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INCORPORATION, REMODELING AND RE-EXPRESSION OF EXOGENOUS GANGLIOSIDES IN HUMAN CANCER CELL LINES *IN VITRO* AND *IN VIVO*

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ABSTRACT

Human neuroblastomas and gliomas express high levels of GD2 ganglioside. Mechanisms for the re-expression of GD2 after the incorporation of an exogenous precursor structure were analyzed using a human heterophilic monoclonal antibody (mAb) together with mouse anti-GD3 and mouse anti-GD2 mAbs. First, mouse anti-GD2 mAb 220-51 was generated and its reactivity was confirmed to be almost identical with that of the well-known mAb 3F8 antibody. As reported previously for GD3 variants, new ganglioside antigens reactive with human mAb 32-27 were analyzed by culturing an astrocytoma cell line AS in the presence of NeuGc-GM3. Analysis of the extracted gangliosides from AS thus cultured revealed a new component detected with mAb 32-27, migrating similarly to GD2. Incorporated NeuGc-GM3 seemed to be converted to NeuAc-NeuGc-type GD3, and then to NeuAc-NeuGc-type GD2 with α2,8-sialyltransferase and β1,4-GalNAc transferase, respectively. In addition, AS was inoculated into nude mice, and glycolipids were extracted from generated tumors. Analysis of the ganglioside components using mAbs indicated that NeuAc-NeuGc-type GD2 was generated in the xenogeneic tumors by incorporating NeuGc-GM3 from mouse blood. These results indicated the presence of a pathway for utilization of exogenous gangliosides for remodeling and re-expression *in vivo*.

Key Words: *Ganglioside, NeuGc, Sialic acid, Recycle, Glycosyltransferase, Astrocytoma*

INTRODUCTION

Gangliosides are sialic acid-containing glycosphingolipids widely expressed in mammals and birds.[1)] In particular, they are enriched in nervous systems and in neurocrest-derived tumors such as malignant melanomas and neuroblastomas.[2)] They have been considered to play important roles in the development, evolution, and expansion of malignant tumors.[3)] In fact, a number of studies indicated that gangliosides are involved in cell proliferation, cell attachment, cell differentiation, and sometimes in cell apoptosis.[4)]

Molecular cloning of the glycosyltransferases responsible for the synthesis of gangliosides has been successful over the last 10 years, and the mechanisms for the synthesis of gangliosides have been well understood.[5)] On the other hand, utilization of exogenous gangliosides or recycling of endogenous gangliosides has not been well understood. This is mainly because it has been very difficult to distinguish the newly synthesized gangliosides from pre-existing ones. It has also been difficult to follow the fates of newly incorporated gangliosides in cultured cells. Therefore, even the life span of some gangliosides on the cell surface could not be determined.

Heterophilic antibodies have been considered to be produced when individuals were

immunized with xenogeneic cells and molecules either by accident or for therapeutic purposes.[6] N-glycolyl neuraminic acid (NeuGc)-containing sialyl compounds have been thought to be candidates for tumor-associated carbohydrate antigens, and many efforts have been made to demonstrate the expression of NeuGc-containing gangliosides in human tumor tissues using antibodies reactive with heterophile antigens (heterophilic antibodies).[7] Despite such persistent efforts, it has been difficult to clearly show the presence of heterophilic antigens in human tumor tissues. These difficulties might have been expected when we realize that the CMP-NeuAc hydroxylase gene (essential for the conversion of NeuAc to NeuGc) is inactivated in humans.[8]

As reported previously, the monoclonal antibodies (mAbs) raised against NeuGc-containing variants of gangliosides have been very useful for the analysis of ganglioside processing after the incorporation of exogenous NeuGc-containing structures.[9-12] In this study, we analyzed the incorporation, processing and re-expression of gangliosides, using a heterophilic human mAb together with anti-gangliosides mouse mAbs.

MATERIALS AND METHODS

Cell culture

Suspension cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO_2. Monolayer cell lines were maintained in Dulbecco's modified Eagle MEM supplemented with 7.5% FCS at 37°C in a humidified atmosphere containing 5% CO_2.

Monoclonal antibodies

MAbs used in this study have been reported previously: anti-GD2 monoclonal antibody (mAb) 3F8[13] was provided by N.-K. Cheung of the Memorial Sloan-Kettering Cancer Center (New York), another anti-GD2 mAb 220-51 was generated in our laboratory by immunizing mice with a neuroblastoma cell line IMR-32; and anti-GD3 mAb R24[14] (mouse IgG3) and anti-GD3 mAb 32-27[9] reactive with GD3 with NeuGc-containing sialic acids were provided by L.J. Old, also of the Memorial Sloan-Kettering Cancer Center. The reaction specificity was summarized in Table 1.

Flow cytometry

Surface expression of gangliosides was analyzed with FACSan (Becton-Dickinson, Mountain View, CA),[15] using mAbs and FITC-labeled anti-mouse IgG (H & L) (Cappel) or anti-human

Table 1 <Reaction specificity of monoclonal antibodies used in this study>

Gangliosides	Monoclonal antibodies			
	mAb 3F8	mAb 220-51	mAb 32-27	mAbR24
NeuAc-type GM3	–	–	–	–
NeuGc-type GM3	–	–	–	–
NeuAc-NeuAc-type GD3	–	–	–	+++
NeuAc-NeuGc-type GD3	–	–	++	++
NeuGc-NeuAc-type GD3	ND	ND	ND	–
NeuGc-NeuGc-type GD3	–	ND	+++	–
NeuAc-NeuAc-type GD2	+++	+++	–	–
NeuAc-NeuGc-type GD2	?	?	?	–

Intensities in ELISA or TLC-immunostaining assay were graded: and presented as follows.
+++, strongly positive; ++, moderately positive; –, negative; ND, not determined.

IgM (Cappel). Control samples were prepared with the second antibody alone.

Extraction of gangliosides

Gangliosides were prepared from glycolipids sequentially extracted from the cultured cell lines or xenogeneic tumor tissues with chloroform: methanol (2:1, 1:1, 1:2) as described previously.[16] Glycolipids were separated with a Florisil column after acetylation, then applied to an ion-exchange column of DEAE-Sephadex (Pharmacia), resulting in the separation into a neutral and an acidic fraction.

Thin layer chromatography (TLC) and TLC-immunostaining

Extracted gangliosides were separated on TLC plates (Merck) with a solvent consisting of chloroform: methanol: 2.5N NH_4OH (60:35:8). For TLC-immunostaining, gangliosides on the TLC plates were blotted onto a PVDF membrane (Millipore) using a Heat-blotter™ (Atto, Tokyo, Japan) as previousely described by Taki *et al.*[17] After being soaked in 5% skim milk in phosphate buffered saline (PBS) over-night, the membrane was stained with individual antibodies at room temperature for one hour followed by a second antibody (biotin-conjugated anti-mouse IgG (or human IgM)). The antibody binding was detected using the ABC kit (Vector, Burlingame, CA) following the manufacturer's instructions.

Addition of ganglioisides into the culture medium

To analyze the incorporation of exogenous gangliosides, cells were cultured with NeuGc-type GM3 extracted from horse erythrocytes as described previously[12] at a range of 0.4~50.0 nmol/ml for two days. The cells were cultured in ITS medium (insulin, transferin, selenium; Becton,Dickinson) in the absence of FCS. After being harvested, cell pellets were served for the glycolipid extraction. The expression of gangliosides was detected with an immune-adherence (IA) assay (for IgM) or an immuno-fluorescence (IF) assay (for IgG)[9].

Transplantation of human tumor cell lines to nude mice

To form xenogeneic tumors, human cancer cell lines (1×10^7) were injected subcutaneously into nude mice. After about 4–6 weeks, tumors 0.5~2.0 cm in diameter were resected and used for ganglioside extraction or tissue culture after mincing.

Generation of an anti-GD2 mAb

To generate anti-GD2 mAb, C3H mice were immunized by subcutaneously injecting 3×10^6 of IMR-32 cells three times every two weeks, three times, and the spleen cells were then fused with a mouse myeloma cell line NS-1 as previously described.[18] Supernatants from the wells with clones were screened by their reactivity with IMR-32, and then the positive supernatants were then screened against GD2 with enzyme-linked immunosorbent assay (ELISA) as described previously.[18]

RESULTS

Generation of anti-GD2 mAb

Among the clones which showed a positive reaction with immunized IMR-32, two also showed a positive reaction with GD2 in ELISA. Their reactivities with various cell lines were then analyzed by flow cytometry. As depicted in Fig. 1, mAb 220-51 showed a very similar reaction pattern with that of mAb 3F8, and mAb 202 also showed a similar reactivity (data not shown). They both strongly reacted with many neuroblastomas and gliomas as well as with some melanomas. Some human lymphotropic virus type I-positive lymphoid cell lines were also positive as described previously.[19]

Reactivity of anti-GD2 mAbs

The reaction specificities of mAb 220-51 and mAb 202 against purified glycolipids were

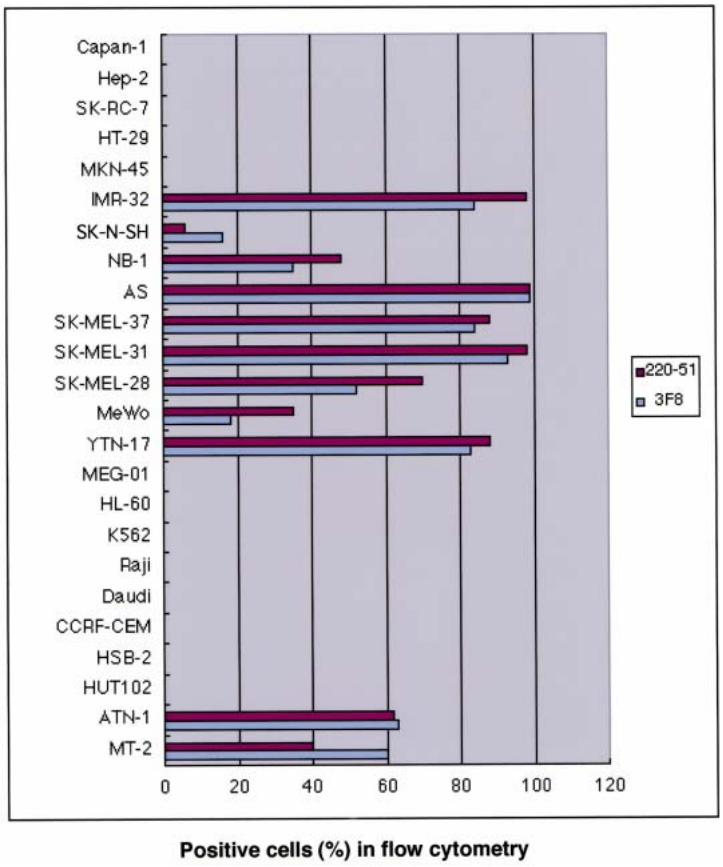

Fig. 1 <Reactivity of mAb 220-51 with human cultured cell lines.> Results of flow cytometric analysis were shown as % positive cells for 24 human cancer cell lines. Derivation of the cell lines was as described in Ref. 9 and Ref 19.

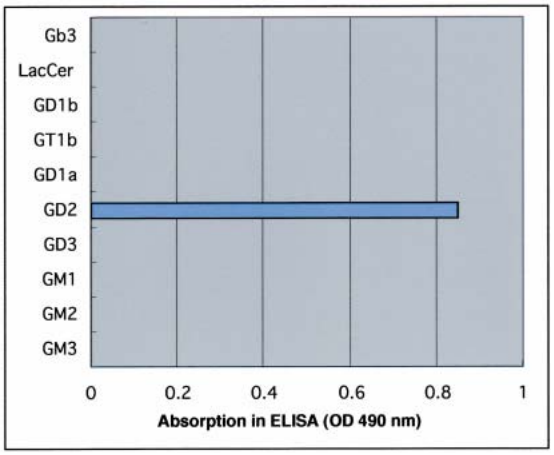

Fig. 2 <MAb220-51 is GD2 specific mAb.> Results of ELISA are summarized. ELISA was performed with the supernatant of the hybridoma using ELISA plates coated with purified glycolipids. Amounts of glycolipids so coated were 20–100 ng/well.

analyzed by ELISA, and revealing that these two mAbs were GD2-specific among gangliosides and neutral glycolipids (Fig. 2, data for mAb 202 not shown). The immunoglobulin class determined using the ELISA kit showed both of them to be class IgG3.

Expression of novel gangliosides in the cells cultured with NeuGc-GM3

As described previously, mAb 32-27 does not react with human cell lines cultured in the absence of FCS, but does react when cells are cultured with an FCS-containing medium.[9] We have also reported that NeuGc-containing sialic compounds should be incorporated into the cells and be converted to novel structures that can be detected by mAb 32-27. When human melanomas were used, NeuGc-type GM3 was taken up into cells and converted to NeuAc-NeuGc-type GD3.[12] An astrocytoma cell line AS was then cultured in the presence of NeuGc-type GM3, and the resulting neo-gangliosides were analyzed by mAb 32-27. As shown in Fig. 3, mAb 32-27 definitely reacted with AS cells when cultured in the presence of NeuGc-type GM3 at higher than 1.0 nmol/ml. The time course of the reactivity of AS with mAb 32-27 was analyzed by culturing AS in the presence of NeuGc-type GM3 (25 nmol/ml), and then removing NeuGc-type GM3 from the culture medium (Fig. 4). A positive reaction appeared 4 hours after the addition of NeuGc-type GM3, and was sustained for more than two weeks after the withdrawal of NeuGc-type GM3.

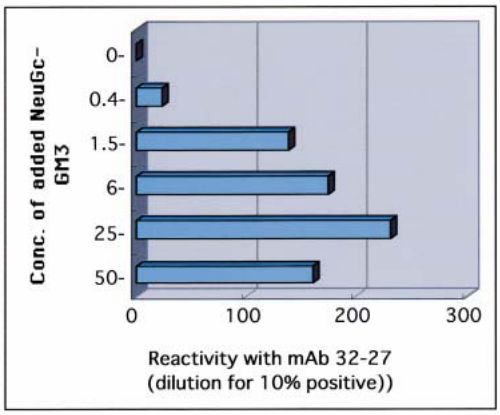

Fig. 3 <Reactivity of AS cells cultured in the presence of NeuGc-type GM3 with mAb 32-27.> AS cells were cultured at the indicated concentration (nmol/ml) of NeuGc-type GM3 for 48 hours, then served for IA assay to detect the binding of mAb 32-27.

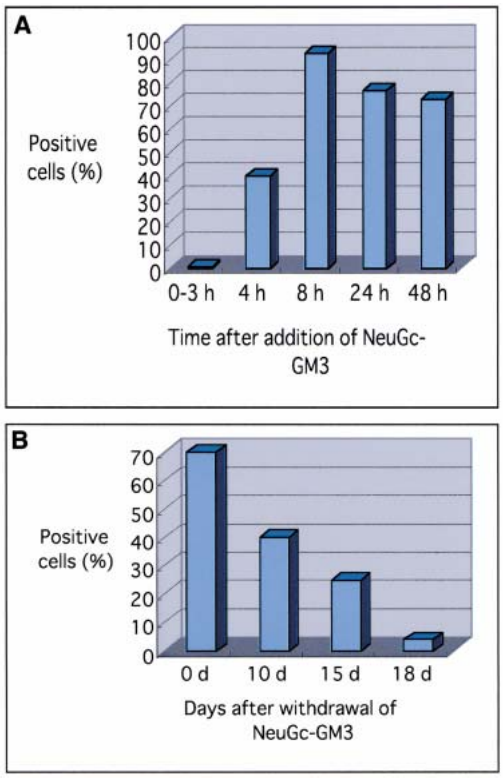

Fig. 4 <Time course of mAb 32-27 reactivity with AS cells cultured in the presence of NeuGc-type GM3.> AS was cultured in FCS free ITS medium, then NeuGc-type GM3 (25 nmol/ml) was added to the culture medium. Cell reactivity with mAb 32-27 was accessed with IA assay after 48 hours.

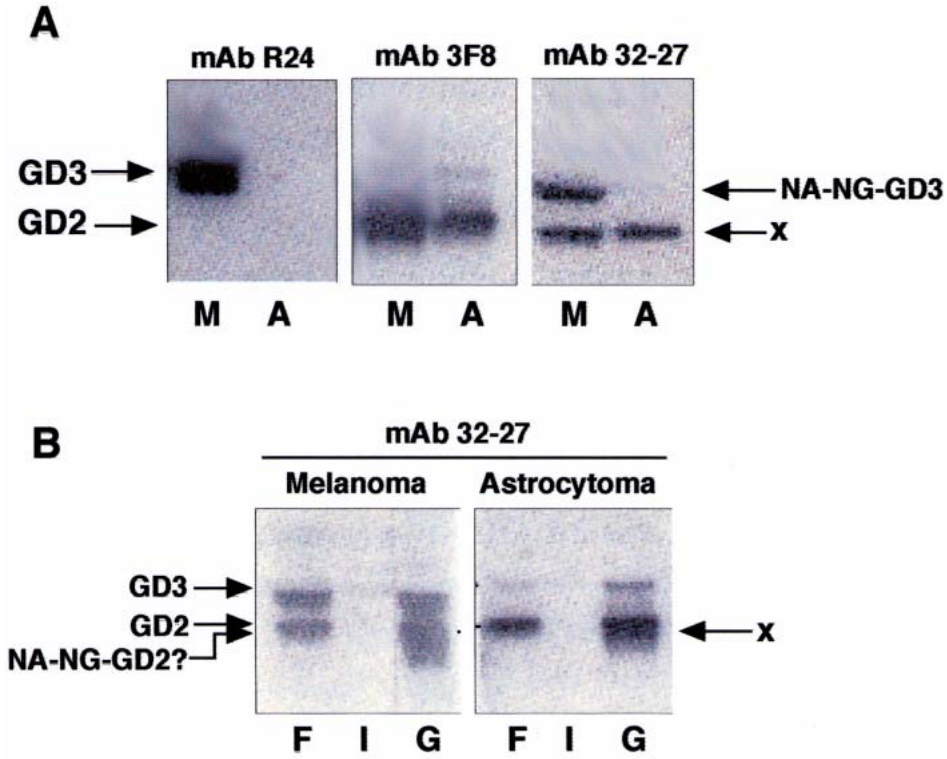

Fig. 5 <TLC-immunostaining of gangliosides extracted from cell lines.> A, TLC-immunostaining of ganglioside fractions from a melanoma line SK-MEL-173 (M) and an astrocytoma line AS (A) using mAbs as indicated. X indicates a novel component analyzed in this study. B, TLC-immunostaining of ganglioside fractions from melanoma (SK-MEL-173) and astrocytoma (AS). Lane F shows gangliosides from cells cultured in regular FCS+ medium. Lane I shows gangliosides from cells cultured in ITS medium alone. Lane G shows gangliosides from cells cultured in FCS-free medium containing NeuGc-type GM3. MAb 32-27 was used for the detection of NeuGc-containining sialyl compounds. NA, NeuAc; NG NeuGc.

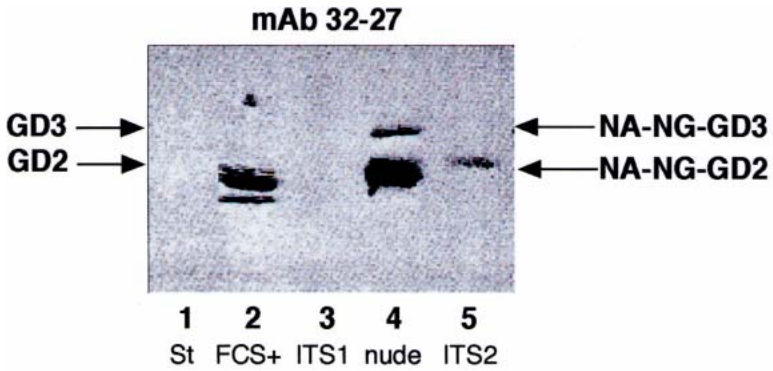

Fig. 6 <TLC-immunostaining of gangliosides from nude mice tumors.> Gangliosides from AS cells of the regular condition with FCS (lane 2), from cells cultured in ITS medium (ITS1) (lane 3), and tumor tissue formed in a nude mouse (lanes 4). Nude mice tumors were resected and transferred to flasks with mincing, and maintained in ITS medium again for more than two weeks (ITS2) (Lane 5). Immunostaining was performed with mAb 32-27. NA, NeuAc; NG NeuGc. Lane 1 shows a ganglioside mixture of bovine brain gangliosides as a standard.

Identification of newly-expressed gangliosides

To clarify the molecular features of the newly-expressed gangliosides in AS cells cultured in the presence of NeuGc-type GM3, TLC-immunostaining was performed using ganglioside fractions prepared from cells cultured in the regular medium with FCS, in ITS medium without FCS, and in ITS medium containing NeuGc-type GM3. As shown in Fig. 5, mAb 3F8 showed a strong band at the migration site of GD2 as expected. MAb 220-51 also showed a similarly strong band at that same site. On the other hand, mAb 32-27 showed no band for the extracts from AS cells cultured without either FCS or NeuGc-type GM3. When AS was cultured with NeuGc-type GM3, a strong band was detected at the migration site of GD2 or just below the GD2 site, suggesting that the band had a GD2-associated structure. A human melanoma cell line, SK-MEL-173, showed two strong bands detected with mAb 32-27 when cultured in the presence of NeuGc-type GM3. The lower band appeared to be identical with the main band in AS. Some of these data depicted in Figs. 5 and 6 were previously reported (see Ref. 12).

Synthesis of NeuGc-containing gangliosides in human tumors in nude mice

We also examined ganglioside changes in the human tumors inoculated in nude mice. AS was injected subcutaneously in the nude mice, and gangliosides from the resulting tumors were extracted and served for TLC-immunostaining. In those fractions, very strong bands reactive with mAb 32-27 were detected at the migration site of GD2 or just below the GD2 site. On the other hand, in the ganglioside fractions extracted from AS cultured in ITS medium after the resection of tumor tissues of nude mice, only a faint band was detected, suggesting that the reactive structures disappeared due to the exhaustion of the supply of NeuGc-sialyl precursors.

DISCUSSION

As reported previously, AS mainly expresses GD2 as gangliosides, and IMR-32 expresses mainly GD2, GM2 and a very low level of GD3. As for mAb 32-27, it can bind very strongly to NeuGc-NeuGc-type GD3 and disialylparagloboside.[10,11] It can also bind to NeuAc-NeuGc-type GD3 and disialylparagloboside.[10,11] Since the AS inoculated in nude mice showed a strong band detected by mAb 32-27 at the migration site of GD2, its positive component seemed to have been generated from the same precursor NeuGc-type GM3 incorporated exogenously as NeuAc-NeuGc-type GD3 was generated[12]. This NeuAc-NeuGc-type GD3 should be further processed to NeuAc-NeuGc-type GD2 in Golgi. An expected synthetic pathway of novel gangliosides in cultured cells and tumors in nude mice was shown schematically (Fig. 7). Here, a dynamic metabolic flow of the incorporation, remodeling, and re-expression of gangliosides was revealed.

The CMP-NeuAc hydroxylase gene is considered to be the gene mainly responsible for the synthesis of NeuGc-containing sialyl compounds. Molecular cloning of human CMP-NeuAc hydroxylase cDNA revealed that an essential portion of the gene has been deleted, resulting in the loss of enzyme activity.[8] Therefore, it seems quite unlikely that NeuGc-type ganglioside antigens are newly synthesized in human cancer tissues. On the other hand, a number of studies demonstrating NeuGc-containing gangliosides in human tumors have been reported.[7] Possible explanations for this discrepancy are either the presence of another source of NeuGc-, or the presence of another NeuGc synthesizing mechanism specific for glycoproteins.

The incorporation of exogenous gangliosides has been studied by a number of investigators mainly using cultured cells *in vitro*.[20] To trace the metabolic changes in the incorporated glycolipids, radiolabeled glycolipids have frequently been used.[20] This approach is quite effective for observing the fates of incorporated glycolipids without essentially modifying their native

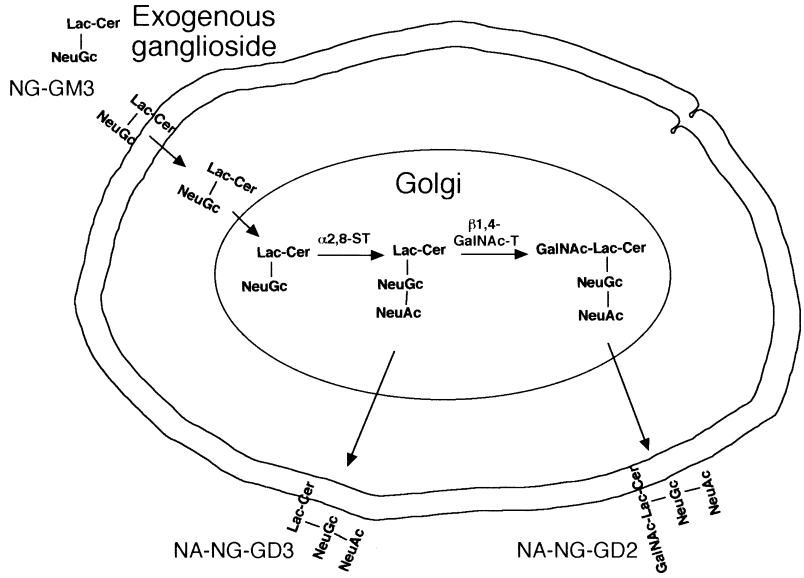

Fig. 7 <A schema of the recycling pathway defined in this study.> Based on the results obtained from studies of NeuAc-NeuGc-type GD2, we proposed a new pathway for the incorporation of NeuGc-type GM3, its re-modeling and re-expression as novel ganglioside.

chemical structures. However, it has been more difficult to assess the re-expression and duration of neo-glycolipids on the cell surface after incorporation and processing in Golgi. Based on the strict specificity of mAb 32-27, we were able to demonstrate here that NeuGc-type GM3 could be incorporated into cultured cells, undergo further sialylation with α2,8-sialyltransferase, and subsequently undergo *N*-acetylgalactosaminylation at the non-reducing end with β1,4-N-acetylgalactosaminyltransferase.

Furthermore, not only in cultured cell lines but also in mouse bodies, very similar transport, processing and re-expression could be found using mAb 32-27. Varki *et al.* recently reported that NeuGc-containing sialyl compounds are taken up from foods and can be converted to neo-antigens detectable by the anti-HD (Hanganutziu-Deicher) heterophile antibody in human bodies and urine.[21] Although their experimental systems involved some ambiguity due to the use of fasting volunteers, the essential mechanisms for the synthesis of HD antigens in human bodies appeared to be quite similar to ours.

Metabolic pathways demonstrated here the exogenous gangliosides might not be confined to exogenous glycolipids. It is highly probable, as shown here, that many glycolipids synthesized *de novo* could be recycled. However, the trafficking pathways and molecules involved in the uptake and recycling of glycolipids, i.e., the transportation machanism of glycolipids from the plasma membrane into Golgi, and of the re-expression of remodeled neo-glycolipids are still poorly understood. Recently, an earthworm-like courier of ceramide was isolated by Hanada *et al*.[22] Therefore, it seems quite likely that transportation molecules involved in the trafficking of glycolipids between individual compartments might be present, and that they may be polymorphic based on the heterogenous structures of corresponding glycolipids. These molecules and mechanisms remain to be further investigated.

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REFERENCES

1) Wiegandt, H.: Ganglioside. In *Glycolipids*, edited by Wiegandt, H., pp. 199–260 (1985), Elsevier, New York.
2) Hakomori, S.: Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res.*, 56, 5309–5318 (1996).
3) Furukawa, K. and Lloyd, K.O.: Gangliosides in Melanoma. In *Human Melanoma*, edited by Ferrone, S., pp. 15–30 (1990), Springer-Verlag, Heidelberg.
4) Hakomori, S.: Glycosylation defining cancer malignancy: new wine in an old bottle. *Proc. Natl. Acad. Sci. USA.*, 99, 10231-10233 (2002).
5) Lloyd, KO. and Furukawa, K.: Biosynthesis and functions of gangliosides: recent advances. *Glycoconj. J.*, 15, 627–636 (1998).
6) Hirabayashi, Y., Kasakura, H., Matsumoto, M., Higashi, H., Kato, S., Kasai, N. and Naiki, M.: Specific expression of unusual GM2 ganglioside with Hanganutziu-Deicher antigen activity on human colon cancers. *Jpn. J. Cancer Res.*, 78, 251–260 (1987).
7) Malykh, YN., Schauer, R. and Shaw, L.: N-Glycolylneuraminic acid in human tumours. *Biochimie*., 83, 623–634 (2001).
8) Kawano, T., Koyama, S., Takematsu, H., Kozutsumi, Y., Kawasaki, H., Kawashima, S., Kawasaki, T. and Suzuki, A.: Molecular cloning of cytidine monophospho-N-acetylneuraminic acid hydroxylase. Regulation of species- and tissue-specific expression of N-glycolylneuraminic acid. *J. Biol. Chem.*, 270, 16458–16463 (1995).
9) Yamaguchi, H., Furukawa, K., Fortunato, SR., Livingston, PO., Lloyd, KO., Oettgen, HF. and Old, LJ.: Cell-surface antigens of melanoma recognized by human monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.*, 84, 2416–2420 (1987).
10) Furukawa, K., Chait, BT. and Lloyd, KO.: Identification of N-glycolylneuraminic acid-containing gangliosides of cat and sheep erythrocytes. ^{252}Cf fission fragment ionization mass spectrometry in the analysis of glycosphingolipids. *J. Biol. Chem*., 263, 14939–14947 (1988).
11) Furukawa, K., Yamaguchi, H., Oettgen, HF., Old, LJ. and Lloyd, KO.: Analysis of the expression of N-glycolylneuraminic acid-containing gangliosides in cells and tissues using two human monoclonal antibodies. *J. Biol. Chem*., 263, 18507–18512 (1988).
12) Furukawa, K., Thampoe, IJ., Yamaguchi, H. and Lloyd, KO.: The addition of exogenous gangliosides to cultured human cells results in the cell type-specific expression of novel surface antigens by a biosynthetic process. *J. Immunol*., 142, 848–854 (1989).
13) Cheung, NK., Saarinen, UM., Neely, JE., Landmeier, B., Donovan, D. and Coccia, PF.: Monoclonal antibodies to a glycolipid antigen on human neuroblastoma cells. *Cancer Res*., 45, 2642–2649 (1985).
14) Pukel, CS., Lloyd, KO., Travassos, LR., Dippold, WG., Oettgen, HF. and Old, LJ.: GD3, a prominent ganglioside of human melanoma. Detection and characterization by mouse monoclonal antibody. *J. Exp. Med*., 155, 1133–1147 (1982).
15) Yamashiro, S., Haraguchi, M., Furukawa, K., Takamiya, K., Yamamoto, A., Nagata, Y., Lloyd, KO., Shiku, H. and Furukawa, K.: Substrate specificity of beta 1,4-N-acetylgalactosaminyltransferase in vitro and in cDNA-transfected cells. GM2/GD2 synthase efficiently generates asialo-GM2 in certain cells. *J. Biol. Chem*., 270, 6149–6155 (1995).
16) Furukawa, K., Clausen, H., Hakomori, S., Sakamoto, J., Look, K., Lundblad, A., Mattes, MJ. and Lloyd, KO.: Analysis of the specificity of five murine anti-blood group A monoclonal antibodies, including one that identifies type 3 and type 4 A determinants. *Biochemistry*, 24, 7820–7826 (1985).
17) Ishikawa, D. and Taki, T.: Thin-layer chromatography immunostaining. *Methods Enzymol.*, 312, 157-159 (2000).
18) Yoshida, K., Hamatani, K., Koide, H., Ikeda, H., Nakamura, N., Akiyama, M., Tsuchiyama, H., Nakayama,

E. and Shiku, H.: Preparation of anti-ras Mr 21,000 protein monoclonal antibodies and immunohistochemical analyses on expression of ras genes in human stomach and thyroid cancers. *Cancer Res.*, 48, 5503–5509 (1988).

19) Furukawa, K., Akagi, T., Nagata, Y., Yamada, Y., Shimotohno, K., Cheung, NK., Shiku, H. and Furukawa, K.: GD2 ganglioside on human T-lymphotropic virus type I-infected T cells: possible activation of beta-1,4-N-acetylgalactosaminyltransferase gene by p40tax. *Proc. Natl. Acad. Sci. USA*., 90, 1972–1976 (1993).
20) Schwarzmann, G.: Uptake and metabolism of exogenous glycosphingolipids by cultured cells. *Semin. Cell Dev. Biol.*, 12, 163–171 (2001).
21) Tangvoranuntakul, P., Gagneux, P., Diaz, S., Bardor, M., Varki, N., Varki, A. and Muchmore, E.: Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc. Natl. Acad. Sci. USA.*, 100, 12045–12050 (2003).
22) Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M. and Nishijima, M.: Molecular machinery for non-vesicular trafficking of ceramide. *Nature.*, 426, 803–809 (2003).