Reduction of glycolipids with D-PDMP, a glucosylceramide synthetase inhibitor, caused cell growth inhibition, enhanced cell adhesion, and facilitated cell motility in human glioma cells

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ABSTRACT

Glycolipid synthesis inhibitor, D-PDMP, not only inhibited the production of glycolipid but also inhibited cell growth in human glioma cell line KG-1C in a cell cycle non-dependent manner. The reduction of glycolipid from the cell membrane allowed us to study the biological functions of glycolipids. The ability of cells to adhere to collagen was enhanced by the reduction of glycolipids, and random cell migration was also activated by the effect of D-PDMP. The results supported our speculation that glycolipids might function in cell growth, adherence and locomotion.

Key words : Glycolipid, Glucosylceramide synthetase, Glioma

INTRODUCTION

The malignant nature of cancer cells is determined, for the most part, by proliferation rate, invasive ability, and metastatic potential. Aberrant oligosaccharide chains, which are expressed on the transformed cells, are thought to play significant roles in determining their biological behavior (1,2). There are a myriad of papers concerning the biological functions of oligosaccharides; however, their fundamental and common function within the cell has yet to be discovered. Most of the glycolipids found in animals are synthesized starting from glucosylceramide (GluCer), to which further carbohydrate residues are added to make complex ones(3). Phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a synthetic analogue of ceramide, can interfere with the synthesis of GluCer, which results in depletion of every glycoli-

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pid(4). Deprivation of glycolipids from the cell membrane with the use of PDMP may alter the behaviors of a cell in various ways which enable us to speculate about the functions of glycolipids. In this communication we examined PDMP's effect on the proliferation, adhesion, and invasive abilities of KG-1C glioma cells.

MATERIALS AND METHODS

CELLS: A human glioma cell line, KG-1C was provided by the Japanese Cancer Research Resources Bank. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) supplemented with 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 1 mM sodium pyruvate without addition of antibiotics at 37℃ in a 5% CO2 atmosphere. The experiments were done with passage levels from 10 to 12.

REAGENTS: A ceramide analogue, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), was synthesized as as inhibitor of glucosylceramide synthetase and was provided by Dr. J Inokuchi, Hokkaido University (4). In this experiment PDMP stock solution (4 mM) was made with distilled water by warming at 40℃ and by sonication. Final working concentrations in the culture media were usually between 5 and 20 uM.

GLYCOLIPID ANALYSIS: After the culture, the cells were scraped from the dishes, and glycolipid analysis was performed according to the method as described elsewhere (5). Briefly, the total lipid was extracted with chloroform/methanol/water (C/M/W=4/8/3, v/v/v) for 2 consecutive-24 hr periods, followed by 4 hr extraction at 40℃. Glycerolipids were decomposed by saponification. Glycolipids obtained were further separated into neutral and acidic fractions by ion-exchange column chromatography (Sephadex A-25, Pharmacia). The glycolipid components were studied with thin layer chromatography (TLC). The total of gangliosides (sialic acid-containing glycolipids) was quantified by resorcinol-HCl method, and were expressed as lipid-bound N-acetyl neuraminic acid (NANA).

CELL PROLIFERATION ASSAY: Two hundred ul of KG-1C cells at 1 x 10^4 /ml (2000 cells) were distributed to flat-bottomed 96-well plates (Falcon 3072, NJ), and incubated for 3 days, and then various doses of D-PDMP were administered into the culture. Tritium-thymidine uptake was measured each day from 24 to 48 hrs after D-PDMP administration by adding 37 KBq of [3H]thymidine (3.1 TBq/mmol, DuPont) for 4 hrs to each well. The

pulsed cells were then harvested on a glass fiber filter and their radioactivities were counted with a scintillation counter (LS6000LL, Beckman, CA).

GROWTH CURVE: D-PDMP from 5 to 20 uM was added to the culture of KG-1C cells to see the growth inhibitory effect. One hundred-thousand KG-1C cells were cultured in separate T-25 cm^2 flasks (Falcon 3014, NJ), and the cell numbers of triplicate flasks with or without addition of D-PDMP were counted each day until day 8 to draw the growth curve. Viability of the cells was also checked with trypan blue-dye exclusion test.

CELL CYCLE ANALYSIS: In order to study the D-PDMP effect on the cell cycle, propidium iodide (PI)-labeled cells were analyzed with FACS(FACScan, Beckton-Dickinson, CA). KG-1C cells in logarithmic growth phase were administered with 10 uM of D-PDMP, and the cells were subjected to PI-labeling at 24 and 48 hrs after D-PDMP administration. The cells were first detached with trypsin, and then reacted with 20 ug/ml of PI (Sigma) with 0.1% Triton X-100 (Wako, Tokyo) in phosphate buffered saline (PBS) at 4℃ for 15 min. The cells were then centrifuged at 1200 rpm for 5 min, and fixed with ice cold 70% ethanol for 30 min. The cells were again centrifuged, and subsequently treated with 7 U of ribonuclease A (Sigma) in 1 ml of PBS at 37℃ for 30 min in a water bath. PI was again added to the cells, for 15 min on ice, and the labeled cells were washed with PBS and analysed by FACS.

ADHESION ASSAY: The effect of D-PDMP on the adhesive ability of KG-1C cells to various extracellular matrix proteins was examined. KG-1C cells cultured in T75-cm^2 flasks were treated with 10 uM of D-PDMP for 48 hrs. The treated cells were trypsinized, and distributed in a hexaplicate manner to the wells of a 96-well plate, which had been coated with collagen type I or IV (Falcon, Biocoat). The cell concentration was adjusted to 5 x 10^5 cells /ml. The plates were cooled on ice during the cell distribution. The plates were then incubated at 37℃ for 60 min. After the incubation the plates were rotated with a horizontal shaker at 220 rpm for 10 min. The detached cells were removed by gentle suction, and each well was washed with PBS twice. The adherent cells were fixed with 4% paraformaldehyde for 30 min, and stained with 0.1% crystal violet. The quantification of the adherent cells was done by spectrophotometric analysis with a microplate reader (BioRad, Model 550) at a wavelength of 595 nm.

INVASION ASSAY: Effect of D-PDMP on the invasive ability of KG-1C

cells was examined with an in vitro assay system (Invasion Chamber, 24-well, Falcon, NJ). Human recombinant epidermal growth factor (EGF) (Wako, Tokyo) was used as a chemoattractant. KG-1C cells with or without prior treatment of 10 uM of D-PDMP for 48 hrs were placed in the upper chamber in 0.1% BSA in DMEM medium, and were then incubated for 48 hrs while the lower chamber contained 10 ng/ml of EGF in the same medium. The cells were first isolated from the lower chamber by a porous membrane (a pore size of 8 um) and on a layered matrix protein, through which they can move across to the lower chamber. Those cells invading through the matrix were counted by taking photomicrographs at different depths from the surface of the matrix after fixation of the cells with 4% paraformaldehyde and staining with 0.5% crystal violet.

RESULTS

GLYCOLIPID ALTERATION OF CULTURED KG-1C CELLS BY D-PDMP: The quantification of gangliosides by the resorcinol-HCl method showed that the total ganglioside content was reduced from 7.9 to 4.2 ug lipid-bound NANA/100 mg wet weight of cells (46.4% reduction) by 24 hr-treatment with D-PDMP, and further down to 56.3% reduction after 48 hr treatment (Table 1). Glycolipid suppression including ganglioside was dose-dependent with D-PDMP (data not shown). The ganglioside profile of the extracted

Table 1 Quantification of ganglioside content after D-PDMP treatment[a].

	pre	24hr	48hr
No treatment	6.68	7.90	7.56
D-PDMPb	n.d.c	4.24	3.30

a) Quantification was done by resolcinol-HCl method. The value is expressed as lipid-bound sialic acid (ug)/100mg wet weight of the cells.
b) D-PDMP was administered on Day 3 to the culture dish at a final concentration of 10uM.
c) n.d.= not done

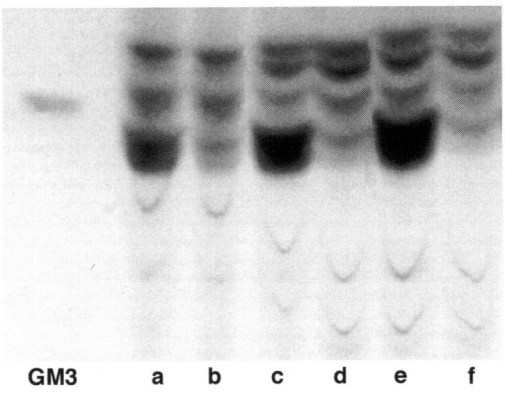

Figure 1. Thin layer chromatograms of gangliosides extracted from KG-1C cells with or without D-PDMP treatment. KG-1C cells were administered with D-PDMP at a final concentration of 10 uM on Day 2. The cells were recovered from the next day until Day 5 to see the effect of D-PDMP on the ganglioside content. The chromatograms clearly showed a marked reduction of GM3 ganglioside content in KG-1C cells.

specimens was shown on TLC (Fig.1).

IN VITRO EFFECT OF D-PDMP ON THE GROWTH OF KG-1C CELLS: The growth curve of RG2 cells showed a lag phase for the first 2 days followed by 3 days of a logarithmic growth phase and a subsequent death phase. (Fig.2) Doubling time was calculated to be 12.5 hr. Tritium thymidine assay showed significant reduction of labeled-thymidine uptake at a concentra-

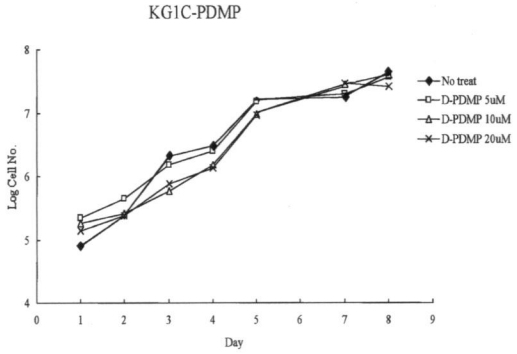

Figure 2. Growth curves of KG-1C cells in vitro with or without D-PDMP treatment. KG-1C cells cultured in T-25cm2 flasks in triplicate were administered with 5-10 uM of D-PDMP on Day 2. Subsequently viable cell numbers of each flask were counted daily with a hemocytometer excluding the cells with Trypan blue staining. D-PDMP at 10 and 20 uM showed significant inhibition of cell growth from Day 3 to Day 5.

tion of 1.25 to 10 uM in a dose-dependent manner (Table 2). The suppression was more significant at 24 hr. Cell cycle analysis with PI labeling revealed no effect of D-PDMP on the ratio of G0 / G1, S, and G2 + M phases (Table 3). The profile of DNA content in FACS analysis did not show the apoptotic pattern (data not shown). From the result of [3H]thymi-

Table 2 D-PDMP effect on the uptake of tritium thymidine by KG-1C cells.

	Dose of PDMP (uM)[a]					
	control	20	10	5	2.5	1.25
time after treatment						
24hr	43785[b]	32965	31376	34966	36796	40117(n.s.)[c]
48hr	12834	8977	10187	12469(n.s.)	13093 (n.s.)	13596(n.s.)

a) PDMP was administered on day 3 of the culture at the final concentrations (uM) indicated.
b) The values were expressed as the average dpm of six wells of a 96-well plate. The standard deviations were within 10% in all samples.
c) n.s.= statistically not significant as determined by Student's t-test as compared with the control.

Table 3 Effect of D-PDMP on the cell cycle of KG-1C cells as determined by PI-labeling[a].

	Pre			24h			48h		
	G0/G1	S	G2+M	G0/G1	S	G2+M	G0/G1	S	G2+M (%)
Control	44.4	36.1	19.5	47.1	35.5	17.4	48.5	30.2	21.4
D-PDMP	-	-	-	48.6	30.3	21.0	46.2	32.5	21.4

a) The culture of KG-1C cells was started in triplicate in T-75cm^2 flasks. On day 3, the cells were detached and subjected to PI-labeling in each flask prior to PDMP administration (Pre). Cell cycle alteration was then studied 24 and 48 hrs after PDMP treatment. The values were expressed as the average percent of the cells of 3 flasks in each cell cycle. The data was representative of 4 independent experiments. Each isoform or the mixture of PDMP was administered at the final concentration of 10 uM. No statistically significant change was noted.

dine uptake, 10 uM of D-PDMP was used as the representative concentration to evaluate the effect on the invasion experiments.

D-PDMP EFFECT ON THE ADHESIVE ABILITY OF KG-1C CELLS: KG-1C cells were pretreated with D-PDMP to see the effect on their adhesive ability to collagen matrices. The remaining cells adhering to the matrices were stained with cresyl violet and their adherence was evaluated by measuring the optical density on the plate reader. The statistical analysis was done by employing the values of optical density. The results indicated that D-PDMP ranging in concentrations from 5 to 20 uM promoted adhesion of KG-1C cells to both collagen type I and IV as compared with untreated cells (Table 4).

Table 4 D-PDMP effect on ability of cells to adhere to collagen matrices[a].

		D-PDMP (uM)		
	control	20	10	5
collagen				
type I	0.38 + 0.01	0.48 + 0.02*	0.49 + 0.05*	0.40 + 0.02**
type IV	0.44 + 0.02	0.54 + 0.04*	0.56 + 0.04*	0.52 + 0.04*

a) The method for adhesion assay is described in the section on Materials and Methods. The values are the mean optical density of 6 wells of a sample ± standard deviation. The statistical analysis was done by Student's t-test. *$p<0.01$. **$p<0.05$.

D-PDMP EFFECT ON THE INVASIVE ABILITY OF KG-1C CELLS: KG-1C cells were pretreated with 10 uM of D-PDMP before assaying their invasive ability on the Matrigel Invasion Chamber. The chemoattractant used was EGF at 10 ng/ml dissolved in the culture media of the lower chamber. The assay was continued for 24 hrs, and the cells invading into the matrix were counted at various depths from the surface of the matrix. The depth was scaled by using the number on the dial for adjusting the focus of the

microscope. The result obtained showed an apparent promoting effect of D-PDMP on the invasion of KG-1C cells when EGF was not added to the

Table 5 D-PDMP effect on the invasive ability of KG-1C cells[a].

	control		D-PDMP	
EGF[b]	(-)	(+)	(-)	(+)
depth[c]				
50	4.9	32.4	23.4	25.5
70	4.4	23.2	20.4	23.8
90	0.9	22.5	22.5	18.0
110	0.0	16.8	16.0	13.8
130	0.0	19.0	11.0	11.7

a) The method for the invasion assay is described in the section on Materials and Methods. The values are expressed as the percentage of the cells having invaded to an indicated depth as compared with the cells remaining on the surface of the matrix.
b) EGF = epidermal growth factor. EGF was used as a chemoattractant in the lower chamber at a concentration of 10 ng/ml.
c) Depth is from the surface of the matrix toward the lower chamber, and is expressed as a scale on the dial of an inversed microscope for focusing.

lower chamber, which might indicate facilitation of a random migration of the cells, and not chemotaxis toward a certain substance (Table 5). EGF successfully promoted the invasion of KG-1C cells, however, D-PDMP did not show any additive effect on the invasion of the cells.

DISCUSSION

Oligosaccharides on the cell surface are considered to play at least some part in determining the biological behavior of a cell in such events as adhesion, invasion, traffic, metastasis, and proliferation (1,2). Most of the glycolipids found in animals are synthesized starting from glucosylceramide (GluCer), to which further carbohydrate residues are added to make complex ones (3). D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), a synthetic analogue of ceramide, can interfere with the synthesis of GluCer, which results in depletion of every type of glycolipid (4).

D-PDMP also causes the accumulation of the glycolipid precursors such as ceramide, sphingosine, and dimethylsphingosine, which have recently been disclosed as the second messengers in signal transduction to induce apoptosis (5). With the use of D-PDMP some of the biological functions of glycolipids can be elucidated by observing the altered cell behavior.

The inhibitory effect of glycolipid synthesis with D-stereoisomer of PDMP on KG-1C glioma cells was consistent with other types of cells previously reported(4,7). A significant reduction of cellular ganglioside content was confirmed. The result of the tritium-thymidine uptake showed dose-dependent suppression of DNA synthesis with D-PDMP at the concentrations of 1.25 to 20 uM. This inhibitory effect of D-PDMP on KG-1C glioma cells was found to be most marked at 24 hr from the administration ($p<0.05$). Actual cell growth in vitro was also suppressed by D-PDMP. Since the number of dead cells counted each day did not differ significantly, the growth inhibitory effect of D-PDMP is considered to be cytostatic rather than cytocidal. It is known that sequential catabolic metabolites of glycolipid precursors induce various biological effects; ceramides suppress mitogenesis and sphingosines enhance cell growth(8); however, the detailed mechanisms of growth control produced by these intracellular messengers has not been fully understood. In the cell cycle analysis, D-PDMP did not cause cell cycle arrest at any phase of KG-1C cells, which indicates that PDMP should act to prolong the lag phase of the growth curve regardless of the cell cycle. Rani et al. (9) reported that DL-PDMP showed growth arrest at G1/S and G2/M strains on IGF-1-stimulated NIH3T3 cells. They found a decrease in the activities of two cyclin dependent kinases. These discrepant results might be due to the different cell strains and/or different growth conditions, as 3T3 cells were under the stimulatory drive of IGF-1. It is necessary to see if the independency of D-PDMP from the cell cycle causing cell growth inhibition might be common to other glioma cells or if it is a glioma-specific phenomenon.

Reduction of ganglioside from the cell surface of KG-1C cells facilitated the cells capability of adhering to collagen matrices. In reports using PDMP to see its effect on cell adhesion, different results were actually obtained. In a study using HL-60 cells, sphingomyelin synthesis was involved in adherence during macrophage differentiation, and the 12-O-tetradecanoylphorbol-13-acetate stimulated a 6- and 12-fold increase in the levels of GM3 and GlcCer during development of an adherent macrophage population(10). And PDMP selectively blocked adherence during macrophage differentiation, and prevented TPA-stimulated effects (10). On the other hand, in another study using Lewis lung carcinoma cells, glycolipid depletion by PDMP reduced the

laminin-mediated attachment (11). They also reported different effects of PDMP on the adhesion to different extracellular matrices such as fibronectin. Thus, glycolipids including gangliosides may not function uniformly at all times. We could confirm that D-PDMP significantly influenced cell adhesive behavior, and that D-PDMP is effective in suppressing the function of glycolipids at particular situations resulting either in the promotion or inhibition of adhesion depending on the intrinsic and extrinsic cellular conditions.

D-PDMP could facilitate the invasion of KG-1C cells when EGF was not added to the culture. The result indicated facilitation of a random migration of the cells, and not chemotaxis towards a certain substance. EGF could successfully promote the invasion of KG-1C cells, however, D-PDMP did not show any effect on the invasion of the cells. The result suggests that different mechanisms are involved in the natural or random migration of the cells and the process of chemotaxis, and that gangliosides might only participate in the former to regulate cell motility. In a study using Lewis lung carcinoma cells, the degree of glycolipid depletion was closely associated with the reduced ability of the cells to invade reconstituted basement membranes (11), which concurred with our results. In the invasion assay, too, there seems to be no general rule to predict the function of glycolipids in any particular cellular behavior.

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