# Docosahexaenoic- and Eicosapentaenoic Acid-bound Lysophospholipids are More Effective in Suppressing Angiogenesis than Conjugated Docosahexaenoic Acid

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**Abstract: Suppression of leukemia, colon cancer, myeloma, and fibrosarcoma to some extent by omega 3 fatty acid bound phospholipids has been reported in the last two decade. However, the anti-angiogenic activity of those phospholipids is still not known. Four kinds of marine phospholipid molecular species i.e. starfish EPA bound diacyl phospholipid (EPA-PC), EPA bound monoacyl phospholipid (EPA-LPC) which was prepare via Lipozyme RMIM mediated partial hydrolysis of EPA-PC, squid DHA bound diacyl phospholipid (DHA-PC), and DHA bound monoacyl phospholipid (DHA-LPC) which was also prepare via Lipozyme RMIM mediated partial hydrolysis of DHA-PC, were subjected to antiangiogenic activity assay by using a piece of rat main artery and a human umbilical cord vein endothelial cell. The lengths of micro vein generated from those tissues after incubation with the above four kinds of phospholipid molecular species were measured and compared. EPA-LPC and DHA-LPC showed strong antiangiogenic activity on the rat main artery tissue, while on the human umbilical cord vein endothelial cells, 100 μM of EPA-LPC in the culture medium, exhibited the most effective suppression on angiogenesis, followed by 100 μM of DHA-LPC. It was concluded that EPA-LPC obtained via Lipozyme RMIM mediated partial hydrolysis of EPA-PC is the most effective omega 3 phospholipid on anti-angiogenesis.**

**Key words:** angiogenesis, phospholipids, lysophospholipids, omega 3

## 1 INTRODUCTION

Angiogenesis, the formation of blood capillary vessel networks, has undesirable consequences in the progression of some diseases. During cancer progression, cancer cells proliferate rapidly, and this accelerated tumor growth requires sufficient blood to supply increased nutrition and oxygen. To effect angiogenesis, cancer cells secrete cytokines which promote vascular endothelial cell growth, migration, and proliferation. For this reason, anti-angiogenic therapeutics are well-established strategies for cancer suppression[1]. In addition to the suppression of tumor growth, the prevention of angiogenesis is important for the treatment of certain chronic inflammatory diseases such as age-related macular degeneration, retinopathy of prematurity and diabetic retinopathy. In addition, the inhibition of vascular endothelial growth factor (VEGF) is also established as a treatment for age-related macular degeneration[2]. Tsuzuki et al. showed robust suppression of angiogenesis by conjugated eicosapentaenoic acid (cEPA) treatment of human umbilical vein endothelial cells (HUVECs)[3] and conjugated docosahexaenoic acid (cDHA) treatment of bovine aortic endothelial cells[4].

We showed that marine phospholipids enriched in squids, such as DHA-bound phosphatidylcholine (PC), are capable of suppressing tumor growth[5-7]. We suggested that these anticancer effects are exerted by modulation of the expression of oncogenes, upregulation of cell differentiating factors, and the limited ability of cancer cells to expel oxidized highly polyunsaturated phospholipids. In addition to these effects, we investigated whether marine

phospholipids or their derivatives mediate angiogenesis suppression.

## 2 EXPERIMENTAL PROCEDURES

### 2.1 Preparation of phospholipid samples

Phospholipids from the Japanese common squid (*Todarodes pacificus*) and Northern Pacific starfish (*Asterias amurensis*) are known to contain DHA and EPA exclusively in the *sn*-2 position of the phospholipid glycerol backbone. When phospholipase $A_1$ or *sn*-1,3 positional specific lipase mediated partial hydrolysis is carried out in these animal phospholipids, fatty acids bound on the *sn*-1 position are preferentially cleaved, leaving DHA or EPA bound on the *sn*-2 position. As a result, the lipids exclusively rich in DHA and EPA can be obtained in the form of *sn*-2 DHA- and EPA-bound lysophospholipids, respectively. The skin of the squid, *T. pacificus*, that was used for the extraction of marine phospholipid was a generous gift from Takeda Syokuhin Co. Ltd. Starfish, *A. amurensis*, was donated by Nemuro fishermen's cooperative. Total lipid was first extracted from the squid's skin and the internal organs of the starfish according to the method described by Bligh & Dyer[8] with a slight modification. The resulting total lipid extract was applied on a low-pressure silica gel PSQ 60B column (3.0 cm×30 cm) using chloroform/methanol (2:3, v/v) as the mobile phase. The flow rate of the mobile phase was 10 mL/min. The eluted fractions were monitored on a thin-layer chromatograph using chloroform/methanol/water (65:25:4, v/v/v) as a developing solvent, and the corresponding PC fractions were collected. Then, the solvent was evaporated to dryness, leaving the purified PC that was rich in DHA (DHA-PC) or EPA (EPA-PC) in the round-bottom flask. Further, 1 g of the PC was dissolved in 100

mL of *n*-hexane, and it was then subjected to a Lipozyme RMIM (a typical *sn*-1,3 positional specific immobilized lipase)-mediated partial hydrolysis by adding water activity (Aw) adjusted Lipozyme RMIM (Aw = 0.44). Argon gas was filled in the head space of the reaction container, after which the reactor was shaken at 75 strokes/min at 40℃ for 8 h. The partial hydrolytic reaction was quenched by passing the reaction mixture through a 0.45-μm polytetrafluoroethylene filter with chloroform/methanol (1:1, v/v) to remove the solid Lipozyme RMIM. The recovered reaction mixtures were then embedded on a silica Sep-Pak cartridge. Free fatty acids were first removed with chloroform/methanol (10:1, v/v), and then the remaining phospholipids (DHA-LPC or EPA-LPC) were recovered with methanol[9, 10]. As shown in **Table 1**, the composition of DHA almost doubled after Lipozyme RMIM-mediated partial hydrolysis. This is because DHA in squid phospholipid is exclusively bound on position *sn*-2. Furthermore, we compared the anti-angiogenic effects of the omega 3-bound phospholipids by adjusting the sample amount based on the mole % of the bound EPA or DHA moiety.

### 2.2 *Ex vivo* angiogenesis assay

Six-week-old male Wistar rats were housed in a room with controlled temperature (24℃ ±1℃) and 12-h light-dark cycle (lights on, 08:00-20:00 h). Rats had free access to food and deionized water and were maintained in accordance with the guide of Hiroshima University Animal Research Committee; the committee had approved this animal experiment. The *ex vivo* angiogenesis assay was performed according to slightly modified version of previously described methods[11, 12]. A single male Wistar rat (body weight, ~200 g) was sacrificed by bleeding from the right femoral artery under anesthesia with diethyl ether. To avoid contamination with blood, the thoracic aorta was

**Table 1** Fatty acid composition of DHA, EPA-bound phosphatidylcholine and lysophosphatidylcholine.

| Fatty acids | DHA-PC (%) | DHA-LPC (%) | EPA-PC (%) | EPA-LPC (%) |
|---|---|---|---|---|
| $C_{14:0}$ | 1.3 | 0.1 | 0.7 | 0.2 |
| $C_{16:0}$ | 32.5 | 0.7 | 4.5 | 0.7 |
| $C_{16:1}$ | 0.7 | − | 1.2 | 0.3 |
| $C_{18:0}$ | 1.3 | 0.4 | 7.8 | 4.6 |
| $C_{18:1}$ | 2.5 | 0.8 | 6.4 | − |
| $C_{20:0}$ | − | − | 8.0 | 4.9 |
| $C_{20:1}$ | 1.8 | 0.2 | 6.1 | 3.4 |
| $C_{20:4}$ | 0.7 | 1.0 | 4.4 | 5.5 |
| $C_{20:5}$ | 6.8 | 9.5 | 41.5 | 52.4 |
| $C_{22:6}$ | 50.9 | 85.7 | 10.6 | 18.5 |
| Others | 1.7 | 1.7 | 8.8 | 9.5 |

removed and washed with RPMI 1640 medium. The artery was then turned inside out and cut into short segments of approximately 1-1.5 mm. A collagen gel (gel matrix solution) was then prepared with 8 vol of porcine tendon collagen solution (3 mg/mL), 1 vol of 10×Eagle's MEM, and 1 vol of reconstitution buffer (80 mM NaOH and 200 mM HEPES). These solutions were mixed gently at 4℃. Each aortic segment was placed in the center of a well on a 6-well culture plate and covered with 0.5 mL of gel matrix solution, which was reconstituted as described above. The solution was allowed to gel at 37℃ for 20 min. The RPMI 1640 medium prepared contained 1% of ITS＋ with sample phospholipids or vehicle (dimethyl sulfoxide, DMSO). The collagen gel was then overlaid with 2 mL of culture medium and incubated for 7 days in a humidified atmosphere containing 5% $CO_2$ in air, at 37℃. Capillary length was estimated by phase-contrast microscopy, by measuring the distance from the cut end of the aortic segment to the approximate mid-point of the capillary. Microscopic fields were photographed using a digital camera. Capillary length was measured using Adobe Photoshop software CS3. Each reported value represents the average of 3-6 culture samples.

### 2.3 Cell culture

HUVECs were purchased from Kurabo Industries. Cells were grown in a humidified incubator with 5% $CO_2$ at 37℃ in HuMedia EG2 medium, which is a modified MCDB 131 medium containing 2% fetal bovine serum, 10 ng/mL recombinant human epidermal growth factor, 1 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 μg/mL amphotericin B, 5 ng/mL recombinant human basic fibroblast growth factor, and 10 μg/mL heparin. Subcultures were obtained by treating the HUVEC culture with Hanks'-based enzyme-free cell dissociation buffer solution. HUVECs passaged 3-7 times were used in this experiment.

### 2.4 HUVEC tube formation assay

The tube formation assay was performed using BD Matrigel™. Briefly, solid gels were prepared on a 96-well tissue culture plate according to the manufacturer's instructions. HUVECs (1×10⁵ cells/mL) in HuMedia EG2 medium containing sample phospholipids (5-100 μM) or vehicle (DMSO) were seeded 100 μl per well onto the surface of BD Matrigel TM. Cells were incubated for 12 h at 37℃ in a $CO_2$ incubator. Tube formation was observed under an inverted light microscope at ×40 magnification. Microscopic fields were photographed using a digital camera. The total length of tube structures in each photograph was measured using Adobe Photoshop software CS3. Each reported value represents the average of 3 samples.

### 2.5 HUVEC chemotaxis assay

The chemotaxis assay was carried out in a modified Boyden chamber[13]. A microporous membrane (8 μm) of 24-well cell culture insert was coated with 0.1% gelatin. HUVECs were suspended in Medium 199 with 0.1% bovine serum albumin (BSA) then seeded in the chamber (2.5× 105 cells/mL). The well was filled with 400 μL of Medium 199 containing 0.1% BSA and 10 ng/mL of human recombinant VEGF with or without the sample phospholipids (5-100 μM). The assembled chamber was incubated for 6 h in a humidified 5% $CO_2$ at 37℃. Non-migrated cells on the upper surface of the membrane were removed by scrubbing with a cotton swab. The cells on the lower surface of the membrane were fixed with methanol and stained with Diff-Quik stain. Migrated cells were counted in 3 fields of each membrane under the microscope at ×200 magnification, and the average number of cells in each field was calculated. The experiment was performed in triplicate.

### 2.6 HUVEC proliferation assay

A HUVEC suspension (1.5×104 cells/mL) was plated onto 96-well plates (100 μL/well), cultured in HuMedia EG2 medium, and incubated for 24 h in a humidified incubator with 5% $CO_2$ at 37℃. The medium was replaced with fresh HuMedia EG2 medium containing sample phospholipids (5-100 μM). After 72 h, 10 μL of WST-8 reagent was added to each well of a 96-well plate and incubated for 4 h. Absorbance was measured at 450 nm using a microplate spectrophotometer. Each reported value represents the average of 3 wells.
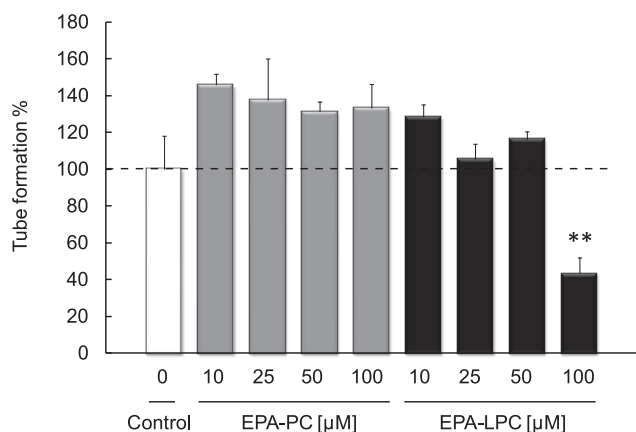
### 2.7 Statistical analysis

Values were presented as means±SD. Data were analyzed by one-factor ANOVA of variance with Turkey-Kramer test to identify significant differences ($p < 0.05$ or $0.01$).

## 3 RESULTS AND DISCUSSION

Angiogenesis involves a series of steps including endothelial cell activation and breakdown of the basement membrane, followed by migration, proliferation, and tube formation of endothelial cells. The primary aim of this study was to obtain evidence for the effects of partially hydrolyzed marine phospholipids on the key steps of angiogenesis, as previously discussed. At concentrations of 10-50 μM, DHA-LPC promoted HUVEC tube formation, but this process was suppressed at 100 μM (**Fig. 1**). Similarly, we observed that EPA-PC moderately promoted HUVEC tube formation, as did EPA-LPC at a low concentration (when less than 50 μM). However, at a concentration of 100 μM, EPA-LPC strongly suppressed HUVEC tube formation (**Fig. 2**). Microscopic analysis of 100 μM EPA- or DHA-treated HUVECs showed that EPA-LPC suppressed tube formation more effectively than DHA-LPC (**Fig. 3**). These results strongly suggest that marine PC-derived LPC sup-
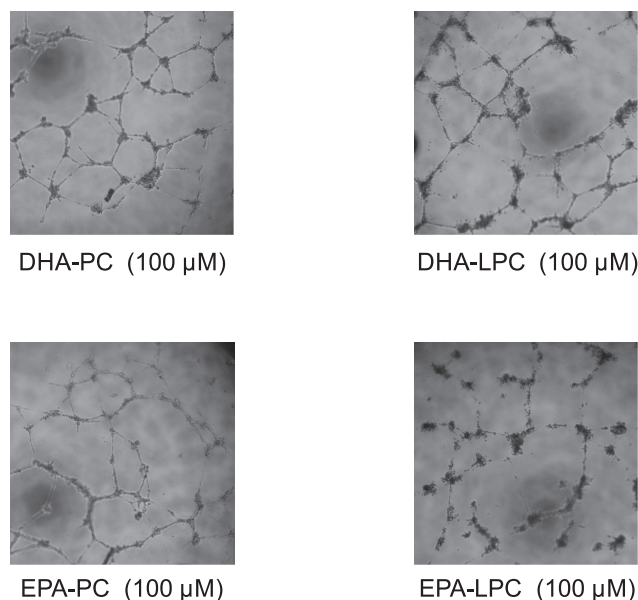
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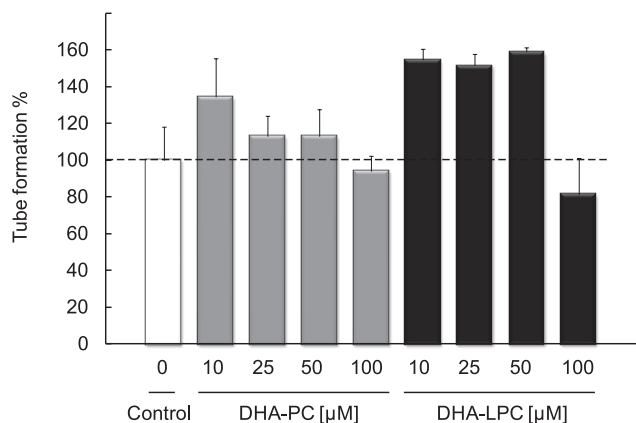
**Fig. 1** Effect of DHA containing PC and LPC on human umbilical vein endothelial cell (HUVEC) tube formation.
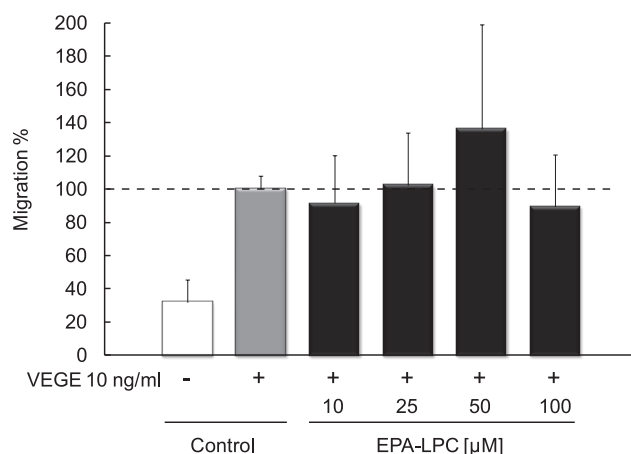


**Fig. 2** Effect of EPA containing PC and LPC on HUVEC tube formation. **P<0.01.



DHA-PC (100 μM)　　　　DHA-LPC (100 μM)

EPA-PC (100 μM)　　　　EPA-LPC (100 μM)

**Fig. 3** Micro scopic views of the effects of EPA or DHA containing phospholipids on HUVEC tube formations (100 μM).



**Fig. 4** Effect of DHA, EPA containing PC and LPC on HUVEC migration.

presses tube formation at 100 μM. Marine-derived LPC did not suppress cell migration, as illustrated in Fig. 4; however, EPA-LPC strongly suppressed the proliferation step of angiogenesis（Fig. 5）. Our results indicate that EPA-LPC is effective in suppressing tube formation and vein endothelial cell proliferation. In experiments using rat aortic segments, DHA-LPC suppressed angiogenesis at concentrations greater than 25 μM, but this did not reach significance（Fig. 6）. On the other hand, 100 μM EPA-LPC significantly（$p<0.05$）suppressed angiogenesis（Fig. 7）. Our results indicate that the anti-angiogenic effect of DHA-LPC and EPA-LPC may be dependent on the species and type of the vein tissues.

cDHA has been well recognized as an effective compound in suppressing angiogenesis[4]. We comparatively evaluated the anti-angiogenic effect of DHA-LPC and cDHA as well as the original DHA-PC（Fig. 8）. On comparison of the micro blood vein lengths, we found that the micro blood vein length was shorter after DHA-PC and DHA-LPC treatment than after cDHA treatment. DHA-LPC was more

effective in suppressing micro vein development than DHA-PC. Endothelial cell proliferation and migration are primarily regulated by members of the VEGF family, and VEGF is known to regulate the generation of tip cells through the Notch-1 signaling pathway[14]. Our subsequent studies will therefore aim to evaluate the effect of DHA-LPC on the Notch-1 signaling pathways as well as on the Ephrin signaling pathway, which orchestrates axon migration in cooperation with VEGF in the early stage of angiogenesis.
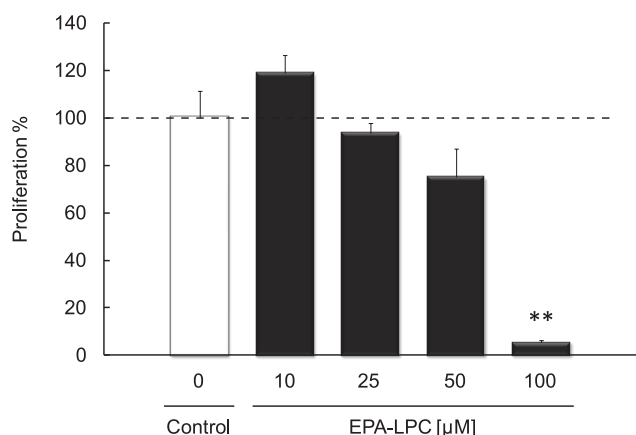
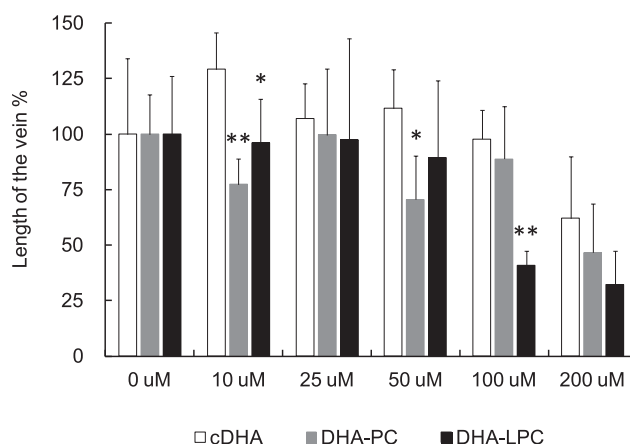**Fig. 5** Effect of EPA containing LPC on HUVEC proliferation. **P<0.01.
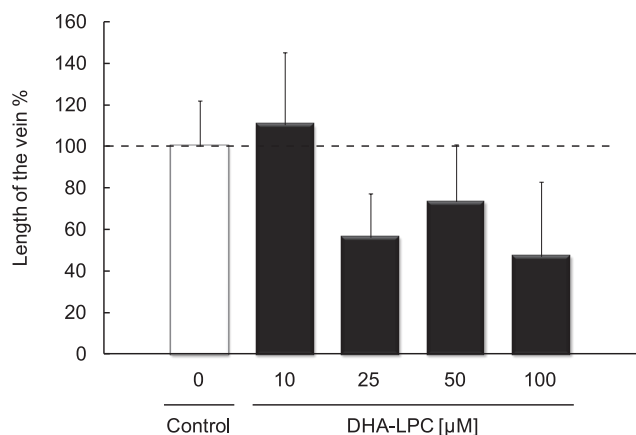


**Fig. 6** Effect of DHA containing LPC on angiogenesis of rat aortic segment.
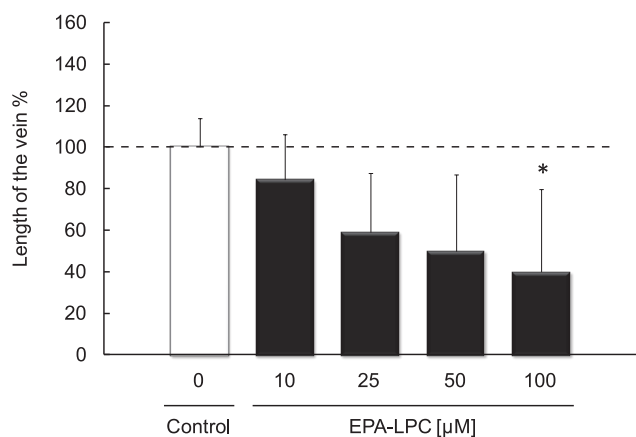


**Fig. 7** Effect of EPA containing LPC on angiogenesis of rat aortic segment. *P<0.05.



**Fig. 8** Effect of DHA-LPC and DHA-LPC in comparison to cDHA on angiogenesis of rat aortic segment. *P<0.05, **P<0.01.

and DHA-LPC, which can be easily obtained or derived from marine phospholipids. We conclude that marine phospholipids rich in DHA-PC and EPA-PC, and their enzyme-derived forms DHA-LPC and EPA-LPC derivatives are anti-angiogenic substances that are more effective, easier to obtain, and more stable than cDHA.

## 4 CONCLUSION

As previously discussed, cDHA is known for its beneficial effects in suppressing angiogenesis. The bioavailability and stability of cDHA, however, is lesser than that of DHA-PC

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