Characteristic Expression of Glycosphingolipid Profiles in the Bipotential Cell Differentiation of Human Promyelocytic Leukemia Cell Line HL-60

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Changes of glycosphingolipids (GSLs) in the bipotential cell differentiation of human promyelocytic leukemia cell line HL-60 cells were investigated by high-performance thin-layer chromatography (HPTLC), with special reference to morphological and functional changes, such as phagocytosis and nitroblue tetrazolium (NBT) reduction. Nine molecular species of neutral GSLs and 13 or more species of sialo-GSLs, ie, gangliosides, were detected on the HPTLC chromatograms for untreated HL-60 cells. The major components were ceramide dihexoside (CDH), GM3, and sialo-paragloboside (SPG). When HL-60 cells were induced to differentiate into both myeloid mature cells and macrophage-like cells in vitro, no new molecular species of GSLs specific for one of the cell differentiations was induced, but distinctive quantitative changes in the GSL composition were definitely observed between the two cell differentiations. During the myeloid differentiation induced by either dimethylsulfoxide (DMSO) or retinoic acid (RA), CDH, paragloboside (PG), and gangliosides having longer sugar moieties characteristically increased with a concomitant decrease of GSLs with shorter sugar chains, such as ceramide monohexoside (CMH) and GM3, and the GSL composition profile of myeloid differentiation-induced HL-60 cells became more similar to that of normal human granulocytes. However, some marked differences were noted between the induced HL-60 cells and the normal granulocytes, especially in the ganglioside compositions. These differences might reflect either some deficiency in the in vitro myeloid differentiation or some leukemic properties of HL-60 cells. In marked contrast to the change of GSL composition during myeloid differentiation, a remarkable increase of GM3, with a concurrent marked decrease of CDH, was observed in the process of cell differentiation into macrophage-like cells with 12-O-tetradecanoyl-phorbol-13-acetate (TPA), which suggested an increase in the biosynthesis of GM3. These results demonstrate that HL-60 cells express distinct GSL profiles, depending not only on maturation stages but also on differentiation directions.

Glycosphingolipids (GSLs) have been known to be located almost exclusively on the outer leaflet of plasma membranes. Although they are minor constituents of cell surface membranes, they show characteristic changes in their composition and biosynthesis during cell development, differentiation, and oncogenic transformation. Therefore, they have been considered to be involved in cellular interaction and cell growth regulation. In addition, they may play important physiologic roles, particularly gangliosides, in special interactions with various bioactive factors, such as bacterial toxins, hormones, and interferons. According to their carbohydrate structure, they are classified into three major series, ie, ganglio, globo, and lacto series, and various cells and tissues have characteristic compositions and structural specificities as to their GSLs.

Recently, Klock et al reported that the structure and composition of neutral GSLs specific for each of the human hematopoietic cell lineages were determined. Using various human leukemia-lymphoma cell lines that were considered to be blocked at certain stages of differentiation, we have also reported that human hematopoietic malignant cells show ganglioside profiles characteristic of their cell lineages and differ-

*The structures of GSLs are as follows: CMH, Gal-Cer or Glu-Cer; CDH, Gal(β1-4)Glc-Cer or Gal(α1-4)Gal-Cer; LTC, GlcNAc(β1-3)Gal(β1-4)Glc-Cer; Glob (globotetraosylceramide), GaINAc(β1-3)Gal(α1-4)Gal(β1-4)Glc-Cer; PG, Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer; GM1, Gal(β1-3)GlcNAc(β1-4) [NeuAc(α2-3)]Gal(β1-4)Glc-Cer; GM2, GalNAc(β1-4) [NeuAc(α2-3)]Gal(β1-4)Glc-Cer; GD1a, [NeuAc(α2-3)]Gal(β1-3)GlcNAc(β1-4) [NeuAc(α2-3)]Gal(β1-4)Glc-Cer; GD1b, Gal(β1-3)GlcNAc(β1-4) [NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer; GD3, NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)Glc-Cer; GTb1, NeuAc(α2-3)Gal(β1-3)GlcNAc(β1-4) [NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer; SPO, NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer. The symbols for gangliosides are essentially based on the nomenclature system of Svennerholm. The abbreviations used are: Gal, α-D-galactose; Glc, α-D-glucose; GaINAc, N-acetyl-α-D-galactosamine; GlcNAc, N-acetyll-α-D-glucosamine; NeuAc, N-acetyl-α-D-neuraminic acid; Cer, ceramide (N-acylephosphosine); DEAE, diethylaminoethyl.
tiation stages and that the ganglioside composition remarkably changes during cell differentiation of mouse myeloid leukemia M1 cells into macrophage-like cells. Results similar to the latter were also reported by Rosenfelder et al. They suggested that gangliosides could serve as complemental differentiation markers for both normal and malignant hematopoietic cells. Such characteristic profiles of cell surface complex carbohydrates have also been demonstrated in glycoproteins among human leukemic cell lines blocked at various stages of myeloid differentiation.

Human promyelocytic leukemia cell line HL-60 cells could be induced to differentiate both into myeloid mature cells and macrophage-like cells and consisted of stem cells that were bipotent with respect to myeloid or macrophage differentiation. In the present study, we investigated changes of GSL composition in the bipotential cell differentiation of HL-60 cells and demonstrated that not only did the cells exhibit bipotency in their functional and morphological differentiation, but also showed distinct changes of GSL composition between two separate pathways of cell differentiation. It is noteworthy that distinctive expression of GSL profiles was observed not only in different maturation stages, but also in different differentiation directions of the bipotent cell that was of hematopoietic origin.

MATERIALS AND METHODS

Chemicals

Retinoic acid (RA) (trans-vitamin A acid) and 12-0-tetradecanoyl-phorbol-13-acetate (TPA) were obtained from Sigma Chemical Co, St. Louis. Neutral GSLs were products of Supelco Inc, Bellefonte, Pa. Gangliosides GM1, GM2, GD1a, and GT1b were isolated and purified from bovine and human brain, and GM3 from dog erythrocytes in our laboratory according to the method of Momoi et al. All other reagents used were of the best grade available and were from Wako Pure Chemicals Co, Tokyo.

Cells and Cell Culture

Human promyelocytic leukemia cell line HL-60 cells and human histiocytic lymphoma cell line U937 cells were grown in Falcon 3024 tissue culture flasks (Becton Dickinson Labware, Oxnard, Calif) in RPMI 1640 (Flow Laboratories, McLean, Va) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Stanmore, NSW, Australia), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 5% carbon dioxide.

For the induction of myeloid differentiation in HL-60 cells, the cells were seeded at an initial concentration of 2 x 10⁶ cells/mL and incubated with either 1.3% dimethylsulfoxide (DMSO) or 1 µmol/L RA. A morphological study of myeloid differentiation-induced HL-60 cells was carried out by Wright-Giemsa staining of cytosin slide preparations prepared with a Shandon Cytospin centrifuge (Shandon Southern Products, Ltd, Astmoor, Runcorn, Cheshire, England). Macrophage-like cell differentiation was induced in the cells with 4 nmol/L TPA. Adherent cells were recovered by scraping with a rubber policeman, after removing nonadherent cells and washing with phosphate-buffered saline (PBS, pH 7.4). The number of adherent cells was expressed as compared to the total number of cells. For lipid analyses, adherent cells and nonadherent cells were combined and harvested. Normal human granulocytes were prepared by the Conray-Ficoll method from heparinized peripheral blood. The contaminating erythrocytes were removed by hypotonic lysis, and the final preparations consisted of more than 98% neutrophils, as determined by Wright-Giemsa staining.

Phagocytic Activity and NBT Reduction

Phagocytic activity of undifferentiated or differentiation-induced cells was measured by counting the number of cells phagocytosing more than five polystyrene latex particles, as described previously. The ability of cells to reduce nitroblue tetrazolium (NBT) dye was assayed according to the method of Collins et al.

Lipid Extraction

For lipid analyses, the cells were collected, washed twice with PBS, and then lyophilized and kept at -80 °C until use. Total lipids were extracted from the lyophilized materials, equivalent to about 2 to 3 x 10⁶ viable cells, with chloroform-methanol (C-M) (1:1, vol/vol) containing 3% (vol/vol) water, and then with C-M (2:1, vol/vol) and C-M (1:2, vol/vol), successively (two hours for each extraction). The extracts were combined and separated into the acidic and neutral lipid fractions by DEAE-Sephadex A-25 (acetate form) column chromatography, essentially as described by Ando et al.

Analysis of Neutral GSLs

Neutral GSLs were purified from the neutral lipid fraction according to the acetylation method described by Saito et al. They were separated on high-performance thin-layer chromatography (HPTLC) plates with a solvent system of C-M-water (65:25:4, vol/vol/vol), sprayed with orcinol-H₂SO₄ reagent, and visualized by heating the plate at 100 °C. Quantitation was carried out by scanning HPTLC chromatograms with a dual wavelength TLC scanner (Shimadzu CS-910) at 540 nm. The hexose content of the total neutral GSLs was determined by the anthrone-H₂SO₄ method with galactose as a standard.

Analysis of Gangliosides

The acidic lipid fraction was subjected to mild alkaline treatment to hydrolyze contaminating phospholipids, and then desalted by gel filtration on Sephadex G-50. The recovered ganglioside fraction was separated on an HPTLC plate with a solvent system of C-M-0.5% CaCl₂ (55:45:10, vol/vol/vol). The gangliosides were sprayed with resorcinol-HCl reagent and visualized by heating the plate at 95 °C and determined quantitatively by the densitometric scanning method, as described previously. Lipid-bound sialic acid in the total ganglioside fraction was estimated by the resorcinol-HCl method as modified by Suzuki.

RESULTS

Differentiation Induction of HL-60 Cells

When HL-60 cells were cultured in the presence of either 1.3% DMSO or 1 µmol/L RA, their growth was suppressed after six days and two days of culture, respectively (Table 1). The cells morphologically dif-
Table 1. Morphological and Functional Differentiation of HL-60 Cells by Chemical Inducers

<table>
<thead>
<tr>
<th>Inducers</th>
<th>Cell Density</th>
<th>Morphology</th>
<th>Phagocytic Activity</th>
<th>NBT Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10⁶ Cells/mL)</td>
<td>Myeloid Mature Cells (%)</td>
<td>Adherent Cells (%)</td>
<td>Phagocytosing Cells (%)</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>8</td>
<td>—</td>
<td>18</td>
</tr>
<tr>
<td>DMSO</td>
<td>3d</td>
<td>8.6 (8.9*)</td>
<td>41</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>6d</td>
<td>27.4 (28.8)</td>
<td>83</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>8d</td>
<td>32.4 (44.6)</td>
<td>92</td>
<td>—</td>
</tr>
<tr>
<td>RA</td>
<td>2d</td>
<td>5.2 (6.0)</td>
<td>60</td>
<td>—</td>
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<tr>
<td></td>
<td>4d</td>
<td>9.3 (14.7)</td>
<td>90</td>
<td>—</td>
</tr>
<tr>
<td>TPA</td>
<td>12h</td>
<td>11.3 (11.4)</td>
<td>—</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>12.8 (15.2)</td>
<td>—</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>36h</td>
<td>11.4 (18.5)</td>
<td>—</td>
<td>74</td>
</tr>
</tbody>
</table>

*The cell density of untreated HL-60 cells after each culture period is shown in parentheses.

For induction of myeloid- and macrophage-like cell differentiations, HL-60 cells were seeded at an initial concentration of 2 x 10⁶ cells/mL and 10⁶ cells/mL, respectively. Cell numbers were counted in hemocytometer and the viability of the cells was determined by the dye-exclusion method with 0.1% erythrosine B in PBS. After each culture period, differential counts were performed on the Wright-Giemsa-stained cytospin smears, with at least 200 cells assessed, and the number of adherent cells was determined as described in Materials and Methods. The percentage of phagocytosing cells and NBT reduction-positive cells was determined by counting at least 200 cells. Myeloid mature cells represent myelocytes and their descendants, ie, metamyelocytes, and banded and segmented neutrophils.

DMSO-induced HL-60 cells were differentiated into myeloid mature cells, with a marked increase in their phagocytic activity and the number of cells positive for NBT reduction (Table 1).

On the other hand, when HL-60 cells were cultured with 4 nmol/L TPA, the cells were induced to differentiate into macrophage-like adherent cells showing prominent pseudopods. The number of macrophage-like adherent cells increased with the culture time, and the phagocytic activity of the cells was also enhanced. However, no significant changes were observed in NBT reduction (Table 1).

GSLs of HL-60 Cells

Both neutral GSLs and ganglioside compositions of HL-60 cells were analyzed by HPTLC. Neutral GSLs of the cells were composed of ceramide monohexoside (CMH) (bands 1 and 2), ceramide dihexoside (CDH) (bands 3, 4, and 5), lactotriaoyl ceramide (LTC; bands 6 and 7), and paragloboside (PG) (bands 8 and 9), which were detected in normal human granulocytes, as reported by Macher et al. and the major component was CDH (Fig 1). They also reported that the greater parts of CMH and CDH were Glc-Cer and Gal-Glc-Cer (lactosylceramide), respectively. On the other hand, gangliosides of the cells consisted of 13 or more molecular species, and the major components were GM3 (bands 1 and 2) and sialo-paragloboside (SPG) (bands 5 and 6). The Rf values of gangliosides designated as bands 7 and 8, band 13, and band 15 were similar to those of the GD3 doublet, GDlb, and GTlb, respectively (Fig 2).

Changes of GSLs During Myeloid Differentiation of HL-60 Cells

When myeloid differentiation was induced with DMSO, no new molecular species of GSLs appeared in the differentiation-induced cells, but characteristic changes in the GSL composition were observed (Figs 1 and 2). During such in vitro differentiation, CDH and PG increased, with a concomitant decrease of CMH, and the composition profile of neutral GSLs resembled that of normal human granulocytes, although the total amount of neutral GSLs in the differentiation-induced cells was lower. Interestingly, among the three CDH molecular species, band 3, which was not found in normal granulocytes, characteristically increased (Figs 1 and 3). On the other hand, the ganglioside molecular species having longer sugar moieties, such as bands 9 and 10, and those that migrated near GDlb (band 13) and GTlb (band 15) also increased, with a concomitant decrease of GM3, and the ganglioside profile of HL-60 cells became more similar to that of normal human granulocytes (Fig 3). However, ganglioside bands 7 and 8, which were one of the major ganglioside constituents of the normal granulocytes (Figs 2 and 3), never increased, but significantly decreased during myeloid differentiation of the cells, and the higher content of SPG was retained in the differentiation-induced cells (Fig 3). When myeloid differentiation was induced with RA, essentially the same changes of GSL composition were observed (data not shown).
Fig 1. An HPTLC chromatogram and the densitometric scanning pattern of neutral GSLs in HL-60 cells and their myeloid differentiation-induced derivatives. The neutral GSL fractions corresponding to $6 \times 10^7$ cells were separated on HPTLC plates, and the chromatograms were scanned with a dual wavelength TLC scanner, as described in the text. Nine orcinol-positive components were detected in untreated HL-60 cells. No new molecular species of neutral GSLs was found in the differentiation-induced derivatives. Neutral GSL components designated as bands 1 and 2, bands 4 and 5, bands 6 and 7, and bands 8 and 9 comigrated with CMH, CDH, LTC, and PG, respectively. Standard LTC was prepared from PG by the $\beta$-galactosidase treatment. Numbers represent neutral GSL components detected in the untreated HL-60 cells.

Fig 2. HPTLC chromatograms and the densitometric scanning pattern of gangliosides in HL-60 cells and their myeloid differentiation-induced derivatives. The ganglioside fractions corresponding to $6 \times 10^7$ cells were separated on HPTLC plates, and the chromatograms were scanned as described in the text. Gangliosides of HL-60 cells and their differentiated derivatives were compared with those of normal human granulocytes. Ganglioside bands 7 and 8, which were among the major ganglioside components in normal human granulocytes, had an $R_F$ value similar to that of the GD3 doublet. Numbers represent ganglioside bands detected in the normal human granulocytes.
Changes of GSLs During Macrophage-like Cell Differentiation of HL-60 Cells

In the HL-60 macrophage-like cell differentiation induced with TPA, no new molecular species of GSLs appeared, but their GSLs showed compositional changes distinct from those observed in the myeloid differentiation of HL-60 cells (Figs 4 and 5). In marked contrast to the changes of GSL composition in the myeloid differentiation, CDH and PG decreased, with a concurrent increase of CMH, and GM3 remarkably increased with no significant changes in the amount of other ganglioside components (Fig 6). The ganglioside composition of macrophage-like differentiation-induced HL-60 cells well resembled that of human monocytoid cell line U937 cells (Figs 5 and 6), although that of normal human monocytes has not yet been elucidated because of the difficulty of collecting enough of the cells for chemical analyses of gangliosides. Similarities of the neutral GSL composition between them were not found (Figs 4 and 6). However, when U937 cells were induced to differentiate into macrophage-like adherent cells with TPA, their neutral GSL profile became similar to that of the macrophage-like differentiation-induced HL-60 cells, except for the higher content of PG in the former. A slight increase of GM3 was also observed in the differentiated U937 cells (data not shown).

DISCUSSION

We have shown that HL-60 were not only bipotent in their functional and morphological differentiation, but also were remarkably different in the GSL composition after two separate inductions of cell differentiation. Differentiation stage-associated differences in the profiles of neutral glycolipids, gangliosides, and glycoproteins have been reported among human leukemia cells and the established human leukemia-lymphoma cell lines of different maturity. Our results indicate that GSL profiles of hematopoietic cells characteristically change, depending on the direction of cell differentiation as well as on the stage of maturation, and suggest that GSL profiles might serve as useful differentiation markers for both normal and malignant hematopoietic cells, implying their maturity and cell lineages.

Previously, we have reported that GM3 was dominant in human myeloid leukemia cell lines assigned to the early differentiation stages by specific surface marker profiles, and that the ganglioside species decreased with a concomitant increase of gangliosides...
Fig 5. HPTLC chromatogram and the densitometric scanning pattern of gangliosides in HL-60 cells and their macrophage-like differentiation-induced derivatives. The ganglioside fractions corresponding to $6 \times 10^7$ cells were developed on HPTLC plates, and each component was assayed by densitometric scanning as described in the text. The ganglioside HPTLC pattern of the differentiated cells was compared with that of U937 cells. Numbers correspond to those in Fig 3.

having longer sugar chains in those cell lines that belonged to the later differentiation stages. It is suggested that the complexity of sugar moieties of gangliosides increases as myeloid cells attain maturation. Macher et al recently reported "large gangliosides" with the general structure, NeuAcα2-3(Galβ1-3,4GlcNAcβ1-3)nGalβ1-4Glcβ1-1Cer, in normal mature granulocytes. The increase in the complexity of sugar moieties during myeloid differentiation has also been observed in neutral glycolipids and in carbohydrate structure of cell surface glycoproteins.

Lee et al reported that the major gangliosides of human acute myelomonoblastic leukemia cells were GM3 and SPG and that the cells contained a small amount of gangliosides having longer sugar moieties. Together with their results, it is suggested that the ganglioside profile with GM3 being dominant is one of the characteristics of monocytoid cells.

Differences in GSL profiles between the two differentiation of the HL-60 cells might reflect those in the enzymatic activities involved in the metabolism of GSLs as reported for glycopeptide synthesis. For example, sialidase (EC 3.2.1.18) is one of the important enzymes involved in ganglioside metabolism. The enzyme activity was stimulated during myeloid differentiation of HL-60 cells, but no significant changes in the activity were observed in the process of macrophage-like differentiation of the cells (unpublished data). A marked decrease of CDH with a simultaneous increase of CMH might indicate that degradation of CDH to CMH occurred, but a remarkable increase of GM3 with no significant changes in
Fig 7. Proposed pathways of GSL synthesis activated in the bipotential cell differentiation of HL-60 cells. The open arrow represents the main synthetic pathway of GSLs stimulated in the myeloid differentiation of HL-60 cells, and the solid arrow represents the activated pathway in the HL-60 macrophage-like cell differentiation.

other gangliosides suggests that sialyltransferase was activated to synthesize GM3 from CDH during cell differentiation into macrophage-like cells. An increase of GM3 with a concomitant decrease of CDH was also observed in the macrophage-like differentiation of U937 cells with TPA. The activation of the synthetic pathway of GM3 from CDH might be one of the phenomena characteristic of the macrophage-like cell differentiation.

Distinctive pathways of GSL synthesis during the two separate differentiations of HL-60 cells are tentatively proposed from the results of chemical analyses in the present study (Fig 7). A metabolic study and further characterization of individual GSLs are now in progress.

Ganglioside bands 7 and 8 consisted of the major ganglioside components of normal granulocytes. However, this ganglioside unexpectedly decreased during myeloid differentiation of HL-60 cells and remained as a minor constituent in the differentiation-induced cells, and the ganglioside molecules having longer sugar moieties found near the origin on HPTLC were not fully increased in the induced cells. The results suggest that the in vitro process of the myeloid differentiation of HL-60 might be defective or that the myeloid differentiation-induced HL-60 cells might be abnormal as myeloid mature cells, despite their morphological assessment. Such defect or abnormality in the myeloid differentiation of HL-60 cells has recently been indicated by others.27,28 The results might also reflect the leukemic origin of the cells as described in studies on fucosylglycopeptides of the cell surface membrane.29

Sialidase activity increased in the process of myeloid differentiation of HL-60 cells,26 and the activity in the differentiated cells was much higher than that of normal human granulocytes, 19.83 nmol/mg protein/h in the former after eight days of culture with DMSO and 1.27 nmol/mg protein/h in the latter, when 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (4MU-NeuAc) was used as a substrate. This might also explain in part the difference in ganglioside composition between them.

Recently, various myeloid- or monocyte/macrophage-specific monoclonal antibodies have been produced, and the differentiation-related antigenic changes of HL-60 cells have been investigated, especially with regard to myeloid differentiation with them.30-34 On the other hand, various GSL molecular species have recently been characterized as cell surface antigens,2 and it has also been reported that the antigenic determinants of the myeloid-specific antigens identified by monoclonal antibodies are sugar sequences found not only on glycoproteins but also on glycolipid molecules with a long carbohydrate chain.35,36 Together with these reports, quantitative changes in GSL composition, as shown in the present study, might be partly implicated in such antigenic changes during myeloid differentiation of HL-60 cells.

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