ORIGINAL

Effects of Phospholipids Containing Docosahexaenoic Acid on Differentiation and Growth of HL-60 Human Promyelocytic Leukemia Cells

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Abstract : Effects of docosahexaenoic acid (DHA)-containing phospholipids (DHA-PLs) on retionic acid (RA)-induced cell differentiation and growth inhibition of the human promyelocytic leukemia cell line, HL-60 were investigated. Cell differentiation judged from NBT reduction assay reached to 50～60% by incubation with 12.5 μM DHA-containing phosphatidylcholines (DHA-PCs) prior to the addition of RA. Differentiation without DHA-PCs remained at 39.2%. By prior incubation with DHA-containing phosphatidylethanolamines (DHA-PEs), differentiation was 70% at 12.5 μM. DHA-PEs induced NBT reducing activity to as much as 20～30%, and inhibited cell growth in the absence of RA. Activity for promoting cell differentiation differed according to DHA-PL molecular species. 1-Palmitoyl (or oleoyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (or phosphoethanolamine) was found most effective molecular species for enhancing RA-induced cell differentiation.

Key Words : docosahexaenoic acid-containing phospholipid, differentiation, cell growth, HL-60.

1 Introduction

Docosahexaenoic acid-containing phospholipids (DHA-PLs) are currently receiving attentions due to their novel physiological functions[1]. DHA-containing phosphatidylcholines (DHA-PCs) with specific acyl groups inhibit 5-lipoxygenase[2] as well as lecithin : cholesterol acyl transferase *in vitro*[3]. Enhancement of discriminatory shock-avoidance learning in rats was also observed after intraperitoneal injection of DHA-PC *in vivo*[4]. These results suggest that the molecular species are important to the functions of DHA-PLs. Physiological functions of DHA-PLs should also be affected by the phospholipid polar base group because *n*-3 highly unsaturated fatty acids (HUFAs) supplemented in cell culture medium are predominantly incorporated into phosphatidylethanolamine (PE), as compared with phosphatidylcholine (PC)[5),6)].

The intention of the present study is to compare the promotional activity of various individual PL molecular species and classes on retinoic acid (RA)–induced cell differentiation, and growth inhibition.

2 Experimental

2・1 Materials

RPMI 1640 medium was obtained from GIBCO (New York, USA) and fetal bovine serum (FBS) from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). All-*trans* retinoic acid (RA), nitroblue tetrazolium (NBT), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (OOPE, purity>99%) were purchased from Sig-

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ma Chemical Co. (St. Louis, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (PPPC, purity >99%) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (OOPC, purity >99%) were purchased from Avanti Polar-Lipids, Inc. (Alabaster, USA). Docosahexaenoic acid (DHA-FFA) and DHA ethyl ester (DHA-EE) were generous gifts from Nippon Chemical Feed Ltd. (Hakodate, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Tokyo Kasei Industry Co., Ltd. (Tokyo, Japan) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Individual DHA-PL molecular species, *i.e.* 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (PDPC), 1-oleoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (ODPC), 1-icosapentaenoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (EDPC), 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (PDPE), 1-oleoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (ODPE) and 1-icosapentaenoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (EDPE) were enzymatically synthesized from soybean lysophosphatidylcholine (LPC) through several steps as previously reported[7),8)]. *sn*-2-Docosahexaenoylated phosphatidylcholine (XDPC), with various fatty chains in the *sn*-1 position was prepared by phospholipase A_2-mediated esterification between DHA-FFA and soy lysophosphatidylcholine, which is a partial hydrolysate of soy PC. DHA-containing phosphatidylethanolamines (DHA-PEs) were prepared from DHA-PCs by phospholipase D-mediated transphosphatidylation[9)]. Fatty acid compositions of DHA-FFA, DHA-EE and these enzymatically synthesized DHA-PLs are shown in **Table 1**.

2·2 Cell culture

HL-60 cells (ATCC CCL-240), obtained from American Type Culture Collection (Rockville, CT, USA), were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 mg/mL penicillin, and 100 IU/mL streptomycin at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. The morphology of HL-60 cells was determined by Wright-Giemsa staining.

2·3 Nitroblue tetrazolium reduction

NBT reduction assay was employed as an indicator of differentiation of HL-60 cells[10)]. Briefly, HL-60 cells were preincubated with PLs (12.5 μM) for 24 h, then RA ethanol solution (final RA concentration corresponds to 100 nM) was added to the culture medium for the NBT reduction assay. The final concentration of ethanol was below 0.1% (vol/vol) in the medium. After another incubation for 24 h, cell-suspended medium (0.5 mL) was mixed

Table 1 Fatty Acid Composition (mol%) of Exogenously Added DHA-containing Lipids Employed.

Fatty acid	PDPC	ODPC	EDPC	XDPC	PDPE	ODPE	EDPE	XDPE	DHA-FFA	DHA-EE
16 : 0	47.0	1.6	0.8	15.8	49.1	1.5	1.9	16.8	0.2	n.d.
18 : 0	0.4	0.3	n.d.	0.6	0.5	0.4	0.6	4.2	n.d.	n.d.
18 : 1	0.7	47.9	n.d.	4.9	0.9	47.5	1.2	7.1	n.d.	n.d.
18 : 2	2.5	2.1	2.4	30.3	1.9	2.7	1.7	24.7	0.2	n.d.
18 : 3	0.2	n.d.	0.8	2.8	0.2	n.d.	0.7	1.1	0.1	n.d.
20 : 5 (EPA)	2.5	2.2	44.7	6.6	2.3	2.6	45.8	1.4	4.5	4.3
22 : 6 (DHA)	46.5	45.9	49.8	39.3	45.1	45.2	45.8	43.0	94.7	94.1
others	0.2	n.d.	1.5	n.d.	n.d.	0.1	2.3	1.7	0.3	1.6

Abbreviations : PDPC, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine ; ODPC, 1-oleoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine ; EDPC, 1-icosapentaenoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine ; XDPC, 2-docosahexaenoylated phosphatidylcholine ; PDPE, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine ; ODPE, 1-oleoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine ; EDPE, 1-icosapentaenoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine ; XDPE, 2-docosahexaenoylated phosphatidylethanolamine ; DHA-FFA, docosahexaenoic acid ; DHA-EE, docosahexaenoic acid ethyl ester ; n.d., not detected.

with 0.5 mL of NBT solution, which contained 2 mg/mL of NBT and 200 ng/mL TPA in phosphate-buffered saline (PBS, pH 7.4), and then incubated for 30 min at 37℃. The cells were washed with aliquots of PBS and suspended in 0.1 mL PBS. HL-60 cells do not produce superoxide anions (O_2^-), but differentiated HL-60 cells produce O_2^-, and show blue-black formazan deposits by NBT reduction. The NBT positive cells that contained intracellular blue-black formazan deposits were counted with a hematocytometer. The results of NBT reduction are based on a positive number of cells over total cells of at least 200 cell counts.

2·4 Determination of cell growth

When HL-60 cells were seeded at a density of 5×10^4 cells per mL, various DHA-PLs dissolved in ethanol were added to this culture medium at a final concentration of 12.5 μM or 62.5 μM. Final concentration of ethanol was below 0.1% (vol/vol). HL-60 cells were grown in this PL-supplemented medium for 120 h. Cell number was determined with a hematocytometer.

2·5 TNBS quenching with supplemented PEs.

TNBS selectively binds to amino groups of PEs, phosphatidylserines, and proteins, and quenches these fluorescent intensity accordingly. Using this technique, the incorporation of PEs into HL-60 cells can be evaluated. The TNBS quenching method of Grunberger *et al.*[11] was employed in this study with minor modification. After preincubating HL-60 cells with 12.5 μM PEs or ethanol as a control for 48 h, 4×10^6 of these cells were washed twice with PBS and then resuspended in 5 mL PBS. These cell suspensions were mixed with 5 mL of 2 μM 1,6-diphenyl-1,3,5-hexatriene (DPH) PBS solution (containing 0.1% tetrahydrofuran), which is a hydrophobic fluorescent probe for cell membranes, and incubated for 30 min at 37 ℃. These HL-60 cells treated with DPH, were divided into two groups, then washed twice with PBS. One group was resuspended in 1 mL TNBS solution (2 mg/mL in cold PBS) and the other in 1 mL cold PBS alone as a control. Incubation was carried out for 1 h at 4℃, then both groups were centrifuged (1200 rpm, 5 min) and suspended again in 3 mL PBS.

Fluorescence intensity (FI, excitation wave length 360 nm, emission wave length 450 nm) was measured by a Hitachi 650-60 fluorescent spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan). Fluorescence quenching (FQ) was determined as follows :

$$FQ\ (\%)=\{(FI\ of\ control-FI\ of\ TNBS\ added)/(FI\ of\ control-FI\ of\ DPH\ free\ cells)\}\times100$$

2·6 Statistical analysis

Statistical analysis was carried out using Student's t-test. Differences between various PLs were tested at $p<0.01$ or $p<0.05$.

3 Results

3·1 Effect of chemical form of DHA on retinoic acid-induced differentiation of HL-60 cells.

Incubation with 100 nM RA alone induced differentiation of HL-60 cells at an NBT reduction level of 39.2%. Preincubation for 24 h with 12.5 μM ODPC prior to RA incubation resulted in a progressive increase in NBT reduction of HL-60 cells of up to 60.2%. In contrast, 25 μM DHA-FFA or DHA-EE increased NBT reduction only 7% more than in the control (**Fig. 1**), and increasing level of NBT reduction was obviously lower than that of ODPC. Morphological changes of HL-60 cells treated for 24 h after adding RA were not significantly different among DHA-PL supplemented and unsupplemented cells.

3·2 Effect of the differences in base groups of DHA-PLs on retinoic acid-induced differentiation of HL-60 cells.

NBT reduction by the cells preincubated with 12.5 μM ODPE before adding RA reached 72.8% (**Fig. 2**). This value was much higher than ODPC, which has the same acyl combination. NBT reduction of HL-60 cells supplemented with OOPC and OOPE without DHA chains prior to RA incubation was about 40%. This was as same as that of RA alone. Unlike

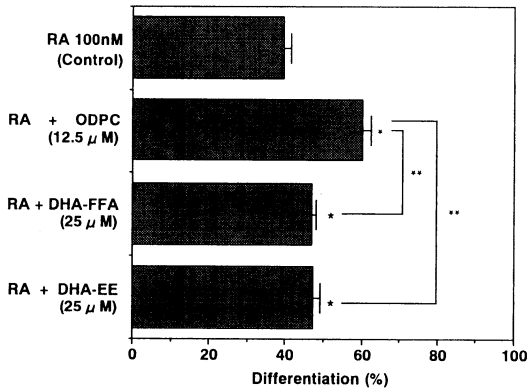

Fig. 1 Effect of DHA-containing Lipid Chemical Structures on Retinoic Acid (RA)-induced Differentiation of HL-60 Cells.
Values represent means ±S.D (n=3).
* $p<0.01$ *vs.* control.
** Significantly different from each other at $p<0.01$.

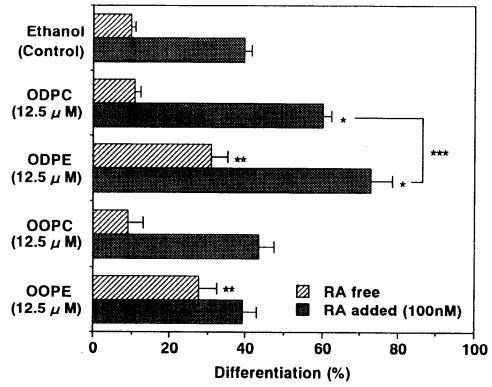

Fig. 2 Comparison of Differentiation of HL-60 Cells Supplemented with PCs and PEs.
Values represent means ±S.D (n=3).
* $p<0.01$ *vs.* control (RA 100 nM).
** $p<0.01$ *vs.* control (RA free).
*** Significantly different from each other at $p<0.01$.

DHA-PCs, ODPE exhibited 30.9% of NBT reduction at a concentration level of 12.5 μM even without RA. OOPE with no DHA chain also induced NBT-reducing activity under the absence of RA.

3·3 Retinoic acid-induced differentiation of HL-60 Cells supplemented with various DHA -PLs.

NBT reduction by the cells preincubated with 12.5 μM PDPC and ODPC before adding RA reached 60.5% and 60.2%, respectively (**Fig. 3**). These values were about 5~10% higher than those of cells supplemented with EDPC and XDPC. On the other hand, supplementation of PPPC or OOPC showed no significant increase against the control.

It is noteworthy that NBT reduction of HL-60 cells supplemented with DHA-containing phosphatidylethanolamines (DHA-PEs) prior to RA incubation reached to 65~70%, and

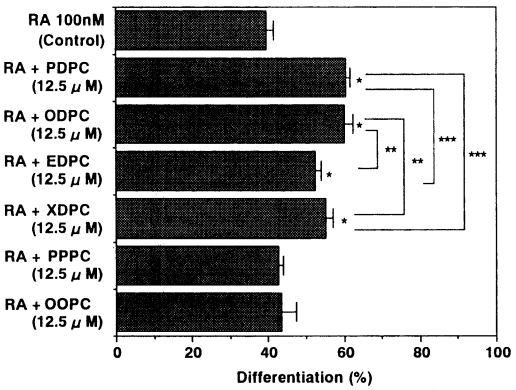

Fig. 3 Effect of Individual DHA-containing PC Molecular Species on Retinoic Acid (RA)-induced Differentiation of HL-60 Cells.
Values represent means ±S.D (n=3).
* $p<0.01$ *vs.* RA 100 nM. Values are significantly different from each other at ** $p<0.01$, *** $p<0.05$.

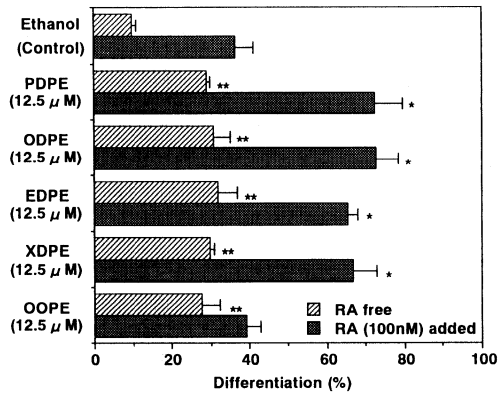

Fig. 4 Effect of Individual DHA-containing PE Molecular Species on Differentiation of HL-60 Cells.
Values represent means ±S.D (n=3).
* $p<0.01$ *vs.* control (RA added).
** $p<0.01$ *vs.* control (RA free).

that NBT reduction of cells supplemented with PDPE and ODPE were higher than those of EDPE and XDPE (**Fig. 4**). However, there were no significant differences in NBT reduction among HL-60 cells supplemented with PEs without RA.

3·4 Cell growth of HL-60 cells supplemented with DHA-PLs.

Table 2 shows the effect of individual DHA-PL molecular species on cell growth. The growth rate of HL-60 cells was not significantly reduced by incubation with 12.5 μM PDPC, ODPC, EDPC, XDPC, PPPC or OOPC. It was notable that 62.5 μM DHA-PCs reduced the cell number from one-fourth to one-fifth that of the control. In contrast, 62.5 μM PPPC and OOPC without DHA chain exhibited no inhibitory effect on cell growth. PDPE, ODPE and OOPE showed a much greater reduction rate (60∼65%) than PDPC, ODPC or OOPC with addition of 12.5 μM.

Table 2 Effect of DHA-containing Phospholipids on Cell Growth of HL-60 Cells.

	Relative cell growth (%)[a)]	
	12.5 μM	62.5 μM
PDPC	87.8±10.5	25.4±2.5
ODPC	76.9± 5.0	20.8±2.8
EDPC	96.6± 3.8	–
XDPC	91.9±11.5	36.4±1.6
PPPC	91.0±13.9	94.1±0.9
OOPC	90.5±11.6	89.0±1.0
PDPE	40.5± 2.9	–
ODPE	34.2± 3.8	–
OOPE	44.3± 5.9	–

a) % against control incubated after 120 h. Values represent means ±S.D (n=3).

Table 3 Fluorescence Quenching (FQ) of HL-60 Cells Supplemented with PEs (12.5 μM).

	FQ (%)
Control (Ethanol)	36.65±4.23
PDPE	46.87±4.12*
ODPE	47.38±2.96*
OOPE	42.07±2.90*

Values represent means ±S.D (n=3).
* $p<0.05$ *vs.* control.

3·5 Incorporation of PEs into HL-60 cell membranes

As shown in **Table 3**, each PEs showed 42~48% TNBS-quenching level, which is clearly distinct from that of ethanol alone (control). It was thus confirmed that PE was incorporated into HL-60 cells.

4 Discussion

The present results demonstrated that DHA-PLs enhance the RA-induced differentiation of HL-60 cells. We measured functional differentiation as judged from sensitive NBT reduction. NBT reduction of HL-60 cells induced by RA (24 h-exposure) was obviously promoted by preincubation with 12.5 μM DHA-PCs, and the rate of NBT reduction (60.5%, PDPC) was comparable to that of HL-60 cells treated with ten times higher RA concentration without any DHA-PLs (data not shown). Promoting effect of DHA-PCs on RA-induced NBT reduction was also observed at 96 h of RA exposure. NBT reduction of HL-60 cells preincubated with PDPC before adding RA reached to 68.5% and this value was higher than cells supplemented with RA alone (53.6%). However, PDPC alone did not increase NBT reduction. Since promoting effect of PDPC on RA-induced NBT reduction was observed at 96 h of RA-exposure incubation which induced morphological changes, it was considered that DHA-PCs have a synergistic effect on RA-induced differentiation. There have been many other reports of various physiological effects of DHA or PLs without DHA fatty chain[12)~15)]. Obermeier *et al.* and Burns *et al.* reported that HUFA increases differentiation of tumor cells in response to RA and 1,25$(OH)_2$-vitamin D_3 addition[16),17)]. In these studies, supplementation with DHA-PCs exerted stronger effect on differentiation than did the DHA-FFA or DHA-EE chemical forms. The effect of PLs without DHA fatty chain was also less pronounced. It is clear that PLs in combination with DHA fatty chain have a higher physiological activity.

A further pattern emerges when the polar head group of PC is changed into ethanolamine. The progressive increase in NBT reduction by DHA-PEs is even higher than that by DHA-PCs, while OOPE did not show a promoting effect on RA-induced NBT reduction. We also observed that expression of surface antigen CD11b induced by RA was enhanced markedly in HL-60 cells supplemented with DHA-PE as compared to DHA-PC (data not shown). Our data suggest that DHA-PEs affect the differentiation of HL-60 cells in response to RA. What is noteworthy is that DHA-PEs induced the NBT-reducing activity of HL-60 cells without addition of RA and inhibited the cell growth at 12.5 μM concentration level, whereas no sig-

nificant change of nucleus and cell morphology was observed at 120 h after adding PDPE. On the other hands, NBT reduction of HL-60 cells supplemented with PDPE prior to RA incubation was constant at about 70% from 24 h to 96 h after RA adding. It was considered that the PDPE increased RA-induced differentiation remarkably in the early time of incubation with RA. Based on these observations, we suggest that the polar base group of PL should have an effect on cell differentiation and on inhibition of cell growth of HL-60 cells, and that these functions of DHA-PEs might be exerted through plural pathways.

Focusing our attention on acyl residues in the *sn*-1 position of DHA-PCs, we note that the acyl residues showed different promoting effects on the RA-induced differentiation. PDPC and ODPC showed a greater effect than EDPC and XDPC. In the DHA-PE classes, the promotional effects on RA-induced differentiation were higher with PDPE and ODPE than with EDPE or XDPE.

PDPC and ODPC were first isolated from rainbow trout embryos by Suzuki *et al.*[18] as phospholipids showing the effect of cell differentiation of tumor cells. The authors suggested that the effect of DHA-PL on differentiation of tumor cells was dependent on their acyl combinations. The present study reconfirms this observations.

At present, the mechanism of promoting effect of DHA-PLs on RA-induced differentiation remains unclear. Stillwell reported that T27A cells fused with ODPC enhanced the permeability of medicinal substances[19]. We demonstrated recently that membrane fluidity and deformability of human erythrocyte are increased by the addition of DHA-PLs[7),20]. PEs easily incorporate into HL-60 cell membrane, as borne out through the TNBS-quenching method carried out in this study. The progressive increase in RA-induced differentiation by supplementation of DHA-PLs might be attributed to either an increase in drug permeability or in cell membrane fluidity as a result of the DHA-PL incorporation. Structual features of DHA-PLs should considerably affect this phenomenon.

The activity of protein kinase C and adenylate cyclase[23] involved in signal transduction was dependent on PL molecular species, as reported by Slater *et al.*[21),22]. They found that increasing the level of phosphatidylserine (PS) unsaturation results in a decrease in PKC-α activity, while, in the case of PC, an increase in unsaturation results in an increase in PKC-α activity[21),22]. DHA-PLs might affect several enzymes and RA-activated transcription factors involved in the differentiation of HL-60 cells.

Choice of fatty chain in position *sn*-2, in combination with the polar base group in the PL molecule, is a critical factor for designing an effective lipid chemical form for RA-induced cell differentiation and cell growth of HL-60 cells. In conclusion, PLs that contain saturated or monounsaturated fatty chain in position *sn*-1, and DHA in position *sn*-2 in combination with ethanolamine as a polar base group, are considered the most beneficial therapeutic lipids against cancer cells. Side effects could be substantially decreased by applying these therapeutic DHA-PLs.

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日本油化学会誌本号掲載 論文要旨

[総説] 逆ミセル溶液における可溶化状態によるコロイド粒子の形態，結晶形および分散性の制御

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逆ミセル系におけるコロイド粒子の形態，結晶形および分散性に及ぼす可溶化水の影響を明らかにするために，種々の分光法を用い，イオンおよび非イオン逆ミセル溶液における水の可溶化状態を Rw (=[水]/[界面活性剤]) の関数として調べた。その結果，それらの逆ミセル内部には，2あるいは3つのタイプの水，すなわち，界面活性剤の極性基への結合水，その水和極性基との結合水，あるいはバルク類似水が種々の割合で存在することがわかった。それらの水の状態と関係づけたミセル状態図から，逆ミセルおよび膨張ミセルおよび W/O マイクロエマルション形成に要する最少 Rw と他のパラメーターとを算出した。そこで，それぞれのタイプの水を含むミセル系でコロイド粒子の合成と分散性について研究した。その結果，Aerosol OT 溶液で $Ge(OC_2H_5)_4$ の加水分解から調製した GeO_2 粒子のサイズおよび形態，および非イオン界面活性剤溶液で $Ca(OH)_2$ 水溶液の可溶化溶液へ CO_2 を吹き込んで調製した $CaCO_3$ 粒子の結晶形は，それぞれ逆ミセル内部における水の状態によって制御されることがわかった。また，TiO_2 粒子の分散性もイオンおよび非イオン逆ミセル系における水の可溶化状態によって制御された。

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[報文] HL-60 ヒト前骨髄性白血病細胞の分化と増殖に及ぼすドコサヘキサエン酸結合型リン脂質の影響

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ヒト前骨髄性白血病細胞 (HL-60 細胞) の分化と増殖に及ぼすドコサヘキサエン酸 (DHA) 結合型リン脂質の影響について検討した。分化誘導剤であるレチノイン酸 (RA) の単独添加に比べ，DHA 結合型ホスファチジルコリンでインキュベーションした後に RA を添加することで分化率が有意に上昇した。このような RA の分化誘導能に対する促進効果は，*sn*-1 位がパルミチン酸又はオレイン酸，*sn*-2 位が DHA である分子種において顕著であった。同一分子種のホスファチジルエタノールアミン (PE) の添加では，ホスファチジルコリンに比べより大きな分化誘導促進効果がみられ，DHA 結合型 PE のみを単独添加した場合においても，強い細胞増殖抑制作用と NBT 還元能の上昇がみられた。以上の結果より，HL-60 細胞の分化誘導および増殖抑制に対して，1-palmitoyl 又は 1-oleoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine が最も効果的であった。

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