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Butyrate-Induced Growth Inhibition and Differentiation in Caco-2 Cells with Hybrid Liposomes

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Abstract: Hybrid liposomes were prepared by mixing glycerophospholipids with glyceroglycolipids. The effects of hybrid liposomes on Caco-2 cells were investigated. Growth inhibition of cells was determined with sodium 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium dye reduction assay. Alkaline phosphatase activity was measured for assessing the cell differentiation induced by sodium butyrate (NaBT). Growth of Caco-2 cells was inhibited at 48 h after addition of all four types hybrid liposomes (PC/DGDG, PC/SQDG, PS/DGDG and PS/SQDG) while at 72 h growth inhibition was significant for all treatments except the hybrid liposome of phosphatidylcholine and digalactosyldiacylglycerol under concentrations of 50 μM glycerophospholipids with 50 μM glyceroglycolipids. Alkaline phosphatase activity of Caco-2 cells increased significantly when cells were treated in combination of hybrid liposomes and NaBT at 72 h, indicating that hybrid liposomes enhanced cell differentiation induced with NaBT.

Key words: hybrid liposome, growth inhibition, differentiation, Caco-2 cells

1 Introduction

Liposomes have recently attracted researchers for their toxicity-reducing character of antitumor drugs (1). Generally, liposome is prepared by reverse-phase evaporation using organic solvents (2). However, trace residual solvent in the final formulation may represent a possible risk for human health. On the other hand, hybrid liposome composed of vesicular and micellar molecules have attracted special interests as a safe drug carrier technology and to enhance the therapeutic efficacy (3-5). Hybrid liposomes are prepared by aqueous buffer solution, which contains phospholipids and surfactants without any organic solvent (6). Remarkably, hybrid liposome showed high inhibition on tumor growth *in vitro* without drugs has been observed (7). The fluorescence probe (fluorescein isothiocyanate) study showed clearly that the hybrid liposomes might be fused with the tumor membrane. Hybrid liposomes would promote the fusion into tumor cells and change the microenvironment of tumor cell membranes (4). As a result, the growth signal should be blocked after changing receptor conformations. Additionaly, no toxicity of hybrid liposome was observed in normal cells *in vitro* and in normal rats *in vivo* without any side effects (8). Interestingly, the hybrid liposomes were found to be stable for 3-5 weeks, having a single distribution of hydrodynamic diameter. The stability of these hybrid liposomes should be advantageous for the clinical application and as drinks containing liposomes for colon cancer high-risk families.

Sodium butyrate (NaBT) is known to produce easily from dietary fiber in intestine. Butyrate has potent effects on growth inhibition and differentiation of different types of cancer cells (9-10). Therefore, in the present study, effects of hybrid liposomes composed of

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glycerophospholipids and glyceroglycolipids on butyrate-induced growth inhibition and differentiation of Caco-2 cells were investigated.

2 Experimental

2·1 Cell Lines and Chemicals

Human colon carcinoma, Caco-2 (HTB-37) and normal human colon, CCD-18Co (CRL-1459) cell lines were obtained from American Type Culture Collection (Rockville, CT, USA). Minimum essential medium (MEM) was obtained from GIBCO (Grand Island, New York, USA), and fetal bovine serum (FBS) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA, USA). L-Serine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2-Amino-2-methyl-1-propanol and sodium butyrate were provided by Sigma Chemicals Company (St. Louis, MO, USA). WST-1 (sodium 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) and 1-methoxy PMS (1-methoxy-5-methyphenazinium methylsulfate) were obtained from Dojindo Laboratories (Kumamoto, Japan). Squid meal glycerophospholipids were generous gifts from Nippon Chemical Feed Co. Ltd., (Hakodate, Hokkaido, Japan).

2·2 Separation of PC from Squid Meal Phospholipids

Phosphatidylcholine (PC) was separated from squid (*Doryteuthiis sibogae*) meal phospholipids on thin layer chromatography (TLC) as previously reported (11). Briefly, phospholipid was dissolved in chloroform and loaded on 20 × 20 cm^2, 0.5 mm thickness preparative glass plates coated with silica gel 60 (Darmstadt, Merck, Germany) at the rate of 100 mg phospholipid per plate. Chromatography was carried out in a glass jar under an air atmosphere in a dark room. The plate was developed with chloroform/ methanol/ water (65:25:4, v/v/v). The band of PC was monitored on a TLC using Dittmer solution near the edge of the TLC plate. The band of PC was then visualized under UV light at 254 or 366 nm in a dark room and compared with an authentic standard. The PC-containing band was scraped off and immediately eluted with methanol. Methanol elutes were evaporated, dissolved in chloroform/methanol/water (10:5:3, v/v/v) and placed in a separating funnel to remove the silica gel. The separating funnel was kept at 4-5℃ for overnight, then the chloroform layer was collected and evaporated approximately at 25℃ to afford the PC.

2·3 Conversion of PS from PC by Transphosphatidylation

Phosphatidylserine (PS) was converted from squid meal PC by phospholipase D-mediated transphosphatidylation as described by Iwasaki *et al*. (12) Briefly, a mixture consisting of 7.2 mg/mL PC, 3.873 M L-Serine, 50 mM acetic acid-sodium acetate buffer (pH 5.6), 36 mg/mL calcium sulfate powder, and 30 units/mL phospholipase D solution was reacted at 40℃ for 24 h with gentle stirring. After 3 h reaction, the calcium sulfate powder (on which the phospholipids were adsorbed) was collected by centrifugation for 5 min at 3,000 rpm. The precipitate was washed with water and resuspended in 20 ml of *n*-hexane/ethanol (2:1 v/v), then 5 mL of water and 0.5 mL of 1 N HCl were added. Subsequently, centrifugation separated the mixture into three phases, i.e., an upper liquid phase, a lower liquid phase, and a precipitate phase. The upper liquid phase was recovered, and the solvent evaporated to afford the PS.

2·4 Extraction and Purification of DGDG and SQDG of *Sargassum horneri*

The brown alga *S. horneri* was collected from the coast of Japan Sea of Toyama prefecture in Japan. Total lipid was extracted from *S. horneri* following the method of Bligh and Dyer (13). Digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) were extracted and purified following the previous study (14). Briefly, the extracted lipid was loaded on a silica gel column chromatography and eluted with chloroform to afford simple lipids. Lipid compositions were monitored on TLC spraying with Orcinol-sulfuric acid compared with authentic lipid standards. Silica gel TLC plate was developed with *n*-hexane/ diethyl ether/ acetic acid (70:30:1, v/v/v) for simple lipids and chloroform/methanol/water (65:25:4, v/v/v) for glycolipid analysis, respectively. Acetone was then applied to the same silica gel column chromatography to collect simple lipids and glycolipids. But there was no simple lipid and negligible amount of monogalactosyldiacylglycerol (MGDG) was present in acetone fraction. Finally, methanol was used to the same silica gel column chromatography to afford glyceroglycolipids (MGDG, DGDG and SQDG). DGDG and SQDG were separated

on TLC coated with silica gel 60. TLC was performed twice for further purification.

2·5 Fatty Acid Composition of PC, PS, DGDG and SQDG

Fatty acids of PC, PS, DGDG and SQDG were converted to methyl ester derivatives individually following the method of Lepage and Roy (15) for fatty acid composition analysis with slight modification. The samples were analyzed following our previous report (11). The identification of fatty acids was established by comparing the peak retention times with authentic standards (Sigma Chemical Co., St. Louis, MO, USA) and following the concept of relative retention potential index, proposed by Takahashi *et al*. (16).

2·6 Preparation of Hybrid Liposomes Consisted of Glycerophospholipids and Glyceroglycolipids

Four types of hybrid liposomes consisted of glycerophospholipids and glyceroglycolipids, i.e. PC/DGDG, PC/SQDG, PS/DGDG and PS/SQDG were prepared. Just in brief, required amount of PC and DGDG were placed in V-shaped vial and mixed well. It was dried flushing with N_2 gas. The V-shaped vial with dried PC and DGDG were placed in a flask and desiccated. The dried PC and DGDG were dissolved in PBS. The content of V-shaped vial was stirred for 30 min at 30 rpm and then 20 min at 1500 rpm for making liposomes. Uniform-sized hybrid liposomes were prepared by passing through a 1-μM pore size membrane filter (AVESTIN, Inc. Ottawa, Canada) several times. Concentrations were adjusted to 50 μM PC/50 μM DGDG. Remaining three types of hybrid liposomes were prepared in the same manner.

2·7 Cell Culture

Both Caco-2 and CCD-18Co cells were grown in MEM supplemented with 10% heat-incubated FBS, 1% non-essential amino acids, 7.5% sodium bicarbonate (29.3 mL/l), 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37℃ in a 5% CO_2-humidified incubator.

2·8 WST-1 Dye Reduction Assay

Caco-2 and CCD-18Co cells were seeded at 1×10^4 cells/mL and 0.2×10^4 cells/mL respectively in 200 μL growth medium per well into 96-well microplates. The plates were incubated for 24 h in the same incubator at 37℃ in a 5% CO_2-humidified incubator. Then, 50 μM PC/50 μM DGDG, 50 μM PC/50 μM SQDG, 50 μM PS/50 μM DGDG and 50 μM PS/50 μM SQDG hybrid liposomes alone, and 1.0 mM NaBT were added. WST-1 (3.3 mg/mL PBS including 7% 1-methoxy PMS) was added at 20 μL in each well containing 200 μL medium with cells, and plates were incubated in the same incubator. Absorbance was measured at 450-650 nm on a precision microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was determined every 24 h following WST-1 dye reduction assay up to 72h.

2·9 Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was measured by using disodium *p*-nitrophenyl phosphate as substrate. We recently reported the method of alkaline phosphatase activity (11). Briefly, Caco-2 cells were seeded at 1×10^4 cells/mL in 15 ml growth medium per petriplate (size, 100 mm). The petriplates were incubated for 24 h at 37℃. Four types of hybrid liposomes and NaBT were added, and then incubated for 72 h at 37℃. Cells were harvested and one-third of total cells were placed in centrifuge tubes and then dissolved in 300 μL PBS containing 0.5% Triton X-100. The dissolved cells were taken at 40 μL per well into 96-well plate. After that, 60 μL of standard assay mixture was added in each well. The plate was then incubated for 30 min at 37℃. The reaction was terminated by adding 100 μL of 0.5 N NaOH to each well. The absorbance due to the formation of *p*-nitrophenol was measured spectrophotometrically at 405-414 nm. Protein was assayed for the remaining two-third of total cells following the method of Lowry *et al*. (17). Enzyme activity was expressed in μmol *p*-nitrophenol/mg cellular protein.

2·10 Statistical Analysis

Data was expressed as means ± standard deviation (SD). Difference among treatment groups were analyzed by ANOVA and Duncan's multiple range test utilizing statistical software package SRISTAT-2.

3 Results

3·1 Fatty Acid Composition

As shown in **Table 1**, the major fatty acids of PC and

PS were 16:0, 20:5 and 22:6. Fatty acid compositions of DGDG and SQDG of *S. horneri* are shown in **Table 2**. The major fatty acids of DGDG were 16:0, 18:4, 20:4, and 20:5; the major fatty acids of SQDG were 16:0, 18:1, 18:4, 20:1, 20:4 and 20:5.

Table 1 Fatty Acid Composition of PC and PS Separated from Squid (*Doryteuthiis sibogae*) Meal Phospholipid.*

Fatty acid	%	
	PC	PS
$C_{16:0}$	35.2	34.6
$C_{18:0}$	1.3	1.9
$C_{18:1}$	2.4	3.2
$C_{18:2}$	1.0	0.3
$C_{20:1}$	0.4	0.3
$C_{20:4}$	1.0	1.0
$C_{20:5}$	9.2	8.5
$C_{22:6}$	42.6	41.3
Others	6.9	8.9

* Previous report (11)

Table 2 Fatty Acid Composition of DGDG and SQDG of the Brown Alga *Sargassum horneri*.*

Fatty acid	%	
	DGDG	SQDG
$C_{14:0}$	1.5	3.1
$C_{16:0}$	12.0	41.6
$C_{16:1}$	2.0	4.0
$C_{16:2}$	—	1.9
$C_{18:0}$	2.8	—
$C_{18:1}$	4.1	14.7
$C_{18:2}$	3.3	2.9
$C_{18:3}$	4.6	0.3
$C_{18:4}$	12.0	5.2
$C_{20:1}$	3.5	6.7
$C_{20:4}$	23.8	6.2
$C_{20:5}$	23.5	4.7
Others	6.9	8.7

* Previous report (14)

3·2 Growth Inhibition Effects of PC/DGDG and PC/SQDG Hybrid Liposomes on Caco-2 Cells

The growth inhibition of Caco-2 cells treated with 50 μM PC/50 μM DGDG and 50 μM PC/50 μM SQDG hybrid liposomes alone and combination with 1.0 mM NaBT are presented in **Fig. 1**. Growth inhibition was significant when treated with 50 μM PC/50 μM SQDG, 50 μM PC/50 μM DGDG + 1.0 mM NaBT, and 50 μM PC/50 μM SQDG + 1.0 mM NaBT at 48 and 72 h (**Fig. 1**). To examine cytotoxic effects of above mentioned hybrid liposomes, normal human colon cell, CCD-18Co was treated under same condition. Result showed that cells viability was more than 100% in all cases under same condition (**Table 3**). Thus, there was no cytotoxic effects on CCD-18Co cell under experimental condition.

3·3 Growth Inhibition Effects of PS/DGDG and PS/SQDG Hybrid Liposomes on Caco-2 Cells

The growth inhibition of Caco-2 cells treated with 50

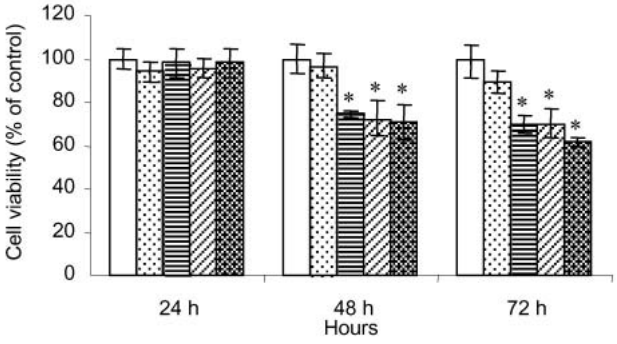

Fig. 1 Effect of PC/DGDG and PC/SQDG Hybrid Liposomes, and in Combination with NaBT on Caco-2 Cells Viability.
□ : Control, ⊡ : 50 μM PC/50 μM DGDG,
▤ : 50 μM PC/50 μM SQDG,
▨ : 50 μM PC/50 μM DGDG + 1.0 mM NaBT,
▩ : 50 μM PC/50 μM SQDG + 1.0 mM NaBT
Caco-2 (1×10^4 cells/mL) were incubated in presence of 50 μM PC/50 μM DGDG and 50 μM PC/50 μM SQDG, and in combination with 1.0 mM NaBT for different time of periods. The bars represent ± SD (n=8).
*$p < 0.01$ vs control

Table 3 Effects of Hybrid Liposomes and Combination of Hybrid Liposomes with NaBT on Growth of CCD-18Co Cell at 72 h.*

Doses	Cell viability (% of control)
Control	100.0 ± 8.3
1.0 mM NaBT	125.4 ± 10.5
50 μM PC / 50 μM DGDG	120.5 ± 12.2
50 μM PC / 50 μM SQDG	113.7 ± 8.7
50 μM PS / 50 μM DGDG	115.5 ± 12.2
50 μM PS / 50 μM SQDG	117.1 ± 6.9
50 μM PC / 50 μM DGDG + 1.0 mM NaBT	105.3 ± 8.3
50 μM PC / 50 μM SQDG + 1.0 mM NaBT	107.8 ± 9.1
50 μM PS / 50 μM DGDG + 1.0 mM NaBT	116.4 ± 10.1
50 μM PS / 50 μM SQDG + 1.0 mM NaBT	114.8 ± 8.8

* CCD-18Co (0.2×10^4 cells/mL) was incubated for 72 h with hybrid liposomes and combination of hybrid liposomes with NaBT. Data are shown as means ± SD (n=8).

μM PS/50 μM DGDG and 50 μM PS/50 μM SQDG hybrid liposomes alone and with 1.0 mM NaBT are presented in **Fig. 2**. Growth inhibition was significant with 50 μM PS/50 μM DGDG, 50 μM PS/50 μM SQDG, 50 μM PS/50 μM DGDG + 1.0 mM NaBT, and 50 μM PS/50 μM SQDG + 1.0 mM NaBT at 72 h (**Fig. 2**). For assessing the cytotoxic effects with above mentioned hybrid liposomes, CCD-18Co cells was treated under same condition. Results showed that there were no cytotoxic effects on CCD-18Co cells under same condition (**Table 3**).

3·4 Differentiation in Caco-2 Cells during PC/DGDG and PC/SQDG Hybrid Liposomes Treatment Induced by NaBT

The ALP activity of Caco-2 cells was estimated to detect the differentiation in presence of 50 μM PC/50 μM DGDG and 50 μM PC/50 μM SQDG hybrid liposomes, and combination with 1.0 mM NaBT. 50 μM PC/50 μM DGDG + 1.0 mM NaBT and 50 μM PC/50 μM SQDG + 1.0 mM NaBT showed significant pro-

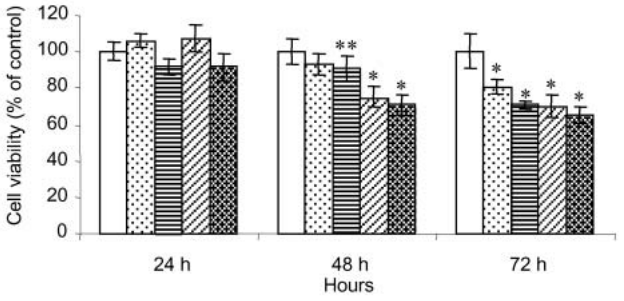

Fig. 2 Effect of PS/DGDG and PS/SQDG Hybrid Liposomes and with NaBT on Caco-2 Cells Viability.
□ : Control, ⊡ : 50 μM PS/50 μM DGDG,
▤ : 50 μM PS/50 μM SQDG
▨ : 50 μM PS/50 μM DGDG + 1.0 mM NaBT,
▩ : 50 μM PS/50 μM SQDG + 1.0 mM NaBT
Caco-2 (1×10^4 cells/mL) were incubated in presence of 50 μM PS/50 μM DGDG and 50 μM PS/50 μM SQDG and in combination with 1.0 mM NaBT for different time periods. The bars represent± SD (n=8).
*$p < 0.01$ vs control, **$p < 0.05$ vs control

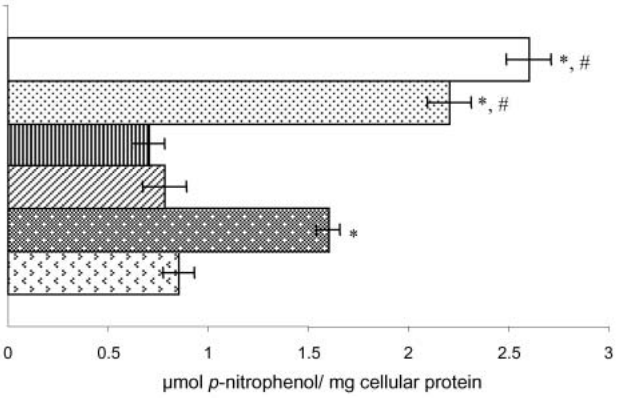

Fig. 3 Effects of PC/DGDG and PC/SQDG Hybrid Liposomes on Differentiation in Caco-2 Cells Induced by NaBT.
□ : Control, ▩ : 1.0 mM NaBT,
▨ : 50 μM PC/50 μM SQDG,
▥ : 50 μM PC/50 μM DGDG,
⊡ : 50 μM PC/50 μM DGDG + 1.0 mM NaBT,
□ : 50 μM PC/50 μM SQDG + 1.0 mM NaBT
Caco-2 (1×10^4 cells/mL) were incubated in presence of 50 μM PC/50 μM DGDG and 50 μM PC/50 μM SQDG hybrid liposomes, 1.0 mM NaBT and in combination of hybrid liposomes with 1.0 mM NaBT for 72 h. Results are means ± SD (n=4).
*$p < 0.01$ vs control, $^{\#}p < 0.01$ vs 1.0 mM NaBT

motion of differentiation in Caco-2 cells (**Fig. 3**), compared with 1.0 mM NaBT alone. Thus, NaBT induced more differentiation with hybrid liposomes (PC/DGDG and PC/SQDG) in Caco-2 cells.

3·5 Differentiation in Caco-2 Cells during PS/DGDG and PS/SQDG Hybrid Liposomes Treatment Induced by NaBT

In order to determine whether the effect of 50 μM PS/50 μM DGDG and 50 μM PS/50 μM SQDG hybrid liposome on differentiation in Caco-2 cells induced by NaBT, the ALP activity was measured. 50 μM PS/50 μM DGDG + 1.0 mM NaBT and 50 μM PS/50 μM SQDG + 1.0 mM NaBT showed significant promotion of differentiation in Caco-2 cells (**Fig. 4**) compared with 1.0 mM NaBT alone. Results indicated that addition of NaBT in combination with glycerophospholipids and glyceroglycolipids to Caco-2 cells led to an increased ALP activity. Thus, NaBT induced more differentiation with hybrid liposomes (PS/DGDG and PS/SQDG) in Caco-2 cells.

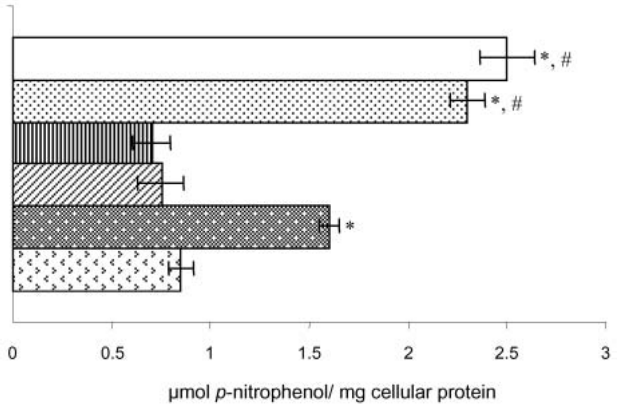

Fig. 4 Effects of PS/DGDG and PS/SQDG Hybrid Liposomes on Differentiation in Caco-2 Cells Induced by NaBT.
□ : Control, ▩ : 1.0 mM NaBT,
▨ : 50 μM PS/50 μM SQDG,
▥ : 50 μM PS/50 μM DGDG,
⊡ : 50 μM PS/50 μM DGDG + 1.0 mM NaBT,
□ : 50 μM PS/50 μM SQDG + 1.0 mM NaBT
Caco-2 cells (1×10^4 cells/ml) were incubated in presence of 50 μM PS/50 μM DGDG and 50 μM PS/50 μM SQDG hybrid liposomes, 1.0 mM NaBT and combination of hybrid liposomes with1.0 mM NaBT for 72 h. Results are means ± SD (n=4).
*$p < 0.01$ vs control, $^{\#}p < 0.01$ vs 1.0 mM NaBT

4 Discussion

The development of novel anticancer drugs is a subject of intensive study in the field of cancer research. Recently, researchers attempted to make hybrid liposomes as cancer chemotherapeutic agents. The growth inhibition of hybrid liposomes against Caco-2 cells was examined in the present study. At 72 h, growth inhibition effects of 50 μM PC/50 μM SQDG hybrid liposome was significant whereas growth inhibition was increased significantly for 50 μM PC/50 μM DGDG when this hybrid liposomes were treated with NaBT. On the other hand, it was attractive that growth inhibition was significant when Caco-2 cells were treated with 50 μM PS/50 μM DGDG and 50 μM PS/50 μM SQDG hybrid liposomes. Growth inhibition could induce the cell cycle arrest at the G1 and S phase. Subsequently, p53 act as a sensor causing G1 arrest of cell damage. Up regulation of p53 may result in the arrest of cells in the G1 phase (18). Hybrid liposomes treated on human lung adenocarcinoma and human stomach tumor *in vitro* and found growth inhibition effects on both cases (4). In addition, Nakano *et al*. (19) also found growth inhibition when hybrid liposome was added to human hepatoma cells *in vitro*. Furthermore, the hybrid liposomes have been found to have a highly inhibitory effect on mice model of carcinoma *in vivo* (20). Matsumoto *et al*. (7) also reported that hybrid liposomes of lipid and micellar molecules inhibit the growth of HL-60 cells. The hybrid liposomes of the present study may have no side effects on normal cells because there was no growth inhibition effect on CCD-18Co under the experimental conditions.

The present results revealed hybrid liposomes composed of glycerophospholipids and glyceroglycolipids promoted the differentiation in Caco-2 cell induced with NaBT. Hybrid liposomes alone did not promote differentiation, but when they were combined with NaBT, the differentiation was increased significantly. Butyrate has been documented to be a differentiating agent and was able to enhance ALP activity in Caco-2 cells (21, 22). In addition, Tochizawa *et al*. (23) noted that phospholipid containing docosahexaenoic acid (DHA) plus retinoic acid promoted differentiation of human leukemic HL-60 cells. Furthermore, Hosokawa *et al*. (24) reported that highly unsaturated fatty acid-phosphatidylethanolamine (HUFA-PE) with dibutyryl

cyclic adenosine monophosphate (dbcAMP) increased the differentiation of HL-60 remarkably. Experimental PC and PS were rich in DHA and eicosapentaenoic acid (EPA). On the other hand, DGDG was rich in arachidonic acid (AA) and EPA as well as SQDG was also moderately rich in AA and EPA, which is caused for promotion of differentiation with NaBT. Chamras *et al*. (25) reported that AA and EPA induced differentiation of MCF-7 human breast cancer cell. These results suggest that hybrid liposomes, which contain both glycerophospholipids and glyceroglycolipids might be considered as potentially useful functional foods for colon cancer patients, because NaBT is produced easily from dietary fiber in intestine.

5 Conclusion

In conclusion, it is suggested that hybrid liposomes prepared with glycerophospholipids and glyceroglycolipids inhibited the growth and promoted differentiation in Caco-2 cells in presence of NaBT.

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