Alteration of glycolipids in *ras*-transfected NIH 3T3 cells

(gangliosides/oncogenes/cholera toxin/cell growth)

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ABSTRACT Glycosphingolipid alterations upon viral transformation are well documented. Transformation of mouse 3T3 cells with murine sarcoma viruses results in marked decreases in the levels of gangliosides GM1 and GD1a and an increase in gangliosylceramide. The transforming oncogenes of these viruses have been identified as members of the *ras* gene family. We analyzed NIH 3T3 cells transfected with human H-, K- and N-*ras* oncogenes for their glycolipid composition and expression of cell surface gangliosides. Using conventional thin-layer chromatographic analysis, we found that the level of GM3 was increased and that of GD1a was slightly decreased or unchanged, and GM1 was present but not in quantifiable levels. Cell surface levels of GM1 were determined by ¹²⁵I-labeled cholera toxin binding to intact cells. GD1a was determined by cholera toxin binding to cells treated with sialidase prior to toxin binding. All *ras*-transfected cells had decreased levels of surface GM1 and GD1a as compared to logarithmically growing normal NIH 3T3 cells. Levels of GM1 and, to a lesser extent, GD1a increased as the latter cells became confluent. Using a monoclonal antibody assay, we found that gangliosylceramide was present in all *ras*-transfected cells studied but not in logarithmically growing untransfected cells. Interestingly, gangliosylceramide appeared when the latter cells became confluent. These results indicated that *ras* oncogenes derived from human tumors are capable of inducing alterations in glycolipid composition.

Viral transformation of cells causes major changes in the glycosphingolipid composition of the cells (for review, see refs. 1 and 2). Two major types of alterations have been observed: (i) glycolipid levels were decreased or lost with precursor accumulation, and (ii) glycolipids that were not present in the untransformed cells were detected. Both of these alterations were observed in mouse 3T3 cells transformed by murine sarcoma viruses (MSV). MSV-3T3 cells exhibited decreased levels of the gangliosides GM1 and GD1a[¶] coincident with a loss of activity of glycosyltransferases involved in their synthesis (4). In addition, the appearance of gangliosylceramide (GgOse₃Cer) was reported in MSV-3T3 cells (5). The underlying mechanism(s) of these alterations is poorly understood.

The active oncogenes of MSV are members of the *ras* oncogene family (6, 7). The human *ras* gene family contains three homologous genes, H-, K- and N-*ras*^{||} (9, 10). They code for a membrane-associated protein of 21 kDa, which possesses guanine nucleotide-binding activity and low GTPase activity. The *ras* protooncogenes are partially homologous with guanine nucleotide-binding (G) proteins involved in membrane signal transduction (11). Although it has been proposed that the N-*ras* protooncogene product may couple certain growth factor receptors to inositol phospho-

lipid breakdown (12), the normal function(s) of the *ras* genes is still unknown.

Using the NIH 3T3 cell transfection assay, *ras* oncogenes have been identified in 10–20% of human tumors (9). Conversion of the *ras* protooncogene to the transforming oncogene most frequently occurs by a mutation at codon 12, 13, or 61 (13). We have investigated the effect of the different *ras* oncogenes (H-, K- and N-*ras*) obtained from human tumors on the glycolipid composition of the *ras*-transfected NIH 3T3 cells. Our purpose was 2-fold: first, to establish that the previously observed changes upon transformation by MSV were due to expression of *ras* oncogenes and, second, to determine if different human *ras* oncogenes could induce similar alterations in glycosphingolipid metabolism.

MATERIALS AND METHODS

Materials. Standard gangliosides were purified as described previously (14). GgOse₃Cer was prepared from GM2 by mild acid hydrolysis. Other neutral glycolipids were from Supelco (Bellefonte, PA). *Vibrio cholerae* sialidase (EC 32.1.18) and cholera toxin were from Calbiochem–Behring. Cholera toxin was iodinated with chloramine-T or Iodo-Gen (Pierce) by Meloy Laboratories (Springfield, VA). ¹²⁵I-labeled protein A and goat anti-mouse IgM were from New England Nuclear. Rabbit anti-mouse IgM was from Miles. Ascites fluid that contained the monoclonal antibody (2D4) to GgOse₃Cer (15) was provided by David Zopf, National Cancer Institute. The antibody was partially purified by chromatography on SP-Trisacryl M (LKB) (16). Thin-layer chromatography plates (high-performance silica gel 60 and aluminum-backed silica gel 60) were from Merck. The high-performance silica gel 60 plates used for anti-GgOse₃Cer overlays were from Baker.

Cells and Cell Culture. NIH 3T3 cell lines transformed by human H-*ras* oncogenes with position 12 or 61 activating lesions were designated T24 (17) and T323-20-1 (18), respectively. 62-2A-1-6 (19) and E25-4-1 (20) were NIH 3T3 transfectants containing human N- and K-*ras* oncogenes. An NIH 3T3 cell line transfected with the human c-H-*ras* protooncogene under murine leukemia virus long terminal repeat influence was designated Proto. This transfectant expressed high levels of the normal H-*ras* p21 product and exhibited the transformed phenotype (S.A.A., unpublished data). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Exponentially growing cultures were utilized,

Abbreviations: MSV, murine sarcoma virus; ¹²⁵I-cholera toxin, ¹²⁵I-labeled cholera toxin; GgOse₃Cer, gangliosylceramide.

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[¶]Glycosphingolipids are designated according to the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (3).

^{||}The recommended designations for these human genes are *HRAS*, *KRAS*, and *NRAS* (8).

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except in some experiments where confluent cultures of control NIH 3T3 cells also were utilized.

Isolation of Glycolipids. Cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS), detached by incubating in PBS/1 mM EDTA/1 mM EGTA, pH 7.4, for 10 min at 4°C, and collected by centrifugation. The cell pellet was suspended in water, and a portion was taken for protein determination (21). Ten milligrams of cell protein was extracted with 4 vol of chloroform/methanol (1:2, vol/vol). Gangliosides were isolated, resolved on high-performance silica gel 60 plates, visualized with resorcinol, and quantified by scanning densitometry (22). Neutral glycolipids in the solvent A fraction from the DEAE-Sephadex column (22) were purified either by acetylation and Florisil column chromatography (23) or by Unisil column chromatography, methanolic alkaline hydrolysis, and phase partitioning (24).

Cholera Toxin Overlay of Gangliosides. A newly developed overlay method was used to detect small amounts of GM1 and GD1a (25). Briefly, gangliosides from 0.5 mg of cell protein were separated on aluminum-backed silica gel sheets. The plate was fixed with 0.4% poly(isobutyl methacrylate) in hexane, dried in air, and overlaid with sialidase (0.1 unit/ml) for 2 hr at 25°C. After washing, the chromatogram was overlaid with ^{125}I -labeled cholera toxin (^{125}I -cholera toxin) (0.5 $\mu\text{Ci}/\text{ml}$; 1 Ci = 37 GBq) for 30 min at 4°C, washed, dried and exposed to Kodak X-Omat AR-2 film.

Analysis of GgOse₃Cer. The glycolipid was identified by immunostaining of thin-layer chromatograms (26). Briefly, neutral glycolipids from 2 mg of cell protein were separated on a high-performance plate, which was then fixed with 0.2% poly(isobutyl methacrylate) in hexane and dried in air. To block nonspecific binding, the chromatogram was soaked in PBS/5% bovine serum albumin and was drained. The chromatogram then was overlaid sequentially with partially purified anti-GgOse₃Cer (diluted 1:100), rabbit anti-mouse IgM (diluted 1:500), and ^{125}I -labeled protein A (0.6 $\mu\text{Ci}/\text{ml}$). The chromatogram was washed with PBS, dried, and exposed to x-ray film. GgOse₃Cer was also quantified by a solid-phase radioimmunoassay (27). Briefly, neutral glycolipids from 0.5 mg of cell protein were adsorbed to the wells of a microtiter plate. After being blocked with 0.25% gelatin, the wells were sequentially incubated with ascites fluid containing anti-GgOse₃Cer (final dilution 1:500) and ^{125}I -labeled anti-mouse IgM (0.037 μCi). After extensive washing, the radioactivity bound to the wells was determined with a Beckman γ -4000 counter. Both diluted antibodies were filtered through a Millex GV filter.

Cholera Toxin Binding to Cells. Cells were grown in 25-cm² flasks and, where indicated, were treated with 0.02 unit of sialidase in 5 ml of Dulbecco's modified Eagle's medium containing 50 mM Hepes (pH 7.2) at 37°C for 2 hr. The cells were released from the flasks by incubation with 2 ml of

0.02% collagenase in PBS for 10 min at 37°C, followed by the addition of 2 ml of 0.01% trypsin in PBS/1 mM EDTA/1 mM EGTA and subsequent incubation for 10 min at 37°C. Proteolysis was stopped by the addition of 5 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Following centrifugation, the pellets were suspended in a Tris-buffered salts solution (28) and were centrifuged again. After suspension in the same buffer, a portion was removed for cell counting using a Coulter Counter (model Z_{B1}). The assay for cholera toxin binding was as described (28). ^{125}I -cholera toxin (0.1 μCi , 10 nM) was incubated with 2.5×10^4 cells in 0.2 ml of the Tris buffer containing 0.1% bovine serum albumin for 45 min at 25°C. Nonspecific binding was determined by adding 0.2 μM unlabeled cholera toxin.

RESULTS

Transfection of NIH 3T3 cells with different *ras* oncogenes as well as the c-H-*ras* protooncogene produced significant alterations in ganglioside composition (Table 1 and Fig. 1). Each of the gangliosides appeared as a doublet, presumably due to differences in the ceramides (29). As compared to untransfected logarithmically growing NIH 3T3 cells, GM3, the least complex ganglioside, was increased 1.5- to 2.3-fold in all transfected cell lines. When the untransfected NIH 3T3 cells became contact-inhibited, substantial changes in gangliosides were also observed, the most noticeable being the increases in GM1 and GD1a. As these two more complex gangliosides were present in only trace amounts in some of the cell lines, alternative procedures were used to identify and quantify them.

By using a cholera toxin overlay technique, the presence of GM1 in the cells was verified (Fig. 2). When the chromatogram was directly overlaid with ^{125}I -cholera toxin, only GM1 was detected in untransfected (Fig. 2A) or transfected (not shown) cells. Treatment of the chromatogram with sialidase prior to the cholera toxin overlay indicated that GD1a was the only ganglioside that was converted to GM1 by the enzyme (Fig. 2B). Thus, both GM1 and GD1a were identified in all of the transfected cells (Fig. 2B). Cholera toxin binding in the overlay technique is generally not quantitative, but decreases in GM1 and GD1a were observed in the *ras*-transfected cells as compared to the untransfected cells.

In order to quantify the levels of GM1 and GD1a on the cell surface, the cells were assayed for their ability to bind ^{125}I -cholera toxin before and after sialidase treatment. Releasing the cells from the culture flask with either chelating agents or collagenase/trypsin as described in *Materials and Methods* had no effect on the amount of toxin that bound (data not shown). The latter method allowed a more accurate determination of cell number. Toxin binding was linear with

Table 1. Ganglioside composition of untransfected and *ras*-transfected NIH 3T3 cells

Cell line	<i>ras</i> gene	GM3		GM2		GM1		GD1a		Total, nmol/mg
		nmol/mg	%	nmol/mg	%	nmol/mg	%	nmol/mg	%	
62-2A-1-6	N	2.33 ± 0.10	81.2	0.10 ± 0.06	3.5	0.03 ± 0.03	1.0	0.41 ± 0.05	14.3	2.87
E25-4-1	K	1.53 ± 0.39	78.9	0.21 ± 0.06	10.8	0.03 ± 0.01	1.5	0.17 ± 0.02	8.8	1.94
T24	H	2.12 ± 0.11	67.7	0.64 ± 0.06	20.4	0.09 ± 0.05	2.9	0.28 ± 0.03	8.9	3.13
T323-20-1	H	1.61 ± 0.09	83.9	0.18 ± 0.04	9.4	0.03 ± 0.02	1.6	0.10 ± 0.05	5.2	1.92
Proto	H	2.04 ± 0.11	62.0	1.12 ± 0.18	34.0	0.03 ± 0.02	0.9	0.10 ± 0.06	3.0	3.29
NIH 3T3										
Logarithmically growing*	—	1.00 ± 0.17	51.5	0.54 ± 0.09	27.8	0.08 ± 0.01	4.1	0.32 ± 0.10	16.5	1.94
Contact-inhibited	—	1.39 ± 0.15	41.6	0.89 ± 0.05	26.6	0.21 ± 0.05	6.3	0.85 ± 0.11	25.5	3.34

Gangliosides were isolated from the cells, separated on high-performance silica gel plates in chloroform/methanol/0.25% CaCl_2 in water (60:35:8, vol/vol), visualized with resorcinol, and quantified by scanning densitometry. Values are the means ± SD (three to six determinations) and are given as nmol of ganglioside per mg of protein. %, percent of total ganglioside.

*Cells were harvested at a density of 1.7×10^4 cells per cm².

FIG. 1. Thin-layer chromatography of gangliosides from *ras*-transfected and untransfected NIH 3T3 cells. Gangliosides were extracted from 2 mg of cellular protein and were visualized with resorcinol. Lanes: 1, T24; 2 and 5, standard gangliosides (1 nmol of each; from top to bottom: GM3, GM2, GM1, GD1a); 3, Proto; 4, T323-20-1; 6, 62-2A-1-6; 7, E25-4-1; 8, logarithmically growing NIH 3T3 cells (1.7×10^4 cells per cm^2); 9, contact-inhibited NIH 3T3 cells.

increasing cell number up to 10^5 cells. As shown in Table 2, all of the *ras*-transfected cells bound substantially less cholera toxin than did rapidly growing untransfected NIH 3T3 cells and thus have less cell surface GM1. After sialidase treatment, the amount of toxin binding increased up to 8-fold. By taking the difference in binding between untreated and sialidase-treated cells, the levels of cell surface GD1a could be calculated. Again, all of the transfected cell lines expressed less GD1a than the untransfected NIH 3T3 cell line. In agreement with thin-layer chromatographic analysis (Fig. 1 and Table 1), the expression of cell surface GM1 and GD1a increased substantially as the NIH 3T3 cells became contact inhibited (Table 2).

Alterations in the neutral glycosphingolipid GgOse₃Cer also were observed in Kirsten sarcoma virus-transformed BALB/c 3T3 cells (5) and *ras*-transfected NIH 3T3 cells (30). By using an antibody to GgOse₃Cer, the identity of GgOse₃Cer was verified by the overlay procedure (Fig. 3). GgOse₃Cer was present in all *ras*-transfected cells, high density logarithmically growing cells, and contact-inhibited untransfected cells. The relative amount of GgOse₃Cer in the cells was determined by antibody binding to neutral glycolipids adsorbed to 96-well microtiter plates. Both anti-GgOse₃Cer and ^{125}I -labeled anti-IgM were at saturating concentrations, and the assay was linear from 1 to 200 pmol (data not shown). As shown in Table 3, the quantitative data confirmed the overlay technique, with the exception of the logarithmically growing untransfected cells. GgOse₃Cer was observed in all

FIG. 2. Detection of gangliosides from *ras*-transfected and untransfected cells by ^{125}I -cholera toxin overlay. Gangliosides extracted from 0.5 mg of cellular protein were separated on aluminum-backed silica gel plates, which were developed in chloroform/methanol/0.25% CaCl_2 in water (60:35:8, vol/vol). Following fixation with poly(isobutyl methacrylate), the plates were overlaid with ^{125}I -cholera toxin (A) or sialidase followed by ^{125}I -cholera toxin (B). Lanes: 1, logarithmically growing NIH 3T3 cells (1.7×10^4 cells per cm^2); 2, contact-inhibited NIH 3T3 cells; 3 and 9, standard gangliosides (0.5 nmol each, from top to bottom as marked by arrows: GM3, GM2, GM1, GD1a); 4, 62-2A-1-6; 5, E25-4-1; 6, T24; 7, T323-20-1; 8, Proto.

Table 2. Binding of cholera toxin to control and sialidase-treated cells

Cell line	Cholera toxin bound, pmol per 10^5 cells		
	Without sialidase	With sialidase	Difference*
62-2A-1-6	0.38 ± 0.07	2.15 ± 0.20	1.77
E25-4-1	0.24 ± 0.07	1.24 ± 0.23	1.00
T24	0.57 ± 0.06	2.76 ± 0.21	2.19
T323-20-1	0.13 ± 0.04	1.08 ± 0.15	0.95
Proto	0.09 ± 0.01	0.56 ± 0.09	0.47
NIH 3T3			
Logarithmically growing†	1.00 ± 0.06	4.00 ± 0.17	3.00
Contact-inhibited	2.40 ± 0.20	6.53 ± 0.20	4.13

Binding of ^{125}I -cholera toxin to control and sialidase-treated cells was determined, and the values given are the means $\pm$ SD of four to six experiments (three determinations per experiment).

*Difference represents the amount of GD1a on the cell surface (see text).

†Cells were harvested at a density of 1.7×10^4 cells per cm^2 .

ras-transfected cell lines and in the contact-inhibited NIH 3T3 cells but not in the rapidly growing untransfected NIH 3T3 cells. The apparent discrepancy between the two techniques for the logarithmically growing untransfected cells may be due to differences in cell density. The cells used for the overlay were harvested at a density of 3.2×10^4 cells per cm^2 , whereas those for the quantitative assay were at 1.7×10^4 cells per cm^2 . GgOse₃Cer may be expressed as a function of cell density with the initiation of synthesis prior to contact inhibition (6×10^4 cells per cm^2). The contact-inhibited cells and the T24-transfected cells had the highest levels of GgOse₃Cer.

DISCUSSION

In the present paper, we demonstrate that NIH 3T3 cells transfected with human *ras* oncogenes express less of the complex gangliosides GM1 and GD1a on their cell surfaces than rapidly growing untransfected NIH 3T3 cells. Cells transfected with a c-H-*ras* protooncogene exhibited an even greater reduction in these two gangliosides. Thus, the expression of either a mutated *ras* gene derived from a human tumor (H-, K- and N-*ras*) or over-expression of a protooncogene appears to be sufficient to alter ganglioside metabolism in NIH 3T3 cells. Similar alterations have been observed in mouse 3T3 cells transformed by MSV (4). It is now known that the oncogenes v-K-*ras* and v-H-*ras* in these retroviruses are homologous with cellular oncogenes derived from human tumors (9).

FIG. 3. Immunostaining of neutral glycolipids from untransfected and *ras*-transfected NIH 3T3 cells by antibody to GgOse₃Cer. Neutral glycolipids extracted from 2 mg of cellular protein were separated on glass-backed silica gel plates, which were developed in chloroform/methanol/water (60:35:8, vol/vol). Following fixation with poly(isobutyl methacrylate) and blocking with bovine serum albumin, the plate was overlaid sequentially with anti-GgOse₃Cer, anti-IgM, and ^{125}I -labeled protein A. Lanes: 1, GgOse₃Cer standard; 2, 62-2A-1-6; 3, E25-4-1; 4, T24; 5, T323-20-1; 6, Proto; 7, high density logarithmically growing NIH 3T3 cells (3.2×10^4 cells per cm^2); 8, contact-inhibited NIH 3T3 cells. Migration of other standard neutral glycolipids is indicated by arrows from top to bottom: lactoceramide (LacCer), globotriaosylceramide (GbOse₃Cer), globotetraosylceramide (GbOse₄Cer).

Table 3. Concentration of GgOse₃Cer in *ras*-transfected cells

Cell line	GgOse ₃ Cer, pmol per mg of protein
62-2A-1-6	1.84 ± 0.14
E25-4-1	2.04 ± 0.24
T24	5.06 ± 0.52
T323-20-1	2.88 ± 0.16
Proto	3.84 ± 0.02
NIH 3T3	
Logarithmically growing*	ND
Contact-inhibited	5.10 ± 0.42

Neutral glycolipids from 0.5 mg of cell protein were mixed with phosphatidylcholine and cholesterol and were adsorbed to wells of a microtiter plate. Following blocking with PBS/0.25% gelatin, the wells were incubated sequentially with ascites fluid containing antibody to GgOse₃Cer and ¹²⁵I-labeled antibody to IgM. After washing, the radioactivity bound to the wells was determined. The amount of GgOse₃Cer was quantitated by comparison with a standard curve, obtained by adsorbing standard GgOse₃Cer to the wells. Values are the means ± range of two experiments (four determinations per experiment). Nonspecific binding to wells coated with just phosphatidylcholine and cholesterol has been subtracted. ND, not detectable.

*Cells were harvested at a density of 1.7×10^4 cells per cm².

In order to identify and accurately measure the levels of cell surface GM1 and GD1a, we used cholera toxin, which is highly specific for GM1 (31) in combination with sialidase, which converts GD1a to GM1 (25, 28). In this regard, an earlier study examined the gangliosides of NIH 3T3 cells transfected with the bladder carcinoma EJ (T24) oncogene (*H-ras*) and the lung carcinoma LX-1 (*K-ras*) (30). Based on conventional thin-layer chromatographic analysis, the authors reported no change in ganglioside composition following transfection. They also were unable to detect any GM1 in untransfected NIH 3T3 cells.

In this same study, GgOse₃Cer was found to increase in the cell lines derived from the *ras*-transfected NIH 3T3 cells (30). We also found GgOse₃Cer to be present in all of the *ras*-transfected NIH 3T3 cells used in our study but not in the rapidly growing untransfected NIH 3T3 cells. Previously, GgOse₃Cer was observed to appear in Kirsten sarcoma virus-transformed BALB/c 3T3 cells (5), due to the *v-K-ras* oncogene. GgOse₃Cer has been purported to be a tumor-specific glycolipid antigen, and antibodies to GgOse₃Cer have been used to protect mice against lymphoma cells bearing the glycolipid (32). Thus, we were rather surprised to find large amounts of GgOse₃Cer in contact-inhibited NIH 3T3 cells. We are unaware of any previous studies that have examined contact-inhibited mouse 3T3 cells for this glycolipid. It does not appear that the presence of GgOse₃Cer is related to cell growth as the nondividing NIH 3T3 cells and the growing *ras*-transfected cells had similar amounts of this glycolipid.

In contrast, the changes in cell surface GM1 and GD1a did correlate with cell growth. The levels were reduced in the growing *ras*-transfected cells and increased as the untransfected cells became contact-inhibited. A role for gangliosides in regulating cell growth has been suggested. Exogenous gangliosides were found to inhibit cell growth and reduce the tyrosine kinase activity associated with certain growth factor receptors (33, 34). More recently, the B or binding subunit of cholera toxin was observed to stimulate resting rat thymocytes (35) and quiescent mouse 3T3 cells (36) to divide. In addition, the B subunit inhibited growth of rapidly dividing 3T3 cells as well as the same *ras*-transfected NIH 3T3 cells used in the present study. It was suggested that gangliosides could function as bimodal regulators of cell growth signals (35, 36). Based on our present results, the ability of a

ganglioside to modulate positive or negative signals may depend on its density on the cell surface. In growing cells with a lower density of surface gangliosides, growth factor receptors may be more responsive to their effectors, whereas in contact-inhibited cells with a higher ganglioside density, the cells may be less responsive.

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