Altered Ganglioside Biosynthesis in Mouse Cell Cultures following Transformation with Chemical Carcinogens and X-Irradiation

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SUMMARY

Chemically and x-ray-transformed subclones of BALB/c 3T3 mouse embryo cells were found to have reduced amounts of the mono- and disialogangliosides galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide (G\textsubscript{M\textsubscript{1}}) and N-acetyleneuraminylgalactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide (G\textsubscript{D\textsubscript{M\textsubscript{1}}}) and increased amounts of N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide (G\textsubscript{D\textsubscript{M\textsubscript{2}}}). The activity of the enzyme UDP-Gal:Gal\textsubscript{M\textsubscript{1}} galactosyltransferase was reduced to between 2.7 and 14.3% of normal in the transformed clones. Other ganglioside glycosyltransferase activities were unaffected. This enzymatic change was consistent with the observed alteration in ganglioside pattern in the transformed cells. The residual galactosyltransferase activity in the transformed cells was kinetically similar to the normal enzyme, suggesting that transformation alters ganglioside biosynthesis by blocking enzyme synthesis at the translational or transcriptional levels.

Glycolipid changes have been observed in a variety of cells transformed by oncogenic viruses (for recent reviews see Refs. 1 and 2). Mouse cell lines established in culture contain a family of gangliosides which is altered in DNA virus-transformed cells (3, 4) due to reduced activity of the enzyme UDP-Gal:Gal\textsubscript{M\textsubscript{1}} galactosyltransferase (5). A similar block in ganglioside biosynthesis was also observed in mouse 3T3 cells transformed by the Moloney strain of murine sarcoma virus, an oncogenic RNA virus (6). More recently, however, the mouse BALB/c 3T3 transformed by the Kirsten strain of murine sarcoma virus were found to accumulate ganglioside G\textsubscript{D\textsubscript{M\textsubscript{1}}} and to lack the enzyme UDP-Gal:Gal\textsubscript{M\textsubscript{2}} galactosyltransferase (7). BALB 3T3 cells in culture can also be transformed by treatment with chemical carcinogens or by high doses of x-irradiation, and the transformed cells are found to be tumorigenic (8). Although it is presumed that malignant transformation arises from changes introduced in the genetic information by such treatment (9), little is known about the biochemical changes that occur in the cell membranes of these transformed cells. Since changes in ganglioside biosynthesis consistently occur in mouse cells transformed by oncogenic viruses, it seemed appropriate to investigate whether similar alterations appeared in mouse cells transformed by nonviral carcinogens.

In this report we describe altered ganglioside patterns and enzyme activities in the BALB/c 3T3 cells transformed by the chemical carcinogens, methylcholanthrene and benzpyrene, and by x-irradiation. The properties of the residual UDP-Gal:Gal\textsubscript{M\textsubscript{2}} galactosyltransferase activity in the transformed cells were compared with those of the normal cell enzyme; the results of these experiments indicate that transformation with these agents affects the synthesis of this glycosyltransferase involved in the biosynthesis of gangliosides.

EXPERIMENTAL PROCEDURE

Materials—Sources of the glycolipid acceptors and sugar nucleotide donors have been described previously (10). Triton CF-54 was purchased from Sigma, Tween 80 from Nutritional Biochemicals, and Nonidet P-40 from Shell Oil Co. Alkaline phosphatase (premium grade) was obtained from Worthington. Sephadex G-25 superfine, Bio-Gel P-2, and Dowex 1-X8 were purchased from Pharmacia, Bio-Rad, and J. T. Baker, respectively.

Cell Lines and Cell Culture—Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum at 37° under 5% CO\textsubscript{2}. Clone A31 is derived from the established mouse embryo cell line BALB 3T3 which is highly contact-inhibited and nontumorigenic (11). All of the transformants were able to form multiple cell layers and to produce colonies in soft agar while the control A31 cells could do neither. Each of the transformants was tumorigenic in newborn BALB/c mice at 10\textsuperscript{3} cells per animal, while the A31 were nontumorigenic at...
Reactions catalyzed by glycosyltransferases involved in ganglioside biosynthesis

The following conditions were used for the various assay systems. Sialyltransferase activity: 12.5 nmol of CHI or 10 nmol of GM3, 20 nmol of CMP-[3H]NAP (7 Ci per mmol), 1.25 μmol of sodium cacodylate buffer, pH 6.3, 100 μg of Triton CF-54, 50 μg of Tween 80, and 100 μg of cell protein (600 × g supernatant), in a final volume of 25 μl. The incubation time was 2 hours at 37°C. N-Acetylgalactosaminyltransferase: 25 nmol of GM3, 5 nmol of UDP-[3H]GalNAc (14 mCi per mmol), 1.25 μmol of sodium cacodylate buffer, pH 7.0, 100 μg of Nonidet P-40, 0.5 μmol of MnCl2, 50 μg of cell protein (600 × g supernatant), in a total volume of 25 μl. The incubation was for 2 hours at 37°C. Galactosyltransferase: 10 nmol of GM3, 50 nmol of UDP-[3H]Gal, 2.5 μmol of sodium cacodylate buffer, pH 5.3, 1 μmol of MnCl2, 200 μg of Triton CF-54, 100 μg of Tween 80, 250 μg of cell protein (600 × g pellet), in a total volume of 50 μl. The incubation was for 3 hours at 37°C.

### Table 1

<table>
<thead>
<tr>
<th>Glycosyltransferase</th>
<th>Sugar nucleotide donor</th>
<th>Acceptor</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialyltransferase I</td>
<td>CMP-NAN</td>
<td>CDH</td>
<td>GM₂</td>
</tr>
<tr>
<td>N-Acetylgalactosaminyltransferase</td>
<td>UDP-GalNAc</td>
<td>GM₃</td>
<td>GM₄</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>UDP-Gal</td>
<td>GM₃</td>
<td>GM₁</td>
</tr>
<tr>
<td>Sialyltransferase II</td>
<td>CMP-NAN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10⁴ cells per animal. A31-MC5-3 and A31-BP-8 (and RP-117-32) are chemically transformed subclones obtained by treating A31 cells with the carcinogens methylcholanthrene and benzopyrene (12). A4 is a transformed subclone of A31 obtained as a survivor of 1200 rads of x-irradiation (6). R₂/B is a subclone selected for absence of thymidine kinase by growing it in medium containing 5-bromodeoxyuridine (13). The cells were between 50% and just confluent when they were harvested. The cells were fed every 3 days; the medium had been changed 3 days prior to harvesting.

**Glycolipid Analysis**—The washed, harvested cells (0.5 ml) were collected by centrifugation, homogenized in 6.5 ml of 0.1 M NaOAc and extracted with 20 ml of chloroform-methanol (2:1, v/v) at 50°C for 30 min. The residue was reextracted with 10 ml of chloroform-methanol (1:2, v/v). The second extract was taken to dryness under nitrogen and combined with the first extraction. The resulting solution was then partitioned by the addition of 0.20 ml of water and the upper aqueous phase removed. The lower, organic phase was washed eight times with chloroform-methanol-water (3:48:47, v/v/v). The upper phases were combined, reduced in volume under N₂, and then lyophilized. The glycolipids were taken up in 0.2 ml of H₂O and chromatographed on a Bio-Gel P-2 (50 to 100 mesh) column (1 × 23 cm). The glycolipids were eluted from the column with water. Fractions of 6 ml were collected; 90 to 100% of the glycolipids were recovered in the second fraction, which was then lyophilized. These modifications of previous procedures (3, 4) allowed more complete recovery of all the glycolipids in the upper phase. Individual gangliosides were separated by thin layer chromatography on Silica Gel G-coated glass plates and detected and quantitated as previously described by direct densitometry with a Zeiss chromatogram scanner (4, 7).

**Enzyme Assays**—The various glycosyltransferase reactions involved in the synthesis of gangliosides in cultured mouse cell lines are indicated in Table 1. The washed packed cells were suspended in 4 volumes of 0.25 M sucrose solution containing 0.1% nercapteran and incubated for four times in a Dry Ice-methanol bath and dispersed by hand homogenization (10). A crude particulate fraction (P₁) was obtained by centrifuging the homogenate at 600 × g for 10 min. This P₁ fraction was then resuspended in the same buffer and the GM₁: UDP-Gal galactosyltransferase activity was measured as described previously (7). Sialyltransferase activities I and II and N-acetylgalactosaminyltransferase were assayed with the 600 × g supernatant fraction as described previously (7, 10). All enzyme activities were assayed under conditions of donor and acceptor saturation and linearity in terms of time of incubation and protein concentration. Each assay was performed at least in duplicate, and net enzymatic activity was determined by the difference in activity with and without added glycolipid acceptor. Sialyltransferase II activity was determined by high voltage electrophoresis (11), whereas the other glycosyltransferase activities were measured by separation of products on Sephadex G-25 (10).

Sugars were determined by gas-liquid chromatography with a gas chromatograph equipped with a flame ionization detector and a 1.5 m column packed with 4% Teflon bonded to Porasil 100 (12) and 4% OV1 (13). The column temperature was 280°C. Gas chromatography—mass spectrometry (14) was used to identify products of the various glycosyltransferase reactions.
measured. Such enzymes could hydrolyze the sugar nucleotide when experiments described above indicated the absence of an activity in the 600 x g supernatants. In addition, there were low levels of this enzymatic activity in the various transformed cell lines. A similar observation was made for the transformed cell lines of MC 5-5 and A31 cells. There was a 2-fold enrichment of galactosyltransferase activity in the P1 fraction of normal cells compared to the whole homogenate (7). A similar enrichment was found for the P1 fractions of the various transformed cell lines. In addition, there were low levels of this enzymatic activity in the 600 x g supernatants.

Sugar Nucleotide Pyrophosphatase Activity Although the mixing experiments described above indicated the absence of an interfering component in the various transformed cell lines, the levels of sugar nucleotide pyrophosphatase activities were also measured. Such enzymes could hydrolyze the sugar nucleotide donors during the glycosyltransferase assays and possibly reduce product formation. The optimum pH for the enzyme catalyzing the hydrolysis of UDP-galactose to galactose 1-phosphate was found to be pH 7.8 (Fig. 2). The activity of this enzyme was similar in the various cell lines at this pH as well as at pH 5.3, that used for the assay of galactosyltransferase (Table V).

However, pyrophosphatase activity was a complicating factor at higher pH values. Although the galactosyltransferase from BALB/C 3T3 cells appears to have a pH optimum of 5.3 (Fig. 3), similar data for the transformed cell lines could not be obtained. The low galactosyltransferase activity in these cells required that the enzyme assays be carried out with large amounts of protein. Sufficient pyrophosphatase activity was present in the latter incubation mixtures to hydrolyze up to 90% of the sugar nucleotide when the assays were done at the higher pH values.

**Kinetic Properties**—In order to determine if the residual galactosyltransferase in the various transformed cell lines was the same as the activity found in the normal BALB/C 3T3 cells, various kinetic and biochemical properties were compared. Michaelis constants were determined for both UDP-galactose and G0M2 (Table VI). There was no significant difference in the values for the normal and transformed cell enzymes.

It is possible that the diminished activity in the transformed cells is the result of the biosynthesis of an enzyme defective in one cell line extracts | Galactosyltransferase activity
---|---|---|---|---
| Separate | Mixed* | Expected | Per cent |
| A31 | 535 | 327 | 998 | 110 |
| RP-8 | 60 | 242 | 275 | 88 |
| MC 5-5 | 14 | 242 | 275 | 88 |
| R4/B | 49 | 299 | 299 | 98 |

*Activity is expressed as picomoles of product formed per mg of protein per hour with P1 fractions, and was determined as described under "Experimental Procedure."

The P1 fraction of each transformed cell line was mixed with an equivalent amount of protein from the normal A31 cell line and assayed for galactosyltransferase activity.

### TABLE III

**Glycosyltransferase activities in normal and chemically and radiation-transformed BALB 3T3 mouse cells**

Enzyme assays were performed on the various cell extracts as described under "Experimental Procedure" and in the legend to Table 1. Each activity was measured in duplicate with and without added glycolipid acceptor and the values were corrected for endogenous incorporation. Endogenous activities were corrected by incubating the labeled substrate with boiled enzyme controls, and the following values were observed: sialyltransferases, 17, 70 pmol per mg of protein per hour; N-acetylgalactosaminyltransferase, 205,308 pmol per mg of protein per hour; and galactosyltransferase, 27, 84 pmol per mg of protein per hour.

<table>
<thead>
<tr>
<th>Glycosyltransferase</th>
<th>BALB 3T3 clone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A31</td>
</tr>
<tr>
<td>Sialyltransferase I</td>
<td>586</td>
</tr>
<tr>
<td>N-Acetylgalactosaminyltransferase</td>
<td>1355</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>511 ± 18</td>
</tr>
<tr>
<td>Sialyltransferase II</td>
<td>348</td>
</tr>
</tbody>
</table>

*Not determined.

*Galactosyltransferase activities were determined in triplicate and the values include standard deviations.
or a few specific amino acid residues resulting in an increased rate of denaturation in the presence of a biochemical stress. Heat denaturation has been used to maximize such defects. There was no appreciable difference in the rate of heat inactivation of the galactosyltransferase activity in the various cell lines (Table VII).

Identification of Enzyme Product—Although the glycolipid product of UDP-Gal:G

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pyrophosphatase activity</th>
</tr>
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<tbody>
<tr>
<td>pH 5.3</td>
<td>pH 7.8</td>
</tr>
<tr>
<td>mmol/mg protein/hr</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>28</td>
</tr>
<tr>
<td>BP-8</td>
<td>25</td>
</tr>
<tr>
<td>MC 5-5</td>
<td>34</td>
</tr>
<tr>
<td>R/B</td>
<td>28</td>
</tr>
</tbody>
</table>

been previously identified as G in enzyme activities obtained from rat brain (15) and normal BALB 3T3 cells (7), it seemed appropriate to confirm that the galactosyltransferase activity in the transformed cell lines was synthesizing this same product. An aliquot (172 µg) of protein of the P fraction from R/B was incubated in the standard assay with additional UDP[14C]-Gal (204 µCi per µmol). The eluant from the Sephadex G-25 column was dried down and applied to a Silica Gel G-coated
plate along with ganglioside standards, and the plate was developed as described under "Experimental Procedure." The radioactive product migrated as a single spot and co-chromatographed with authentic GM1. The radioactive material was eluted from the silica gel with chloroform-methanol-water (10:10:3, v/v/v) and counted by liquid scintillation spectroscopy. Based on the recovery of radioactive product (1291 net dpm) and the specific radioactivity of the UDP[¹⁴C]Gal (45 μCi per μmol), an activity of 36 pmol per mg of protein per hour was calculated which corresponds very well with the value of 40 pmol per mg of protein per hour in extracts of these cells (Table III). After correcting for the recovery of GM3 from the thin layer chromatogram (80 to 90%), it is apparent that the synthesis of GM3 from endogenous GM2 is minimal in the transformed R4/β cells. Since this cell line contains the highest concentration of endogenous GM2 of the cell lines analyzed, the amount of endogenous GM2 utilized as acceptor in the galactosyltransferase assay in other cell lines would also be expected to be only a minor fraction of that obtained in the presence of exogenous GM2.

Discussion

When cultured cells of various species are transformed by oncogenic viruses there is an alteration of their glycolipid composition which is typified by a loss of components with more complex oligosaccharide chains (1, 2, 16-18; see also ref. 19 and 20). These changes in glycolipid metabolism were first shown to be due to a block in glycolipid biosynthesis by Cumar and co-workers (5). In mouse cells transformed by DNA viruses (1, 5) and an RNA virus, Moloney sarcoma virus (6), there is a loss of a specific aminosugar transferase involved in ganglioside biosynthesis. More recently, mouse cells transformed by another RNA virus, the Kirsten sarcoma virus, have lost all UDP-Gal:GM3 galactosyltransferase activity and accumulate GM3 (7). We now report a block of this galactosyltransferase in mouse cells transformed by chemical carcinogens and by x-irradiation.

The consistent feature of these changes in the glycolipid metabolism of established mouse cells is a specific block in the biosynthetic pathway which results in the reduction of the major ganglioside of these mouse cells, G0. Although increased levels of sialidase activity have been reported in virus transformed hamster cells when compared to untransformed primary or established hamster cells (21), no differences were detected when ganglioside sialidase activities were measured in normal and transformed mouse cells (5). An extreme decrease in sugar nucleotide pyrophosphatase activity was also observed in transformed cells after transformation by viruses and chemicals (22). We, however, observed similar UDP-galactose pyrophosphatase activity in the normal and transformed mouse cells used in our experiments. Since Sela et al. (22) used UDP-N-acetylhexosamine as the substrate in their studies, we also measured the UDP-N-acetylglactosamine pyrophosphatase activity in the various mouse cell lines. Cells from clone R4/β had the same activity as clone A3I, whereas BP-8 had one-half and NC 5-5 twice the activity as the A3I controls.

Since the same specific enzyme change, reduced UDP-Gal:GM3 galactosyltransferase activity, is now found in mouse cells following transformation by an RNA tumor virus, two different chemical carcinogens, and x-irradiation, the results suggest that a common biochemical change can occur when cells are transformed by various carcinogenic agents. Since all of the transformed cell lines were derived from the same clonal normal line, it would appear that the reduction in enzyme activity is related to the transformation process. It is important to note that all of the transformed cell lines were selected on the basis of their altered morphology and growth behavior. We cannot say whether transformed cells selected on some other basis would have the same or different alteration in ganglioside metabolism. However, when cultured mouse (6) or chicken (23) cells were quantitatively transformed by RNA tumor viruses, all of the cells appeared to undergo a reduction in ganglioside biosynthesis.

Whether there is a direct relationship between malignant transformation and altered ganglioside metabolism is still unclear. Revertants from DNA virus-transformed mouse cells which had returned to a low saturation density in culture had regained their complex gangliosides and aminosugar transferase activity (24). In contrast, when revertants were obtained from polyoma virus and dimethylnitrosamine-transformed hamster cells, which have low levels of this enzyme compared to normal hamster cells, the revertants did not regain this aminosugar transferase activity (25). However, using a different selection process, Nigram and co-workers were able to demonstrate a correlation between UDP-GalNAc:GM3 N-acetylgalactosaminyltransferase activity and phenotypic properties as well as tumorigenicity in SV40-transformed hamster cells (26).

Previous studies have indicated the murine sarcomas induced by chemical carcinogens in vivo exhibit cell surface tumor-specific transplantation antigens that are not cross-reacting (27). In addition, when a clonal population of mouse cells was transformed by 3-methylcholanthrene, all of the transformed subclones examined exhibited no cross-reactivity (28). In this light, our findings of the same alterations in membrane-bound glycolipids in a clonal population transformed by different chemical agents take on added significance; even independently isolated clones transformed by the same chemical, A3I clone BP-113-32, and clone 1P-8 have reduced galactosyltransferase activity.

In all of our previous studies as well as those reported here, we have never been able to detect an inhibitor of glycosyltransferase activity in transformed cells or an activator or cofactor in normal cells (5-7). A similar observation was made for the reduced N-acetylgalactosaminyltransferase activity in transformed hamster cells (25). In addition, the residual galactosyltransferase activity in the chemical and x-ray transformed mouse cells has kinetic properties and heat stability similar to this enzyme activity from normal mouse cells. These observations suggest that the transformation process interferes with ganglioside biosynthesis either at the genome or the level of protein synthesis. In support of this conjecture, the kinetic properties of the UDP-GalNAc:GM3 N-acetylgalactosaminyltransferase were found to be similar in normal and DNA tumor virus-transformed mouse cells, which characteristically have reduced levels of aminosugar transferase activity. It is realized that these data are preliminary, and that isolation of these enzymes in pure form for physical, chemical, and immunological analyses will be necessary in order to conclusively establish the level at which transformation impairs ganglioside biosynthesis.

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