Alteration of Ganglioside Composition by Stable Transfection with Antisense Vectors against GD3-Synthase Gene Expression†

Guichao Zeng, Donna D. Li, Luoyi Gao, Stéphane Birklé, Erhard Bieberich, Akira Tokuda,‡ and Robert K. Yu*

Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0614

Received March 23, 1999; Revised Manuscript Received May 6, 1999

ABSTRACT: Gangliosides are ubiquitous components of mammalian cells. Their expression is frequently altered in many tumor types. We previously showed that alteration of the ganglioside composition often resulted in changes in cellular morphology and differentiation of cultured cells. In this study, we targeted sialyltransferase gene expression by the antisense knockdown experiment, and the results showed that inhibition of the expression of gangliosides GD3 and *O*-acetylated GD3 (OAc-GD3) in the neuroblastoma F-11 cells greatly reduced the tumor growth in nude mice. The sense and antisense vectors containing either a 5′ end fragment or the entire sequence of the cDNA coding for GD3-synthase were prepared and used in separate experiments to transfect the F-11 cells which express high levels of gangliosides GD3 and OAc-GD3. Single clones were isolated and expanded. Both the activity of the GD3-synthase and the concentrations of GD3 and OAc-GD3 in the antisense-transfected cells were dramatically decreased as a result of transfection with the antisense expression vectors. Further characterization of the antisense-transfected cells showed reduced rates of cell growth and neurite formation and changes in cellular morphology. When the cells were inoculated in athymic nude mice, the tumor growth rate was remarkably suppressed although the tumor incidence was not affected by the altered ganglioside composition. These results indicate that the tumor-associated ganglioside(s) is(are) involved in regulation of tumor growth, probably through the stimulation of angiogenesis of the tumor.

Gangliosides are sialic acid-containing glycosphingolipids (GSLs) found primarily in the plasma membrane of virtually all vertebrate tissues. They constitute part of the glycocalyx network surrounding the cell surface and are crucial in determining the properties and functions of cells. Specific changes in the composition and metabolism of gangliosides have been observed during cell proliferation (*1*, *2*), cell cycle phase (*3*), brain development (*4*), differentiation (*5*−*9*), and neoplasms in various cell types (*10*−*24*). The significance of the transient appearance of cell surface glycolipids may be related to factors governing cellular growth, migration, differentiation, and transformation. Many studies have revealed tumor-specific expression of gangliosides in various cancers (*10*−*24*). Altered ganglioside expression in tumor cells is usually associated with invasive growth and metastatic potential of the tumors (*25*−*28*). Disialoganglioside GD3 is overexpressed in many types of tumors (*11*, *13*, *29*−*37*), whereas the expression of *O*-acetylated GD3 (OAc-GD3) is restricted in particular cancers such as melanoma, pheochromocytoma, and breast cancer (*29*, *36*−*39*). Furthermore, gangliosides are shed from these cancer cells into the local environment and eventually into the blood stream (*40*). Since the expression of gangliosides is associated with malignant transformation (*26*), it is of clinical significance to study changes in ganglioside distribution during cellular transformation and to alter the expression of the tumor-associated gangliosides in cancer cells.

The ganglioside composition can be altered by manipulating the activity of the sialyltransferases which, in turn, is controlled largely at the level of gene expression (*41*−*43*). Using synthetic antisense oligodeoxynucleotides to GM2-synthase and GD3-synthase[1] cDNA sequences, we effectively down-regulated the enzyme activities in HL-60 cells, resulting in an accumulation of the precursor, GM3, in the cells which also underwent monocytic differentiation (*42*). We have also applied the same strategy to primary cerebellar neurons and altered the ganglioside composition in the cells (*44*). In addition, we introduced the influenza virus C hemagglutinin-esterase gene, which specifically hydrolyzes the *O*-acetyl esters from the sialic acid moiety of gangliosides, into F-11 neuroblastoma cells (*43*) and hamster melanoma cells (*28*), resulting in a decreased OAc-GD3 expression and an altered cellular morphology. Therefore, the antisense knockout technique promises to be a powerful tool to

† This work was supported by National Institutes of Health Grant NS 11853 and American Cancer Society Grant IRG-105.

* To whom correspondence should be addressed. Tel: 804-828-9762. Fax: 804-828-1473. E-mail: rkyu@hsc.vcu.edu.

‡ Present address: Second Internal Medicine, Faculty of Medicine, Fukui Medical University, 23-3 Shimoaigetsu Matsuoka-cho, Fukui 910-1193, Japan.

[1] Abbreviations: GD3-synthase, CMP-NeuAc:GM3 α2−8 sialyltransferase; GM3, NeuAcα2−3Galβ1−4Glcβ1−1′Cer; GD3, NeuAcα2−8NeuAcα2−3Galβ1−4Glcβ1−1′Cer; GD1a, NeuAcα2−3Galβ1−3GalNAcβ1−4Gal(3−2α NeuAc)β1−4Glcβ1−1′Cer; GD1b, Galβ1−3GalNAcβ1−4Gal(3−2αNeuAc8−2αNeuAc)β1−4Glcβ1−1′Cer; GT1b, NeuAcα2−3Galβ1−3GalNAcβ1−4Gal(3−2αNeuAc8−2αNeuAc)β1−4Glcβ1−1′Cer.

10.1021/bi9906726 CCC: $18.00
Published on Web 06/06/1999

investigate the roles of individual gangliosides in various cellular events and to provide a potential therapeutic approach for cancer treatment.

Our goal of the present experiments was to investigate further the role of ganglioside GD3 in tumorigenesis by the antisense knockdown procedure to suppress the expression of GD3 in the neuroblastoma F-11 cells. F-11 cells are hybrid cells derived from mouse neuroblastoma N18TG-2 cells and rat dorsal root ganglion cells (*45*). These cells show many properties characteristic of dorsal root ganglion neurons (*46*) and express high levels of gangliosides GD3 and OAc-GD3 (*43*). They also exhibit strong tumorigenicity in nude mice. In this study we established single clones of the neuroblastoma F-11 cells by stable transfection of antisense expression vectors in the cells, which resulted in a knockdown of expression of the GD3-synthase gene. We further showed that the concentrations of ganglioside GD3 as well as OAc-GD3 in the antisense-transfected cells decreased specifically. Morphologically, the antisense-transfected cells had smaller soma and retracted dendritic processes than the control cells. In addition, the tumor growth rate of the antisense-transfected neuroblastoma F-11 cells in nude mice was drastically decreased.

EXPERIMENTAL PROCEDURES

Cell Culture. The F-11 neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Stock cultures were passaged weekly and supplied with fresh medium every 3 days. Single clones of the stably transfected cells were isolated and maintained in the media described above supplemented with 500 μg/mL Geneticin. For neurite measurement, a photograph was randomly taken after cells had been passaged for 3 days. The summation of the length of all the neurite from 200 cells was obtained from the photograph, and data were presented as μm/cell.

Construction of Antisense Expression Vector and Transfection. The rat GD3-synthase cDNA was generated by PCR amplification. The template was the total RNA from F-11 cells, and the two primers were 5′ GAGGCTGCCACCACT-GACCCTGGGAC (sense) and 5′ TCTTCTGCAGTCCC TAGGAAGTGGGC (antisense), derived from −38 to −13 (*47*) and from 1080 to 1115 (*44*), respectively. To construct the sense and antisense vectors, the PCR product was ligated into a mammalian expression vector pCR3.1 (Invitrogen, San Diego, CA) which contained cytomegalovirus (CMV) promoter and neomycin resistance gene. *Escherichia coli* was transformed by the plasmids, selected clones were harvested, the bacteria were lysed by alkali treatment, and the plasmids were purified. The plasmids containing the cDNA in the forward (5′ to 3′) and the reverse (3′ to 5′) orientations were confirmed by DNA sequencing and referred to as the sense and the antisense GD3-synthase plasmids, respectively. These plasmids were then transfected into F-11 cells using LipoFectin (Gibco BRL, Gaithersburg, MD) as described by the manufacturer. The transfected cells were plated in 10-cm culture dishes 48 h after transfection. Isolation of single clones of the stable transfectants were accomplished by adding Geneticin (G418 sulfate; Gibco BRL, Gaithersburg, MD) to the culture medium at 500 μg/mL. G418-resistant transfected clones appeared in about 2 weeks, and more than a dozen of single clones were pooled later on and expanded. In addition, we have previously obtained a 530-bp fragment of the 5′ end of the GD3-synthase cDNA which was inserted in pCRII vector (containing no neomycin resistance gene; Invitrogen, San Diego, CA) in either forward (sense) or reverse (antisense) orientations (*44*). In a separate experiment, the antisense construct of the 530-bp fragment was cotransfected with pRSV-Neo plasmid into F-11 cells, and over 10 single clones of the stable transfectants resistant to G418 were isolated and propagated.

Analysis of Gangliosides. The ganglioside isolation procedures were described previously (*48*). Briefly, the total lipids were extracted from the cells with chloroform:methanol (1:1, v/v) and chloroform:methanol:water (30:60:8, v/v/v) (solvent A), successively. The combined extracts were adjusted into the ratio of solvent A and applied to a DEAE-Sephadex A-25 column (acetate form, bed volume, 2 mL). The column was eluted with 15 mL of solvent A to remove neutral lipids. The ganglioside fraction was then eluted with 15 mL of chloroform:methanol:0.8 M sodium acetate (30:60:8, v/v/v) (solvent B). After drying, the sample was redissolved in 0.5 mL of solvent A and desalted. The ganglioside fractions were analyzed by high-performance thin-layer chromatography (HPTLC) using plates of silica gel 60. The plates were developed with chloroform:methanol:0.2% aqueous $CaCl_2 \cdot 2H_2O$ (50:45:10, v/v/v) (solvent C). Gangliosides were visualized by spraying the plate with the resorcinol-HCl reagent followed by heating the covered plate at 100 °C for 30 min. For autoradiography, cells were cultured in 35-mm dishes and labeled with 0.5 μCi of [^{14}C]-galactose (New England Nuclear, 5.75 mCi/mmol) in 1 mL of medium for 24 h before harvest as described previously (*42*). For TLC plate immunostaining, the plates were fixed by 0.1% poly(isobutyl methacrylate) in *n*-hexane and treated with PBS/1% BSA at room temperature for 1 h. They were then overlaid with an antibody-containing supernatant (1:50 diluted in PBS/0.1% BSA) overnight at 4 °C. After three washes with PBS, monoclonal antibody binding was detected by stepwise incubation with biotinylated goat anti-mouse antibodies (1:2000 dilution) for 1 h at room temperature, followed by streptavidin-horseradish peroxidase complex diluted at 1:2000 for 45 min at room temperature. After extensive washing with PBS, the bound peroxidase was visualized by addition of a chloro-4-naphthol solution.

Immunocytochemistry of GD3 in Transfected Cells. Cultured F-11 cells in 35-mm dishes were fixed with 4% paraformaldehyde in PBS for 15 min and washed with PBS three times. After incubation in PBS with 3% nonimmunized mouse serum, a mouse monoclonal antibody R24 diluted 1:100 in PBS was applied at 4 °C for 12−36 h. The dishes were washed three times with PBS, and FITC-conjugated anti-mouse immunoglobulin (Cappel, West Chester, PA) at a dilution of 1:200 was applied for 2 h at room temperature to visualize the labeled sites. The fluorescence was determined by a Nikon microscope, model UFX-IIA. The monoclonal antibody R-24 (recognizing GD3) was a generous gift from Dr. Kenneth Lloyd, Memorial Sloan-Kettering Cancer Center, New York, NY.

Assay of Sialyltransferase Activities. F-11 cells were cultured to 80% confluence. Cells were then washed with

PBS and harvested by scraping from the plastic surface. The activities of GM3-synthase and GD3-synthase were determined according to the procedures described previously (*49*, *50*). The radioactive substrates used in the enzyme assay were CMP-[^{3}H]-*N*-acetylneuraminic acid (New England Nuclear, 19.7 mCi/mmol). After the reactions were completed, the glycolipid products were separated by Sephadex G-50 gel filtration and their radioactivities measured by liquid scintillation counting. Protein contents of the enzyme extracts were determined by Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA).

Cell Growth and Thymidine Incorporation. Cells were inoculated in 35-mm dishes at the cell density of 2×10^3 cells/dish, and the growth kinetics of the transfected F-11 cells were determined by counting the cells from triplicate dishes every day. DNA synthesis was determined by [^{3}H]-thymidine incorporation as described previously (*51*). Briefly, cells were plated at a density of 5×10^4 cells/dish and incubated for 4 h with 0.5 μCi of [^{3}H]thymidine (50 Ci/mmol) (Amersham, Arlington Heights, IL). Cells were then washed in PBS, and 1 mL of lysing solution (50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 150 mM NaCl, and 0.6% sodium dodecyl sulfate) was added to each dish. The dishes were left at room temperature for 10 min. The lysate was precipitated by addition of 1 mL of 20% trichloroacetic acid at 4 °C for 20 min and collected on Whatman GF/C filters (Whatman, Clifton, NJ). The filters were washed three times with 5% trichloroacetic acid and dried by ethanol and acetone. Radioactivity was determined by a scintillation counter. The protein contents of the cell lysates were determined using a Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA).

In Vivo Tumor Growth. Female athymic nude mice (Harlan Sprague−Dawley, Inc.) were housed under specific pathogen-free conditions and used at 4 weeks of age. Cells from exponential cultures of F-11 transfectants and untransfected F-11 cells were inoculated subcutaneously behind the anterior forelimb. Each mouse was inoculated on both sides with 5×10^5 cells in 0.25 mL of PBS per site. After xenografts became visible, the sizes of xenografts were determined daily by externally measuring tumors in two dimensions. The volume (V) of the xenograft was calculated by the following equation: $V = (L \times W^2) \times 0.5$, where L is the length and W is the width of a xenograft.

RESULTS

Ganglioside Composition of Transfected F-11 Cells. Expression vectors containing the 5′ 530-bp fragment or the entire sequence of the GD3-synthase cDNA spanning the translation start site in the sense and antisense orientations were transfected into F-11 cells, respectively, in two separate experiments as described under Experimental Procedures. Over 10 single clones were isolated from each transfection paradigm. Ganglioside analysis of the stably transfected clones showed that the level of GD3 was greatly reduced in at least two single clones of antisense cotransfected cells (clones 2-1-8 and 2-1-19) and four single clones of one antisense vector-transfected cells (clones 3-2-2, 3-2-7, 3-4-9, and 3-4-21) (data not shown). Clones 2-1-19 and 3-4-9 were designated as F-11-II (antisense transfectants cotransfected with the antisense vector containing the 530-bp fragments and the pRSV-Neo plasmid), and F-11-III (antisense transfectants transfected with the antisense vector containing the entire cDNA), respectively. Single clones of sense-transfected cells were isolated from F-11 cells transfected with the sense vectors containing either the 530-bp fragment or the entire cDNA. HPTLC analysis of sense-transfected clones showed no changes in their ganglioside composition (data not shown). One of the single sense-transfected clones was used and referred to as F-11-I (sense transfectants transfected with the sense vectors containing the entire cDNA).

Table 1: Ganglioside Composition of F-11 Cells Transfected with Sense or Antisense Expression Vectors[a]

ganglioside	F-11 cells	F-11-I cells	F-11-II cells	F-11-III cells
GM3	20.7 ± 1.5	23.2 ± 2.2	33.6 ± 2.0	54.7 ± 2.4
GM1	15.5 ± 1.5	8.3 ± 1.2	16.9 ± 1.2	11.6 ± 1.3
OAc-GD3	16.8 ± 1.1	15.2 ± 2.0	4.7 ± 2.4	1.0 ± 0.3
GD3	24.5 ± 1.3	29.2 ± 1.4	7.3 ± 1.1	2.7 ± 1.4
GD1a	7.9 ± 0.8	4.6 ± 1.3	12.0 ± 2.1	9.6 ± 1.2
GD1b	9.4 ± 1.4	12.8 ± 1.2	12.4 ± 0.9	10.2 ± 0.5
GT1b	5.2 ± 1.2	6.4 ± 2.2	11.2 ± 2.2	8.5 ± 0.7

[a] The ganglioside composition was analyzed by HPTLC and subjected to densitometry. Values are presented as percentages (mean ± SD) of three determinations and therefore refer to the relative amounts of each ganglioside in the sample. The content of gangliosides in F-11 cells was estimated to be 0.82 ± 0.08 μg (mean ± SD) of lipid-bound sialic acid/mg of protein. The content of gangliosides of the transfected F-11 cell lines was the same as that of the untransfected F-11 cells. F-11, untransfected F-11 cells; F-11-I, sense-transfected F-11 cells; F-11-II and F-11-III, F-11 cells transfected with antisense vectors containing the 5′ end of 530-bp fragment or the entire sequence of the GD3-synthase cDNA, respectively.

The stable transfected clones F-11-I, F-11-II, and F-11-III cells, together with untransfected F-11 cells, were then analyzed for the composition of gangliosides by HPTLC (Table 1). An autoradiogram of the ganglioside composition of the untransfected F-11 cells, sense-transfected cells, and antisense-transfected cells is shown in Figure 1. Untransfected F-11 cells expressed high levels of gangliosides GD3 and OAc-GD3, which contributed to 25% and 17% of total gangliosides, respectively (Table 1), the same as we reported previously (*43*). However, the amount of ganglioside GD3 was dramatically decreased in the antisense-transfected F-11-II and F-11-III cells (Table 1). A decrease in the amount of GD3 and OAc-GD3 was not observed in the sense-transfected F-11-I cells (Table 1). Inhibition of the synthesis of gangliosides GD3 usually resulted in an accumulation of the precursor GM3 and/or an increased synthesis of the "a" series gangliosides such as GM1 and GD1a (Table 1; *42*). However, it should be noted that the amounts of GD1b and GT1b, the minor "b" series gangliosides present in F-11 cells (*43*), were not reduced in antisense-transfected cells (Table 1). This may be due to continuous conversion of the remaining amount of ganglioside GD3, or nonspecificity of glycosyltransferases.

Confirmation of the above results was further achieved by immunostaining of the GD3 bands on TLC plates with the monoclonal antibody R24 specific to ganglioside GD3. Figure 2A shows that GD3 in the antisense-transfected F-11-II and F-11-III cells was drastically reduced, compared to that of the untransfected F-11 and the sense-transfected F-11-I cells. Ganglioside GD3 on the cells surface was also determined by immunostaining using the specific antibody

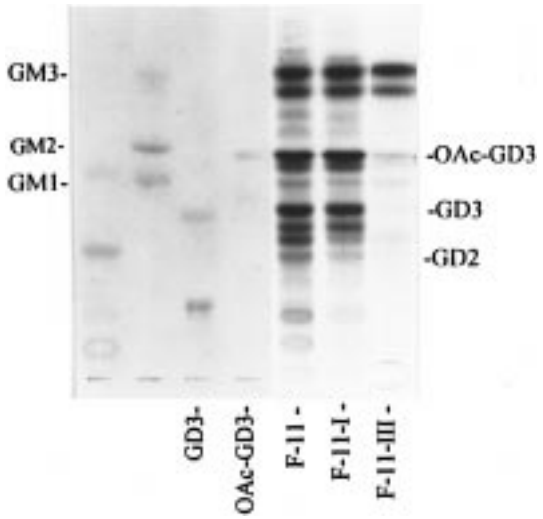

FIGURE 1: Autoradiograms of ganglioside pattern of F-11 neuroblastoma cells. Cells were cultured in 35-mm dishes and labeled with 0.5 μCi of [^{14}C]galactose (New England Nuclear, 5.75 mCi/mmol) in 1 mL of medium for 24 h before harvest as described (*42*). Each lane contained the total ganglioside fraction (containing 3100 $\pm$ 380 cpm) corresponding to 1 $\times$ 10^6 cells. Standard gangliosides (lanes from left to right: total rat brain gangliosides; GM3, GM2, and GM1; GD3 and GD1b; *O*-acetylated GD3) were revealed by the resorcinol hydrochloride reagent.

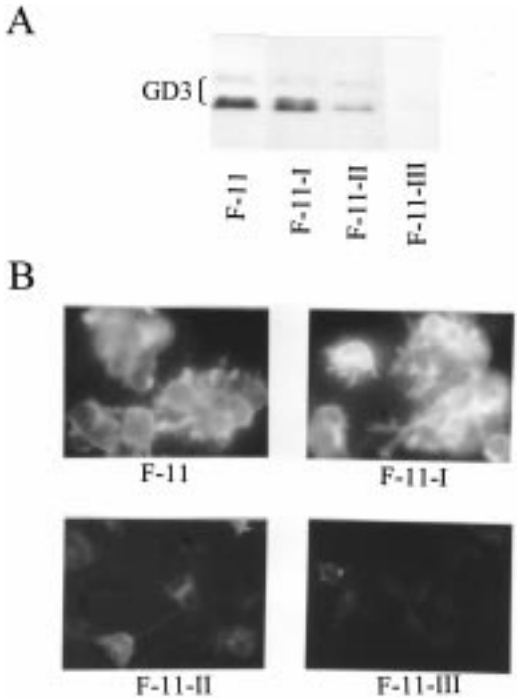

FIGURE 2: Immunoassay of ganglioside GD3 in antisense-transfected F-11 cells. A: Immunostaining of TLC plate with the mouse monoclonal antibody R24 specific to ganglioside GD3. Three micrograms of gangliosides of each sample used in HPTLC overlay immunostaining. The binding of the antibody to ganglioside GD3, which normally appears as two bands, is shown. B: Immunostaining of the cell surface ganglioside GD3 using the mouse monoclonal antibody R24. Magnification: 400×. F-11, untransfected F-11 cells; F-11-I, sense-transfected F-11 cells; F-11-II and F-11-III, antisense-transfected F-11 cells. (Reduced to 60% of original for publication.)

R24. As shown in Figure 2B, binding of the antibody to both antisense-transfected F-11-II and F-11-III cells was almost undetected while the binding to the sense-transfected F-11-I

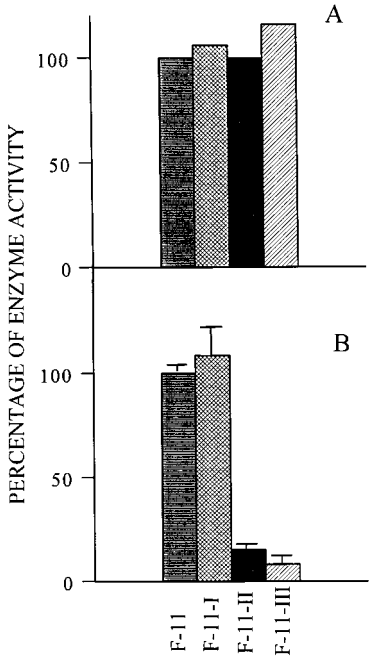

FIGURE 3: Determination of the activities of GM3-synthase and GD3-synthase in antisense-transfected F-11 cells. The activities of GM3-synthase (A) and GD3-synthase (B) in untransfected F-11, sense-transfected F-11-I, and antisense-transfected F-11-II and F-11-III cells were determined as described in Experimental Procedures. The enzyme activities in transfected F-11 cells were presented as percentages (mean $\pm$ SD) of those in the untransfected F-11 cells, which were designated as 100%; 100% was equal to 1.82 $\pm$ 0.12 and 2.42 $\pm$ 0.18 nmol/mg of protein/h for GM3-synthase and GD3-synthase activities, respectively.

cells was the same as that to the parental F-11 cells (Figure 2B). Therefore, the decrease in the concentration of GD3 was a result of the transfected antisense expression vectors targeting the GD3-synthase gene. In addition, the level of OAc-GD3 in both F-11-II and F-11-III cells was also reduced (Table 1), consistent with the diminished expression of its precursor GD3 in these cell lines.

Activities of Sialyltransferases in Transfected Cells. GD3-synthase activity of the transfectants was then determined in order to confirm the specific inhibition of its gene expression. F-11 cells and the transfected F-11 cells were cultured to 70−80% confluence and harvested for enzyme analysis. Aliquots of cell extracts were used to determine the activity of GM3-synthase which was used as a control. The results are shown in Figure 3. The antisense-transfected F-11-II and F-11-III cells had a 85% and 92% reduction in the activity of GD3-synthase, respectively, when compared with the untransfected F-11 cells, while the activity of the enzyme in the sense-transfected F-11-I cells was not suppressed (Figure 3B). On the other hand, there was no difference in the activities of the GM3-synthase in all four cell lines (Figure 3A). Therefore, the expression of the GD3-synthase gene was specifically inhibited by the antisense expression vectors against the GD3-synthase gene.

Morphological and Growth Characteristics of the Antisense-Transfected F-11 Cells. Suppression of the expression of the gangliosides GD3 as well as OAc-GD3 in the antisense-transfected F-11 cells resulted in morphological changes as shown in Figure 4. The antisense-transfected cells appeared to have an elongated shape, a smaller soma size,

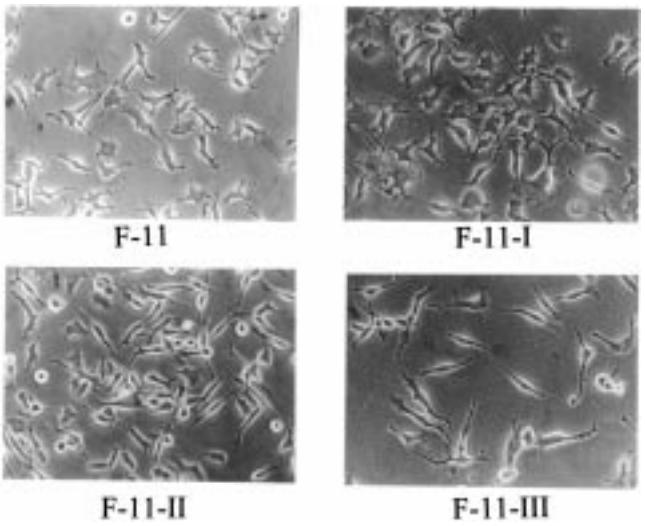

FIGURE 4: Morphological changes in antisense-transfected F-11 cells. Single clones of transfected F-11 cells were selected by G418 and maintained in culture in the medium containing G418 for months. Cells were passaged every week, and fresh medium was added every 3 days. Photograph was taken after cells had been passaged for 3 days. The antisense-transfected F-11-II and F-11-III cells appeared to have elongated cell shape and suppressed neurites and processes. Magnification: 200×. (Reduced to 60% of original for publication.)

and suppressed neurite growth (Figure 4C,D) when compared with the untransfected and the sense-transfected F-11 cells (Figure 4A,B). Quantitative measurement of the neurites was carried out 3 days after cells were passaged. A total of 200 cells for each cell line were counted. The length of neurites of the untransfected F-11 cells and the sense-transfected F-11-I cells was 105.4 ± 12.4 and 106.1 ± 4.2 μm/cell, respectively, while that of the antisense-transfected F-11-II and F-11-III cells was 40.5 ± 6.4 and 50.8 ± 1.1 μm/cell, respectively, indicating that new neurite growth in the antisense-transfected cells was greatly suppressed. Then, the growth properties of the transfected cells were determined. The increase in cell number of the antisense-transfected F-11 cells in the cultures was reduced to about 70% of the control cells (Figure 5B). Similarly, the rate for DNA synthesis in the antisense-transfected F-11 cells was reduced to 80% of that of the control cells when determined by [^{3}H]thymidine incorporation (Figure 5A).

Tumor Growth. We inoculated exponentially growing cells of untransfected F-11 cells, sense-transfected F-11-I cells, and antisense-transfected F-11-II and F-11-III cells into athymic nude mice. In initial experiments, we inoculated 4 × 10^6, 1 × 10^6, 5 × 10^5, or 2.5 × 10^5 cells/site into nude mice. Since F-11 cells exhibit strong tumorigenicity and a very high tumor growth rate in nude mice, 5 × 10^5 cells/site were inoculated into nude mice to follow the progression of xenograft formation. The xenografts became visible in 4−5 days after inoculation and grew to their maximal volume in 2 weeks. Xenografts were found in all sites inoculated with all four types of cells, indicating that the tumor incidence was not changed by the altered ganglioside composition. However, as shown in Figure 6, the tumor growth rate of antisense-transfected F-11-II and F-11-III cells was greatly reduced. The average volume of the tumors formed by the antisense-transfected F-11 cells was two-thirds

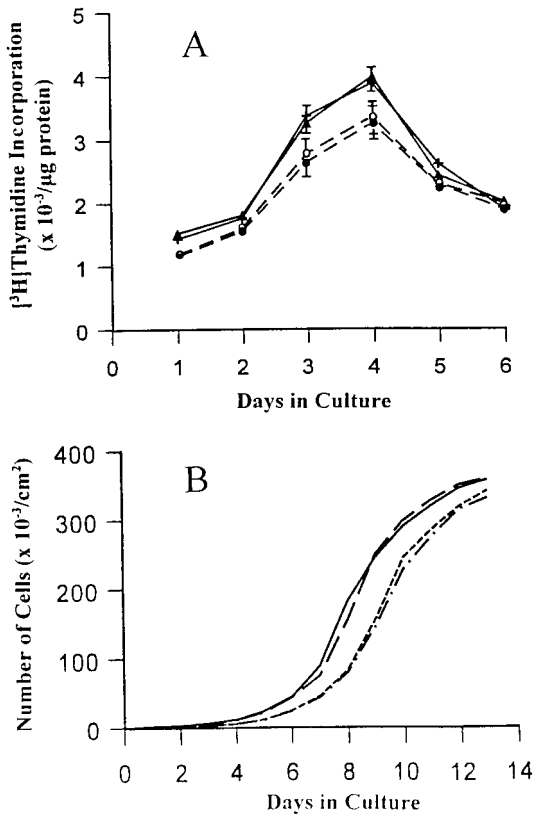

FIGURE 5: Cell growth and DNA synthesis in antisense-transfected F-11 cells. A: Rate of DNA synthesis in F-11 cells (+), sense-transfected F-11-I cells (▲), antisense-transfected F-11-II cells (○), and F-11-III cells (●) was determined by [^{3}H]thymidine incorporation. DNA synthesis was presented as mean values of CPM/μg of protein from three determinations. B: Rate of cell growth was determined by counting the cell number in triplicate dishes on the day as indicated: F-11 cells (—), sense-transfected F-11-I cells (— —), antisense-transfected F-11-II cells (- - -), and F-11-III cells (— · —).

smaller than that formed by the untransfected F-11 cells or the sense-transfected F-11-I cells at day 13 (Figure 6).

DISCUSSION

Since the first report on the changes in glycosphingolipid composition in tumor cells (*52*), extensive studies have been undertaken to elucidate the functional role of gangliosides in the biology of cancer. Among all the gangliosides, GD3 is frequently overexpressed in many tumor cells of neuroectodermal or epithelial origin, such as glioma, medulloblastoma, neuroblastoma, melanoma, head and neck tumors, breast cancer, and teratoma cell lines (*11*, *13*, *29*−*37*). GD3 has also been shown to be characteristic of immature neuroectodermal cells (*53*). In addition, OAc-GD3 has been considered as a tumor-associated antigen in certain types of tumors (*29*, *36*−*39*). The abundance of GD3 and OAc-GD3 in many tumors of neuroectodermal origin should stimulate the development of new approaches for tumor diagnosis, vaccine construction, and tumor immunotherapy (*54*). Monoclonal antibodies directed against GD3 and GD2 have been used in the immunotherapy of patients with metastasizing melanoma and neuroblastoma, respectively (*55*−*59*). However, inadequate response rates have often been observed probably owing to binding of the antibody to free circulating gangliosides coupled with the known poor penetration of

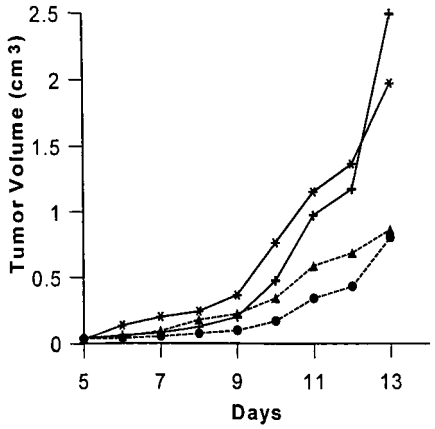

FIGURE 6: Xenograft growth curves of antisense-transfected F-11 cells. Exponentially growing cells (5×10^5) of F-11 (+), sense-transfected F-11-I (*), antisense-transfected F-11-II (▲), and F-11-III (●) were subcutaneously inoculated behind the anterior forelimb of 4-week-old female athymic nude mice. Six mice were inoculated at both sides of the back with each type of cells in two separate experiments. Tumors were measured externally in two dimensions using calipers. Tumor volume was calculated according to the equation: $V = (L \times W^2) \times 0.5$, where L is the length and W the width of a tumor. The mean values and the standard deviations (SD) of the tumor volumes at day 13 were F-11, 1.92 ± 0.13 cm^3; F-11-I, 2.51 ± 0.52 cm^3; F-11-II, 0.87 ± 0.32 cm^3 (vs F-11 or F-11-I, $p \leq 0.001$); and F-11-III, 0.86 ± 0.09 cm^3 (vs F-11 or F-11-I, $p \leq 0.001$).

monoclonal antibody macromolecules in general into tumor tissue (*59*). Another potential approach, as we present in this report, is to target the expression of sialyltransferase genes to alter the expression of tumor-associated gangliosides in cancer cells and thus to suppress tumor growth.

The F-11 cell line is a hybrid of embryonic rat dorsal root ganglion (DRG) cells with mouse neuroblastoma cell line N18TG-2 (*45*). These cells express three major lacto-series gangliosides: i.e., GM3, GD3, and OAc-GD3 (Table 1; *43*). Ganglio-series gangliosides, including GM1, GD1a, GD1b, and GT1b, are also expressed in much lower concentrations. The expression of high levels of lacto-series gangliosides, especially GD3 and OAc-GD3 in F-11 cells, reflects the fact they originated from neuroectodermal cells (*43*). In addition, F-11 cells showed strong tumorigenicity in nude mice. Xenografts became visible in nude mice as early as 4 days after inoculation with even a low dose of F-11 cells (2.5×10^5 cells/site, data not shown) and grew to a volume of over 2 cm^3 within 2 weeks, the maximum size that mice could bear. Therefore, F-11 cells present a useful model for investigating the roles of gangliosides GD3 and/or OAc-GD3 in tumor growth in vivo. In this study, we constructed two antisense expression vectors containing either the 5′ fragment or the entire sequence of the GD3-synthase cDNA. The results demonstrated that ganglioside GD3, as well as its down stream product OAc-GD3, were selectively inhibited in the F-11 cells transfected with either of the two antisense expression vectors. The altered ganglioside composition in the antisense-transfected F-11 cells resulted in morphological changes, characterized by suppressed neurite processes and reduced cell growth properties in culture. This is in contrast with our previous study, in which OAc-GD3 in F-11 cells was specifically destroyed by introducing the influenza virus C hemagglutinin-esterase gene into the cells with a concomitant increase in the level of GD3 (*43*). Similar results were obtained with hamster melanoma cells (*28*). These esterase-transfected cells exhibited elongated neurites or cellular differentiation (*43*). However, indirect evidence obtained by Mendez-Otero and Friedman has suggested that OAc-GD3 may be involved in cell differentiation (*60*). Our present observation that a decrease in both GD3 and OAc-GD3 concentrations resulted in suppressed neurite outgrowth (Figure 3C,D) is consistent with the previous reports (*43*, *60*). On the other hand, it was reported that introduction of the exogenous GD3-synthase cDNA in neuroblastoma cells induced biosynthesis of GD3 and the "b" series gangliosides, resulting in cholinergic differentiation and neurite sprouting (*41*, *61*). Our previous results also showed that a decrease in OAc-GD3 and an increase in GD3 in the F-11 cells were accompanied by cellular differentiation (*28*, *43*). However, a recent report showed that mouse embryonic stem cells with a disrupted GD3-synthase gene undergo neuronal differentiation in the absence of the "b" series gangliosides (*62*). In addition, a high ratio of the "b" series gangliosides to the "a" series gangliosides is often found in proliferating cells and many tumors (*10*, *29*−*35*). Our present data showed that the decrease in the amounts of ganglioside GD3 did not stimulate the proliferation of the neuroblastoma F-11 cells either in vitro or in vivo. How ganglioside GD3 is involved in neuronal differentiation remains to be elucidated.

Another interesting finding in this investigation is that the tumor growth rate in the F-11 cells was greatly reduced after both GD3 and OAc-GD3 had been down-regulated. The reduced tumor growth rate of the antisense-transfected F-11 cells was most likely due to a specific reduction of the ganglioside GD3. This is because inoculation in nude mice of an esterase-transfected F-11 subclone in which ganglioside OAc-GD3 was reduced to 30% while GD3 was increased to 150% (*43*) did not show a reduced tumor growth rate in nude mice. The volume of xenografts of the esterase-transfected F-11 cells and their parental F-11 cells on day 12 after inoculation was 1.41 ± 1.1 and 1.63 ± 0.72 cm^3 (mean value $\pm$ SD), respectively (unpublished data). Similar results have been reported with human melanoma cell lines deficient in synthesis and transport of GD3 (*63*). These mutant cell lines showed more elongated cell morphology, slower growth rates in culture, and lower tumor growth rate and tumor incidence in nude mice than did the parent cell line. This is consistent with our present results except that we did not observe changes in tumor incidence after GD3 synthesis was inhibited. The mechanism involving ganglioside GD3 in tumor growth is unknown. However, it has been suggested that there is a complex relationship between polypeptide growth factors and membrane-bound gangliosides (*64*−*70*). In particular, the production of vascular endothelial growth factor (VEGF), an angiogenic factor, is stimulated by either ganglioside GD3 or TGF-β in human glioma cells, suggesting that both ganglioside GD3 and TGF-β may act as indirect angiogenic factors by stimulating VEGF secretion (*69*). In addition, formation of new vessels induced by angiogenic factors can be stimulated or repressed in the cornea by reduction or enhancement of the GM3:GD3 ratio of tissue gangliosides, respectively (*70*). Our results showed that the decrease in the level of ganglioside GD3 slightly reduced the growth rate of the antisense-transfected F-11 cells in culture (Figure 5) while greatly suppressed the growth of xenografts of the cells in nude mice (Figure 6).

The possibility that GD3 may stimulate angiogenesis merits serious consideration, although other mechanisms exist, such as effects of GD3 on growth factor receptors and/or the cell adhesion.

REFERENCES

1. Hakomori, S. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1741−1747.
2. Robbins, P. W., and Macpherson, I. (1971) *Nature 229*, 569−5670.
3. Chatterjee, S., Sweeley, C. C., and Velicer, L. F. (1975) *J. Biol. Chem. 250*, 61−66.
4. Yu, R. K. (1994) *Prog. Brain Res. 101*, 31−44.
5. Fishman, P. H., Simmons, J. L., Brady, R. O., and Freese, E. (1974) *Biochem. Biophys. Res. Commun. 59*, 292−299.
6. Simmons, J. L., Fishman, P. H., Freese, E., and Brady, R. O. (1975) *J. Cell Biol. 66*, 414−424.
7. Patt, L. M., Itaya, K., and Hakomori, S. I. (1978) *Nature 273*, 379−381.
8. Huberman, E., Heckman, C., and Langenbach, R. (1979) *Cancer Res. 39*, 2618−2624.
9. Xia, X. J., Gu, X. B., Sartorelli, A. C., Yu, R. K., and Santorelli, A. C. (1989) *J. Lipid Res. 30*, 181−188.
10. Ravindranath, M. H., Tsuchida, T., Morton, D. L., and Irie, R. F. (1991) *Cancer (Philadelphia) 67*, 3029−3035.
11. Cheresh, D. A., Varki, A. P., Varki, N. M., SlalIcup, W. B., Levine, J., and Reisfeld, R. A. (1984) *J. Biol. Chem. 259*, 7453−7459.
12. Carubia, J. M., Yu, R. K., Macala, L. J., Kirkwood, J. M., and Varga, J. M. (1984) *Biochem. Biophys. Res. Commun. 120*, 500−504.
13. Ren, S. L. Scarsdale, J. N., Ariga, T., Zhang, Y., and Yu, R. K. (1992) *J. Biol. Chem. 267*, 12632−12638.
14. Hamilton, W. B., Helling, F., Lloyd, K. O., and Livingston, P. O. (1993) *Int. J. Cancer 53*, 566−573.
15. Higashi, H., Hirabaynshi, Y., Fukui, Y., Naiki, M., Matrumoto, M., Ueda, S., and Kato, S. (1985) *Cancer Res. 45*, 3796−3802.
16. Dohi, T., Ohta, S., Hanai, N., Yamaguchi, K., and Oshima, M. (1990) *J. Biol. Chem. 265*, 7880−7885.
17. Ogata, S., Ho, I., Chen, A., Dubois, D., Maklanvky, I., Singhal, A., Hakomori, S.-I., and Itzkowitz, S. H. (1995) *Cancer Res. 55*, 1869−1874.
18. Nusson, O., Brezicka, F. T., Holragren, J., Sorenson, S., Svennetholm, L., Yngvason, F., and Lindholm, L. (1989) *Cancer Res. 46*, 1403−1407.
19. Brezicka, F.-T., Oiling, S., Nusson, O., Bergh, J., Holmgren, J., Sisrenson, S., Yngvason, F., and Lindholm, L. (1989) *Cancer Res. 49*, 1300−1305.
20. Murayama, K., Levery, S. B., Schirrmacher, V., and Hakomori, S.-I. (1986) *Cancer Res. 46*, 1395−1402.
21. Schulz, O., Cheresh, D. A., Varki, N. M., Yu, A., Staffileno, L. K., and Reisfeld, R. A. (1984) *Cancer Res. 44*, 5914−5920.
22. Saito, M., Yu, R. K., and Cheung, N.-K. V. (1985) *Biochem. Biophys. Res. Commun. 127*, 1−7.
23. Wu, Z.-L., Schwartz, F., Seeger, R., and Ladisch, S. (1986) *Cancer Res. 46*, 440−443.
24. Ye, J. N., and Cheung, N.-K. V. (1992) *Int. J. Cancer 50*, 197−201.
25. Hakomori, S. (1985) *Cancer Res. 45*, 2405−2414.
26. Hakomori, S. (1990) *Cancer Res. 56*, 5309−5318.
27. Yogeeswaran, G. (1983) *Adv. Cancer Res. 38*, 289−350.
28. Birklé, S., Ren, S., Slominski, A., Zeng, G., Gao, L., and Yu, R. (1998) *J. Neurochem.* (in press).
29. Marquina, G., Waki, H., Fernandez, L. E., Kon, K., Carr, A., Valiente, O., Perez, R., and Ando, S. (1996) *Cancer Res. 56*, 5165−5171.
30. Portoukalian, J., David, M. J., Shen, X., Richard, M., and Dubreuil, C. (1989) *Biochem. Int. 18*, 759−765.
31. Ren, S.-L., Ariga, T., Scarsdale, N., Zang, Y.-J., Slominski, A., Livingston, P. O., Ritter, C., Kushi, Y., and Yu, R. K. (1993) *J. Lipid Res. 34*, 1565−1572.
32. Yates, A., Thompson, D. K., Boesel, C. P., Albrightson, C., and Hart, R. W. (1979) *J. Lipid Res. 20*, 428−436.
33. Miyatake, T., and Ariga, T. (1981) *Jpn. J. Pediat. Surg. 13*, 509−515.
34. Honsik, C. J., Jung, C., and Reisfeld, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9893−9897.
35. He, X., Wikstrand, C., Fredman, P., Mansson, B., and Svennerholm, L. (1989) *Acta Neuropathol. 79*, 317−325.
36. Dippold, W. G., Lloyd, K. O., Li, L. T., Ikeda, H., Oettgen, H. F., and Old, L. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6114−6118.
37. Nudelman, E., Hakomori, S., Kannagi, R., Levery, S., Yeh, M. Y., Hellstrom, K. E., and Hellstrom, I. (1982) *J. Biol. Chem. 257*, 12752−12756.
38. Thurin, J., Herlyn, M., Hindsgaul, O., Stromberg, N., Karisson, K.-A., Elder, D., Steplewski, Z., and Koprowski, H. (1985) *J. Biol. Chem. 260*, 14556−14563.
39. Cheresh, D. A., Reisfeld, R. A., and Varki, A. P. (1984) *Science 225*, 844−846.
40. Li, R., and Ladisch, S. (1991) *Biochim. Biophys. Acta 1083*, 57−64.
41. Kojima, N., Kurosawa, N., Nishi, T., Hanai, N., and Tsuji, S. (1994) *J. Biol. Chem. 296*, 30451−30456.
42. Zeng, G., Ariga, T., Gu, X.-B., and Yu, R. K. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8670−8674.
43. Ariga, T., Blaine, G. M., Yoshino, H., Qi, Y., Dawson, G., Kanda, T., Zeng, G., Kasama, T., Kushi, Y., and Yu, R. K. (1995) *Biochemistry 34*, 11500−11507.
44. Zeng, G., Gao, L., Ariga, T., and Yu, R. K. (1996) *Biochem. Biophys. Res. Commun. 226*, 319−323.
45. Platika, D., Boulos, M. H., Baizer, L., and Fishman, M. C. *Proc. Natl. Acad. Sci. U.S.A. 82*, 3499−3503.
46. Francel, P. C., Miller, R. J., and Dawson, G. (1987) *J. Neurochem. 48*, 1632−1639.
47. Zeng, G., Gao, L., and Yu, R. K. (1998) *Biochim. Biophys. Acta 1397*, 126−130.
48. Ariga, T., Yoshida, K., Nemoto, K., Seki, M., Miyatani, N., and Yu, R. K. (1991) *Biochemistry 30*, 7953−7961.
49. Gu, X. B., Gu, T. J., and Yu, R. K. (1990) *Biochem. Biophys. Res. Commun. 166*, 387−393.
50. Preuss, U., Gu, X., Gu, T., and Yu, R. K. (1993) *J. Biol. Chem. 268*, 26273−26738.
51. Zeng, G., Donegan, J., Ozer, H. L., and Hand, R. (1984) *Mol. Cell. Biol. 4*, 1815−1822.
52. Hakomori, S. I., and Murakami, W. T. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 254−261.
53. Seyfried, T. N., and Yu, R. K. (1985) *Mol. Cell. Biochem. 68*, 3−10.
54. Ritter, C., Boosfeld, B., Markstein, E., Yu, R. K., Ren, S.-L., Stallcup, W. B., Oettgen, H. F., Old, J., and Livingston, P. O. (1990) *Cancer Res. 50*, 1403−1410.
55. Houghton, N. A., Mintzer, D., Cordon-Cardo, C., Welt, S., Fliegel, B., Vadgann, S., Carswell, E., Melamed, M., Oettgan, F., and Old, L. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1242−1246.
56. Irie, R. F., and Morton, D. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8694−8698.
57. Marx, J. L. (1989) *Science 245*, 813−815.
58. Frost, J. D., Hank, J. A., Reaman, G. H., Frierdich, S., Seeger, R. C., Gan, J., Anderson, P. M., Ettinger, L. J., Cairo, M. S., Blazar, B. R., Krailo, M. D., Matthay, K. K., Reisfeld, R. A., and Sondel, P. M. (1997) *Cancer 80*, 317−333.
59. Dippold, W., Bernhard, H., and Meyer zum Buschenfelde, K. H. (1994) *Eur. J. Cancer 30A*, 137−144.
60. Mendez-Otero, R., and Friedman, J. E. (1996) *Eur. J. Cell Biol. 71*, 192−198.
61. Liu, H., Kojima, N., Kurosawa, N., and Tsuji, S. (1997) *Glycobiology 7*, 1067−1076.
62. Kawai, H., Sango, K., Mullin, K. A., and Proia, R. L. (1998) *J. Biol. Chem. 273*, 19634−19638.
63. Nakano, J., Raj, B. K., Asagami, C., and Lloyd, K. O. (1996) *J. Invest. Dermatol. 107*, 543−548.

64. Hoon, D. S., Okun, E., Banez, M., Irie, R. F., and Morton, D. L. (1991) *Cancer Res. 51,* 5687−5693.
65. Hoon, D. S., Banez, M., Okun, E., Morton, D. L., and Irie, R. F. (1991) *Cancer Res. 51,* 2002−2008.
66. Meuillet, E., Cremel, G., Dreyfus, H., and Hicks, D. (1996) *Glia 17,* 206−216.
67. Bremer, E. G., Schlessinger, J., and Hakomori, S. (1986) *J. Biol. Chem. 261,* 2434−2440.
68. Weis, F. M., and Davis, R. J. (1990) *J. Biol. Chem. 265,* 12059−12066.
69. Koochekpour, S., Merzak, A., and Pilkington, G. J. (1996) *Cancer Lett. 102,* 209−215.
70. Ziche, M., Morbidelli, L., Alessandri, G., and Gullino, P. M. (1992) *Lab. Invest. 67,* 711−715.

BI9906726