Synergistic Effects of Highly Unsaturated Fatty Acid-containing Phosphatidylethanolamine on Differentiation of Human Leukemia HL-60 Cells by Dibutyryl Cyclic Adenosine Monophosphate

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Highly unsaturated fatty acid-containing phospholipid (HUFA-PL) has many nutritional and medical applications. We investigated the effect of HUFA-PL on differentiation of human leukemia HL-60 cells induced by dibutyryl cyclic adenosine monophosphate (dbcAMP). HUFA-containing phosphatidylethanolamine (HUFA-PE), such as salmon testis PE, significantly enhanced dbcAMP-induced cell differentiation. A combined treatment of 200 µM dbcAMP with 50 µM HUFA-PE increased the nitroblue tetrazolium (NBT)-reducing activity, which is an indicator of differentiation, to a level comparable to that in the case of 500 µM dbcAMP treatment. In contrast, HUFA-lyso PE (a monoacyl form) did not exert an enhancing effect on dbcAMP-induced differentiation. The enhancing effect of HUFA-PE was suppressed by a protein kinase C inhibitor, staurosporine, while a protein kinase A inhibitor, H-8, did not suppress the enhancing effect. These findings suggest that HUFA-PE might enhance dbcAMP-induced differentiation through modulation of the protein kinase C signaling pathway in HL-60 cells.

Key words: Differentiation — Highly unsaturated fatty acid — Phospholipid — Dibutyryl cAMP — HL-60 cells

A human promyelocytic leukemia cell line, HL-60, has been used as a model system for studying leukemia cell differentiation. HL-60 cells are induced to differentiate into granulocyte-like cells by retinoic acid (RA),1) dimethyl sulfoxide2) and dibutyryl cyclic adenosine monophosphate (dbcAMP)3) or into monocyte/macrophage-like cells by 1α,25-dihydroxyvitamin D34) and phorbol 12-myristate 13-acetate.5) These differentiation-inducing drugs are often used in combination with other compounds to reduce side effects without losing the therapeutic effects.6, 7) ω-3 highly unsaturated fatty acids (HUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have many physiological functions. In vitro, the effect of HUFAs on cell proliferation and differentiation has been well documented.8−10) When HUFAs are incubated with tumor cells, they are incorporated into cell membrane phospholipid (PL) and affect cellular functions. It has been reported that HUFAs enhance differentiation of leukemia cells induced by RA10) or 1,25(OH)2-vitamin D3 with interferon-γ.11) An exogenous supplement of HUFA-containing PL also affects the membrane environment and influences various cellular functions.12−14) We previously reported that the enhancing effect of DHA-PL on RA-inducing differentiation was stronger than that of DHA.15)

Since signal transduction enzymes such as protein kinase C (PKC)16,17) and adenosine cyclase18) are affected by specific molecular species of PLs, the membrane modification by HUFA-PL may be effective to enhance the sensitivity of leukemia cells to differentiation-inducing drugs. Furthermore, the medical benefits may differ depending on the chemical forms of HUFA-PLs.

In this study, we investigated the effect of several chemical forms of HUFA-PLs on differentiation of HL-60 cells induced by dbcAMP. In addition, we evaluated the influence of HUFA-PL treatment on dbcAMP permeability and the contribution of protein kinases to elucidate the mechanisms of the enhancing effect of HUFA-PL on dbcAMP-induced differentiation.

MATERIALS AND METHODS

Materials Human promyelocytic HL-60 cells (ATCC CCL-240) were obtained from American Type Culture Collection (Rockville, CT). RPMI 1640 medium was obtained from GIBCO (Grand Island, New York), and fetal bovine serum (FBS) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA). DbcAMP, nitroblue tetrazolium (NBT), 12-O-tetradecanoylphorbol-13-acetate (TPA), staurosporine, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO). H-8 (N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) was obtained from Seikagaku

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Co. (Tokyo). A cyclic AMP enzyme immunoassay (EIA) kit, “Biotrak,” was purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ).

**Preparation of phospholipids** Extraction of lipids from salmon testis and salmon muscle was carried out according to the method of Bligh and Dyer with slight modifications. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) classes were separated via silica gel column chromatography from the extracted total lipid. Salmon testis lyso PE (salmon testis LPE) and salmon testis lyso PC (salmon testis LPC) were prepared by partial hydrolysis of salmon testis PE and PC with lipase. 1-Oleoyl-2-docosahexaenoyl-phosphoethanolamine (ODPE) was enzymatically synthesized from soybean LPC through several steps as previously reported. The fatty acid compositions of phospholipids used in this study are shown in Table I.

**Cell culture** HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. For each experiment, HL-60 cells were seeded at a density of 5×10⁴ cells/ml. DbcAMP as a differential inducer and H-8 or staurosporine were dissolved in phosphate-buffered saline (PBS) or ethanol, respectively and added to the culture medium. The final concentration of ethanol was below 0.1% (v/v). For the experiments with protein kinase inhibitors, HL-60 cells were incubated with H-8 or staurosporine for 24 h before dbcAMP and salmon testis PE were added to the culture medium.

**Nitroblue tetrazolium reduction** NBT reduction assay was employed as an indicator of HL-60 cell differentiation. After incubation of HL-60 cells with phospholipid and/or dbcAMP, the cell pellet was separated from the culture medium by centrifugation at 500g for 5 min and rinsed 3 times with PBS. The cells were suspended in 0.5 ml of NBT solution containing 2 mg/ml of NBT and 200 ng/ml TPA in PBS, then incubated for 30 min at 37°C. The cells were washed with aliquots of PBS and resuspended in 0.1 ml of PBS. HL-60 cells do not produce superoxide anions (O₂⁻), but when they differentiate, the cells start to produce O₂⁻, then form blue-black formazan deposits by reducing the NBT reagent. For each determination, at least 200 cells were counted using a hemacytometer. The number of NBT-positive cells containing intracellular formazan deposits was expressed as a percentage of the viable cell number.

**Determination of cell growth** HL-60 cells were seeded at a density of 5×10⁴ cells per milliliter and were preincubated for 24 h. Both HUFA-PL and dbcAMP were then added to the culture medium and incubated for another 72 h. Viable cell number was determined by the trypan blue dye exclusion method.

**Fatty acid composition of phospholipids in HL-60 cells** HL-60 cells were incubated with 100 μM dbcAMP and/or 50 μM PEs for 72 h. Cells were washed twice with PBS, and then total lipids were extracted by following the procedure of Folch et al. To separate PL fraction from the total lipids, a chloroform solution of the extracted total lipid was applied to a “Sep-Pak” silica cartridge (Waters Associates Co., Ltd., Milford, MA). The total PL fraction was eluted with methanol after the non-polar lipid fraction was eluted with chloroform:methanol:formic acid (65:25:10, v/v/v) and with a second developmental solvent of chloroform:methanol:formic acid (65:25:10, v/v/v). The spots corresponding to PE and PC were scraped off and were eluted with methanol. The PE and PC in the total PL fraction were separated by two-dimensional thin layer chromatography with a first developing solvent of chloroform:methanol:acetic acid (65:25:10, v/v/v) and with a second developmental solvent of chloroform:methanol:formic acid (65:25:10, v/v/v). The spots corresponding to PE and PC were scraped off and were eluted with methanol. The PE and PC thus obtained were methylated according to the method described by Prevot and Mordret. Then, they were analyzed for fatty acid composition with a Hitachi 163 gas chromatograph equipped with a flame ionization detector (Hitachi Co., Ltd., Tokyo), a G-300 column (1.2 mm×40 m, Chemicals Inspection and Testing Institute, Tokyo) and a D-2500 gas chromatograph.

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**Table I. Fatty Acid Composition of the Supplemented Phospholipids**

|            | Salmon testis PE | Salmon muscle PE | Salmon testis LPE | ODPE | Soy PE | Salmon testis PC | Salmon testis LPC |
|------------|------------------|------------------|------------------|------|-------|-----------------|------------------|
| C₁₆:₀      | 11.2             | 16.1             | 0.5              | 1.4  | 14.2  | 35.3            | 5.6              |
| C₁₆:₁      | 0.6              | 0.7              | 0.3              | —    | 0.2   | 2.6             | 1.7              |
| C₁₈:₀      | 3.6              | 5.7              | 0.4              | —    | 4.1   | 1.0             | 0.1              |
| C₁₈:₁      | 20.5             | 10.0             | 0.3              | 45.8 | 14.8  | 21.6            | 14.4             |
| C₁₈:₂      | 1.6              | 0.9              | —                | 1.4  | 63.3  | 3.5             | 0.4              |
| C₂₀:₄      | 4.5              | 9.0              | 6.4              | 1.4  | —     | 1.9             | 3.6              |
| C₂₀:₅ (EPA)| 30.7             | 9.0              | 42.4             | —    | —     | 14.2            | 37.2             |
| C₂₂:₆ (DHA)| 20.3             | 46.5             | 44.7             | 49.1 | —     | 12.3            | 31.4             |

**Abbreviations:** PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; ODPE, 1-oleoyl-2-docosahexaenoyl-PE; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.
integrated. Helium was used as a carrier gas at a flow rate of 10 ml/min.

**Intercellular cAMP determination** HL-60 cells were incubated with 200 µM dbcAMP for 72 h after 24 h preincubation with 50 µM HUFA-PE. Cells were then washed three times with Hanks’ balanced salt solution containing 500 µM IBMX. Intercellular cAMP was measured with a commercially available kit (“Biotrakenhanceing,” Amersham Pharmacia Biotech Inc., Piscataway, NJ).

**Statistical analysis** Statistical comparisons between two groups were performed using Student’s t test. A difference was considered significant at P<0.01.

**RESULTS**

**Effect of salmon testis PE on differentiation of HL-60 cells induced by dibutyryl cAMP**

Salmon testis PE, which has 30.7% EPA and 20.3% DHA in fatty acid composition, enhanced the NBT-reducing activity of HL-60 cells induced by 100 µM dbcAMP in a dose-dependent manner (Table II). When HL-60 cells were treated with a combination of 50 µM salmon testis PE and 100 µM dbcAMP, NBT-reducing activity increased to more than 40%, while NBT-reducing activity induced by dbcAMP alone remained 11.6±1.1%. Salmon testis PE itself did not directly induce NBT reduction. When 200 µM dbcAMP and 50 µM salmon testis PE were combined, the NBT-reducing activity of HL-60 cells exceeded 80%. This value corresponded to the value when the cells treated with 500 µM dbcAMP without salmon testis PE (Fig. 1).

**Effect of dibutyryl cAMP and salmon testis PE on the growth of HL-60 cells**

The effects of dbcAMP and salmon testis PE on growth of HL-60 cells were examined. Two hundred micromolar dbcAMP itself inhibited the cell growth (Fig. 2). Viable cell number was 61.8% of the control after 72 h incubation with 200 µM dbcAMP (total incubation time, 96 h). Salmon testis PE did not show any inhibitory effect on cell growth at 50 µM. A notable feature is that when 200 µM dbcAMP and 50 µM salmon testis PE were combined, a synergistic inhibitory effect on cell growth was observed. Viable cell number decreased to 27.3% of the control.

**Effect of phospholipid chemical form on differentiation of HL-60 induced by dibutyryl cAMP**

We compared the combined effect of various PL chemical forms on dbcAMP-induced differentiation (Fig. 3). ODPE, which has 45.8% oleic acid at the sn-1 position and 49.1% DHA at the sn-2 position, showed the highest enhancing effect in this study. NBT-reducing activity induced by 100 µM

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**Table II. Synergistic Effect of Salmon Testis PE on Differentiation of HL-60 Cells Induced by DbcAMP**

| Treatment | NBT reducing activity (%) |
|-----------|---------------------------|
| 100 µM dbcAMP | 11.6±1.1 |
| 100 µM dbcAMP+10 µM salmon testis PE | 22.2±1.9 |
| 100 µM dbcAMP+25 µM salmon testis PE | 34.0±3.9 |
| 100 µM dbcAMP+50 µM salmon testis PE | 42.9±1.1 |

HL-60 cells (5×10⁴ cells/ml) were incubated with 100 µM dbcAMP and/or salmon testis PE for 72 h after preincubation for 24 h. Data are shown as means±SD (n=3).

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**Fig. 1.** Effect of salmon testis PE on differentiation of HL-60 cells induced by dbcAMP. HL-60 cells (5×10⁴ cells/ml) were incubated with 100, 200 and 500 µM dbcAMP for 72 h after preincubation with 50 µM salmon testis PE for 24 h. Data are shown as means±SD (n=3). *P<0.01 vs. dbcAMP-treated cells.

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**Fig. 2.** Effect of salmon testis PE and dbcAMP on growth of HL-60 cells. HL-60 cells (5×10⁴ cells/ml) were incubated with 200 µM dbcAMP and/or 50 µM salmon testis PE for 72 h after preincubation for 24 h. Data are shown as means±SD (n=3). ○, control; ▲, salmon testis PE 50 µM; ■, dbcAMP 200 µM; ●, dbcAMP 200 µM+salmon testis PE 50 µM.
dbcAMP with 50 µM ODPE increased to 46.2%. This corresponded to almost two-fold increase as compared with 100 µM dbcAMP treatment without ODPE. Salmon muscle PE, which contains DHA at 46.5%, also expressed the enhancing effect on differentiation induced by dbcAMP. But the enhancing effect of soy PE, which does not contain HUFA, was far less than that of salmon testis PE or ODPE. HUFA at the sn-2 position of PL might crucial for the enhancing effect on differentiation.

In contrast to salmon testis PE, the enhancing effect of salmon testis PC was weak. Monoacyl PLs such as salmon testis LPE and salmon testis LPC did not show any enhancing effect on dbcAMP-induced differentiation of HL-60 cells in spite of their high levels of HUFA (87.1% and 68.6%, respectively) (Fig. 4).

**Incorporation of HUFA-PE into HL-60 cells** The PL fatty acid composition of HL-60 cells was analyzed by gas chromatography. In HL-60 cells treated with ODPE and dbcAMP together, the level of DHA in the fatty acid composition of total PL showed nearly a three-fold increase compared to that of the control. DHA increased to 20.6±5.0% from 7.8±1.0% (control) as shown in Table III, while DHA in PC remained at a level of 1% increase (data not shown).

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**Intracellular cAMP concentration** Cyclic AMP concentration in HL-60 cells treated with 200 µM dbcAMP and/or or 50 µM salmon testis PE were measured with the EIA kit. The antibody used in this EIA kit recognized dbcAMP equivalent to cyclic AMP (cAMP). When HL-60 cells were incubated with 200 µM dbcAMP for 24 h after preincubation with 50 µM salmon testis PE after 24 h incubation. The intercellular cAMP concentration was signifi-

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**Table III. Fatty Acid Composition of PE Class in HL-60 Cells Differentiated with DbcAMP and ODPE Combination**

| Acyl   | Control | DbcAMP+ODPE |
|--------|---------|-------------|
| C16:0  | 7.4±0.3 | 7.8±2.0     |
| C16:1  | 7.1±0.7 | 2.2±0.5     |
| C18:0  | 15.0±0.7| 12.6±3.2    |
| C18:1  | 25.8±0.5| 37.8±1.6    |
| C18:2  | 3.4±0.6 | 3.0±0.4     |
| C20:4  | 13.2±1.4| 6.4±2.3     |
| C22:6 (DHA) | 7.8±1.0 | 20.6±5.0    |

HL-60 cells (5x10^4 cells/ml) were incubated with/without 50 µM PLs and 100 µM dbcAMP for 72 h after preincubation for 24 h. Data are shown as means±SD (n=3). * P<0.01 vs. control.
cantly higher than that of the control (Fig. 5). HL-60 cells incubated with 200 µM dbcAMP and salmon testis PE for 72 h tended to have higher cAMP concentration than that of cells treated with dbcAMP alone, though the difference was not significant.

**Effects of protein kinase inhibitors on differentiation of HL-60 cells induced by dbcAMP**  
To elucidate the contribution of protein kinase A (PKA) and PKC to the expression of the enhancing effect of HUFA-PE on dbcAMP-induced differentiation, we investigated the effects of their inhibitors on dbcAMP-induced differentiation. A PKA inhibitor H-8 decreased the NBT-reducing activity induced by dbcAMP and salmon testis PE (Fig. 6). After incubation for 72 h with H-8 supplementation, the NBT-reducing activity of HL-60 cells decreased to 68.0% from 85.3% (without H-8). However, the enhancing effect of salmon testis PE on dbcAMP-induced differentiation was not impaired by the addition of 20 µM H-8.

In contrast, PK C inhibitor, staurosporine, suppressed the enhancing effect of salmon testis PE (Fig. 7). The NBT-reducing activity of HL-60 cells treated with a combination of 200 µM dbcAMP and 50 µM salmon testis PE and with 5 nM staurosporine decreased to 12.5%.

**DISCUSSION**

HUFA-modification of cell membrane PLs results in a marked increase in the sensitivity of leukemia cells to drugs. In the current study, we observed the enhancing effect of HUFA-PE on differentiation of HL-60 cells induced by dbcAMP. When HL-60 cells were incubated with 200 µM dbcAMP together with 50 µM salmon testis PE, the NBT-reducing activity increased to approximately 85%, up almost two-fold compared to that of HL-60 cells incubated with 200 µM dbcAMP alone. The combination of dbcAMP and salmon testis PE also showed cell growth arrest of HL-60 cells, while salmon testis PE alone did not directly inhibit the cell growth. It is known that terminal differentiation of HL-60 cells induced by some drugs is accompanied by cell growth arrest. Our data suggest...
that HUFA-PE potentiates the response of HL-60 cells to dbcAMP.

The enhancing effect on dbcAMP-induced differentiation was remarkably affected by the molecular species of PL. Salmon testis PC or soy PE which does not bind HUFA showed much lower effects than of salmon testis PE or ODPE. No enhancing effect of salmon testis LPE contained HUFA at 87.1% was observed at all. These results indicate that PL should be in diacyl form with HUFA at the sn-2 position and with ethanolamine as a polar head for the enhancing effect on dbcAMP-induced differentiation.

Burns et al. reported that uptake of adriamycin was greater in DHA-enriched L1210 cells as compared to 18:1-enriched cells. In the present study, the supplemented ODPE was incorporated into the PE class in HL-60 cells. However, the intracellular cAMP level in HL-60 cells treated with a combination of dbcAMP and HUFA-PE was not significantly higher than that of cells treated with dbcAMP alone. Namely, the permeability of dbcAMP did not increase much, even though the supplemented ODPE was incorporated into HL-60 cells. We therefore presume that other factors also contribute to the expression of the enhancing effect.

It is known that PKA and PKC play important roles in the differentiation of HL-60 cells induced by dbcAMP. It has also been reported that PE facilitates PKC activation by phosphatidylserine and HUFA potentiates PKC activity. To elucidate the contribution of protein kinases to the expression of the enhancing effect, we further investigated the effects of PKA and PKC inhibitors on dbcAMP-induced differentiation, respectively. A protein kinase A inhibitor, H-8, decreased the NBT-reducing activity induced by dbcAMP. However, the enhancing effect of salmon testis PE remained. In contrast, staurosporine which is a nonspecific PKC inhibitor remarkably suppressed the enhancing effect. These results suggest that PKC might be more involved than PKA in the signaling pathway of the enhancing effect of HUFA-PE on dbcAMP-induced differentiation. Since staurosporine inhibits tyrosine kinase and phosphorylase kinase, other protein kinases as well as PKC may also be important for the regulation of signaling pathways in cell differentiation.

We can sum up by saying that HUFA-PE enhances the differentiation of HL-60 cells induced by dbcAMP. PL should be in the diacyl form with HUFA at the sn-2 position and with ethanolamine as a polar head for the enhancing effect on dbcAMP-induced differentiation. Since a PKC inhibitor, staurosporine, suppressed the enhancing effect, it is suggested that PKC might play an important role in expressing the enhancing effect of HUFA-PE on dbcAMP-induced differentiation.

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