka* and Koichi Furukawa*

*Department of Biochemistry II, Nagoya University Graduate School of Medicine, Nagoya, Japan
†Department of Immune System, Institute for Environmental Medicine, Nagoya, Japan
‡Department of Biomedical Science, College of Life and Health Science, Chubu University, Kasugai, Aichi, Japan
§Instrumental Analysis Research Center for Life Science, Tokyo Medical and Dental University, Tokyo, Japan

Abstract
GM2/GD2 synthase gene knockout mice lack all complex gangliosides, which are abundantly expressed in the nervous systems of vertebrates. In turn, they have increased precursor structures GM3 and GD3, probably replacing the roles of the depleted complex gangliosides. In this study, we found that 9-\(O\)-acetyl GD3 is also highly expressed as one of the major glycosphingolipids accumulating in the nervous tissues of the mutant mice. The identity of the novel component was confirmed by neuraminidase treatment, thin layer chromatography-immunostaining, two-dimensional thin layer chromatography with base treatment, and mass spectrometry. All candidate factors reported to be possible inducer of 9-\(O\)-acylation, such as bitamine D binding protein, acetyl CoA transporter, or \(O\)-acetyl ganglioside synthase were not up-regulated. Tis21 which had been reported to be a 9-\(O\)-acylation inducer was partially down-regulated in the null mutants, suggesting that Tis21 is not involved in the induction of 9-\(O\)-acyl-GD3 and that accumulated high amount of GD3 might be the main factor for the dramatic increase of 9-\(O\)-acyl GD3. The ability to acetylate exogenously added GD3 in the normal mouse astrocytes was examined, showing that the wild-type brain might be able to synthesize very low levels of 9-\(O\)-acyl GD3. Increased 9-\(O\)-acyl GD3, in addition to GM3 and GD3, may play an important role in the compensation for deleted complex gangliosides in the mutant mice.

Keywords: 9-\(O\)-acyl GD3, brain, ganglioside, knockout, neuron, \(O\)-acylation.

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GD3 ganglioside has been considered to be associated with cell proliferation and cell activation, as it is exclusively expressed in the fetal brain tissues at the proliferative stage of development (Yu et al. 1988). It can be induced in human T lymphocytes by stimulating with Concanavalin A or interleukin 2 (Yamashiro et al. 1995). It can also be induced in fibroblasts introduced with oncogenic ras gene (Nakaishi et al. 1988). GD3 has been considered to be a melanoma-associated tumor antigen (Furukawa and Lloyd 1990), and been used as a target of antibody therapy in melanoma patients (Houghton et al. 1985). Recently, we demonstrated that GD3 expression actually enhanced the malignant properties of melanoma cells via increased tyrosine phosphorylation of adaptor molecules such as p130Cas or paxillin (Hamamura et al. 2005).

Besides GD3, a derivative of GD3 with \(O\)-acylated sialic acid has been reported to be also associated with tumors. In particular, 9-\(O\)-acetyl GD3 was found in some of human melanomas (Cheresh et al. 1984) and in other tumor tissues (Kohla et al. 2002), suggesting that this structure is involved in the development and evolution of cancer (Schauer and Kamerling 1997; Angata and Varki 2002).
7(9)-O-acetyltransferase, the enzyme responsible for forming O-acetylated gangliosides, was demonstrated in bovine submandibular glands in 1970 (Schauer 1970). Recently, many attempts have been made to identify the gene without success, suggesting that a single gene product may not be able to catalyze the 9-O-acetylation of sialic acids. These attempts resulted in the identification of a putative acetyl-CoA transporter (named AT-1) cDNA (Kanamori et al. 1997) or of a cDNA for a novel transcriptional factor (or AGS, O-acetyl ganglioside synthase) (Ogura et al. 1997). Vitamin D-binding protein (vit-D bp) was also defined as a candidate for 9-O-acetylation-causing factor (Shi et al. 1988). Recently, Satake et al. identified Tis21 as an inducer of 9-O-acetylation of GD3 under the expression of GD3 (Satake et al. 2002). This gene was originally identified as a promoting factor of cell proliferation, and appears to also act as an inducing factor of the O-acetyltransferase.

In the mutant mice of GM2/GD2 synthase lacking all complex gangliosides, GM3 and GD3 accumulated at extremely high levels (Takamiya et al. 1996). Recently, we detected an additional band migrating between GM3 and GD3 in thin layer chromatography (TLC). This component could only be detected in the preparation without alkaline treatment, suggesting it is alkaline sensitive. Based on the sensitivity to neuraminidase treatment and alkaline treatment, TLC-immunostaining, and mass spectrometry, the band was identified as 9-O-acetyl GD3. Mechanisms for the neo-expression of 9-O-acetyl GD3 in the mutant mice were analyzed.

Materials and methods

Knockout (KO) mice

The generation of the KO mice of GM2/GD2 synthase (Takamiya et al. 1996) and GD3 synthase (Okada et al. 2002) was previously reported. Maintenance and genetic typing of these mice were performed according to the directions of the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT). This study was approved by the Committee for Animal Experiment of Nagoa University Graduate School of Medicine.

Extraction of glycolipids and TLC

Glycolipid extraction was performed as described previously (Funukawa et al. 1985). The brief and quick extraction was performed as previously described (Miyazaki et al. 1997), in which alkaline treatment step was skipped. Extracts with chloroform/methanol were directly applied to DEAE Sephadex™ A-50 (Amersham Biosciences AB, Uppsala, Sweden) ion exchange column chromatography after desalting as described previously (Miyazaki et al. 1997). TLC was performed usually with chloroform/methanol/0.2% CaCl₂ (55 : 45 : 10), and bands were detected with resorcinol spray.

Neuraminidase treatment of gangliosides

In order to clarify the core structures of individual bands in TLC, ganglioside mixtures were digested with a neuraminidase (Clostridium perfringens, Sigma, St Louis, MO, USA), and the products were desalted and analyzed in TLC/resorcinol spray.

TLC-immunostaining

Using an anti-9-O-acetyl GD3 monoclonal antibody (mAb) Jones (Blum and Barnstable 1987) (kindly provided by Dr Colin Barnstable at Rockefeller Institute, New York) or mAb GMR2 (anti-9-O-acetyl GD3) (Seikagaku Kogyo, Tokyo), TLC-immunostaining was performed. After development of TLC, glycolipids were heat-blotted with an automated heat blotter (Atto, Tokyo) as described in the manufacturer’s instruction, and soaked in 2% bovine serum albumin/PBS for the blocking at 4°C over night. The membrane was stained with the antibodies at 2-5 μg/mL for 2 h at 25°C, then detected with horseradish peroxidase-labeled anti-mouse IgG (H & L) antibody or anti-mouse μ antibody combined with Western Lightning™ chemiluminescence reagent (PerkinElmer LAS, Inc., Boston, MA, USA). TLC-immunostaining with mAb R24 (anti-GD3) was also performed. Detection of antibody binding was also performed with Immunostaining HRP-1000™ (Seikagaku Kogyo, Tokyo).

Two-dimensional TLC

Two-D TLC was performed by changing the direction of TLC –90°. For the standard condition, an identical neutral solvent system was used to confirm the diagonal distribution of all components. Then, alkaline-sensitivity was analyzed by exposing TLC plates to NH₃ vapor for 2 h in a sealed box between the first and the second development.

Mass spectrometry

Mass spectrometry was performed to confirm the chemical structure of the new band, using the excised band from PVDF membrane on which gangliosides were transferred from an HP-TLC plate. Secondary ion mass spectrometry (SIMS) of the glycolipids was performed as described previously (Taki et al. 1995; Kasama et al. 1996). Negative ion mass spectra of glycolipids were recorded on a TSQ 700 quadrupole mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a cesium ion gun as follows. The glycolipid band on the PVDF membrane was excised (1.5 mm in diameter) and placed on a sample tip of mass spectrometer, and few microliters of triethanolamine (Wako Pure Chemical Industries, Osaka, Japan) was added as the SIMS matrix. The matrix with sample was bombarded with primary ion beam of Cs⁺ at 20 KeV. The ion multiplier was kept at 1.2 KV and the conversion dynode at 20 KV. The spectrum was accumulated several tens of scans.

Reverse transcription–polymerase chain reaction

Expression levels of candidate genes for the inducing factor of O-acetylation were examined with total RNA from the nervous tissues of the wild-type (WT), GM2/GD2 synthase KO, and GD3 synthase KO mice. Total RNA 4 μg was served for reverse transcription in 1 l L mixture at 37°C for 90 min followed by 3 min incubation at 95°C. Usually, PCR was performed in 50 μL of reaction mixture using cDNA (2.5 μL), 2 mM dNTP, 25 mM MgCl₂, Taq polymerase (0.25 μL), and 5 μM primers with a time course of 94°C for 3 min and 30 cycles of (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min), then at 72°C for 7 min. Actually, PCR for AGS gene was performed at 60°C for annealing.
The products were analyzed by electrophoresis and ethidium bromide (EtBr) fluorescence under UV. Primer sequences used for PCR were as follows, vitD-bp: sense primer, 5'-CCAACCCTGAA-AACCCTTAG-3', anti-sense primer, 5'-GAGGCTGCTGATTCGACG-3'; AT-1: sense primer, 5'-AACACTGTGCTAAATCTGGG-3', anti-sense primer, 5'-CCAATTGTTTGGCATTG-3'; Tis21: sense primer, 5'-GTCATGTGACC-3', antisense primer, 5'-GATCTGAATGCTGCTACCAC-3'; glyceraldehydes 3-phosphate dehydrogenase, (GAPDH): sense primer, 5'-GTCAGTGGTGACCT-3', anti-sense primer, 5'-GTCAGTGGTGACCT-3'.

Uptake and expression of exogenous gangliosides by cultured astrocytes

Astrocytes from mouse brain were prepared as described previously (Mizuno et al. 2005). Astrocytes passaged more than three times were plated in microtiter plates (500 cells/well), and incubated overnight. Then, GD3 was dried in a glass tube, and was resuspended in plain minimum essential medium with rigorous vortex. GD3 solution was added to the cells in the plates after washing twice with plain medium. Ganglioside expression was analyzed next day by an immunofluorescence assay. Serially diluted antibodies with PBS containing 3% fetal calf serum was added to the cells, and incubated for 1 h at 25°C. After washing twice, FITC-conjugated second antibodies (goat anti-mouse IgG or anti-mouse lambda chain) were added and incubated for 30 min. After washing, cells were examined under a fluorescence microscope.

Results

TLC of WT and KO mice brain gangliosides

Ganglioside fractions were extracted as previously reported (Furukawa et al. 1985). Then, gangliosides were also prepared with a brief method without alkaline treatment, where acidic fractions were directly isolated from chloroform/methanol extracts using DEAE SephadexTM A-50 ion-exchange column (Miyazaki et al. 1997). In the standard separation, only GM3 and GD3 could be found in the extracts from the mutant mice (Takamiya et al. 1996) (Fig. 1a). In contrast, the brief preparation resulted in the appearance of a new band between GM3 and GD3, showing similar band intensity to that of GM3 (Fig. 1b). This TLC pattern was essentially same in cerebrum and cerebellum (Fig. 1c). The new band was present persistently from 16 to 70 weeks after birth (Fig. 1d).

Neuraminidase treatment

In order to clarify the structures of the bands in TLC, ganglioside mixtures were digested with a neuraminidase, and the products were analyzed in TLC. Gangliosides from WT mice brain became a single band at the migration level of GM1 (data not shown), corresponding to the previous reports (Yamamoto et al. 1995). On the other hand, no GM1-like band could be found in the null mutants. In stead, a definite band was found at just above GM1 site, showing identical migration behavior to the original migration site of the novel band (Fig. 2). This result suggested that the unknown component was neuraminidase-resistant. An intense band above GM3 was newly found in the KO extracts after the neuraminidase treatment (Fig. 2). This brown band distinct from violet bands with resorcinol was found only in the KO

![Fig. 1](image1.png) (a) TLC of brain gangliosides from the WT and KO mice prepared via alkaline treatment. (b) TLC of brain gangliosides from the WT and KO mice prepared by non-alkaline condition. (c) Ganglioside fractions from cerebrum and cerebellum tissues of a KO mouse. Ten mg tissue-derived gangliosides were applied for all samples. (d) Ganglioside fractions of the KO brains at different ages were compared in TLC/resorcinol. The new band was indicated by X in b–d.

![Fig. 2](image2.png) Neuraminidase treatment of gangliosides from the KO mouse brain. Gangliosides were incubated in the presence or absence of neuraminidase and the products were analyzed in TLC after desalting. ST, bovine brain gangliosides. TLC was developed with a solvent system of chloroform: methanol: 0.2% CaCl2 (55 : 46 : 10). Bands were visualized by resorcinol spray. The new band was indicated by X.

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sample, suggesting the product was a neutral (non-sialylated) glycolipid, lactosylceramide derived from GM3 and GD3.

**TLC-immunostaining**

To identify the new band, TLC-immunostaining using mAb Jones was performed. An intensely stained band was found at the same migration site as the new band indicated by X in Fig. 3a and 3b. This result indicated that the unknown band was 9-O-acetyl-GD3.

**Comparison of TLC-immunostaining pattern between the wild-type and the KO mice**

To clarify whether the wild-type mice express some levels of 9-O-acetyl-GD3, further TLC-immunostaining was performed using acidic fractions from WT and KO mouse brains as shown in Fig. 4(a), TLC-immunostaining was performed with anti-GD3 mAb R24 and anti-9-O-acetyl GD3 mAb GMR2. mAb R24 stained GD3 in bovine brain gangliosides (moderately), WT mouse brain extracts (weakly), and in the KO mice brain (strongly) (Fig. 4(b)). As expected, mAb GMR2 stained a strong band of probably 9-O-acetyl-GD3 at the site of X. No corresponding band was detectable in the WT sample. When, double amounts of gangliosides were stained with longer incubation with the substrate, a very faint band appeared at the migration site of X, suggesting that very low amount of 9-O-acetyl-GD3 was present in the brain of the WT mice (Fig. 4(c)). Additional bands (indicated as a and b) were formed. Using acidic fractions from WT and KO mouse brains as shown in Fig. 4(a), TLC-immunostaining was performed with a chemiluminescence detection system.

![Fig. 3](image)

**Fig. 3** The new component was 9-O-acetyl GD3 as analyzed by TLC-immunostaining. (a and b) TLC-immunostaining of ganglioside fractions from a KO mouse with mAb Jones. (a) TLC pattern of gangliosides from a KO mouse as well as bovine brain to show the migration sites of bands. After TLC, primulin-stained bands were marked with a pencil, then transferred to PVDF by heat-blotting. (b) After blotting, bands were stained with mAb Jones (ascites, 1 : 100 dilution) and a chemiluminescence detection system.

![Fig. 4](image)

**Fig. 4** The new component was detectable even in the WT mouse brain extracts. To clarify whether 9-O-acetyl-GD3 was expressed in the WT mouse brain, TLC-immunostaining was rigorously performed. (a) Resorcinol pattern of the acidic fractions from WT and KO mice. BBG is a standard (bovine brain gangliosides). (b) TLC-immunostaining of the samples as shown in (a) with mAb R24 and mAb GMR2. mR24 was used at 1 : 100 dilution of ascites, and mAb GMR2 was at x10 dilution of the commercial product (1 µg/mL). (c) Twice amounts of gangliosides were applied, and incubated with the substrate at the final step for longer time, leading to the detection of a faint band at the site of X. TLC-immunostaining with mAb Jones also showed same results.
GM2/GD2 synthase knockout resulted in overt expression of 9-O-acetyl GD3

observed under this condition, whereas the identity of them was not known.

2D TLC
In order to further investigate the nature of the new band in the null mutants, we examined the 2D TLC pattern of gangliosides from WT and KO mice brains. After TLC of gangliosides using C: M: 0.2% CaCl2 (55 : 45 : 10), the TLC plates were exposed to NH3 vapor for 2 h. Then, TLC was developed by turning the direction 90° using the same neutral solvent. As expected, all ganglioside components were aligned on the diagonal line in WT gangliosides as well as bovine brain gangliosides (Fig. 5a), indicating all these spots consisted of alkaline-resistant structures. When gangliosides from KO mice were developed with a neutral solvent system in both first and second dimension, three spots were found on the diagonal (Fig. 5b). On the other hand, KO mice samples showed a non-diagonal pattern, i.e., spots were found on the diagonal (Fig. 5b). When gan-

expression levels of candidate genes for the inducing factor of O-acetylase
In order to clarify involvement of several genes that were previously reported to be associated with O-acetylation of GD3 in the neo-expression of 9-O-acetyl GD3 in the mutant mice, RT-PCR was performed. None of the genes examined, i.e., Tis21, vitD-bp, AT-1, and AGS, showed up-regulation in GM2/GD2 synthase gene KO mice compared to the WT mice (Fig. 7). In particular, the expression level of Tis21 that had been reported to be expressed under GD3 expression rather decreased in the KO brain (Fig. 7d). Since it has been suggested that Tis21 was induced by GD3 expression leading to the induction of machinery for 9-O-acetylation, the mRNA expression levels in GD3 synthase KO mice were also examined. However, no definite changes in the expression levels of Tis21 could be observed (Fig. 8).

Uptake and acetylation of GD3 in astrocytes
In order to clarify whether WT or other mutant mouse brain had enzyme activity to synthesize 9-O-acetyl-GD3, expression of 9-O-acetyl-GD3 as well as GD3 was analyzed after addition of GD3 to the culture medium of astrocytes. When nothing was added to the medium, strong expression of GD3 in the astrocytes from the KO mice were observed (Fig. 9(a)). WT astrocytes also expressed GD3 at a fairly high level. However, in the presence of GD3 in the medium, expression of GD3 in WT astrocytes was significantly repressed whereas in KO astrocytes it was not affected. In order to investigate the underlying mechanism of this differential GD3 expression between WT and KO astrocytes, we examined the expression levels of multiple genes that were previously reported to be associated with O-acetylation of GD3. Among them, Tis21 was the most significantly increased in KO astrocytes (Fig. 7d).

Fig. 5 The new component was 9-O-acetyl GD3 as analyzed by 2D TLC. 2D TLC of gangliosides extracted from a KO mouse brain showing an alkaline-labile component. (a) Gangliosides from bovine brain as a standard. Gangliosides from a WT mouse showed a similar pattern (data not shown). (b and c) Gangliosides from a KO mouse.

Alkaline treatment of the plate was done between the 1st and the 2nd development in (c), but not in (b). For all plates, the 1st and the 2nd developments were done with the identical solvent system of chloroform : methanol: 0.2% CaCl2 (55 : 45 : 10). Resorcinol spray was used for the spot detection.
level. As expected, astrocytes from the double KO mice (GM2/GD2 synthase and GD3 synthase) showed no GD3. As for 9-O-acetyl-GD3, the KO-derived astrocytes showed a low, but definite level of 9-O-acetyl-GD3 as detected by mAb GMR2. WT samples showed marginal expression. When exogenous GD3 (25 µM) was added, high levels of GD3 was observed in both the double KO- and the WT-derived astrocytes (Fig. 9(b)). Low levels of 9-O-acetyl-GD3 were also found in both types of mice. Addition of half amount of GD3 resulted in essentially same effects with slightly less intensity (Fig. 9(c)). In Fig. 10, pictures of cells observed under fluorescence microscopy were shown.

Discussion

9-O-acetyl GD3 has been considered to be associated with tumors. Thus, 9-O-acetyl GD3 was found in some of human melanomas (Cheresh et al. 1984; Ritter et al. 1990) and basal cell sarcomas (Fahr and Schauer 2001), suggesting this structure is involved in cancer evolution (Schauer and Kamerling 1997; Angata and Varki 2002), though no direct evidence has been shown. On the otherhand, 9-O-acetylation in influenza C protein transgenic mice resulted in the arrest of cell division at the early stage of development (Varki et al. 1991).

7(9)-O-acetyltransferase, the enzyme responsible for the acetylation of gangliosides was first detected in bovine submandibular glands (Schauer 1970). Recently, many trials have been performed to identify the gene mainly by expression cloning system, resulting in failure. These results suggest that a single gene product may not be able to catalyze the reaction of 9-O-acetylation onto sialic acids or sialic acid-nucleotides. Thus, the regulatory mechanisms for the synthesis of O-acetylated sialic acids is not well understood because of the unavailability of the cDNA of the enzyme coding 9-O-acetylase. The active form may exist as a molecular complex of several components.

In the mutant mice of GM2/GD2 synthase gene, 9-O-acetyl GD3 is expressed at an extremely high level. In the early reports, gangliosides were isolated via alkaline treatment, resulting in the elimination of O-acetyl compounds (Fig. 1a) (Takamiya et al. 1996). Here, we demonstrated the identity of the new band using multiple approaches. First of all, the component was sialidase-resistant, while two major bands were completely digested and converted to lactosylceramide. Second, the band was specifically detected by an
anti-9-O-acetyl GD3 mAb in TLC-immunostaining. Third, the component showed altered migration in TLC after alkaline treatment, resulting in the identical migration with that of GD3. Fourth, mass spectrometry of the component
revealed that it contained O-acetyl base in GD3. Taken together, the unknown band found in the brain tissues from the mutant mice turned out to be 9-O-acetyl GD3.

Mechanisms for the neo-synthesis of 9-O-acetyl GD3 in the mutant mice were analyzed by examining the changes in the expression levels of mRNAs for genes that had been reported to be associated with 9-O-acetyl GD3. Rat AGS (Ogura et al. 1996) containing high homology with mouse fat globule membrane protein was cloned by expression cloning using GD3 synthase-transfected CHO cells, and was considered as a putative O-acetyltransferase. This gene was reported to be up-regulated in stellate cells during liver fibrogenesis (Lee et al. 2003). AT-I was also isolated by expression cloning as a membrane protein required for the formation of O-acetylated gangliosides, and concluded to be a putative acetyl-CoA transporter gene (Kanamori et al. 1997). Shi et al. also isolated cDNA clones by expression cloning using CHE-FcD as a detection probe, and identified that one of them was vit-D bp (Shi et al. 1988). This could induce 9-O-acetylation on COS cells expressing α2,6-linked sialic acids. As these authors pointed out, it is unlikely that the products of all these genes act as an O-acetyltransferase.

![Fig. 10](image-url) Pictures of the immunofluorescence assay as shown in Fig. 9. Pictures were taken with identical conditions, i.e., fluorescence samples were at 3 s of the exposure, and DIC was at 1 s exposure. Results of mAb R24 (1:100 dilution) and mAb GMR2 (1:3 dilution) were shown. Since the KO (β4KO) already showed positive reaction for 9-O-acetyl-GD3 before the GD3 treatment, we did not try to add GD3 (they had extremely high amounts of GD3).
Recently, Satake et al. reported that GD3-specific 9-O-acetyltransferase was induced by Tis21, expression of which was enhanced by the transfection of GD3 synthase cDNA into CHO cells (Satake et al. 2002). They concluded that GD3 expression triggered Tis21 expression. This hypothesis was confirmed here in the mutant mice exhibiting accumulation of GD3 as well as GM3, and this seemed not the case in the mutant mice. In the RT-PCR, none of mRNAs examined showed significant up-regulation. Surprisingly, Tis21 showed rather a reduced level in the mutant mice, suggesting that high expression of 9-O-acetyl GD3 suppressed the Tis21 expression probably as a feed back inhibition.

Accumulation of the precursor GD3 might be a crucial factor in the enhanced synthesis of 9-O-acetyl GD3, and the synthetic activity itself (9-O-acetyltransferase) might not be changed, although no definite evidence could be shown. The results of TLC-immunostaining with WT mouse brain extracts suggested that even WT mice could synthesize 9-O-acetyl-GD3, though at an extremely low level. Martin et al. reported that specific sialyltransferases influence the in vivo regulation of O-acetylation, i.e., loss of O-acetylation in ST6GalI and ST3GalI null mice (Martin et al. 2002). This seems quite reasonable, because these enzymes are indispensable to synthesize the precursors or acceptors of the O-acetylation. By analogy, accumulation of GD3, the precursor of 9-O-acetyl GD3, could lead to the neo-synthesis of 9-O-acetyl GD3 based on its high level in the mutant mice of GM2/GD2 synthase.

Since over-expression of GD3 might have induced Tis21 gene expression, leading to the induction of 9-O-acetylation of GD3 (Satake et al. 2002), Tis21 expression in GD3 synthase KO mice was examined to clarify whether GD3 is essential for the induction of Tis21. Contrary to expectation, Tis21 mRNA levels were not altered in the GD3 synthase KO mice, indicating that Tis21 mRNA was not affected even in the absence of GD3. Exogenously added GD3 was reported to induce O-acetyltransferase in CHO cells (Chen et al. 2006). Whether neo-expression of 9-O-acetyl GD3 is really dependent on the neo-expression of an O-acetyltransferase because of GD3 or the accumulation of GD3 merely prompts the synthesis of 9-O-acetyl GD3 remains to be investigated. It seems more likely that O-acetylase was induced directly or indirectly by the high levels of GD3 regardless of Tis21, or the existing level of O-acetylase was sufficient to increase 9-O-acetyl GD3 levels in the presence of huge amount of GD3. At least we can conclude that Tis21 is probably not an inducing factor of such an O-acetyltransferase in GM2/GD2 synthase KO mice.

As Chen reported (Chen et al. 2006), it might be possible that accumulated GD3 induced synthetic machinery of 9-O-acetyl-GD3 in the KO mice. As shown in Fig. 9 and 10 together with the results in Fig. 4, low levels of 9-O-acetyl-GD3 were already found in the WT mouse brain. Therefore, it seems reasonable that the expression of 9-O-acetyl-GD3 in the KO mouse brain became overt because of the increased synthesis based on the increased precursor structure.

Although 9-O-acetyl GD3 or 9-O-acetylated sialic acid-containing mucins have been considered to be involved in the malignant features of tumor cells, their roles in cell proliferation and invasion have not been clearly demonstrated. A significant role for the O-acetylation of GD3 in the induction of apoptosis has also been reported (Malisan et al. 2002). However, it is not clear whether modulation of GD3 is important or 9-O-acetyl sialic acid itself has a unique function. The mutant mice lacking complex gangliosides showed much milder phenotypes than expected (Takamiya et al. 1996). Accumulation of GM3 and GD3 was considered to compensate the functions of the lost structures, and to attenuate the morphological and functional defects because of the lack of the complex gangliosides. Neo-synthesis of 9-O-acetyl GD3 might also play an important role in the attenuation of the abnormal phenotypes of GM2/GD2 synthase KO mice. In fact, 9-O-acetyl GD3 was appeared to participate in neuronophilic as well as gliophilic migration of subventricular zone explants (Miyakoshi et al. 2001), and granule cell migration in the developing cerebellum (Santiago et al. 2001), suggesting its roles in the protection of neuronal death or reconstruction of the degenerated nervous system in the KO mice (Sugiura et al. 2005). In order to investigate these mechanisms, it seems critical to define the enzyme and other essential molecules responsible for the O-acetylation of sialic acids.

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References

Angata T. and Varki A. (2002) Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. Chem. Rev. 102, 439–470.

Blum A. S. and Barnstable C. J. (1987) O-acetylation of a cell-surface carbohydrate creates discrete molecular patterns during neural development. Proc. Natl Acad. Sci. USA 84, 8716–8720.

Chen H. Y., Challa A. K. and Varki A. (2006) 9-O-acetylation of exogenously added ganglioside GD3. The GD3 molecule induces its own O-acetylation machinery. J. Biol. Chem. 281, 7825–7833.

Cheres D. A., Reisfeld R. A. and Varki A. P. (1984) O-acetylation of disialoganglioside GD3 by human melanoma cells creates a unique antigenic determinant. Science 225, 844–846.

Fahr C. and Schauer R. (2001) Detection of sialic acids and gangliosides with special reference to 9-O-acetylated species in basaliomas and normal human skin. J. Invest. Dermatol. 116, 254–260.

Furukawa K. and Lloyd K. O. (1990) Gangliosides in melanomas, in Human Melanoma, From Basic Research to Clinical
Application (Ferrone, S., ed.), pp. 15–30. Springer-Verlag, New York.

Furukawa K., Claussen H., Hakomori S., Sakamoto J., Look K., Lundblad A., Mattes M.J. and Lloyd K. O. (1985) Analysis of the specificity of five murine anti-blood group A monoclonal anti-bodies, including one that identifies type 3 and type 4 A deter-minants. Biochemistry 24, 7620–7826.

Hamamura K., Furukawa K., Hayashi T. et al. (2005) Ganglioside GD3 promotes cell growth and invasion through p130Cas and paxillin in malignant melanoma cells. Proc. Natl Acad. Sci. USA 102, 11041–11046.

Houghton A. N., Mintzer D., Cordon-Cardo C., Welt S., Fliegel B., Vadhán S., Carswell E., Malamed M. R., Oettgen H. F. and Old L. J. (1985) Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma. Proc. Natl Acad. Sci. USA 82, 1244–1246.

Kanamori A., Nakayama J., Fukuda M.N., Stallcup W.B., Sasaki K., Hamamura K., Furukawa K., Clausen H., Hakomori S., Sakamoto J., Look K. and Lundblad A. (1989) Microscale analysis of glycosphingolipids by TLC blotting/second ion mass spectrometry: a novel blood group A-active glycosphingolipid and changes in glycosphingolipid expression in rat mammary tumour cells with different metastatic potentials. Glycoconj. J. 13, 461–469.

Kohla G., Stockfleth E. and Schauer R. (2002) Gangliosides with O-acetylated sialic acids in tumors of neuroectodermal origin. Neurochem. Res. 27, 583–592.

Lee S. H., Seo G. S., Park P. H., Choi J. Y., Park Y. N., Kim H. K., Cha K. S. and Sohn D. H. (2003) Increased expression of O-acetyl disialo-ganglioside synthase during rat liver fibrogenesis relates to stellate cell activation. Biochem. Biophys. Res. Commun. 303, 954–961.

Malisan F., Franchi L., Tomassini B. et al. (2002) Acetylation suppresses the proapoptotic activity of GD3 ganglioside. J. Exp. Med. 196, 1535–1541.

Martin L. T., Marth J. D., Varki A. and Varki N. M. (2002) Genetically altered mice with different sialyltransferase deficiencies show tissue-specific alterations in sialylation and sialic acid 9-O-acetylation. J. Biol. Chem. 277, 32930–32938.

Miyakoshi L. M., Mendez-Otero R. and Hedin-Pereira C. (2001) The 9-O-acetyl GD3 gangliosides are expressed by migrating chains of subventricular zone neurons in vitro. Braz. J. Med. Biol. Res. 34, 669–673.

Miyazaki H., Fukumoto S., Okada M., Hasegawa T. and Furukawa K. (1997) Expression cloning of rat cDNA encoding UDP-galactose:GD3 beta1,3-galactosyltransferase that determines the expression of GD1b/GM1/GA1. J. Biol. Chem. 272, 24794–24799.

Mizuno T., Kuno R., Nitta A., Nabeshima T., Zhang G., Kawanokuchi J., Wang J., Jin S., Takeuchi H. and Suzumura A. (2005) Protective effects of 9,10-dihydroxy-7,8-epoxy-9,10-goldenone against neuronal cell death induced by activated microglia and astrocytes. Brain Res. 1066, 78–85.

Nakashii H., Sanai Y., Shibuya M. and Nagai Y. (1988) Analysis of cellular expression of gangliosides by gene transfection. II: Rat Y1 cells transformed with several DNAs containing oncogenes (fes, fps, ras & src) invariably express sialosylparagloboside. Biochem. Biophys. Res. Commun. 150, 766–774.

Ogura K., Nara K., Watanabe Y., Kohno K., Tai T. and Sanai Y. (1996) Cloning and expression of cDNA for O-acetylation of GD3 ganglioside. Biochem. Biophys. Res. Commun. 225, 932–938.

Okada M., Itoh M., Haraguchi M. et al. (2002) b-series Ganglioside deficiency exhibits no definite changes in the neurogenesis and the sensitivity to Fas-mediated apoptosis but impairs regeneration of the lesioned hypoglossal nerve. J. Biol. Chem. 277, 1633–1636.

Ritter G., Boosfeld E., Markstein E., Yu R. K., Ren S., Stallcup W. B., Oettgen H. F., Old L. J. and Livingston P. O. (1990) Biochemical and serological characteristics of natural 9-O-acetyl GD3 from human melanoma and bovine buttermilk and chemically O-acetylated GD3. Cancer Res. 50, 1403–1410.

Santiago M. F., Berredo-Fiuho M., Costa M. R., Gandra M. G., Cavalcante L. A. and Mendez-Otero R. (2001) Expression and function of ganglioside 9-O-acetyl GD3 in postmitotic granule cell development. Mol. Cell. Neurosci. 17, 488–499.

Satake H., Chen H. Y. and Varki A. (2002) Genes modulated by expression of GD3 synthase in Chinese hamster ovary cells. Evidence that the Tis21 gene is involved in the induction of GD3 9-O-acetylation. J. Biol. Chem. 278, 7942–7948.

Schauer R. (1970) Biosynthesis of N-acetyl-O-acetylgangliosamines. I. Incorporation of (14C) acetate into sections of the submaxillary salivary gland of ox and horse. Hoppe Seylers Z. Physiol. Chem. 351, 595–602.

Schauer R. and Kamerling J. P. (1997) Chemistry, biochemistry and biology of sialic acids, in Glycoproteins II, Vol. 29b (Montreuil J., Vliegenthart J. F. G. and Schachter H., eds), pp. 243–402, Elsevier, Amsterdam.

Shi W.-X., Channas R. and Varki A. (1988) Induction of sialic acid 9-O-acetylation by diverse gene products: implications for the expression cloning of sialic acid O-acetyltransferases. Glycobiology 8, 199–205.

Sugiyama Y., Furukawa K., Tajima O., Mii T., Honda T. and Furukawa K. (2005) Sensory nerve-dominant nerve degeneration and remodeling in the mutant mice lacking complex gangliosides. Neuroscience 135, 1167–1178.

Takamiya K., Yamamoto A., Furukawa K. et al. (1996) Mice with disrupted GM2/GD2 synthase gene lack complex gangliosides but exhibit only subtle defects in their nervous system. Proc. Natl Acad. Sci. USA 93, 10662–10667.

Taki T., Ishikawa D., Handa S. and Kasama T. (1995) Direct mass spectrometric analysis of glycosphingolipid transferred to a polyvinylidene difluoride membrane by thin-layer chromatography blotting. Anal. Biochem. 225, 24–27.

Varki A., Hooshmand F., Diaz S., Varki N. M. and Hedrick S. M. (1991) Developmental abnormalities in transgenic mice expressing a sialic acid-specific 9-O-acetylesterase. Cell 65, 65–74.

Yamamoto A., Yamashiro S., Takamura K., Atsuta M., Shiku H. and Furukawa K. (1995) Heterogeneity in the expression pattern of two ganglioside synthase genes during mouse brain development. J. Neurochem. 65, 2417–2424.

Yamashiro S., Okada M., Haraguchi M., Furukawa K., Lloyd K. O., Shiku H. and Furukawa K. (1995) Expression of alpha 2,8-sialyltransferase (GD3 synthase) gene in human cancer cell lines: high level expression in melanomas and up-regulation in activated T lymphocytes. Glycoconj. J. 12, 894–900.

Yu R. K., Macala L. J., Taki T., Weinfield H. M. and Yu F. S. (1988) Developmental changes in ganglioside composition and synthesis in embryonic rat brain. J. Neurochem. 50, 1825–1829.