Ganglioside GM₃ Can Induce Megakaryocytoid Differentiation of Human Leukemia Cell Line K562 Cells¹

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ABSTRACT

The role of acidic glycosphingolipids in cell growth and differentiation was investigated using the multipotent leukemia cell line K562. When GM₃ was added to cell culture media, the growth of K562 cells was remarkably inhibited and the cells were shown to have megakaryocytic morphology. Ultrastructural study demonstrated that K562 cells treated with GM₃ had platelet peroxidase-positive structures, which were considered to be the specific marker of megakaryocyte. Furthermore, AP-3 directed against an epitope present on membrane glycoprotein Illa reacted with the GM₃-treated cells. Free N-acetyllactosaminic acid, GM₁, GM₂, GD₃, and a mixture of bovine brain gangliosides containing GD₃, and GT₃ did not affect growth of K562 cells or show morphological changes. According to chemical analyses, GM₃ content increased in megakaryocytoid differentiation induced by tetradecanoylphorbol-13-acetate, whereas GD₃ decreased in erythroid differentiation induced by hemin. Enzymatic analysis showed that the GM₃ increase during megakaryocytoid differentiation was a result of the sialyltransferase activation. These results indicated that exogenous GM₃ induced differentiation of K562 cells into a “GM₃-rich” lineage, i.e., mainly megakaryocytoid differentiation was a result of the sialyltransferase activation. The role of acidic glycosphingolipids in cell growth and differentiation was further investigated using the multipotent leukemia cell line. Previously we demonstrated that GM₃ ganglioside, a minor membrane component, has a crucial role in not only the differentiation induction but also the determination of the differentiation direction in pluripotent K562 cells.

INTRODUCTION

Gangliosides, sialic acid-containing glycosphingolipids (GSLs), are a structurally varied class of complex carbohydrates and are ubiquitous membrane components in vertebrates, localized almost exclusively on the outer leaflet of the plasma membrane (1, 2). They have been proposed to play important roles in cellular interactions, differentiation, and oncogenesis (3), especially in cell to cell interaction (4), neurite outgrowth (5, 6), and cell growth (7, 8) in various cell systems. Changes in glycosphingolipids during hematopoietic cell differentiation were also studied using human leukemia cell line HL-60 cells and were found to be dependent on the directions of differentiation (9, 10). This suggested a specific role of GSLs in differentiation induction of leukemia cells. Previously we demonstrated that exogenous ganglioside GM₂ and neolacto-series gangliosides induced monocytic and granulocytic differenti-
an equal volume of Dulbecco's modified Eagle's minimal essential medium and Ham's F-12 medium of 2-fold concentration and was added to the culture medium to the desired ganglioside concentration (11).

Evaluation of Cell Differentiation. For morphological assessment of cell differentiation, cytospin slides were prepared with a Shandon cytopin centrifuge (Shandon Southern Products, Cheshire, England) and stained with Wright-Giemsa staining solutions. Non-specific esterase activity was determined using a-naphthyl butyrate and naphthol AS-D-chloroacetate as substrates (24). Hemoglobin staining was conducted by the method using o-dianisidine (3,3'-dimethoxybenzidine).

For PPO analysis (25), untreated and ganglioside-treated cells were fixed with a mixture of 4% paraformaldehyde and 1% tannic acid in phosphate buffer (pH 7.4) for 1 h and then incubated in the medium containing 10 mg of 3,3'-diaminobenzidine tetrahydrochloride in 10 mM Tris-HCl buffer (pH 7.6) with 0.01% hydrogen peroxide for 60 min to detect the activity of platelet peroxidase. The specimens were then washed several times in phosphate buffer (pH 7.4) which contained 4% glucose and postfixed for 1 h in 1% osmium tetroxide. After postfixation, the specimens were dehydrated and embedded in Epoxy resin. Ultrathin sections were examined under an electron microscope.

Untreated K562 cells, chemical inducer-treated cells, and GM1-treated cells were examined for expression of membrane glycoprotein Gp IIIa with flow cytometry, an Ortho Spectrum III, using a monoclonal antibody AP-3 (26), which was a generous gift from Dr. Peter J. Newman (The Blood Center of Southeastern Wisconsin, Milwaukee, WI).

Ganglioside Analysis of K562 Cells. Gangliosides from K562 cells treated with chemical inducers were prepared by chloroform/methanol extraction and DEAE-Sephadex A-25 chromatography (20) and separated on an HPTLC plate with a solvent system of chloroform/methanol/0.5% CaCl2(OH)2 (50:50:10, v/v/v). The HPTLC plates were visualized by heating the plates at 95°C (27) followed by quantitation using a CS-9000 flying-spot scanner (Shimadzu, Kyoto, Japan).

GM3, GM2, and GD1a were characterized by the thin layer chromatography-immunostaining method (28). After separation of gangliosides on an HPTLC plate, the plate was coated with 0.4% polyisobutylmethacrylate (Aldrich, Milwaukee, WI). The plate was reacted with resorcinol/HCl reagent and gangliosides were visualized by heating the plates at 95°C (27) followed by quantitation using a CS-9000 flying-spot scanner (Shimadzu, Kyoto, Japan).

GM3 Synthetic Activity Assay. K562 cells (5-10 x 10^7) treated with chemical inducers were homogenized in 1-2 ml of 0.25 M sucrose in 25 mM sodium cacodylate buffer, pH 6.9, with 1 mM EDTA using a Polytron homogenizer (Kinetamika GmbH, Littau-Luzern, Switzerland) with a PTA10S generator for 30 s at a maximum speed. After centrifugation with 700 x g for 10 min, the supernatant was spun at 105,000 x g for 60 min using a TL-100 ultracentrifuge (Beckman, Palo Alto, CA). The pellet was resuspended in about 100 µl of homogenization buffer using a Potter-type homogenizer.

GM3 synthetic activity was assayed as follows: 10 nmoi of LacCer and 2.5 µl of CMP-[sialic acid]-dilabeled NeuAc were added in a microtube and then dried. CMP-NeuAc (4.8 mmol), sodium cacodylate buffer (pH 6.2, 3.75 mmol), Triton CF-54 (75 µg), and enzyme preparation (100-200 µg protein) were incubated in a total volume of 25 µl for 3 h. The radioactive product was separated from other radioactive compounds using reverse-phase adsorption column chromatography as described previously (30). Radioactivity was counted in liquid scintillation spectrometry.

Protein Assay. Protein was determined by an Amido-Schwarz dye-binding method (31) using bovine serum albumin as a standard.

RESULTS

Effect of Exogenous Gangliosides on Cell Growth and Cell Differentiation in K562 Cells. To examine the effect of exogenous gangliosides on growth of K562 cells, gangliosides were added to the cell culture medium. According to ganglioside analyses of K562 cells, GM3, GM2, and GD1a were found to be the major components. Therefore, we examined GM3, GM2, GM1, GD1a, a bovine brain ganglioside mixture, and free NeuAc for their bioactivities. The final concentrations of these gangliosides were adjusted to 50 µM. As shown in Fig. 1A, cell growth was completely inhibited only when treated with exogenous GM3. Other gangliosides and free NeuAc had no effect on growth of K562 cells. For morphological examination, K562 cells were stained by Wright-Giemsa procedures after 6 days of incubation with 50 µM GM3. Changes were found in the morphology of K562 cells treated with GM3. Forty % of GM3-treated cells showed megakaryocytoid cell morphology (Fig. 2B) and another 40% showed the morphology, which was distinct from megakaryocyte and other hematological cells, with large cytoplasm and 4 nuclei (Fig. 2C). Ten % of the cells showed macrophage-like morphology (Fig. 2D). Unlike GM3, other ganglioseries gangliosides and free NeuAc had no effect on morphology of K562 cells.

When K562 cells were cultured with various concentrations of GM3, cell growth was inhibited markedly in a dose-dependent manner (Fig. 1B) and was completely inhibited with 50 µM GM3. During the culture period, K562 cells displayed >90% viability. This indicated that the suppressive effect of GM3 on cell growth was not due to a cytotoxic effect or a result of selective enrichment for differentiated cells but was due to an inhibition of the proliferation of the leukemic cells by the enforced terminal differentiation.

Ultrstructural Morphology. Untreated K562 cells seemed to have lobulated nucleus with a smooth outline and with nucleoli (Fig. 3A). No chromatin condensation was observed. The cytoplasmic organelles were not well developed and the cytoplasm contained only a few mitochondria, monosomes, and polysomes. No rough endoplasmic reticulum was found. On the other hand, GM3-treated K562 cells showed a low nuclear/cytoplasmic ratio and their nuclei showed lobular structures with nuclear clefts and little chromatin condensation (Fig. 3B). Rough endoplasmic reticulum and granules in the cytoplasm were recognized. These results showed that GM3-treated cells became mature and differentiated. Untreated K562 cells were negative for PPO (Fig. 3A). After stimulation with GM3, however, cells with a PPO-positive reaction in rough endoplasmic
Ganglioside GM3 Induces K562 Cell Differentiation

Fig. 2. Differentiation induction of K562 cells with exogenous GM3. A, untreated K562 cells cultured in the serum-free medium for 6 days (Wright-Giemsa stain). B-D, K562 cells cultured in the serum-free medium with 50 μM exogenous GM3 for 6 days (Wright-Giemsa staining). Original magnification, ×400.

Fig. 3. Electron microscopic observation of K562 cells after treatment with exogenous GM3. A, untreated K562 cells cultured in serum-free medium. B, K562 cells cultured in the serum-free medium with 50 μM exogenous GM3; arrows, PPO-positive structures. Bars, 1 μm.

reticulum were detected (Fig. 3B). These were considered typical cells of megakaryocytic origin induced by exogenous GM3.

Expression of Differentiation Markers in K562 Cells. Markers for differentiation were analyzed with hemoglobin staining, esterase double staining, and membrane glycoprotein expression. As shown in Table 1, TPA-treated K562 cells expressed Gp IIIa recognized by monoclonal antibody AP-3, whereas o-dianisidine-positive cells did not increase during the TPA treatment. o-Dianisidine-positive cells were markedly increased after hemin treatment, while AP-3-positive cells showed only a little increase. TPA induced K562 cells into a megakaryocytic lineage and hemin induced the cells into an erythroid lineage because Gp IIIa was specific for platelets or megakaryocytoid cells (32) and o-dianisidine reactivity was specific for an erythroid lineage as previously described (16, 18). On the other hand, none of the cells was stained by o-dianisidine after GM3 treatment; however, the cells having o-naphthyl butyrate esterase activity, which was completely inhibited by NaF, increased 3-fold as much as the control. Furthermore, in GM3-treated K562 cells, Gp IIIa-positive cells increased 2-fold as much as the control.

Analysis of Gangliosides from K562 Cells. We analyzed ganglioside composition of K562 cells after treatment with chemical inducers. Fig. 4 shows the ganglioside patterns of TPA- and hemin-treated K562 cells as well as nontreated cells. The compounds corresponding to GM3, GM2, and GD1a, were characterized by the immunostaining method using specific anti-

Table 1 Expression of differentiation markers in K562 cells

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<th>Hemoglobin staining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Esterase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AP-3&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>(o-dianisidine)</td>
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<td>Control</td>
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<td>GM3</td>
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<sup>a</sup> o-Dianisidine positive cells were classified into three groups, i.e., ++, strongly stained; +, normally stained; ±, faintly stained; −, not stained.
<sup>b</sup> % positive cells; mean of two determinations. SD < 10%.
<sup>c</sup> ND, not done.
There were two components located between GM2 and GD1a, which corresponded to α2 → 3 and α2 → 6 sialosylparaglobosides when judged by their mobilities on HPTLC. These two were decreased in all treatments.

Enzymatic Basis for GM3 Increase in Megakaryocytoid Differentiation. To assess the enzymatic background of GM3 increase in TPA-treated K562 cells, GM3 synthase activities were determined. Fig. 5B clearly shows that the sialyltransferase activities increased in TPA-treated K562 cells and decreased in hemin-treated cells. GM3 synthase activities corresponded well to GM3 contents in the control K562 cells, TPA-treated cells, and hemin-treated cells. Furthermore, GM3 synthase activities were elevated in a time-dependent manner in TPA-treated K562 cells, and the radioactive product after the enzymatic reaction was clearly identified as GM3 on HPTLC plates (data not shown).

DISCUSSION

Ganglioside profiles of leukemia cells showed specific patterns and were thought to be biochemical markers for human hematopoietic cell lines (33). Moreover, they were dependent upon both differentiation stages and directions of differentiation (9). In the human myelogenous leukemia cell line HL-60, we previously demonstrated that GM1 increased during monocytic differentiation, while neolacto-series gangliosides characteristically increased during granulocytic differentiation (10). Furthermore, we reported that exogenous ganglioside GM1 could induce monocytic differentiation (11) and neolacto-series gangliosides could induce granulocytic differentiation (12) of HL-60 cells. These results indicated that gangliosides in the cell surface membrane may play critical roles in regulation of the growth and differentiation of leukemia cells.

The K562 cell line was derived from a patient with chronic myeloid leukemia in acute blastic crisis (13). It was reported that K562 cells had glycophorin as a marker of erythroid cells (14) and synthesized embryonic hemoglobin in response to chemical inducers, sodium butyrate, and hemin (15, 16), indicating that the cells had potential to differentiate into an erythroid lineage. It was also reported that K562 cells had antigens recognized by H5 and anti-M1 as markers of myeloid cells (18, 34, 35). On the other hand, when induced by TPA, K562 cells expressed Gp IIb/IIIa and a moderate number of induced cells became positive for PPO (18, 19), indicating that the cells had potential to differentiate into a megakaryocytic lineage. Furthermore, K562 cells could be differentiated into erythroid, granulocytic, and monocytic lineages spontaneously in long-term culture (17). K562 cells, however, did not have lymphocyte markers (36). On the basis of these studies, it was considered that K562 cells were derived from pluripotent hemopoietic stem cells and that the cells could potentially differentiate into erythroid, monocytic, granulocytic, and megakaryocytic lineages. In our experiments, we investigated two differentiation lineages, megakaryocytoid and erythroid lineages, which were induced by chemical inducers, TPA and hemin, respectively, because monocytic and granulocytic differentiation in long-term culture seemed spontaneous (17) and were difficult to subject to repetitive analyses.

Suzuki et al. (37) have reported that GM3, GM2, and GD1a were the major components of gangliosides in K562 cells. Confirming these molecular structures by immunostaining, we found that the cells had minor components in addition, α2 → 3 and α2 → 6 sialosylparaglobosides. However, the structures of these two acidic glycolipids were yet to be characterized more clearly. After induction of differentiation by TPA and hemin, the two minor gangliosides decreased, whereas the major GM2 and GD1a increased. However, GM1 behaved in a totally different manner in both lineages. While GM2 increased in erythroid differentiation induced by hemin, GM1 increased in megakaryocytic differentiation induced by TPA, indicating that the megakaryocytic, but not the erythroid, lineage was “GM3-rich.” It was previously reported that human platelets had GM3 as a major component of gangliosides (38). While sialosylparagloboside was the major component, GM1 was only 25.7% of the total lipid-bound sialic acid in human erythrocytes (39).
Our present results indicate that about 40% of acidic glycosphingolipids consist of GM₃ in nontreated K562 cells. This suggests that the GM₃ content in human hematopoietic cells decreased along with erythrocytic differentiation. A major component of gangliosides in human mature monocytes was GM₃ (40), and GM₃ was increased along with monocytic differentiation of human leukemia HL-60 cells (10). On the other hand, sialosylparaglobosides and other neolacto-series gangliosides were major components in human mature granulocytes (41), and the neolacto-series gangliosides were increased along with granulocytic differentiation of human leukemia HL-60 cells as reported earlier (9). Consequently, megakaryocytic and monocytic lineages can be considered as GM₃-rich lineages in the differentiation of human hematopoietic cells, among erythrocytic, monocytic, granulocytic, and megakaryocytic cells.

Using K562 cells, we examined whether or not several gangliosides could inhibit cell growth. Among them, only GM₃ could inhibit growth of the cells, while other gangliosides, GM₁, GM₂, GD₁₃, a bovine brain ganglioside mixture, and free NeuAc had no effect. According to the morphological studies on the basis of Wright-Giemsa staining, electron microscopy, and cytochemistry, the growth inhibition by GM₃ was thought to be the result of terminal differentiation of K562 cells. Moreover, membrane glycoprotein (GP IIIa) expression (26) was also utilized to evaluate cell differentiation. As a result of findings from PPO staining, GP IIIa expression, and the morphological studies, it was considered that about 40% of GM₃-treated K562 cells were differentiated into a megakaryocytic lineage. On the other hand, about 10% of GM₃-treated K562 cells showed macrophage-like morphology and expressed α-naphthyl butyrate esterase activity. These findings indicate that GM₃ could induce differentiation into a monocytoid lineage, as well as a megakaryocytoid lineage, in a small population of K562 cells. We have already reported that GM₃ could induce differentiation into a monocytic lineage in HL-60 and U-937 cells (11). In K562 cells, however, it was shown that exogenous GM₃ could induce differentiation not only into monocytic but also into megakaryocytic lineage. From the studies using HL-60 cells, it was hypothesized that GM₃ induced differentiation of the cells into a GM₃-rich lineage and neolacto-series gangliosides induced differentiation of the cells into a "neolacto-series ganglioside-rich" lineage (11, 12). The present studies support the assumption that some molecular species of gangliosides may have critical roles in determining the direction of differentiation of leukemic cells; exogenous GM₃ induces the differentiation of K562 cells into GM₃-rich lineages. The difference in directions of differentiation between GM₃-treated K562 cells and HL-60 cells might be explained by the fact that the differentiation direction was also dependent on their potential and stages of differentiation.

There have been several reports that gangliosides had physiological effects in cell growth and differentiation. Ganglioside GQ₁₀, was reported to have nerve growth factor-like activities through the effect upon protein kinase (5, 42). It was reported that GM₃ and GM₁, which were decreased in transformed Swiss 3T3 cells, inhibited cell growth (7, 43). These findings indicated that cell surface gangliosides play a role as a regulator or an inhibitor of cell growth via deactivating growth factor receptor kinases. However, in hematopoietic cells the mechanism of cell differentiation by gangliosides was not yet clear. We analyzed GM₃ metabolism in HL-60 cells and suggested that GM₃ incorporation into the cells was mediated by a receptor-like mechanism (44). Furthermore, it was reported that there was a ganglioside-specific binding protein in rat brain (45). Likewise, a GM₃-specific binding protein might exist also in hematopoietic cells, but it remains to be elucidated more clearly. On the other hand, the GM₃ increase in monocytic differentiation of HL-60 cells induced by TPA was the result of an activation of LacCer:NeuAc α2 → 3sialyltransferase as previously reported (46). Our present results also indicate that the GM₃ increase in megakaryocytic differentiation of K562 cells is based on an activation of the same sialyltransferase, while the GM₃ decrease in erythrocytic differentiation is the result of a low level of sialyltransferase activity. Together with the observations reported earlier (46), GM₃ levels and synthetic activity levels and the direction of differentiation induced by TPA, which is related to protein kinase C (47), correspond well with each other in leukemic cell differentiation. In order to understand more clearly these relationship and the mechanism of cell differentiation by gangliosides, it is of interest to determine the sialyltransferase activity in GM₃-treated K562 or HL-60 cells and to elucidate whether protein kinase C directly activates GM₃ synthase activity. Although these remain to be elucidated, GM₃ synthase and its product, GM₃, might play important and critical roles in megakaryocytic differentiation of K562 cells as well as in monocytic differentiation of HL-60 cells.

ACKNOWLEDGMENTS

We give special thanks to Dr. Y. Enomoto, Department of Pathology, School of Medicine, Keio University, for the ultrastructural analysis, and to Dr. M. Ohta in our laboratory and Dr. J-Q. Zhou now in Peking, China, for their assistance with cytochemical studies. We also thank Drs. K. Motoyoshi, S. Kitagawa, M. Ohta, and Y. Furukawa in our laboratory for their valuable discussions.

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