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Regulation of glycolipid synthesis in HL-60 cells by antisense oligodeoxynucleotides to glycosyltransferase sequences: Effect on cellular differentiation

(gangliosides/gene expression/cell maturation)

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ABSTRACT **Treatment of the human promyelocytic leukemia cell line HL-60 with antisense oligodeoxynucleotides to UDP-*N*-acetylgalactosamine:β-1,4-*N*-acetylgalactosaminyltransferase (GM2-synthase; EC 2.4.1.92) and CMP-sialic acid:α-2,8-sialyltransferase (GD3-synthase; EC 2.4.99.8) sequences effectively down-regulated the synthesis of more complex gangliosides in the ganglioside synthetic pathways after GM3, resulting in a remarkable increase in endogenous GM3 with concomitant decreases in more complex gangliosides. The treated cells underwent monocytic differentiation as judged by morphological changes, adherent ability, and nitroblue tetrazolium staining. These data provide evidence that the increased endogenous ganglioside GM3 may play an important role in regulating cellular differentiation and that the antisense DNA technique proves to be a powerful tool in manipulating glycolipid synthesis in the cell.**

The composition of gangliosides in cells undergoes dramatic changes during cellular growth, differentiation, and oncogenic transformation, suggesting a specific role of gangliosides in the regulation of these cellular events (3–5). The human promyelocytic leukemia HL-60 cells can be induced to mature along either the monocyte/macrophage or the granulocyte pathway by exposure to different inducers of differentiation. When HL-60 cells are induced to monocytic differentiation by "phorbol 12-*O*-tetradecanoate 13-acetate" (TPA), the activity of CMP-*N*-acetylneuraminic acid:lactosylceramide sialyltransferase (GM3-synthase; EC 2.4.99.9) and the amount of its product GM3 increase (6). Additionally, exogenously administered gangliosides are known to affect the morphological and biochemical properties of cultured cells (7). Administration of GM3 induces HL-60 cells to monocytic differentiation (8) and enhances differentiation of oligodendrocytes cultured from developing rat brain (9). Exogenously added GM3 promotes process formation by cultured bovine oligodendrocytes and stimulates the release of glycoproteins into the medium (10). These results suggest that ganglioside GM3 may be involved in the cellular differentiation process, perhaps by regulating growth factor receptor activities (4, 11, 12). It has not been demonstrated, however, that the effect of exogenously added gangliosides reflects the biological function of endogenous gangliosides.

In the ganglioside biosynthetic pathways, GM3 is a unique intermediate in that it can be further converted to GM2 in the a-series or GD3 in the b-series by the action of UDP-*N*-acetylgalactosamine:β-1,4-*N*-acetylgalactosaminyltransferase (GM2-synthase; EC 2.4.1.92) or CMP-sialic acid:α-2,8-sialyltransferase (GD3-synthase; EC 2.4.99.8), respectively (Fig. 1). It has been demonstrated (13–15) that the developmental changes in brain ganglioside composition are correlated with changes in the activities of GM2-synthase and GD3-synthase. These two enzymes are situated at one of the branching points in the ganglioside biosynthetic pathways and function as key regulatory enzymes in governing ganglioside biosynthesis during cellular growth and differentiation (5). With the availability of the cDNA sequences for human GM2-synthase (16) and GD3-synthase (17), it is in principle possible to modulate the enzyme activities by antisense DNA techniques and change the level of endogenous GM3 for investigating the function of ganglioside GM3 in cells. Here, we report that the accumulation of endogenous GM3 caused by suppressing the expression of the genes coding for the GM2- and GD3-synthases could result in cellular differentiation as judged by the morphological changes and the increase in the number of nitroblue tetrazolium (NBT)-positive cells.

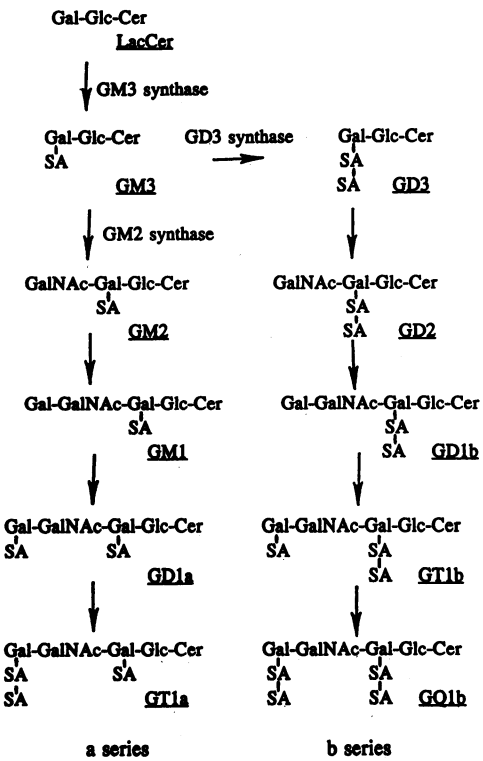

FIG. 1. Ganglioside biosynthetic pathways. SA, *N*-acetylneuraminic acid; Gal, galactose; Glc, glucose; GalNAc, *N*-acetylgalactosamine; Cer, ceramide.

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Abbreviations: NBT, nitroblue tetrazolium; TPA, "phorbol 12-*O*-tetradecanoate 13-acetate." The nomenclature used for gangliosides and glycosyltransferases is based on the abbreviations according to Svennerholm (1) and Basu *et al.* (2).

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MATERIALS AND METHODS

Cell Culture. HL-60 leukemia cells were cultured in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum (56°C for 20 min) supplemented with penicillin (100 international units/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37°C with 5% CO_2/95% air. For the various experiments, cells from exponentially growing cultures were inoculated at 1–2 × 10^5 cells per ml of fresh RPMI 1640 medium containing a reduced concentration (5%) of fetal bovine serum with or without other agents.

Treatment of Cells with Oligodeoxynucleotides, GM3, or TPA. The synthetic oligodeoxynucleotides were complementary to either the sense or the antisense strand of the 21 nucleotides encoding human GM2- or GD3-synthase (16, 17) at the 5′ end and including the initiator codon ATG. They were 5′-GACAGGATGTGGCTGGGCCGC-3′ (GM2-synthase sense oligodeoxynucleotide), 5′-GCGGCCCAGCCACATC-CTGTC-3′ (GM2-synthase antisense oligodeoxynucleotide), 5′-AGAGGGGCCATGGCTGTACTG-3′ (GD3-synthase sense oligodeoxynucleotide), and 5′-CAGTACAGCCATG-GCCCCTCT-3′ (GD3-synthase antisense oligodeoxynucleotide). Cells were grown as described above but in a 24-well culture plate to minimize consumption of oligodeoxynucleotides. In this experiment, cells were resuspended in serum-free RPMI 1640 medium containing the oligodeoxynucleotides at 30 μM at 37°C for 30 min and then fetal bovine serum was added to 5%. Wickstrom *et al.* (18) reported that 1–2% of a 15-base oligomer is taken up and this level remains about the same up to 24 h in HL-60 cells although degradation of oligodeoxynucleotides in RPMI 1640 medium with 10% fetal bovine serum is completed by 8 h. Therefore, to obtain efficient hybridization arrest, the medium containing the oligodeoxynucleotides was changed every 2 days. Ganglioside GM3 was purified from bovine adrenal medulla (19). Treatment of HL-60 cells with GM3 was performed as described by Nojiri *et al.* (8) except that in some experiments RPMI 1640 medium with 5% fetal bovine serum was used to replace the synthetic medium (20). TPA at 3–5 nM was used to induce monocytic differentiation of HL-60 cells as described (21).

Assay of Glycosyltransferase Activities. HL-60 cells were treated with 5 nM TPA, 50 μM GM3, or either sense or antisense oligomers to GM2- and GD3-synthases (each oligomer at 30 μM) for 4 days, respectively. The activities of GM3-synthase, GM2-synthase, and GD3-synthase were determined as described (22–24). Under the assay conditions, the synthase activities were linear with respect to time, substrates, and protein added. The radioactive substrates used in the enzyme assay were CMP-*N*-[^{3}H]acetylneuraminic acid (New England Nuclear, 19.7 mCi/mmol; 1 Ci = 37 GBq) for GM3 and GD3 synthesis and UDP-*N*-acetyl[1-^{14}C]galactosamine (New England Nuclear, 60 mCi/mmol) for GM2 synthesis. After the reactions were completed, the glycolipid products were separated by Sephadex G-50 gel filtration, and their radioactivities were measured by liquid scintillation counting.

Analysis of Glycosphingolipids. HL-60 cells were treated with 5 nM TPA, 30 μM GM2-synthase antisense oligomer, or a combination of 30 μM GM2- and 30 μM GD3-synthase antisense oligomers for 4 days, respectively. HL-60 cells cultured in the absence of agents were used as control. Cells were labeled with 0.25 μCi of [^{14}C]galactose (New England Nuclear, 5.75 mCi/mmol) in 0.5 ml of medium for 24 h before harvest. The acidic and neutral glycolipids were separated on a DEAE-Sephadex column (25). The individual acidic glycolipids were then resolved on a high-performance thin-layer chromatographic plate with the developing system of $CHCl_3$/CH_3OH/aqueous 0.2% $CaCl_2 \cdot 2H_2O$, 50:45:10 (vol/vol). The glycolipid bands containing the ^{14}C label were visualized by exposure to an x-ray film.

Measurement of Cellular Proliferation. Cell numbers were determined by counting the trypan blue-excluding cells in a hemocytometer as described (21). Maturation of HL-60 leukemia cells was evaluated by NBT staining. The test was based on Segal's method (26).

RESULTS AND DISCUSSION

We designed antisense oligodeoxynucleotides complementary to either the sense or the antisense strand of the 21 deoxynucleotides encoding human GM2- or GD3-synthase (16, 17) at the 5′ end, which also included the initiator codon ATG. Treatment of HL-60 cells with the oligodeoxynucleotides antisense to GM2- and/or GD3-synthase sequences resulted in a dra-

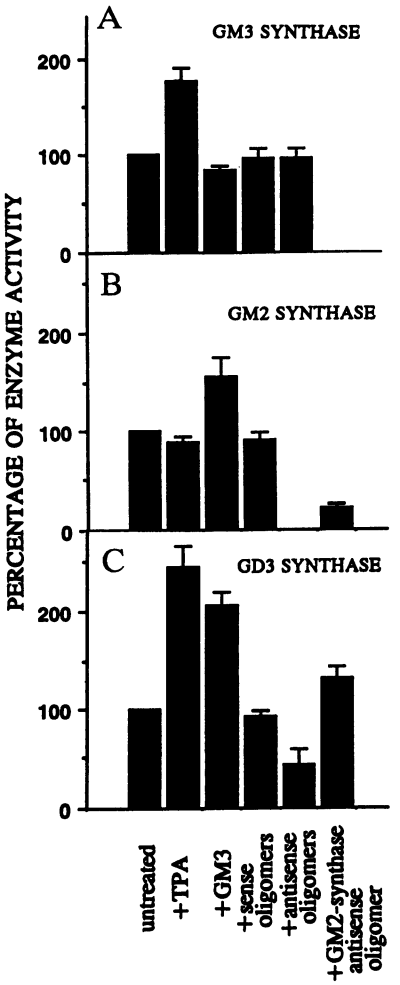

FIG. 2. Effect of TPA, exogenous GM3, or GM2- and GD3-synthase antisense oligodeoxynucleotides on the activities of ganglioside synthases in HL-60 cells. HL-60 cells were treated with 5 nM TPA, 50 μM GM3, 30 μM GM2- or 30 μM GM3-synthase sense oligomers, 30 μM GM2- or 30 μM GD3-synthase antisense oligomers, or 30 μM GM2-synthase antisense oligomer alone for 4 days, respectively. The activities of GM3-synthase (*A*), GM2-synthase (*B*), and GD3-synthase (*C*) in the treated and untreated cells were determined. The enzyme activities were expressed as percentages of the total activities from HL-60 cells cultured in the absence of differentiation-inducing agents. Each value represents the mean ± SD of at least three determinations. One hundred percent was equal to 1.36 ± 0.12 nmol per mg of protein per h, 3.62 ± 0.06 nmol per mg of protein per h, and 1.73 ± 0.18 nmol per mg of protein per h for GM3-, GM2-, and GD3-synthase activities, respectively. The activity of GM2-synthase in the presence of GM2- and GD3-synthase antisense oligomers was not determined (*B*), nor was the activity of GM3-synthase in the presence of GM2-synthase antisense oligomer (*A*).

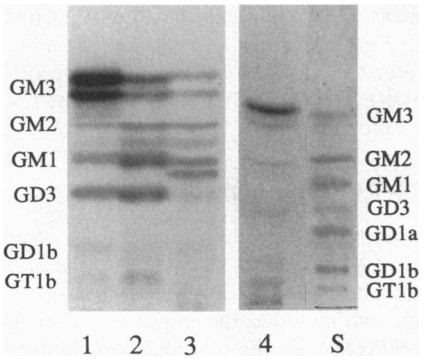

FIG. 3. Autoradiograms of gangliosides isolated from HL-60 cells. HL-60 cells were treated with 5 nM TPA (lane 1), 30 μM GM2-synthase antisense oligomer (lane 2), or a combination of 30 μM GM2- and 30 μM GD3-synthase antisense oligomers (lane 4) for 4 days. HL-60 cells cultured in the absence of agents were used as control (lane 3) (GM1 appeared as a doublet due to heterogeneity in the ceramide portion). Each lane contained the total ganglioside fraction (containing 2700 ± 250 cpm) corresponding to 10^6 cells. Lane S shows a standard ganglioside mixture used as reference and was revealed by the resorcinol hydrochloride reagent.

matic reduction (60–80%) of the activity of GM2- and/or GD3-synthases (Fig. 2). Sense oligodeoxynucleotides to GM2- or GD3-synthase sequences, which served as controls, failed to alter the activity of GM2- or GD3-synthase, and both the sense and the antisense oligomers had no effect on the activity of GM3-synthase. These results clearly indicated that the antisense oligodeoxynucleotides suppressed the expression of GM2- and GD3-synthases specifically and efficiently. Fig. 2 also shows that the activity of GM3-synthase increased when HL-60 cells were treated with TPA, as reported (6, 21).

The composition of HL-60-cell gangliosides after exposure to TPA, the GM2-synthase antisense oligomer alone, or a combination of GM2- and GD3-synthase antisense oligomers was studied by [^{14}C]galactose incorporation experiments. At the end of ^{14}C labeling, gangliosides were extracted from the cells. The ^{14}C incorporation in gangliosides in all samples was 2700 ± 250 cpm per 10^6 cells (equal to 213 ± 19 pmol of radioactive galactose), indicating that the total amounts of newly synthesized gangliosides were not affected by the treatments. However, the distribution of the ^{14}C incorporation, i.e., the ganglioside composition after the treatment with antisense oligomers or TPA, was significantly altered as analyzed by thin-layer chromatography and autoradiography (Fig. 3). The

Table 1. Distribution of radiolabeled gangliosides in HL-60 cells treated with antisense oligodeoxynucleotides to GM2- and GD3-synthase sequences or TPA

	% of total gangliosides			
Ganglio-sides	Untreated cells	Cells treated with antisense oligomer to GM2-synthase	Cells treated with antisense oligomers to GM2- and GD3-synthases	Cells treated with TPA
GM3	24 ± 4	32 ± 5	66 ± 3	66 ± 5
GM2	19 ± 1	12 ± 2	7 ± 1	5 ± 2
GM1	44 ± 5	22 ± 2	3 ± 1	8 ± 3
GD3	5 ± 2	21 ± 1	6 ± 3	13 ± 5
GD1b	2	4	5	3
GT1b	0	8	12	2

Ganglioside composition of each sample from autoradiography was subjected to densitometry. The area under each peak in the densitometer tracing was calculated and total area under all the peaks in each sample was designated as 100%. The percentage therefore refers to the relative amounts of each ganglioside in the sample. Values are percentages (mean ± SD) of three determinations.

ganglioside composition of each sample from autoradiography was then subjected to densitometry and the data obtained from three determinations were expressed as percentages, which are shown in Table 1. Treatment of HL-60 cells with GM2- and GD3-synthase antisense oligomers resulted in an increase in the proportion of GM3, which was accompanied by a drastic decrease in the levels of GM2 and GM1. These data indicate that suppression of GM2- and GD3-synthases by the antisense oligomers could lead to the accumulation of the common substrate GM3 of the two enzymes. A remarkable accumulation of GM3 was also achieved by TPA induction, presumably by stimulation of the activities of GM3-synthase (Figs. 2 and 3 and Table 1). When HL-60 cells were treated with GM2-synthase antisense oligomer alone, both GM3 and GD3 increased (Fig. 3, lane 2, and Table 1). The increase in GD3 concentrations was even more striking than that in GM3. It has been reported that the increase in the amount of GM3 ganglioside during induced HL-60 cell differentiation can be affected by the increased amount of the metabolic precursor LacCer, which in turn may result in an increase of the activity of GM3-synthase (27). Our results showed an increase in the activity of GD3-synthase when ganglioside synthesis in the a-series was blocked (Fig. 2). Besides, there was no significant change in the amount of GD2 and GD1b between the treated and untreated cells, and gangliosides in the b-series were only

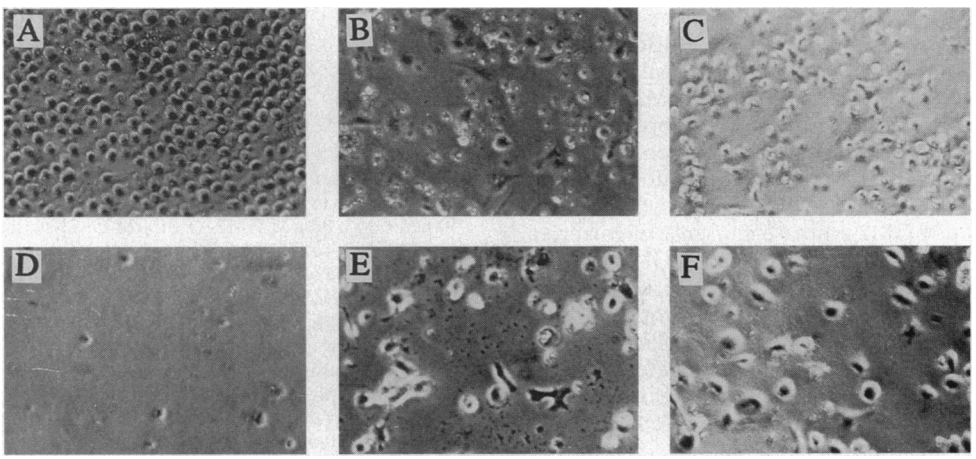

FIG. 4. Morphological changes and surface attachment of HL-60 cells induced by the antisense oligonucleotides. HL-60 cells were cultured in the absence of inducing agents (*A* and *D*) or exposed to 3 nM TPA (*B* and *E*) for 3 days or 30 μM GM2- and 30 μM GD3-synthase antisense oligodeoxynucleotides (*C* and *F*) for 6 days. Photography was taken without washing (*A–C*) or after removal of nonadherent cells by washing with PBS (*D–F*).

minor components in HL-60 cells (Fig. 3, lane 3). The striking increase in the concentration of GD3 is most likely due to the conversion of the accumulated precursor GM3 to GD3 by the increased activity of GD3-synthase. This observation again indicates the effectiveness of employing antisense DNA technology in modulating the activity of GD3- and/or GM2-synthase and thereby achieving the goal of regulating ganglioside expression in HL-60 cells.

Previous studies from several laboratories showed that exposure of HL-60 cells to 5 nM TPA or 50 μM GM3 strongly inhibited cellular growth (21, 28). In the present investigation, however, we found that addition of the GM2- and GD3-synthase antisense oligodeoxynucleotides to HL-60 cells only had a slight effect on cellular growth (ref. 24 and data not shown). Treatment of the cells with TPA for 3 days resulted in cell attachment (>90%) to the surface of the culture plate (Fig. 4 *B* and *E*). About 10% adherent cells were found after incubation of the cells with 30 μM antisense oligomers for 6 days (Fig. 4 *C* and *F*). Adherent cells were not seen in untreated HL-60 cells (Fig. 4 *A* and *D*), cells treated with the corresponding sense oligomers, or cells treated with exogenous GM3, which was added in either a synthetic serum-free medium (20) or a medium containing 5% fetal bovine serum (data not shown). On the other hand, HL-60 cells treated with the antisense oligomers showed morphological changes characteristic of monocytic differentiation (Fig. 4 *C* and *F*). After exposure of HL-60 cells to TPA and the sense and the antisense oligomers to GM2- and GD3-synthases, the degree of differentiation was monitored by measuring the ability of differentiated cells to reduce soluble NBT to insoluble blue-black formazan. As shown in Fig. 5, we found strong NBT-positive cells in the culture containing the GM2- and GD3-synthase antisense oligomers. Significant increases in NBT-reducing activity were observed in cultures treated with either the oligomers or TPA as shown in Table 2.

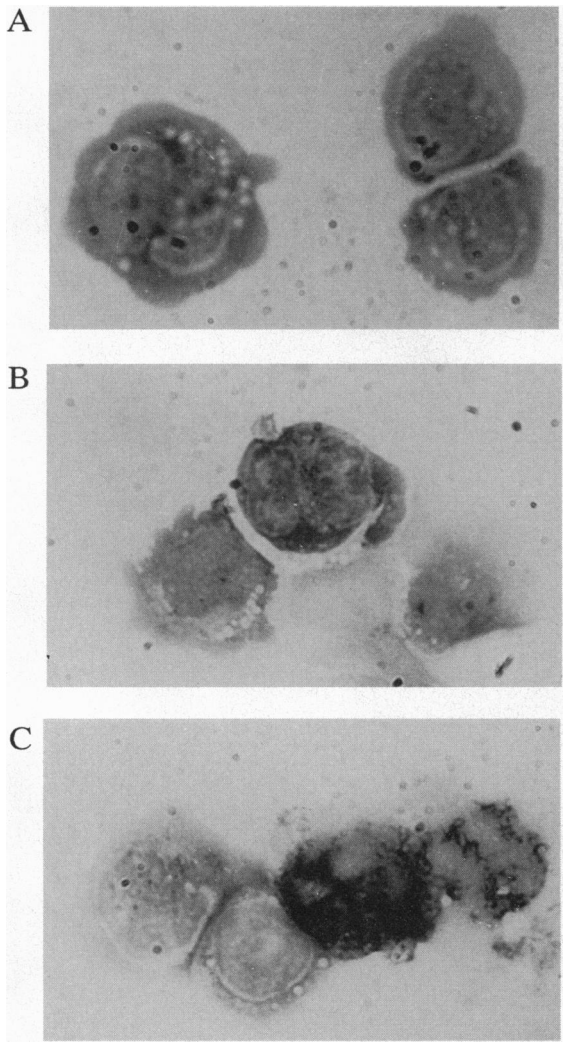

FIG. 5. NBT staining. (*A*) Untreated HL-60 cells. (*B*) HL-60 cells treated with the sense oligomers for 6 days. (*C*) HL-60 cells treated with the antisense oligomers for 6 days.

Table 2. NBT-positive HL-60 cells treated with the oligodeoxynucleotides and TPA

Cells	% NBT-positive cells	
	Day 3	Day 6
Untreated	4.8 ± 0.5	5.5 ± 0.3
Treated with sense oligomer to GM2- and GD3-synthases	4.4 ± 0.8	4.9 ± 1.1
Treated with antisense oligomers to GM2- and GD3-synthases	7.3 ± 0.9*	9.6 ± 1.5†
Treated with TPA	12.4 ± 2.5†	13.3 ± 2.7†

HL-60 cells were treated with the sense oligomers, the antisense oligomers, or TPA. The percentage of NBT-positive cells was ascertained for at least 200 cells by using a hemocytometer. Values are the mean ± SD of three determinations. *, $P < 0.02$; †, $P < 0.01$ vs. untreated cells.

In conclusion, we have induced HL-60 cells to monocytic differentiation with oligodeoxynucleotides antisense to GM2- and GD3-synthase sequences as judged by morphological changes, cell attachment to the surface of culture plates, and NBT staining. These differentiation-associated changes were accompanied by an increase in the concentration of GM3 and a decrease in the concentrations of more complex ganglioseries gangliosides. Since the common feature of the cellular differentiation induced in HL-60 cells by TPA (6, 21), exogenously added GM3 (21, 28), or the antisense oligodeoxynucleotides, observed in the present study, is a remarkable increase in cellular GM3 concentrations, our data suggest that the increase in endogenous GM3 may be associated with the cellular differentiation process. GM3 has been shown to induce oligodendrocyte differentiation (9). Additionally, GM3 is capable of retarding cellular growth by inhibiting epidermal growth factor-dependent receptor autophosphorylation in cultured fibroblasts (11, 12) and modulating several protein kinase systems in cultured brain microvascular endothelial cells (29). Whereas the precise mechanism by which cell surface GM3 may trigger cellular differentiation remains to be defined (30), our present investigation nonetheless demonstrates a strategy to control glycolipid biosynthesis that uses defined antisense DNA techniques. This strategy should prove to be a powerful tool in elucidating the functional role of glycolipids in cellular differentiation and growth.

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