Genetic and Differential Basis for the Differentiated Expression of G_{M2} and G_{D2} Gangliosides in Human Cancer Cell Lines

Shuji Yamashiro, Shutian Ruan, Keiko Furukawa, Tadashi Tai, Kenneth O. Lloyd, Hiroshi Shiku, and Koichi Furukawaa

ABSTRACT

Using β1,4-N-acetylgalactosaminyltransferase (EC 2.4.1.92) complementary DNA, the correlation of gene expression, enzyme activity, and expression of ganglioside antigens was analyzed in 20 human tumor cell lines. In many lines, G_{M2} and/or G_{D2} were the most complex structures examined. Northern blot analysis revealed 5.2- and 3.0-kilobase mRNAs in almost all cell lines expressing G_{M2} and/or G_{M3}. Some melanoma lines, however, showed no bands although they expressed fairly high levels of G_{D2}. These cell lines expressed very high levels of α2,8-sialyltransferase and the resulting product, G_{D3}, Semiquantitative RT-PCR demonstrated that even cell lines with no bands in Northern blot contained 0.4-2.5% of mRNA level in the highest expressing cell line. These results indicate that G_{D2} expression on individual cell lines is regulated not only by the expression level of the N-acetylgalactosaminyl transferase but also by the amount of its precursor structure i(.,,.i and «2,8-sialyltransferase present in the cells. β1,4-N-acetylgalactosaminyltransferase activities and mRNA levels generally correlated quite closely. A few lines, however, showed lower enzyme activities than expected from their mRNA levels, indicating the possibility that the enzyme is being regulated by translational or posttranslational modification such as phosphorylation and glycosylation as well as by transcriptional regulation. Depending on their patterns of ganglioside synthesis and expression, the lines examined were classified into 6 groups which were characteristic of different tumor cell types.

INTRODUCTION

Gangliosides, glycosphingolipids containing sialic acids, have been studied as characteristic amphipathic molecules expressed in brain and other normal tissues and also as tumor markers of the neuroectoderm-derivated malignant cells (1-3). Carbohydrate structures on gangliosides are synthesized by the sequential catalytic reactions of glycosyltransferases. For the biosynthesis of major gangliosides, three pathways have been considered to lead to a, b, and c series gangliosides (4-6). GalNAc-T (EC 2.4.1.92) which catalyzes the synthesis of G_{M3} and G_{D2} from G_{M2} and G_{D3}, respectively, plays a key role in providing the carbohydrate structures utilized in the formation of characteristic gangliosides for individual tissues and tumor cells. Although the genes of a number glycosyltransferases have been recently cloned (7, 8), the regulatory mechanisms controlling expression of these genes have not yet been well studied, except for α2,6-sialyltransferase (9, 10). The correlation of mRNA expression of glycosyltransferase genes and enzyme activity have also not been investigated. Since the β1,4-GalNAc-T gene became available by cDNA cloning (11), the regulatory mechanisms for the expression of G_{M2}/G_{D2} and other complex gangliosides can now be analyzed more precisely than ever. In this study, we have analyzed β1,4-GalNAc-T gene expression and corresponding enzyme activity in a panel of human cancer cell lines and correlated those results with the expression level of relevant gangliosides.

The results show that the differential expression of G_{M2} and G_{D2} in those malignant cells is regulated by the enzyme activity of β1,4-GalNAc-T and also by the amount of available precursor structures. The results indicate that the translational or posttranslational regulation of the transferase gene is also important.

MATERIALS AND METHODS

Northern Blot Analysis

Preparation of Poly(A)* RNA. Poly(A)* RNA was prepared as described previously (11) according to Badley et al. (12). One × 10^6 cells were pelleted in 50 ml centrifuge tube and lysed in 15 ml of lysis buffer consisting of 0.2 mM NaCl, 0.2 mM Tris-HCl (pH 7.5), 1.5 mM MgCl2, 2% SDS, and 200 μg/ml proteinase K. The lysate was homogenized by passing through a plastic syringe fitted with 18- and 21-gauge needle and incubated at 45°C for 2 h. The NaCl concentration of the lysate was adjusted to 0.5 mM by addition of 0.95 ml of 5 M NaCl. The lysate was poured into a 50-ml centrifuge tube with prequilliated olio (dT) cellulose and rocked gently at 37°C for 1 h. The oligo (dT) cellulose was washed 4 times with 10 ml of binding buffer (0.5 M NaCl, 0.5 mM Tris-HCl, pH 7.5), resuspended in 1 ml of binding buffer, and poured into a small column. It was washed until the A260 of the flow-through was ≤ 0.05. Poly(A)* RNA was eluted with eluting buffer (0.01 M Tris-HCl, pH 7.5). The RNA was precipitated with 0.15 volumes of 2 M sodium acetate and 2.5 volumes of ethanol at -70°C until solid. The precipitate was pelleted and washed in 80% ethanol and then was dissolved in 50 μl of diethylpyrocarbonate-treated H2O.

Northern Blot Analysis. Poly(A)* RNA, 10 μg, was electrophoresed on 1.25% formaldehyde agarose gel, blotted onto a nylon membrane (Genescreen Plus; DuPont/NEN), and then fixed by baking for 2 h at 80°C. Blots were incubated in hybridization buffer consisting of 50% formamide, 10% dextran sulfate, 1 mM NaCl, 1% SDS, 50 mM Tris (pH 8.0), and 100 μg/ml of denatured salmon sperm DNA for 3 h at 42°C. Hybridization was performed by adding random primed probes at 3 X 10^5 cpm/ml at 42°C for 16 h. The probes were radioabeled using the Multiprime DNA labeling system (Amerham, Arlington Heights, IL). Blots were washed as described previously (11) and then exposed to X-ray film at -70°C for 48-72 h. The relative amount of each mRNA was determined by an image analyzer BAS2000 (Fuji, Tokyo, Japan).

β1,4-N-Acetylgalactosaminyltransferase Assay

The membrane fractions of cells were prepared as described by Thampoo et al. (13). Briefly, cells were lysed in ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride using a nitrogen cavitation apparatus (Parr Instrument Co., Milone, IL) at 400 psi for 30 min. Nuclei were removed by low centrifugation and supernatant was centrifuged at 105,000 g for 1 h at 4°C. The pellet was resuspended in ice-cold 100 mM cacodylate-HCl buffer, pH 7.2. Enzyme assay was performed as described previously (14). The reaction mixture contained in a volume of 50 μl: 100 mM sodium cacodylate-HCl (pH 7.2) (Wako Junyaku, Osaka, Japan); 10 mM MnCl2, 0.3% Triton X-100, 0.25% formaldehyde.
were mixed in a glass tube and dried in a nitrogen flow. Mn2<, detergent, and CF-54 (Sigma, St. Louis, MO); 325 /µM GM3 (for GM2 synthesis); 400 ¡µM

Cells dioactivity and the remainder was analyzed by TLC and fluorography. GD3 of chloroformimethanol (1:1). One fifth of product was used for counting ra

ml of water. The bound glycolipid is eluted with 2 ml of methanol and 3 ml

Sep-Pak cartridge (Waters, Millford, MA). The cartridge was washed with 15

by the addition of 2 ml H2O. The sample was applied three times to CIS

buffer. The glycolipid acceptor, donor, and inhibitor of pyrophosphatase

as well as membrane fraction were separately prepared with cacodylate-HCl

buffer. The sample was applied three times to CIS

panel as determined by reactivity with specific

GGR12. Percentage of positive cells showing

stronger fluorescence intensity than that of negative

control is shown.

Fig. 1. Structures and synthetic pathway of major gangliosides analyzed in this study.

CF-54 (Sigma, St. Louis, MO); 325 µM Gm3 (for Gm2 synthesis); 400 µM

UDP-GalNAc (Sigma); UDP-[14C]GalNAc (3.5x10^3 dpm) (NEN); 10 mM

CDP-choline (Kohjin Co., Tokyo, Japan); and membranes containing 200 µg

protein. Gm5 was dissolved in chloroform/methanol (1:1), UDP-GalNAc was

in 70% ethanol, and CDP-choline was in 50% ethanol. MnCl2, Triton CT-54,

protein. GM1 was dissolved in chloroform/methanol (1:1), UDP-GalNAc was

CDP-choline (Kohjin Co., Tokyo, Japan); and membranes containing 200 fig

UDP-GalNAc (Sigma); UDP-[14C]GalNAc (3.5x10^5 dpm) (NEN); 10 HIM

GD1b, GM1, GD3, GD2, GD1a, GT1b, asialoGM2

lines, ATN-1, HUT102, and MT-2, were presented by Dr. Naoe (Nagoya University, Branch Hospital, Nagoya, Japan), Dr. Gallo (NIH), and Dr. Miyoshi (Kochi University, Kochi, Japan), respectively. NK like line, YTN17 was pre

presented by Dr. J. Yodoi in Kyoto University. MEG-01 (megakaryoblastic leuk

emia line) was presented by Dr. Saito (Nagoya University).

Flow Cytometry

Cells were incubated with monoclonal antibodies (5-10 µg/ml) or 2-fold dilution of culture supernatant of hybridoma for 45 min on ice. After being washed twice with phosphate-buffered saline, the cells were incubated with 100 µl of 50-fold diluted fluorescein isothiocyanate-conjugated anti-mouse IgG (Cappel, Westchester, Philadelphia, PA) for 30 min on ice. After washing twice, the cells were examined with a FACScan (Becton-Dickinson, Mountain View, CA) as described previously (11).

RT-PCR

RT. Single-strand cDNA was synthesized with oligo (dT)14 primer as de

scribed (15). Total RNA (3 µg) was dissolved in 50 µl of 50 mM Tris-HCl (pH 8.3) containing 75 mM KCl, 3 mM MgCl2, 10 mM dithiotreitol, 0.5 mM dATP, dGTP, dCTP, dTTP, 0.5 µg oligo-d(T)14, and 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Grand Island, NY) and then incubated for 90 min at 37°C.

PCR. PCR was performed basically according to the previous report (16). The PCR primers used for ßl,4-GalNAc-T gene were a M2T1-1 sense primer (5'AGGGC GGCTGTCGAGATCT-3'), and a M2T-AS2 antisense primer (nucleotides 677-696), 5'GGTCTGGAAGCTTTGCTG-3'.

Five µl of reverse transcription products were diluted in a 50-µl reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, all 4 deoxynucleotide triphosphates (0.2 mM each), and 0.002% gelatin, and PCR was done by adding 0.325 µg M2T1-2 sense primer, 0.325 µg M2T-AS2 antisense primer, and 1 units of Taq DNA polymerase (Wako Junyaku). Samples were subjected to 15, 20, 25, and 30 cycles of amplification. Each cycle consisted of 3 steps: (a) denaturation for 1 min at 94°C; (b) primer annealing for 1 min at 55°C; and (c) polymerization for 2 min at 72°C.

Southern Blot Analysis

Ten µl of RT-PCR products were electrophoresed on a 10% polyacrylamide gel and treated with an alkaline solution (0.4 x NaOH-0.6 M NaCl) prior to transfer onto nylon filters. Filters were prehybridized in a solution containing 1 M NaCl, 1% SDS, 10% dextran sulfate, 50 mM Tris-HCl (pH 7.5), and 100 µg/ml of denatured salmon sperm DNA. Samples were hybridized overnight at 65°C in the same buffer containing 1 x 10^6 cpm/ml full length cDNA probe labeled with [32P]dCTP using a multiprime DNA labeling system (Amersham) as described previously (15), exposed to an imaging plate, and analyzed by using the BAS2000 Bio-Image Analyzer.
Monoclonal Antibodies

mAb used in this study were as follows. mAb 10-11 (anti-Gm2, mouse IgM; Ref. 17) was a gift from Dr. P. O. Livingston, Sloan-Kettering Cancer Center, New York. mAb 3F8 (anti-Gm1, IgG2a; Ref. 18) was provided by Dr. N. K. Cheung, Sloan-Kettering Cancer Center. mAb R24 (anti-Gm2, IgG2a; Ref. 19) was presented by Dr. L. J. Old, Sloan-Kettering Cancer Center. mAb 2D4 (anti-asiago GM2) (IgM; Ref. 20) was obtained from American Type Culture Collection. Anti-Gm1, mAb GMR17 (IgM), anti-Gm2, mAb GGR12 (IgG), anti-Gm1, mAb GMB16 (IgM), anti-GT1b, and mAb GMR5 (IgM) were described previously (21, 22).

RESULTS

Ganglioside Expression Analyzed by Monoclonal Antibodies. Ganglioside expression on the panel of cell line was analyzed by flow cytometry. Structures and synthetic pathway of main gangliosides studied here were summarized in Fig. 1. The monoclonal antibodies used were able to detect the main ganglioside structures metabolically close to GM2 and GD2 and have been demonstrated to be specific for individual structures (17-22). As shown in Fig. 2, many of the cell lines examined expressed GM2, GD3, or GI52. These cell lines did not express more complex structures, except that GI5la was detected in several lines. Myelogenous leukemia lines expressed low level of GM2, and Raji and T-ALL lines showed no ganglioside expression except for low level of GM1 and GI5la in Raji. Human T lymphotropic virus type I lines (MT-2, HUT102, and ATN-1) showed GM2 and/or GD2 expression as reported (16). Neuroblastoma and glioma lines also showed GM2 and/or GD2 expression but no or very low level of GD3. Melanoma lines expressed high level of GD3, and some of them also expressed high level of GM2 and/or GD2. The data on the neuroblastomas and melanomas were consistent with those derived previously by the TLC analysis of extracted gangliosides (23).

Expression of ß1,4-GalNAc-T Gene Analyzed by Northern Blot Hybridization. Poly(A)+ RNA was subjected to Northern blot analysis using a whole length of M2T1-1 clone as the probe. As shown in Fig. 3, except for some of melanoma lines, all cell lines expressing GM2 or GD2 showed two bands at 5.2 and 3.0 kilobases. A band at approximately 2.0 kilobases was also detectable in several samples, but the intensity was much weaker than the larger bands. Among melanoma lines, SK-MEL-23, SK-MEL-28, and MeWo showed no bands although they express some GD2. In SK-MEL-23 especially, the GD2 expression was fairly high in both flow cytometry and TLC of extracted gangliosides (data not shown).

RT-PCR Analysis. In order to compare the relative amount of mRNA, semiquantitative RT-PCR assay was performed. The correlation of intensity in Northern blot hybridization of RT-PCR products with serially diluted mRNA of SK-MEL-31 was shown in Fig. 4. Twenty and 25 cycles of amplification were performed. Good correlation between amounts of RNA and intensity of amplified bands detected radioisotopically was obtained in both preparations, although slightly broader range of RNA dilution was covered by 20-cycle amplification. Therefore, using the standard curve from 20-cycle amplification, relative mRNA level was determined in 17 lines and summarized in Table 1. The cell lines showing mRNA levels less than 3% of SK-MEL-31 in this analysis were, except for SK-MEL-23, negative in Northern blot analysis.

Enzyme Activity of ß1,4-GalNAc-T. Since the enzyme synthesized by the ß1,4-GalNAc-T cDNA clones we isolated is capable of converting GM2 to GM2 as well as Gm1 to Gm2 (11), we analyzed the enzyme activity in cell lines using GM1 as an acceptor. In Fig. 5, the products of enzyme reaction by using GM2 and GD3 as acceptors were shown. As summarized in Fig. 6, the enzyme activity ranged from 0

Fig. 4. Standard curve for semiquantitative analysis of ß1,4-GalNAc-T mRNA. Total RNA of SK-MEL-31 was serially (10-fold) diluted by using total RNA of NALM-6 and subjected to RT and PCR. The PCR products of 20 and 25 cycles were analyzed by Southern blotting using oligo nucletide probe and quantified by an image analyzer as described in “Materials and Methods.” Results of 20 cycle PCR are shown.
Table 1 Expression of β1,4-GalNAc-T-gene determined by Northern blotting and RT-PCR

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Tumor type</th>
<th>Northern blot</th>
<th>RT-PCR (20 cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEG-01</td>
<td>Megakaryoblastic leukemia</td>
<td>5.7</td>
<td>ND</td>
</tr>
<tr>
<td>K562</td>
<td>Erythroleukemia</td>
<td>19.3</td>
<td>9.8</td>
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<tr>
<td>HL-60</td>
<td>Promyelocytic leukemia</td>
<td>10.8</td>
<td>11.9</td>
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<tr>
<td>Ara10</td>
<td>Myeloma</td>
<td>7.5</td>
<td>11.2</td>
</tr>
<tr>
<td>YTN-17</td>
<td>NK-like</td>
<td>32.3</td>
<td>115.4</td>
</tr>
<tr>
<td>Raji</td>
<td>Burkitt lymphoma</td>
<td>3.1</td>
<td>4.0</td>
</tr>
<tr>
<td>P12/Ichikawa</td>
<td>T-ALL*</td>
<td>0</td>
<td>2.3</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>T-ALL</td>
<td>0</td>
<td>2.1</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>T-ALL</td>
<td>0</td>
<td>0.4</td>
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<tr>
<td>MT-2</td>
<td>HTLV-I†</td>
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<td>63.5</td>
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<td>HUT102</td>
<td>HTLV-I‡</td>
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<td>ND</td>
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<tr>
<td>ATN-1</td>
<td>ATL§</td>
<td>36.7</td>
<td>40.4</td>
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<tr>
<td>SK-NSH</td>
<td>Neuroblastoma</td>
<td>16.8</td>
<td>18.3</td>
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<tr>
<td>IMR-32</td>
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<td>44.4</td>
<td>16.7</td>
</tr>
<tr>
<td>AS</td>
<td>Astrocytoma</td>
<td>69.0</td>
<td>115.4</td>
</tr>
<tr>
<td>MeWo</td>
<td>Melanoma</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>SK-MEL-31</td>
<td>Melanoma</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>SK-MEL-23</td>
<td>Melanoma</td>
<td>0</td>
<td>8.8</td>
</tr>
<tr>
<td>SK-MEL-37</td>
<td>Melanoma</td>
<td>24.0</td>
<td>4.0</td>
</tr>
<tr>
<td>SK-MEL-28</td>
<td>Melanoma</td>
<td>0</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Not determined.
† T-cell acute lymphocytic leukemia.
§ Human T-lymphotropic virus type I-positive.
‡ Adult T-cell leukemia.

Ganglioside expression (Gm2/GD2), GalNAc-T activity, and relative intensity of mRNA band in Northern blot analysis are summarized in Fig. 6. Since two different mRNA bands showed similar proportion of intensity in each cell line except SK-MEL-37, it seemed that products from both transcripts might be involved in the synthesis of Gm2 and/or GD2.

Comparison of Gm2/GD2 Synthase and GD3 Synthase Activities.

In a smaller panel of cell lines (8 samples) the enzyme activities of the β1,4-GalNAc-T were directly compared with levels of α2,8-S-T (GD3
synthase). As shown in Table 2, there was a considerable degree of variation in the proportions of the two enzymes in the various cell lines.

DISCUSSION

Although a number of genes coding for glycosyltransferases have recently been isolated (7, 8), precise studies on the correlation between gene expression and enzyme activity, and resulting carbohydrate products have not been reported. As shown in Fig. 5, the products directly synthesized by β1,4-GalNAc-T are apparently GM2 and GD2 gangliosides (11). (Fine-substrate specificity of the enzyme will be reported elsewhere.) Moreover, as summarized in Fig. 2, GM2 and GD2 gangliosides are the final products of the synthetic pathway in almost all the cell lines tested. It was, therefore, possible to analyze the correlation of gene expression, enzyme activity, and the relevant ganglioside expression in these cells.

Carbohydrate structures expressed on the cell surface are primarily determined by the combined activities of glycosyltransferases needed for the each step of the synthesis (7). The activity of a glycosyltransferase should be partly or entirely dependent on the expression of the corresponding gene. Results shown in this report indicated that expression levels of GM2 and GD2 generally correspond to the β1,4-GalNAc-T activity. However, as shown in some melanoma lines, GD2 could be expressed at a fairly high level without definite GalNAc-T activity or the mRNA expression of the transferase gene, although minimal levels of the gene expression seemed to take place (Table 1). This is presumably because melanoma lines contain very high level of α2→8sialyltransferase (S-T2) and synthesize high amount of GD2, the precursor of GD2. Consequently, the amount of precursor structure present in the individual cell lines is also a very important factor in determining the ganglioside profile. This means that the expression of the glycosyltransferase responsible for the synthesis of the precursor structure is equally important (14).

The representative patterns of major ganglioside synthetic pathway in the cell lines studied are shown in Fig. 8. Pattern A represents the simplest pattern and corresponds to MOLT-3, CCRF-CEM, or P12/Ichikawa T-cell lines. Pattern B is typical for melanoma tissues from patients and some melanoma lines. However, melanoma cell lines frequently showed some level of GM2 and GD2 expression (3, 24, 25). Therefore, patterns D or F represent the actual ganglioside profile of many melanoma cell lines. On the other hand, neuroblastoma lines and glioma lines corresponded to pattern C or E in which β1,4-GalNAc-T is very high and GM2 and GD2 are dominant in comparison with the precursors GM3 and GD3, as observed previously (14). Myelogenous leukemia lines belong to this group also. This point was confirmed by comparing the β1,4-GalNAc-T and α2,8-S-T activities in 8 of the cell lines (Table 2).

In addition to the factors taken into consideration in proposing the patterns shown in Fig. 8, other parameters could be important in

| Table 2 Comparison of GM2/GD2 synthase and GD3 synthase activities |
|-----------------------------|-----------------------------|
| **Cell lines** | **β1,4-GalNAc-T (pmol/mg/h)** | **α2,8-S-T (pmol/mg/h)** | **Ganglioside pattern** |
| SK-MEL-28 | 0 | 1921 | F (B) |
| SK-MEL-23 | 12 | 1213 | F |
| MeWo | 32.5 | 789 | F |
| SK-MEL-31 | 923 | 1502 | D |
| SK-MEL-37 | 376 | 463 | D |
| IMR-32 | 709 | 162 | E |
| HL-60 | 192.5 | 1 | C |
| Raji | 0 | 1 | A |

* Classified as shown in Fig. 8.
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