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AN ACIDIC GLYCOSPHINGOLIPID, MONOSIALO-GANGLIOSIDE GM3, IS A POTENT PHYSIOLOGICAL INDUCER FOR MONOCYTIC DIFFERENTIATION OF HUMAN PROMYELOCYTIC LEUKEMIA CELL LINE HL-60 CELLS

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A remarkable increase in monosialo-ganglioside GM3 was observed during the monocytic differentiation of HL-60 cells induced by 12-O-tetradecanoyl-phorbol-13-acetate(TPA). On the other hand, when the cells were cultured with exogenously-added ganglioside GM3 in serum-free conditions, their differentiation along a monocytic lineage was demonstrated with simultaneous complete growth inhibition. Other gangliosides such as ganglioside GM1 showed no effects on cell differentiation, exhibiting instead stimulatory actions on the cell growth. These results indicate that a physiologically-existent, membranous ganglioside GM3, which specifically increases during monocytic cell differentiation, might play a primary role as a trigger in the monocytic cell differentiation.

Glycosphingolipids (GSLs) are located almost exclusively on the outer leaflet of plasma membranes (1), and have been considered to be involved in cellular interactions and cell growth regulations, changing characteristically as to their composition and biosynthesis during cell development, differentiation and oncogenic transformation (2), although they are minor constituents of cell surface membranes. In addition, acidic GSLs, gangliosides, have been recently shown to exhibit special receptor-functions for some bioactive factors

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Symbols and abbreviations used are as follows: GM3(ganglioside GM3), NeuAc(α2-3)Gal(β1-4)Glc-Cer; SPG(sialosylparagloboside), NeuAc(α2-3)-Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer; Gx, the ganglioside molecule having N-acetyllactosamine moieties which is one of the major components in normal human mature granulocytes, but of which the structure has not yet been determined; SnHC(sialosyl nor-hexaosyl ceramide), NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer; LGx, the heterogenous larger gangliosides having longer sugar-moieties which were located near the origin on HPTLC (3); Gal, α-D-galactose; Glc, α-D-glucose; GlcNAc, N-acetyl-α-D-glucosamine; NeuAc, N-acetyl-α-D-neuraminic acid; Cer, ceramide (N-acylsphingosine); TPA, 12-O-tetradecanoyl phorbol-13-acetate; DMSO, dimethylsulfoxide.

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such as bacterial toxins, hormones and interferons (2). GSLs are classified into three major series, i.e. the ganglio-, globo- and lacto-series, according to their carbohydrate structure, and various cells and tissues have characteristic compositions and structural specificities as to their GSLs. We and other investigators have recently reported that human (3-6) and murine (7) hematopoietic malignant cells show ganglioside profiles characteristic of their cell-lineages and differentiation-stages, and that gangliosides can serve as complemental differentiation-markers for both normal and malignant hematopoietic cells (3-8). It was also reported that the enzymic activity of sialidase [EC 3.2.1.18; N-acetylneuraminosyl glycohydrolase], the hydrolytic enzyme participating specifically in the catabolism of sialo-compounds such as sialoglycoproteins and gangliosides (9), changed closely in association with the differentiation of HL-60 cells into granulocytic mature cells (10).

The HL-60 cell line is of human acute promyelocytic leukemia cell origin (11). In continuous cultures, it is a heterogeneous population of cells of which approximately 90% express phenotypic characteristics of either myeloblasts or promyelocytes (12). The cells undergo morphological and functional differentiation in response to the addition of a wide range of chemicals to the culture medium (8,12,13). Differentiation proceeds along either the myeloid or monocytic pathway, depending on the chemical inducer used (14,15). HL-60 cells have been recently demonstrated to show distinct sialo-GSLs profiles, depending not only on the differentiation-stages but also on the differentiation-directions (3). In a search for some physiological functions of such a remarkably-changed sialo-GSL molecule in the cell differentiation processes, we have investigated an exogenous addition of purified ganglioside GM3 to HL-60 cell cultures, and found that this acidic GSL molecule itself is a potent inducer for monocytic differentiation of HL-60 cells.

MATERIALS AND METHODS

Cell Culture: Human promyelocytic leukemia cell line HL-60 cells were grown in Falcon 3024 tissue culture flasks (Becton Dickinson Labware, Oxnard, CA) in serum-free medium at 37°C in a humidified atmosphere of 5% carbon dioxide according to the culture method described by Breitman et al.(16). The serum-free synthetic medium (DME/F12 medium) was composed of equal volumes of Dul-

becco's modified Eagle's minimum essential medium (MEM) and Ham's F12 medium supplemented with 30nM selenium dioxide, 1.2mg/ml sodium bicarbonate, 15mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.2), 5μg/ml insulin (Sigma Chemical Co.,St. Louis) and 5μg/ml transferrin (Sigma Chemical Co., St. Louis).

Cell Differentiation and Morphological Assessment: Induction of cell differentiation of HL-60 cells into monocytic or granulocytic mature cells was performed with 4nM TPA or 1.3% dimethylsulfoxide (DMSO), respectively, as previously reported (13). For morphological assessment of the cells, cytospin slide preparations were prepared using a Shandon Cytospin centrifuge (Shandon Southern Instruments, Inc., Sewickley, PA) and stained with Wright-Giemsa staining solution.

Cytochemical Evaluation of Cell Differentiation: Phagocytic activity of undifferentiated or differentiation-induced cells was measured by counting the number of cells that phagocytosed more than five polystyrene latex particles as described previously (13,18). Nonspecific esterase (α-naphthyl butyrate esterase) activity was detected cytochemically by the esterase double-staining method of Li et al.(19). The appearance of monocyte-specific surface-antigens on uninduced and induced HL-60 cells was investigated by cytofluorometry in Ortho Spectrum III, using authorized monoclonal antibodies such as OKM1 and OKM5, which were recognized to be reactive with human peripheral blood monocytes (20-22).

Analysis and Preparation of Gangliosides: Monosialoganglioside GM1 was isolated from a ganglioside mixture, which was prepared from bovine brain gray matter according to the method of Momoi et al.(23), by prolonged incubation with sialidase (9), and then purified by combined column chromatographies on DEAE-Sephadex and Iatrobeads (24). Ganglioside GM3 was prepared from stromata of dog erythrocytes and highly purified (its purity was higher than 95%, as judged by densitometric scanning of the HPTLC chromatogram) by the same combination of DEAE-Sephadex (A-25) and Iatrobeads column chromatographies used for purification of ganglioside GM1. Other gangliosides, GD1a and GT1b, were also purified from bovine brain in our laboratory essentially according to the method described above. Column chromatographic fractionation of individual glycolipids was monitored by analytical TLC (23). Gangliosides were separated on high-performance thin-layer chromatography (HPTLC) plates (Merck, Damstadt) with a solvent system of chloroform-methanol-0.5% $CaCl_2$(55:45:10, v/v/v), sprayed with resorcinal-HCl reagent, visualized by heating the plate at 95°C, and determined quantitatively by densitometric scanning, as described previously (3,4,23). Lipid-bound sialic acid in gangliosides was estimated by the resorcinol-HCl method as modified by Suzuki (25).

Treatment of HL-60 Cells with Gangliosides: The cells were seeded at an initial concentration of 1.5 x 10^5 cells/ml and grown in the presence or absence of 50 nmol/ml of either ganglioside GM3, GM1 or a bovine brain ganglioside mixture. Cell growth was monitored by determining the cell concentration after a given culture time, and viability was checked by the Erythrosine B dye-exclusion test; higher than 90% viability being constantly maintained throughout the culture periods.

RESULTS

As shown in Table 1, the ganglio-series acidic GSL with a shorter sugar chain, ganglioside GM3, characteristically increased with a concomitant decrease in the lacto-series gangliosides having longer sugar-moieties, such as SPG, Gx, SnHc and LGx during the monocytic differentiation induced by TPA.

Table 1. Changes in acidic glycosphingolipid composition during cell differentiation of HL-60 cells into monocytic or granulocytic mature cells

Inducer	Treatment period	Acidic glycosphingolipids(μg-lipid bound NeuAc/10^8cells)					
		GM3	SPG	Gx	SnHc	LGx	(total)
None		1.40	1.06	0.225	0.225	0.450	3.36
TPA	12h	1.93	0.825	0.200	0.250	0.275	3.48
	24h	2.49	0.613	0.150	0.175	0.282	3.71
	36h	3.68	0.663	0.188	0.180	0.289	5.00
DMSO	3d	0.88	1.44	0.220	0.492	0.608	3.64
	6d	0.72	1.28	0.120	0.640	0.850	3.61

Values represent means of two determinations in three separate experiments. Standard errors of means were constantly less than 10%. Induction of cell differentiation of HL-60 cells into monocytic or granulocytic mature cells was performed as described in MATERIALS AND METHODS. Acidic glycosphingolipids were quantitatively determined as described in the text.

The results are in marked contrast to those found during myeloid differentiation induced by DMSO where the latter ganglioside species significantly increased with a concurrent decrease in ganglioside GM3 (Table 1).

To allow clear and unambiguous analyses of the effects of sialoglycosphingolipids, we first transferred the HL-60 cells to serum-free culture conditions according to the method of Breitman et al.(16), and could maintain the cells in good growth conditions without any loss of differentiation potentiality. As shown in Fig. 1, ganglioside GM3 preparation was highly pure, which

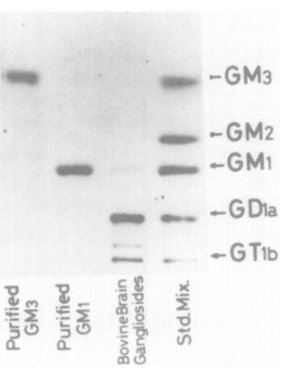

Fig. 1. High performance thin-layer chromatogram of gangliosides. From left to right, purified ganglioside GM3 (1.0 μg-lipid bound NeuAc), purified ganglioside GM1 (1.0 μg-lipid bound NeuAc), bovine brain gangliosides (1.0 μg-lipid bound NeuAc) and a standard mixture of gangliosides GM3 (1.0 μg-NeuAc), GM2 (0.6 μg-NeuAc), GM1 (0.6 μg-NeuAc), GD1a (0.45 μg-NeuAc) and GT1b (0.34 μg-NeuAc). HPTLC was carried out as described in MATERIALS AND METHODS.

Table 2. Growth inhibition and monocytic differentiation of HL-60 cells with ganglioside GM3 in comparison with the effects of other gangliosides

Addition	Culture periods (days)	Cell growth (viable cells per ml)	Positive cells (%)			
			Phagocytic activity	Nonspecific esterase	OKM1	OKM5
None	1	1.5×10^5	14.5	19.0	9.0	1.4
	6	8.5×10^5	16.5	23.5	10.6	1.9
GM3	6	2.5×10^5	65.0	82.5	35.7	11.7
GM1	6	10.7×10^5	10.0	18.5	8.5	1.0
Brain gangliosides	6	10.2×10^5	11.0	18.0	8.0	0.9

Values represent means of duplicate experiments. Standard deviations were less than 10%.

HL-60 cells were grown and treated with gangliosides as described in MATERIALS AND METHODS, and the differentiation-induced phenotypes were assayed as described in the text.

showed a single band on HPTLC and was identified to be NeuAc-type GM3 (N-acetyl-α-D-neuraminosylgalactosylglucosyl ceramide) in higher than 95% purity.

When HL-60 cells were cultured with ganglioside GM3 for 6 days, cell growth was markedly inhibited with a significant enhancement of the phagocytic activity (Table 2) and a remarkable induction of the activity of the monocytic marker-enzyme, α-naphthyl butyrate esterase, which was detectable cytochemically (19) (Table 2, Fig. 2D). Simultaneously, morphological differentiation, reflected by such a remarkable decrease in the nuclear-cytoplasmic ratio and the cytoplasmic basophilia, was observed with this ganglioside GM3 treatment (Fig. 2). Cytofluorometric studies clearly demonstrated that mature monocyte-specific surface antigens that reacted with monoclonal antibodies such as OKM1 (20) and OKM5 (21,22) appeared increasingly on the surface of HL-60 cells during their cultivation with ganglioside GM3 (Table 2), supporting the view that HL-60 cells are induced by this acidic sialo-GSL to differentiate along the monocytic lineage. The mature granulocyte-specific surface antigen detectable by monoclonal antibody OKB2 (26) showed a tendency to decrease during this monocytic differentiation (data not shown). In contrast, other ganglio-series acidic sialo-GSLs such as ganglioside GM1 and a brain ganglioside

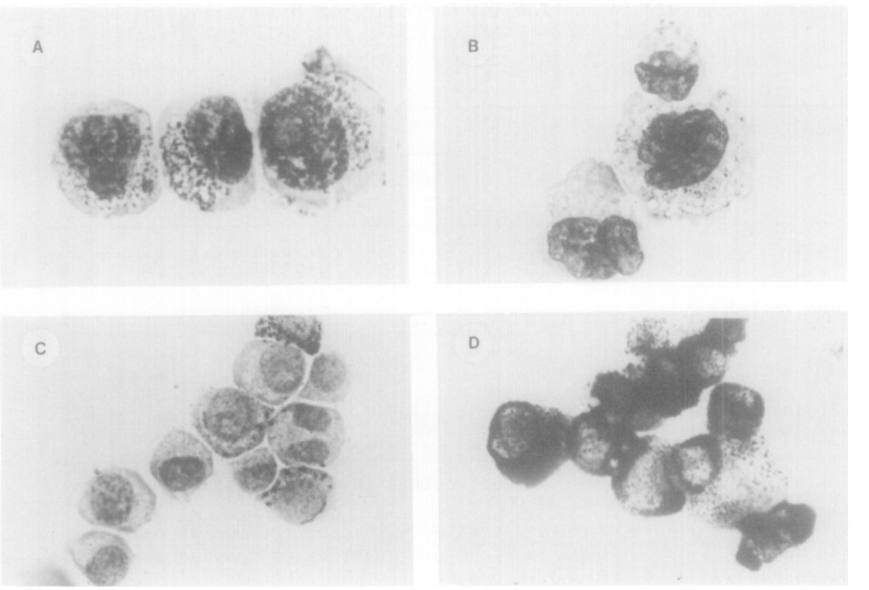

Fig. 2. Differentiation of HL-60 cells into monocytic mature cells induced by monosialoganglioside GM3. Wright-Giemsa (A, B) and α-naphthyl butyrate esterase (C, D) stainings were performed as described in MATERIALS AND METHODS. A, HL-60 cells maintained in serum-free synthetic medium (DME/F12 medium) without any additions. B, HL-60 cells, cultured in the serum-free medium with 50nmol/ml ganglioside GM3 for 6 days, which differentiated morphologically into monocytic mature cells. C, HL-60 cells grown in the serum-free DME/F12 medium, some of which were slightly stained for α-naphthyl butyrate esterase activity. D, HL-60 cells treated with ganglioside GM3 (50nmol/ml) for 6 days which differentiated into monocytic mature cells. All the cells were strongly stained for α-naphthyl butyrate esterase activity.

mixture had no significant effects on the induction of both phagocytic and non-specific esterase activities in HL-60 cells without any increase in the monocyte-specific surface antigens on their membranes (Table 2). These gangliosides showed, instead, stimulatory activities toward the growth of HL-60 cells in serum-free cultures. Free sialic acid (NeuAc, N-acetyl-α-D-neuraminic acid) showd no effects on either the growth or differentiation of HL-60 cells (data not shown).

DISCUSSION

It should be of great interest that a physiologically present and differentiation-dependently increasing lipophilic membrane component, monosialoganglioside GM3, itself exhibited a definite differentiation-inducible activity on human hematopoietic malignant cells. As to the sialic acid residue,

ganglioside GM3 comprised two molecular species, one being a NeuAc-type and the other being a NeuGly (N-glycolyl-α-D-neuraminic acid)-type. Ganglioside GM3 was first discovered both by Yamakawa and Suzuki (27) and Klenk and Wolter (28) in horse erythrocyte stromata, and its molecular structure was determined to be N-glycolyl-α-D-neuraminosylgalactosylglucosyl ceramide (NeuGly(α2-3)Gal(β1-4)Glc-Cer) (29,30). Thereafter, NeuAc-type ganglioside GM3 (NeuAc(α2-3)Gal(β1-4)Glc-Cer) was detected in various kinds of mammalian tissues including dog erythrocytes (17). Our GM3 preparation was of dog erythrocyte origin and was found to be almost all (more than 95%) of the NeuAc-type, and the recent report by Yasue et al. (31) clarified that the majority of dog erythrocyte ganglioside GM3 was of the NeuAc-type with NeuGly-type GM3 being detectable in erythrocytes only from dogs of Asian origin (Shiba dogs).

As for GSLs in general, extensive studies on their molecular structures have been performed, and so many molecular species of GSLs have been recently discovered in mammalian tissues, some of which are surface markers specific for malignant transformations and cell-differentiations (2). However, less information is available on the physiological functions of GSLs in mammalian cells. Recently, Tsuji et al. (32) reported that the tetrasialyl acidic GSL, ganglioside GQ1b, specifically functions as a very potent nerve growth factor. It was also reported by Goldenring et al. (33) that polysialyl gangliosides stimulated a protein-kinase system, which might be calmodulin-dependent phosphorylation, but inhibited phospholipid-dependent protein-kinase C activity. Our present study demonstrated that a ubiquitous acidic GSL molecule, monosialo-ganglioside GM3, exhibited an important function in a physiological sense as a specific potent inducer for the monocytic cell differentiation of human malignant hemopoietic cells. It should be emphasized that a particular acidic GSL molecule having specific sugar-moieties, which dramatically increased during monocytic differentiation induced by chemical agents, functions as a potent trigger for cell differentiation along the monocytic lineage. Similar monocytic differentiation-induction by ganglioside GM3 was also ob-

served for human monocytoid cell line U937 cells, and furthermore, for freshly obtained human myeloid leukemia cells as demonstrated by the chemical inducer (18). Further work on the effects of GSLs on hemopoietic cell differentiation will be reported elsewhere.

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