Ganglioside $G_{D2}$ in Small Cell Lung Cancer Cell Lines: Enhancement of Cell Proliferation and Mediation of Apoptosis

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ABSTRACT

Expression levels of gangliosides and glycosyltransferase genes responsible for their synthesis in human lung cancer cell lines and a normal bronchial epithelial cell line were analyzed. Both non-small cell lung cancers and small cell lung cancers (SCLCs) mainly expressed $G_{M2}$ and $G_{M1}$, whereas only SCLCs expressed b-series gangliosides, such as $G_{D2}$, $G_{D1b}$, and $G_{T1d}$. Accordingly, many SCLC cell lines showed up-regulation of the $G_{D3}$ synthase gene. Consequently, we introduced $G_{D3}$ synthase cDNA into a SCLC line with low expression of b-series gangliosides and analyzed the effects of newly expressed gangliosides on tumor phenotypes. The transfectant cells expressing high levels of $G_{D2}$ and $G_{D3}$ exhibited markedly increased growth rates and strongly enhanced invasion activities. Addition of anti-$G_{D2}$ monoclonal antibodies into the culture medium induced apoptosis of SCLC cells strongly suggested the usefulness of $G_{D2}$ as a target for the therapy of disastrous cancer, although the precise mechanisms for apoptosis remain to be clarified.

INTRODUCTION

Acidic glycophospholipids and gangliosides are widely expressed in many tissues and organs in vertebrates (1) and have been suggested to be involved in the regulation of development and differentiation as recognition molecules or signal modulators (2). However, there are some ganglioside species with relatively simple structures that show very restricted expression in normal tissues and markedly enhanced expression in particular malignant tumors, i.e., tumor-associated antigens. In the analysis of ganglioside expression in various human tumors, characteristic patterns have been demonstrated depending on individual tumor types, such as melanomas (3, 4), sarcomas (5), astrocytomas (6), and epithelial cancers (7). Among tumor-associated glycolipids, distosyl ganglioside $G_{T1}$ has been considered a human melanoma-specific antigen (8) and has been used as a target molecule in antibody immunotherapy (9). Distosyl ganglioside $G_{D2}$ has also been considered a neuroblastoma-associated antigen (10). It has also been used as a target of antibody therapy (11–13). We reported previously that $G_{D2}$ was specifically expressed in human T-cell lymphotrophic virus type I-infected cells under the regulation of the $p40^{\text{DP}}$ transactivator (14). However, the implications of the expression of these tumor-associated glycolipids have never been clarified to date, except for a few studies describing indirect evidence about the role of $G_{D3}$ in the growth of melanoma cells (15, 16). Many epithelial cancers have a high incidence and are very much resistant to current therapies. In particular, lung cancer is one of the most frequent and most malignant diseases because we have no efficient way to suppress tumor growth and kill tumor cells in patients. In trials to search for tumor-specific antigens of human lung cancer cells, various carbohydrate antigens have been defined. Expression of sialyl Lewis X in NSCLCs was reported (17), whereas SCLC cells mainly expressed gangliosides, such as $G_{D1}$ (18), fucosyl-$G_{M1}$ (19–21), $G_{D3}$ (21, 22), 9-O-acetyl-$G_{D3}$ (22), and $G_{D2}$ (23). Some of them have been used as targets of immunotherapy (24–26) or immunomodulation (27).

Recently, a number of glycosyltransferase genes were isolated, and their substrate specificities and expression patterns were investigated. Availability of these transferase genes and information about their structures and functions enable further analysis of the implications of those tumor-associated carbohydrate structures in malignant phenotypes of lung cancer cells. We are now able to directly analyze the roles of glycoconjugates expressed on tumor cells and the regulatory mechanisms for their expression.

In the present study, we analyzed the expression of gangliosides and the glycosyltransferase genes responsible for their synthesis in human lung cancer cell lines. Because b-series gangliosides were specifically found in SCLC cells with up-regulation of the $G_{D3}$ synthase gene and $G_{M2}/G_{M1}$ were broadly detected in the majority of lung cancer cell lines, we tried remodeling of a ganglioside profile by introducing the $G_{D3}$ synthase gene into a b-series-negative SCLC cell line. Using newly established transfectant cells, we have demonstrated the important role of ganglioside $G_{D2}$ in the cell proliferation of SCLC cells. Moreover, the binding of anti-$G_{D2}$ mAbs could induce apoptotic death of $G_{D2}$-expressing SCLC cells, suggesting the usefulness of anti-$G_{D2}$ antibodies or their derivatives in the therapy of SCLC patients.

MATERIALS AND METHODS

Cell Lines. All human lung cancer cell lines were provided by Dr. T. Takahashi (Aichi Cancer Center Research Institute, Aichi, Japan) and were maintained in RPMI 1640 containing 10% FBS in a humidified 5% CO$_2$ atmosphere at 37°C. The immortalized human bronchial epithelial cell, BEAS-2B, was obtained from the American Type Culture Collection and was cultured on human fibronectin-coated plastic dishes in serum-free small airway cell growth medium (Clonetics) in a humidified 5% CO$_2$ atmosphere at 37°C.

Flow Cytometry. Cell surface expression of gangliosides was analyzed by FACScan (Becton Dickinson) using various anti-ganglioside mAbs. The mAbs used were as follows: anti-$G_{M3}$, M2590 (mouse IgM; Japan Biotest Research), anti-$G_{M1}$, M2590 (mouse IgM; Japan Biotest Research), and anti-$G_{D3}$, M2590 (mouse IgM; Japan Biotest Research).

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The abbreviations used are: NSCLC, non-small cell lung cancer; mAb, monoclonal antibody; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MAPK, mitogen-activated protein kinase; PI, propidium iodide; RT-PCR, reverse transcription-PCR; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-N,N,N′,N′-tetramethylrhodamine; z-VAD-fmk, z-Val-Ala-Asp-fluoromethyl ketone.

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Institute, Tokyo, Japan); anti-GM2, 10-11 (mouse IgM; provided by Dr. P. O. Livingston, Memorial Sloan-Kettering Cancer Center, New York, NY); anti-GD1a, 92-22 (mouse IgM); anti-GD3, R-24 (mouse IgG3; provided by Dr. L. J. Old, Memorial Sloan-Kettering Cancer Center); anti-GD2, 220-51 (mouse IgG3); anti-GD1b, 370 (mouse IgM); anti-GT1b, 549 (mouse IgM); anti-\(\text{A}_2\), 2D4 (mouse IgM; American Type Culture Collection); and anti-\(\text{A}_1\), 229 (mouse IgM). Unless described otherwise, the antibodies were generated in our laboratory (28). The cells were incubated with mAbs for 45 min on ice and then stained with FITC-conjugated goat antimouse IgM or IgG (Cappel, Durham, NC). To analyze GM1, cells were incubated with a biotin-conjugated cholera toxin B subunit (List Biological Laboratories, Campbell, CA) for 45 min on ice and then stained with FITC-conjugated avidin (EY Laboratories, San Mateo, CA). Control cells for flow cytometry were prepared using the second antibody alone. For quantification of positive cells, the CELLQuest program was used.

Quantitative Real-Time RT-PCR Analysis. The quantitative RT-PCR analysis was performed using TaqMan One-Step RT-PCR Master Mix Reagents kit (PE Biosystems). The TaqMan probe contained a reporter dye at the 5′ end (FAM) and a quencher dye at the 3′ end (TAMRA; PE Biosystems). Target-specific PCR amplification results in cleavage and release of the reporter dye from the quencher-containing probe by nuclease activity of AmpliTaq Gold (PE Biosystems). Thus, the fluorescence signal generated from released reporter dye is proportional to the amount of PCR product. To compare the relative expression levels of glycosyltransferase genes in various lung carcinoma cells, total RNA was extracted from each cell line with the guanidine isothiocyanate/cesium chloride ultracentrifugation method, and real-time RT-PCR was performed using gene-specific primers in each sample according to the manufacturer’s protocol. The primers used for \(a_2,8\)-sialyltransferase (GD3 synthase; Ref. 29) were a forward primer (GenBank L32867, nucleotides 456-476), 5′-CATTCCAGCTGCCATTGAAGA-3′, and a reverse primer (nucleotides 1306-1286), 5′-CGGATCTACGAGGAAAGTTGC-3′. The primers for \(b_1,4\)-N-acetylgalactosaminyltransferase (GM2 synthase; Ref. 30) were a forward primer (GenBank NM-001478, nucleotides 1163-1184), 5′-GGACATGAGGCTGCTTTCACTA-3′, and a reverse primer (nucleotides 1130-1128), 5′-CGGATCTACGAGGAAAGTTGC-3′. The TaqMan probes used were 5′-FAM-ATCTATTTGACGGCCACAGCCACTTCT-TAMRA-3′ for GD3 synthase and 5′-FAM-AGCCCAGTACACTCATCAGCGCTCTAGTCAC-TAMRA-3′ for GM2 synthase. For glyceraldehyde-3-phosphate dehydrogenase, Predeveloped TaqMan Assay Reagents Control kit (PE Biosystems) was used according to manufacturer’s protocol. RT-PCR reactions were performed on an ABI PRISM 7700 Detector (PE Biosystems). The relative expression level was deduced from a standard curve constructed using the positive control sample and normalized against the expression level of glyceraldehyde-3-phosphate dehydrogenase in each sample.

Gene Transfection and Selection. Human \(a_2,8\)-sialyltransferase cDNA clone pD3T-31 (29) was subcloned into pMIKneo vector (Manyama; Tokyo Medical Dental University) to obtain the pMIKneo/D3T-31. SCLC SK-LC-1 cells used for cDNA transfection were plated in a 60-mm plastic tissue culture plate (Falcon) at a density of \(6 \times 10^5\) cells/4 ml/plate. After 24 h, the medium was removed, and the cells were washed twice with serum-free DMEM. The pMIKneo/D3T-31 vector (4 \(\mu\)g) was mixed with Lipofectamine (Life Tech-
and then added to the plate. After a 6-h incubation, the medium was changed to the regular one as described above. Stable transfectants were selected with 250 μg/ml G418 (Life Technologies, Inc.).

Glycolipid Extraction and TLC. Glycolipids were extracted as described previously (31). Briefly, glycolipids were extracted from 400 μl of packed cells using chloroform/methanol (2:1, 1:1, and 1:2), sequentially. TLC was performed on high-performance TLC plates (Merck, Darmstadt, Germany) using a solvent system of chloroform:methanol:2.5N NH₄OH (60:35:8) and sprayed by resorcinol. For standards, acid glycolipids from human melanoma SK-MEL-37 cells were used.

Antibody Purification. The anti-GD2 mAb (220-51) was purified using a protein G affinity column, and the concentration of the protein was determined by Lowry’s method (32).

MTT Assay. For cell proliferation assay, transfectant cells and control cells (1 × 10⁴ cells/well) were prepared in 48-well plates in serum-containing medium and cultured for 6 days. For cell growth inhibition assay, transfectant cells and control cells (5 × 10⁴ cells/well) were seeded in 48-well plates in serum-containing medium and treated with various anti-GD2 mAbs diluted to the indicated concentrations for 3 days. Freshly prepared medium containing antibodies was used for medium exchange on days 1 and 2. The anti-GD2

Fig. 3. Correlation between the expression levels of gangliosides and those of glycosyltransferases in the lung cancer cell lines. Surface expression of gangliosides was analyzed as described in Fig. 1. Left, numbers are individual cell lines presented in Fig. 1. Percentages of positive cells are shown. Expression levels of the GD3 synthase gene and the GM2/GD2 synthase gene were measured with a quantitative real-time RT-PCR analyzer system as described in “Materials and Methods.” A: a, SCLC; b, NSCLC. Percentages of b-series positive cells (left) were the sum of those of GD3⁰, GD2⁰, and GT1b-positive cells. The relative expression level of the GD3 synthase gene (right) is presented as a percentage of that of melanoma SK-MEL-37 cells, which were regarded as 100%. B: a, SCLC; b, NSCLC. The percentages of complex ganglioside-positive cells (left) were the sum of those of G3M⁰, GM1⁰, GD3⁰, GD2⁰, GD1b⁰, and GT1b⁰-positive cells. The relative expression level of the GM2/GD2 synthase gene (right) is presented as a percentage of that of the SCLC line ACC-LC-171 cells, which were regarded as 100%.

Fig. 4. Remodeling of ganglioside expression by gene manipulation. A, surface expression of gangliosides in transfectant cells. A vector control cell line (C-8; top) transfected with pMIKneo vector alone and a GD3 synthase gene transfectant cell line (D-18; bottom) transfected with pMIKneo/D3T-31 were used for flow cytometry as described in “Materials and Methods.” Filled gray lines, the controls with the second antibody alone. B, TLC of glycolipids extracted from SK-LC-17 cells (LC-17), C-8, and D-18 are presented. TLC was performed as described in “Materials and Methods.”

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were added. The color reaction was quantitated using an automatic plate reader, Immuno-Mini NJ-2300 (Nihon InterMed, Tokyo, Japan), at 590 nm with a reference filter of 620 nm as reported previously (33). MTT assays were carried out in triplicate. To analyze the growth-suppression effects of mAb 220-51 in SCLC lines, cells (×10^4 cells/well) were prepared in 96-well plates in serum-containing medium and treated with 40 μg/ml of mAb 220-51 for 3 days and then used for the MTT assay. In this case, lysis buffer (n-propylalcohol with 0.1% NP40 and 4mM HCl) was added to each well without aspiration of the medium.

**In Vitro Invasion Assay.** The Boyden chamber in vitro invasion assay was performed as described previously (34) with some modifications. In brief, Matrigel (Becton Dickinson) was diluted with ice-cold PBS (250 μg/ml), added to each filter (190 μg/filter; polyethylene terephthalate membrane, 8-μm pore size, 23.1 mm in diameter; Falcon 3092), and allowed to polymerize and dry overnight. The polymerized and dried Matrigel membranes were reconstituted with serum-free medium. The lower chamber (6-well plate; Falcon 3502) was filled with serum-free medium (3 ml) before the chamber was assembled. Cells (×10^6 cells/well) were added to serum-free medium in the upper chamber and incubated for 18 h in a humidified 5% CO₂ atmosphere at 37°C. After incubation, the cells on the upper surface of the filter were removed completely by wiping with a cotton swab. The filters were then fixed in ethanol and stained with Giemsa (Wako). The number of cells migrating to the lower surface of the filter was counted in 10 fields under ×200 magnification, and the mean of the number in each field was calculated. Assays were carried out in triplicate.

**Western Immunoblotting.** Cells (×10^5 cells) were plated in 60-mm tissue culture plates in serum-containing medium and then treated with either 40 or 60 μg/ml of mAb 220-51 for the appropriate times in the individual experiments. After treatment, cells were harvested with 0.02% EDTA, washed twice with ice-cold PBS, and solubilized in lysis buffer [20 mM Tris-HCl (pH 7.4), 1% NP40, 10% glycerol, 50 mM NaCl, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 20 units of aprotinin]. Soluble proteins (50 μg/lane) were subjected to either 10 or 15% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with 5% dry milk in PBS overnight at 4°C, washed with PBS containing 0.05% Tween 20, and then incubated with antibodies reactive with either MAPK, phospho-MAPK (New England Biolabs), or active caspase-3 (PharMingen). Bands were detected with peroxidase-conjugated antirabbit IgG (New England Biolabs) combined with an ECL kit (Amersham Pharmacia Biotech).

**Analysis of Apoptotic Cells.** Cells (×10^5 cells) were plated in 60-mm tissue culture plates in serum-containing medium and then treated with either 20 μg/ml of various anti-CD133 mAbs or 20 μg/ml of irrelevant mAbs (10-11 and R24) for 24 h at 37°C. For analysis of the time course and dose dependency of the antibody effects, cells were treated with mAb 220-51 diluted appropriately. After treatment, cells were harvested with trypsin/EDTA, resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂], and then incubated with FITC-conjugated Annexin V and 1 μg/ml PI (Annexin V-FITC kit; Bender Medsystems), according to the manufacturer’s protocol. The numbers of apoptotic cells were monitored with a FACScan and determined using the CELLQuest program. For analysis of sub-G₁ DNA content, cells (1.5 × 10^6 cells) were cultured with or without 100 μM z-VAD-fmk (Peptide Institute, Inc., Osaka, Japan) in the presence of mAb 220-51 (30 μg/ml). After 48 h, cells were fixed with 70% ethanol and incubated with 4 μg/ml propidium iodide (4 μg/ml citric acid, 192 μM Na₂HPO₄) for 30 min at room temperature. After centrifugation, cells were resuspended in PBS containing 0.05% Tween 20 and incubated with antibodies reactive with either MAPK, phospho-MAPK (New England Biolabs), or active caspase-3 (PharMingen). Bands were detected with peroxidase-conjugated antirabbit IgG (New England Biolabs) combined with an ECL kit (Amersham Pharmacia Biotech).

**DNA Fragmentation Assay.** Cells (×10^6 cells) were treated with 60 μg/ml of mAb 220-51. After 48 h, cells were harvested and lysed in 100 μl of lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100] for 10 min at 4°C. After centrifugation, the supernatants were collected, and 2 μl of RNase (10 mg/ml) and 2 μl of Proteinase K (10 mg/ml) were added. After incubation for 1 h at 37°C, the fragmented DNA was precipitated in 2-propanol and electrophoresed at 50 V for 1.6 h on a 1% agarose gel containing 0.2 μg/ml ethidium bromide in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gel was observed under UV light.

**Statistical Analysis.** Significance was determined using the Student t test.
RESULTS

Ganglioside Typing of Lung Cancer Cell Lines. Expression of gangliosides in 44 lung cancer cell lines was analyzed by flow cytometry (Fig. 1, A–D). GM2 and GM1, belonging to the a-series gangliosides, were expressed at significant levels in almost all of the lung cancer cell lines, whereas the b-series gangliosides, such as GD2, GD1b, and GT1b, were found characteristically in SCLC lines. These b-series gangliosides were not expressed or were expressed at very low levels, if present, in non-SCLC lines. A bronchial epithelial cell line, BEAS-2B, expressed no or very low levels of gangliosides except for GM1 (Fig. 1E).

Gene Expression of Glycosyltransferases in the Lung Cancer Cell Lines. To analyze the correlation between the expression levels of gangliosides and those of glycosyltransferase genes, expression levels of the GD3 synthase gene and the GM2/GD2 synthase gene were measured with a quantitative real-time RT-PCR analyzer system (PE Biosystems). The expression level of the GD3 synthase gene was well correlated with that of b-series gangliosides, i.e., high levels of the GD3 synthase gene expression were shown in the SCLC cell lines (Fig. 2 and Fig. 3A). As for the GM2/GD2 synthase gene, its expression levels were well correlated with those of complex gangliosides (Fig. 3B). This gene was broadly expressed in all of the lung cancer lines examined, whereas SCLC lines showed slightly higher expression levels.

Remodelling of Ganglioside Expression by Gene Manipulation. To analyze the effect of b-series gangliosides dominantly expressed in the SCLC cell lines on malignant cancer phenotypes, a cDNA expres-
sion vector of GD3 synthase was introduced into a SCLC line, SK-LC-17, which did not express b-series gangliosides. Newly established transfectant cell lines showed definite and high expression of b-series gangliosides in the flow cytometric analysis (Fig. 4A). In contrast, GM2 and GM1 levels were reduced dramatically because of the shift of the synthetic flow from a-series to b-series. Control lines transfected with a vector alone showed no change in the ganglioside profile compared with the parent cell, i.e., GM2 and GM3 expressions were observed (Fig. 4A). These findings were confirmed by TLC of ganglioside fractions extracted from the individual cell lines (Fig. 4B).

As for GM3, its expression could not be detected in the flow cytometry, presumably because of the low accessibility of the antibody. However, high expression in the vector control and the parent cell and marked reduction in the transfectant lines, because of the consumption as a substrate of the transfected enzyme, were exhibited in TLC. When the expression levels of the GD3 synthase gene in the transfectants were analyzed by real-time RT-PCR, an ~1400-fold increase in the expression level, compared with that of the vector control, was observed (data not shown).

Changes in Phenotypes of GD3 Synthase Gene Transfectant Cells. In the MTT assay, the transfectant cells expressing b-series gangliosides exhibited significantly enhanced proliferation compared with control lines (Fig. 5A). Cell growth, as presented by absorbance at 590 nm, was ~2-fold or greater after 4–6 days of culture. Invasion activity as analyzed by the Boyden chamber method was markedly increased in the transfectant lines, i.e., 483 ± 117 cells were found for the transfectants and 98.7 ± 26.3 cells in the controls, which had penetrated the Matrigel layer and come out of the reverse side of the filter (Fig. 5, B and C).

Suppression of Cell Growth by Anti-GD2 Antibodies. Effects of anti-GD2 mAbs on cell growth were then examined by adding antibodies to the culture medium. The increased cell growth after GD3 synthase gene transfection was strongly suppressed in the presence of an anti-GD2 mAb 220-51. The suppression effects were dependent on the concentration of the added antibody and became significant, even at 5 μg/ml on day 3 (Fig. 6A). Activation (phosphorylation) levels of MAPK were also reduced along with the time course after antibody addition (Fig. 6B). In vector control cells, neither suppression of cell proliferation nor reduction in MAPK activation were observed (Fig. 6, A and B). These suppression effects of anti-GD2 mAb on the cell growth were also detected in SCLC lines (NCI-N417 and ACC-LC-171; Fig. 6C), which primarily expressed high levels of GD2 but not in a non-GD2-expressing line, ACC-LC-170 (data not shown). Other anti-GD2 mAbs than 220-51, including KM666 (mouse mAb) and KM1138 (mouse-human chimeric mAb), also showed significant and similar growth suppression activity for the transfectant cells (Fig. 7). The nonspecific mouse IgG3 fraction showed no effects (data not shown).

Apoptosis Induction by Anti-GD2 mAbs. To clarify the induction of apoptosis during the growth suppression of SCLC cell lines with anti-GD2 mAbs, double staining of cells with Annexin V (FITC) and PI was performed. Transfectant lines treated with anti-GD2 mAb were 72.2% positive for Annexin V binding and 76.9% positive for PI staining at 24 h after the addition of mAbs (Fig. 8A), indicating the induction of apoptotic cell death. A vector control line, C-8, showed no staining for both reagents. The transfectant cells treated with anti-GD2 mAb showed marked shrinkage of cytoplasm and formation of spaces between cells, whereas vector control cells showed no change (Fig. 8B). When other anti-GD2 mAbs were added, similar morphological changes (data not shown) and double staining with Annexin V and PI were found (Fig. 9A). However, nonrelevant antibodies, such as anti-GM2 mAb or anti-GM3 mAb, did not induce these changes (Fig. 9B). During the time course, the Annexin V-positive population increased after 0.5 h, and then the PI-positive fraction expanded after 1 h incubation (Fig. 10, A and B). This apoptosis induction depended on the antibody concentration (Fig. 10C). To investigate the sensitivity to the apoptosis induction with the anti-GD2 mAb in cell lines with various levels of GD2 expression, GD2 expression levels and the percentage of positive Annexin V and PI after the antibody treatment were compared in six lung cancer cell lines. Not only high GD2 expressers such as ACC-LC-171 and NCI-N417 but also those with moderate GD2 levels showed apparent apoptosis (Fig. 11). To confirm the DNA degradation in the anti-GD2 mAb-treated cells, cytoplasmic DNA was prepared from 3 × 105 cells and used for agarose gel electrophoresis. Only cells treated with the mAb showed definite DNA ladder formation (Fig. 12A) and also activation of caspase-3 (Fig. 12B). This apoptotic process was partially inhibited by a caspase inhibitor, z-VAD-fmk (Fig. 12C), which suggested that apoptosis induced by anti-GD2 mAb was mediated via the caspase-3-dependent pathway and some independent pathway.

**DISCUSSION**

Despite much effort to elucidate the biological functions of glycosphingolipids, few definite molecular functions have been demonstrated to date. Current ways to modulate carbohydrate structures on cells have provided only indirect information about the roles of glycoconjugates, because methods to modulate carbohydrate chains harbor substantial limitations in the specificity of their effects. Successful cloning of glycosyltransferase genes dramatically changed this situation and improved the limitations in the analysis of carbohydrate functions. In particular, application of gene knockout technology has clearly demonstrated in vivo functions of products of individual glycosyltransferases (35), although the expected outcome has not necessarily been obtained because of various unknown reasons.
Remodeling of ganglioside profiles in cultured cells has shown their roles in the regulation of cell proliferation and differentiation (36, 37). Then, gene targeting of galactosyl-ceramide synthase revealed GalCer functions in the stability of myelin (38) and spermatogenesis (39). GM2/G D2 synthase gene knockout mice lacking complex gangliosides exhibited their roles in the regulation of the nervous system (40), spermatogenesis (41), and T-cell functions (42). However, roles of gangliosides in various malignant phenotypes in tumors have never been clearly demonstrated by glycosyltransferase gene manipulation, although many glycolipid antigens have been considered as tumor markers or have been used as target molecules of antibody therapy (9, 10).

G D2 studied in this paper is one of the melanoma-associated glycolipid antigens (8). In particular, G D2 is up-regulated in advanced and vertically metastatic melanoma cells (43), suggesting that G D2 plays roles in malignant features of melanomas such as metastasis (44). Actually, G D2 and G D3 have been considered to modify integrin functions (45). Moreover, an anti-G D2 immune reaction against melanoma cells in melanoma patients was reported by Watanabe et al. (46) and Cahan et al. (47). These findings suggest that G D2 is one of the target molecules for immunotherapy of melanoma patients. In addition to melanoma, G D2 has been considered to be a neuroblastoma-associated antigen, and anti-G D2 mAb has been tried in a therapy for neuroblastoma patients (48). We also reported previously the specific expression of GD2 on human T-cell lymphotrophic virus type 1-infected lymphocytes under regulation of p40ras transactivator (14). Despite a number of studies indicating the tumor-specific expression or malignant phenotype-associated expression of G D2, no definite studies to demonstrate the significant roles of G D2 in tumor cell proliferation have been reported. The findings demonstrated in the

![Fig. 9](image_url)  
**Fig. 9.** Anti-G D2 mAb-specific apoptosis induction in the transfectant cells. A, double staining of the transfectants treated with various anti-G D2 mAbs using Annexin V and PI. D-18 was treated with 20 μg/ml of mAb 220-51 (left), KM666 (middle), or KM1138 (right) for 24 h and stained with Annexin V (top) and PI (bottom) and then analyzed by flow cytometry as described in "Materials and Methods." Filled gray lines, negative controls with neither Annexin V nor PI. B, D-18 was treated with 20 μg/ml of mAb 220-51 (left), mAb 10-11 (anti-G D2 mAb; middle), or mAb R24 (anti-G D3 mAb; right) for 24 h. After the treatments, they were stained as described in A.

Fig. 10. The time course and dose dependency of the anti-G D2 mAb effects in the transfectant cells. D-18 was treated with 20 μg/ml of mAb 220-51 for the times indicated and used for double staining with Annexin V and PI. A, the positive percentages of Annexin V; ■, the positive percentages of PI. B, two-dimensional presentation of double staining with Annexin V and PI. Borders were set to make double-negative cells 95% at 0 h, and the percentages of individual fractions are shown. C, D-18 was treated with mAb 220-51 diluted at the concentrations indicated for 12 h and then used for double staining with Annexin V and PI. Results are presented as in A.

![Fig. 11](image_url)  
**Fig. 11.** Apoptosis induction in lung cancer cell lines with various levels of G D2 expression by an anti-G D2 mAb. Cells (8 × 10^5 cells/well) were seeded in a six-well plate in serum-containing medium and treated with 40 μg/ml of mAb 220-51 for 24 h and used for double staining with Annexin V and PI. A, two-dimensional presentation of the double staining of ACC-LC-171 cells with Annexin V and PI (left, nontreated cells; right, treated cells). B, GD2 expression levels (left) and positive percentages (right) stained with Annexin V (■) and PI (□) of six lung cancer cell lines are shown.
present study strongly suggest that GD2 actually exerts important roles to enhance cellular proliferation signals as shown in Fig. 6B.

Kasahara et al. (49) reported recently that GD3 on primary cultured cerebellar neurons mediated activation of a Src family tyrosine kinase, lyn, in cooperation with the TAG-1 molecule. Fukumoto et al. (37) reported that introduction of the GD3 synhase gene into the rat pheochromocytoma cell line PC12 resulted in the continuous activation of nerve growth factor receptor TrkA and its downstream signal molecule, MAPK. These alterations in the signal molecules appears to induce a marked enhancement of cell proliferation. These findings suggest that GD2 on the cell surface of SK-LC-17 is associated with some growth factor receptors and modulates its functions, resulting in an observed increase in cell growth. Identification of GD2-associated receptors or signal molecules remains to be investigated.

Apoptosis induced by anti-GD2 mAbs is very interesting and provides a possibility for the application in immunotherapy of SCLC. Gangliosides, including GD2, are expressed on the outer layer of plasma membrane; therefore, it is unclear how GD2 can mediate the apoptosis signal triggered by the binding of anti-GD2 mAb. Binding of anti-GD2 mAb to GD2 might cause clustering of GD2 molecules, resulting in the modulation of unknown neighboring molecules and in the activation of subsequent intracellular molecules that emit a signal for apoptosis. No studies have been performed on the induction of apoptosis of tumor cells using antiglycolipid mAbs except for anti-GD2 mAb by Nakamura et al. (50). However, they showed just a decrease of tumor cell layer volume in multicellular heteroscleroids after treatment with anti-GD2 mAb. Apoptosis of SCLC cells induced by anti-GD2 mAb, as demonstrated in the present study, should be the first example in which apoptosis induction was definitely shown using antiglycolipid mAbs. The molecular mechanisms for this apoptosis are now under investigation in our laboratory and should provide information necessary for the application of anti-GD2 mAbs in therapies against drug-resistant malignant tumors.

Although this study was performed with lung cancer cell lines, GD2 expression in tumor specimens of SCLC has been reported (27), suggesting that GD2 in SCLC is worth being focused on as a target of chemoinmunotherapy. Of course, many devices in the modification of antibodies, in the modes of antibody treatment, e.g., in combination with vaccines in the adjuvant setting (11), or in the controlling of side effects (51) remain to be achieved.

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