Reduced Trisialoganglioside Synthesis in Chemically but not mos-transformed Mouse Epidermal Cells

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ABSTRACT

A specific decrease in the net de novo synthesis ([1-14C]-glucosamine incorporation) of cell surface trisialoganglioside (GT) occurs in preneoplastic mouse JB6 epidermal cells in response to tumor-promoting phorbol esters, mezerein, or epidermal growth factor, all of which promote neoplastic transformation in JB6 cells, but not in response to the bladder promoter sodium cyclamate, a nonpromoter in JB6 cells. The ganglioside showing elevated synthesis after mezerein or epidermal growth factor exposure is monosialoganglioside 1, whereas disialoganglioside 1b synthesis is elevated after phorbol ester exposure. Primary mouse epidermal cells and putatively initiated epidermal cell lines selected for their resistance to induction of terminal differentiation by high calcium are resistant to promotion of anchorage-independent transformation by 2-week exposure to 12-O-tetradecanoylphorbol-13-acetate. In both cell types, little or no decrease in GT synthesis occurs in response to short-term 12-O-tetradecanoylphorbol-13-acetate exposure, thus extending further our previous observation that this GT response is restricted to promotable cells. A decreased synthesis of GT also occurs consistently in cell lines transformed by 12-O-tetradecanoylphorbol-13-acetate or N-methyl-N-nitro-nitrosoguanidine as compared with their nontransformed counterparts but not in cell lines transformed by a cloned integrated murine sarcoma provirus containing the oncogenic sequence v-mos. Thus, reduced cell surface GT synthesis may be important both in the induction and in the maintenance of the chemically transformed but not viral oncogene mos-transformed phenotype in mouse epidermal cells.

INTRODUCTION

Gangliosides are integral components of plasma membrane which are involved in regulating cellular growth and differentiation (16). When exogenously incorporated into normal or virus-transformed cells, certain gangliosides reduce or delay cell growth (2), decrease saturation density (19, 20), or modulate the binding of growth factors to cell surface receptors (4). In neuronal cells, gangliosides induce axon formation (24). We have reported previously that JB6 mouse epidermal cells when treated with the tumor promoter TPA (2) showed (a) promotion of anchorage independence and tumorigenicity (6, 7) and (b) a pronounced decrease in the de novo synthesis of GT (presumably GT1b) as measured by [1-14C]glucosamine incorporation (28). The reduced GT synthesis occurred with an onset of 4 hr and a maximal effect at 24 hr of TPA exposure (28). Both events were antagonized by the antipromoter retinoic acid (8, 28). This decrease occurred consistently in TPA-sensitive promotable clonal cell lines but not in T~ variants (29), suggesting that this GT synthesis decrease is required for induction of transformation in JB6 cells. Further support for this possibility was obtained from the observation that, when GT was added to cells treated with TPA, induction of anchorage-independent transformation was inhibited (29). This inhibitory activity was shown by GT but not by any other sialo-glycoconjugates tested (29). In this paper, we report the results of our experiments to determine (a) whether the GT response generalizes to other promoters of transformation, (b) whether the lack of GT response generalizes to other independently derived promotion-insensitive cells, and (c) whether reduced GT synthesis extends to neoplastically transformed cells.

MATERIALS AND METHODS

Cells. Promotable JB6 mouse epidermal cells (7, 10) were grown as described previously (28). The development and characterization of JB6 transformed lines are described by Colburn et al. (5, 7, 11). Primary epidermal cells from 1- to 3-day-old BALB/c mice were cultured in low-calcium (0.07 mM CaCl2 containing) medium 199 obtained from NIH, as described by Yuspa et al. (33). Putatively initiated epidermal cell lines, LC7 (Footnote 3, Ref. 17) and 308 (32) that were resistant to induction of terminal differentiation by a shift from low- to high-calcium-containing medium, were kindly supplied by Dr. Henry Hennings and Dr. Stuart Yuspa of the National Cancer Institute. Line 308 was derived after in vivo exposure to 7,12-dimethylbenz(a)anthracene and line LC7 after in vitro exposure to sodium cyclamate, a P~ cell line (12), was used as recipient for transfecting pMl v-mos DNA kindly supplied by Dr. Donald Blair of the National Cancer Institute. The pMl, a pBR 322 subclone of lambda (30), is a cloned Moloney sarcoma virus DNA with terminally redundant sequences. Transfection was carried out using calcium phosphate DNA precipitates and carrier DNA from P" cells (9). The v-mos DNA-tranfected cells showed anchorage-independent growth in 0.33% agar. These colonies were individually plucked from agar, and 2 of them were grown as clonal lines designated C125/pm1-2 and C125/pm1-6.

Chemicals. Mezerein and TPA were obtained from Chemical Carcinogenesis, Eden Prairie, MN. EGf was kindly supplied by Dr. Bruce Magun of the University of Arizona. Sodium cyclamate was purchased from Sigma Chemical Co., St. Louis, MO. Ganglioside standards were purchased from Supelco, Inc., Bellefonte, PA, and Silica Gel G thin-layer chromatography plates were from Brinkman Instruments, Inc., Westbury, NY. [1-14C]Glucosamine (55 mCi/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, IL. All solvents used were of analytical grade from J. T. Baker Chemical Co., Phillipsburg, NJ. Gom was a generous gift of Dr. Robert Yu of Yale University. HPTLC plates were purchased from E. Merck for the identification of unknown gangliosides. The identification of GT was based on HPTLC separation using 2 solvent systems (26).

Analysis of Ganglioside Synthesis. Cells were treated with TPA or other promoters at indicated concentrations for 24 hr during logarithmic growth with [1-14C]glucosamine (5 μCi/ml) labeling for the terminal 4 hr.

1 To whom requests for reprints should be addressed.

2 H. Hennings, personal communication.
Ganglioside Synthesis in Transformed Cells

Table 1
Effect of nonphorbol promoters on [1-14C]glucosamine incorporation into gangliosides of JB6 cells

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Mezerein (10 ng/ml)</th>
<th>EGF (1.0 ng/ml)</th>
<th>Sodium cyclamate (0.1%, 0.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G(T)</td>
<td>18.0</td>
<td>18.0</td>
<td>20.0</td>
</tr>
<tr>
<td>G(010)</td>
<td>16.0</td>
<td>18.0</td>
<td>26.0</td>
</tr>
<tr>
<td>G(011)</td>
<td>15.0</td>
<td>16.0</td>
<td>24.0</td>
</tr>
<tr>
<td>G(012)</td>
<td>14.0</td>
<td>16.0</td>
<td>22.0</td>
</tr>
<tr>
<td>G(013)</td>
<td>13.0</td>
<td>16.0</td>
<td>20.0</td>
</tr>
<tr>
<td>G(014)</td>
<td>12.0</td>
<td>15.0</td>
<td>18.0</td>
</tr>
<tr>
<td>G(015)</td>
<td>11.0</td>
<td>14.0</td>
<td>16.0</td>
</tr>
<tr>
<td>G(016)</td>
<td>10.0</td>
<td>13.0</td>
<td>14.0</td>
</tr>
<tr>
<td>G(017)</td>
<td>9.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>G(018)</td>
<td>8.0</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>G(019)</td>
<td>7.0</td>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>G(020)</td>
<td>6.0</td>
<td>9.0</td>
<td>6.0</td>
</tr>
<tr>
<td>G(021)</td>
<td>5.0</td>
<td>8.0</td>
<td>4.0</td>
</tr>
<tr>
<td>G(022)</td>
<td>4.0</td>
<td>7.0</td>
<td>2.0</td>
</tr>
<tr>
<td>G(023)</td>
<td>3.0</td>
<td>6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>G(024)</td>
<td>2.0</td>
<td>5.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>G(025)</td>
<td>1.0</td>
<td>4.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Total gangliosides</td>
<td>100.0 (4432)*</td>
<td>100.0 (2552)</td>
<td>100.0 (1521)</td>
</tr>
</tbody>
</table>

Table 2
Effect of TPA exposure on ganglioside synthesis in mouse epidermal primary cells and JB6 promotable mouse epidermal cell lines

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Primary culture</th>
<th>JB-6 promotable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>TPA treated</td>
<td>TPA treated</td>
</tr>
<tr>
<td>G(T)</td>
<td>36.0</td>
<td>38.0</td>
</tr>
<tr>
<td>G(010)</td>
<td>46.0</td>
<td>37.3</td>
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<tr>
<td>G(011)</td>
<td>2.6</td>
<td>11.8</td>
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<tr>
<td>G(012)</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>G(013)</td>
<td>9.3</td>
<td>5.3</td>
</tr>
<tr>
<td>G(014)</td>
<td>2.2</td>
<td>3.6</td>
</tr>
<tr>
<td>G(015)</td>
<td>2.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Total gangliosides</td>
<td>100.0 (4473)*</td>
<td>100.0 (2431)</td>
</tr>
</tbody>
</table>

RESULTS

Effects of Nonphorbol Tumor Promoters on Ganglioside Synthesis. Three nonphorbol agents which have tumor-promoting activity in mouse skin or other systems were examined for their activity in inducing ganglioside changes, particularly reduced synthesis of G(T) in JB6 cells (Table 1). These agents include mezerein, a second-stage mouse skin tumor promoter (27) and promoter of transformation in JB6 cells (12); EGF, a mouse skin cocarcinogen (25) and promoter in JB6 cells (12); and sodium cyclamate, a bladder tumor promoter (18) and nonpromoter in JB6 cells (14). All 3 compounds produced a 2- to 4-fold distributional increase in incorporation of [1-14C]glucosamine into the G(T) band, which by comparison with authentic G(010) on HPTLC analysis appears to contain G(011). The G(T) response to mezerein and sodium cyclamate appears to be biphasic. All 3 agents produced at least a 2-fold increase in incorporation into G(010) at the higher concentrations tested. Only mezerein and EGF produce appreciable decreases in the synthesis of G(T). In contrast to TPA (Table 2), mezerein and EGF produced striking increases in G(010) synthesis. This phenomenon was not observed in sodium cyclamate-treated cells. TPA, mezerein, and EGF, at the concentrations shown in Table 1 and Table 2, showed high activity for promotion of transformation in JB6 cells (12), while sodium cyclamate showed none (14). Thus, the ganglioside change which correlates best with transformation-promoting activity for these 4 compounds is the G(T) synthesis decrease.

Ganglioside Synthesis Response to TPA in Primary Epidermal Cultures and in "Initiated" Calcium-resistant Epidermal Cells. We next sought to ascertain whether the ganglioside responses to promoters observed in the relatively homogeneous

* L. Srinivas, unpublished data.
of promoter-induced preneoplastic progression, we studied the effects of TPA on putatively initiated mouse epidermal cells LC7 (Footnote 3; Ref. 17) and 308 (32), selected for resistance to high-calcium-induced terminal differentiation (Table 3). These 2 cell lines were not promotable to anchorage independence by 2-week exposure to TPA. GT was the major ganglioside being synthesized in LC7 and 308 cells. These cells showed no distributional decrease in GT synthesis and an overall synthesis of gangliosides that increased in response to TPA. In fact, the absolute incorporation of [1-14C]glucosamine into GT was increased 2-fold in 308 and 9-fold in LC7 cells by TPA treatment. The relative synthesis of both G0,4a and G1 was decreased in both calcium-resistant lines in response to TPA.

**Ganglioside Synthesis in Neoplastically Transformed Cells.**

The ganglioside synthesis profile of transformed cells was determined to investigate whether reduced GT synthesis was associated with the neoplastically transformed phenotype (Chart 1). In the JB6-derived TPA-induced transformants, namely, T6274, R681, and RT101, GT accounted for only 2 to 7% of the precursor incorporation into gangliosides, as contrasted with 62% into parental JB6 cells. In JB8 and VB8, 2 transformants paired with the nontransformant JB1 by virtue of similar origin via N-methyl-N-nitro-nitrosoguanidine exposure of primary cultures, there was also a substantial though less extensive decrease in GT synthesis. Thus, from Chart 1, it can be concluded that, for this set of cell lines, reduced GT synthesis is consistently associated with the chemically transformed phenotype. Since overall synthesis decreased on transformation, the absolute decreases were even more pronounced. Increased G0,1a synthesis also appears to be associated with chemical transformation in mouse epidermal cell lines.

Having obtained data implicating reduced GT synthesis in both induction and maintenance of chemical transformation in JB6 cells, we sought to determine whether reduced GT synthesis was associated with another mode of transformation, namely, that induced by transfection of a viral oncogene mos. JB6 C1 25, a promotion-insensitive JB6 clonal cell line (12), was transfected with pml Moloney mos DNA (9, 30), and the transformed cells were cloned from soft agar. When these transformed cell lines were analyzed, GT synthesis as a percentage of total ganglioside synthesis was found to be about 35% lower than was untransfected controls (Chart 2). The incorporation of [1-14C]glucosamine into total gangliosides was elevated in the mos transformants in contrast to the chemical transformants shown in Chart 1, yielding a small absolute increase in incorporation into GT. Go,4a was the ganglioside the synthesis of which was consistently and substantially elevated in mos transformants, as was also observed for chemically transformed cells.

**DISCUSSION**

The nonphorbol promoters of transformation in JB6 cells messed in EGF, like phorbol diesters, produced a substantial

**Table 3**

<table>
<thead>
<tr>
<th>Gangliosides</th>
<th>Untreated</th>
<th>TPA treated</th>
<th>Untreated</th>
<th>TPA treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0 (Gb)</td>
<td>20.7</td>
<td>9.6</td>
<td>33.0</td>
<td>16.2</td>
</tr>
<tr>
<td>G1</td>
<td>74.8</td>
<td>76.8</td>
<td>47.0</td>
<td>80.0</td>
</tr>
<tr>
<td>G0,10</td>
<td>1.5</td>
<td>9.7</td>
<td>4.3</td>
<td>0.5</td>
</tr>
<tr>
<td>G0,1a</td>
<td>3.1</td>
<td>0.8</td>
<td>4.9</td>
<td>0.1</td>
</tr>
<tr>
<td>G0,1b</td>
<td>0.1</td>
<td>1.3</td>
<td>5.2</td>
<td>2.5</td>
</tr>
<tr>
<td>G0,2</td>
<td>0.2</td>
<td>1.8</td>
<td>3.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Total gangliosides

100.0 (6099) a 100.0 (12452) 100.0 (1279) 100.0 (8955)

* Numbers in parentheses, total cpm incorporated into gangliosides per 10^6 cells.

1. JB6
2. JB1
3. T6274
4. R681
5. RT101
6. VB8
7. JB8

![Chart 1](chart_1.png)
The putatively initiated calcium-resistant mouse epidermal cells showed neither a decrease in G\textsubscript{T} synthesis nor promotion of anchorage-independent transformation in response to short-term exposure to TPA. This observation suggests that the resistance of these cells to promotion of transformation by 2-week exposure to TPA may be attributable to their lack of G\textsubscript{T} response. The calcium-resistant phenotype, which also characterizes JB6 P\textsuperscript{-} cells, may represent an early stage, and the "G\textsubscript{T}-responsive" phenotype, which characterizes promotion-sensitive cells, may represent a later stage in preneoplastic progression.

The ganglioside synthesis profiles of N-methyl-N-nitro-nitrosoguanidine- or TPA-transformed epidermal cell lines when compared with nontumorigenic JB6 or JB1 cells showed consistently reduced G\textsubscript{T} synthesis. The occurrence of low G\textsubscript{T} synthesis in transformed cells is compatible with the possibility that reduced G\textsubscript{T} synthesis is a required biochemical event for maintaining the tumor phenotype. Reduced net G\textsubscript{T} synthesis could occur as a result of a specific hydrolysis by a neuraminidase. It has been reported that viral transformation of cells can produce increased neuraminidase activity in chick embryo fibroblasts (23). This increased activity might lead to the simplified ganglioside profile found in various transformed cells (2, 3, 16). We have preliminary evidence to show the presence of a neuraminidase with some degree of specificity for G\textsubscript{T} in JB6 cells. Such an enzyme activity could be due to the existence of a specific protein activator, as reported for G\textsubscript{M\textsubscript{3}} and G\textsubscript{M\textsubscript{2}} degradation (22). Whether a neuraminidase is activated or induced due to TPA action or is active in transformed cells needs to be established.

In order to determine whether reduced G\textsubscript{T} synthesis generalizes to viral oncogene-transformed JB6 cells, 2 clonal lines of pm1 Moloney mos DNA-transfected, anchorage-independent transformants of a JB6 P\textsuperscript{-} cell line were studied. These differed from TPA-transformed JB6 cells in showing a smaller magnitude of distributional reduction in G\textsubscript{T} synthesis relative to nontransformants. These were similar to chemically transformed cells in showing a substantial increase in G\textsubscript{D\textsubscript{10}} synthesis. The total ganglioside synthesis was increased by about 2-fold after mos transformation. Thus, G\textsubscript{T} synthesis underwent an absolute increase after mos transformation. These results suggest that viral mos DNA transfection induces transformation by a different mechanism than does TPA, as reflected in the ganglioside synthesis by these transformants and/or that the basis for maintenance of neoplastic transformation is different in the 2 cases. Current studies in our laboratory are concerned with characterization and purification of genes that specify sensitivity to promotion of neoplastic transformation by tumor promoters (9). Initial evidence suggests that these genes are different from the oncogenes, including mos, that have been described to date (not shown).

That tumor promoters produce some of the same biochemical changes that are constitutively expressed in tumor cells has been observed frequently (31). Among these changes, at least 2 have been observed in JB6 cells as well as other systems, namely, reduced collagen synthesis (13, 14, 15) and reduced fibronectin synthesis and release (1, 15). Our current observations regarding reduced G\textsubscript{T} synthesis in chemical transformants also follow this pattern. However, the reduced G\textsubscript{T} response differs from the collagen and fibronectin synthesis responses in JB6 cells in that the G\textsubscript{T} response is specific for promotion-sensitive cells.
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We thank Edmund Wendel and Roy Sims for excellent technical assistance; Dr. Federico Bertolero, National Cancer Institute, for primary epidermal cultures; Dr. Henry Hennings and Dr. Stuart Yuspa of the National Cancer Institute for calcium-resistant cells; Dr. Donald Blair, National Cancer Institute, for the pM1 DNA; Catherine Talmadge for carrying out transfection of pM1 Moloney mos DNA; Dr. Howard Holden, Donald Blair, and Dr. Henry Hennings of the National Cancer Institute for their critical review of the manuscript; and Beverly Bales for typing.

REFERENCES

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