PRELIMINARY REPORT

Suppression by Eicosapentaenoic Acid of Oxidized Low-Density Lipoprotein and Lysophosphatidylcholine-Induced Migration in Cultured Rat Vascular Smooth Muscle Cells

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The migration of medial smooth muscle cells into the intima is proposed to be an initial process of intimal thickening in atherosclerotic lesions. The present study was designed to determine whether pretreatment with the antiatherogenic agent eicosapentaenoic acid (EPA) inhibits the migration induced by oxidized low-density lipoprotein (LDL) and its major phospholipid component, lysophosphatidylcholine (lyso-PC), in cultured rat vascular smooth muscle cells (VSMCs) using Boyden's chamber method. The effects of EPA pretreatment on angiotensin II (Ang II)– and platelet-derived growth factor BB (PDGF BB)–induced migration were also examined in these cells. Oxidized LDL and lyso-PC induced migration in a concentration-dependent manner. EPA pretreatment clearly suppressed oxidized LDL (200 μg/mL)– and lyso-PC (10^{-5} mol/L)–induced migration between 40 and 160 μmol/L. EPA pretreatment also suppressed Ang II (10^{-7} mol/L)– and PDGF BB (5 ng/mL)–induced migration at a concentration of 80 and 160 μmol/L. However, in a trypan blue exclusion test, dead cells stained with trypan blue were not found 24 hours after treatment with EPA. These results suggest that EPA suppresses VSMC migration induced by oxidized LDL and lyso-PC, as well as Ang II and PDGF BB. These preliminary data concerning the effects of EPA may partly explain the antiatherosclerotic effects of this agent.

EICOSAPENTAENOIC ACID (EPA), which is one of the n-3 polyunsaturated fatty acids and plentiful in marine lipids, has been shown to exert its antithrombotic and antiatherogenic action through the modulation of various cell functions related to atherosclerosis.[1-5] We have previously shown that oxidized low-density lipoprotein (LDL) and its major phospholipid component, lysophosphatidylcholine (lyso-PC), stimulate vascular smooth muscle cell (VSMC) migration.[6,7] The present study was designed to determine whether pretreatment with EPA inhibits migration induced by oxidized LDL and its lyso-PC moiety in cultured rat VSMCs using the Boyden chamber method.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), trypsin, Versine, penicillin, and streptomycin were purchased from GIBCO Laboratories (Grand Island, NY). Human LDL, n-3 EPA, lyso-PC (palmitoyl), and bovine serum albumin (BSA) were purchased from Sigma Chemical (St Louis, MO). Type I collagen was purchased from Koken (Tokyo, Japan). Flasks and multiple plates were purchased from Becton Dickinson (Mountain View, CA). Diff-Quick staining solution was purchased from Green-cross (Tokyo, Japan).

LDL Oxidation

LDL oxidation was performed as previously reported.[6] LDL was oxidized at a concentration of 500 μg/mL by exposure to 10 μmol/L $CuSO_4$ for 24 hours at room temperature, followed by dialysis at 4°C for 24 hours against 3 changes of phosphate-buffered saline. The extent of lipid peroxidation was estimated as for thiobarbituric acid–reactive substances. Tetramethoxypropane was used as a standard, and the results are expressed as nanomoles of malondialdehyde equivalents per 100 μg protein. The mean degree of oxidation for native LDL and oxidized LDL was 0.3 ± 0.1 and 4.1 ± 0.4 nmol malondialdehyde equivalents/100 μg protein, respectively.

VSMC Culture

Rat VSMCs were grown from aortic explants of Sprague-Dawley rats and cultured in DMEM containing 10% FCS, penicillin (50 U/mL), and streptomycin (50 μg/mL) as previously described.[8] Cells were identified as VSMCs according to morphologic and growth characteristics.[8]

Migration Assay

VSMC migration was measured by a modification of Boyden's chamber method using microchemotaxis chambers (Neuro Probe, MD) and polycarbonate filters (Nucleopore, MD) with a 5.0-μm pore diameter, as previously reported.[9] Cultured VSMCs were trypsinized and suspended at a concentration of approximately 5.0×10^5 cells/mL in DMEM. The cell number was determined with an electronic cell counter (model ZB1; Coulter Electronics, Hialeah, FL). A volume of 200 μL cell suspension was placed in the upper chamber, and 40 μL medium (0.4% BSA containing 20, 50, 100, or 200 μg/mL oxidized LDL or 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} mol/L lyso-PC) was placed in the lower chamber. The chamber was incubated at 37°C under 5% CO_2 in air for 6 hours.

Next, the effects of 3 concentrations (40, 80, and 160 μmol/L) of EPA on oxidized LDL– or lyso-PC–induced migration were examined. Cells were cultured in the absence or presence of 40, 80, and 160 μmol/L EPA for 24 hours, and the cell monolayers were gently washed twice with serum-free medium. Then, a volume of 200 μL of the cell suspension was placed in the upper chamber and 40 μL medium containing 200 μg/mL oxidized LDL or 10^{-5} mol/L lyso-PC in the presence or absence of EPA was placed in the lower chamber.

In addition, the effects of 3 concentrations (40, 80, and 160 μmol/L) of EPA on angiotensin II ([Ang II] 10^{-7} mol/L)– or platelet-derived

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growth factor BB ([PDGF BB] 5 ng/mL)–induced migration were examined as already mentioned.

Migration activity was calculated as the mean number of migrating cells observed in 4 high-power fields and is given as the mean value of 4 measurements.

Calculations and Statistical Analysis

The statistical significance of differences in the results was evaluated by 1-way ANOVA, and *P* values were calculated by Scheffe's method.[10] Values are expressed as the mean ± SD.

RESULTS AND DISCUSSION

The effects of various concentrations of oxidized LDL and lyso-PC on VSMC migration are shown in Fig 1A and B. Oxidized LDL and lyso-PC induced migration in a concentration-dependent manner. By contrast, the migration-stimulatory effects of high (10^{-4} mol/L) lyso-PC were clearly weak. This concentration of lyso-PC appeared cytotoxic, since cell viability estimated by the trypan blue exclusion test was significantly reduced and apparent morphologic cell injury was observed.

The effects of pretreatment with various concentrations of

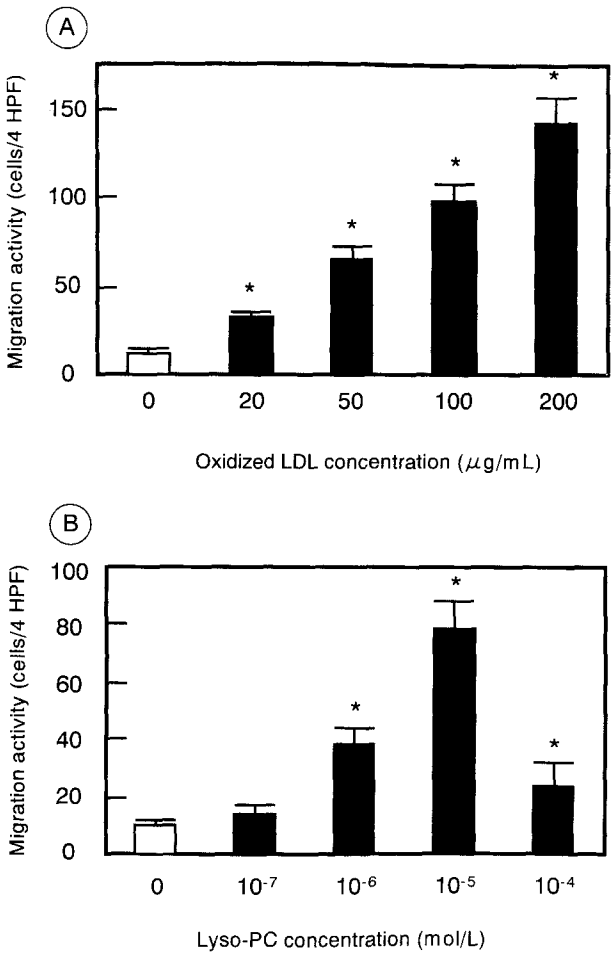

Fig 1. Concentration-dependent effects of oxidized LDL (A) and lyso-PC (B) on rat VSMC migration. Values are the mean ± SD of 4 measurements. Migration activity is expressed as the number of cells per 4 high-power fields (4 HPF). *$P < .05$ *v* control.

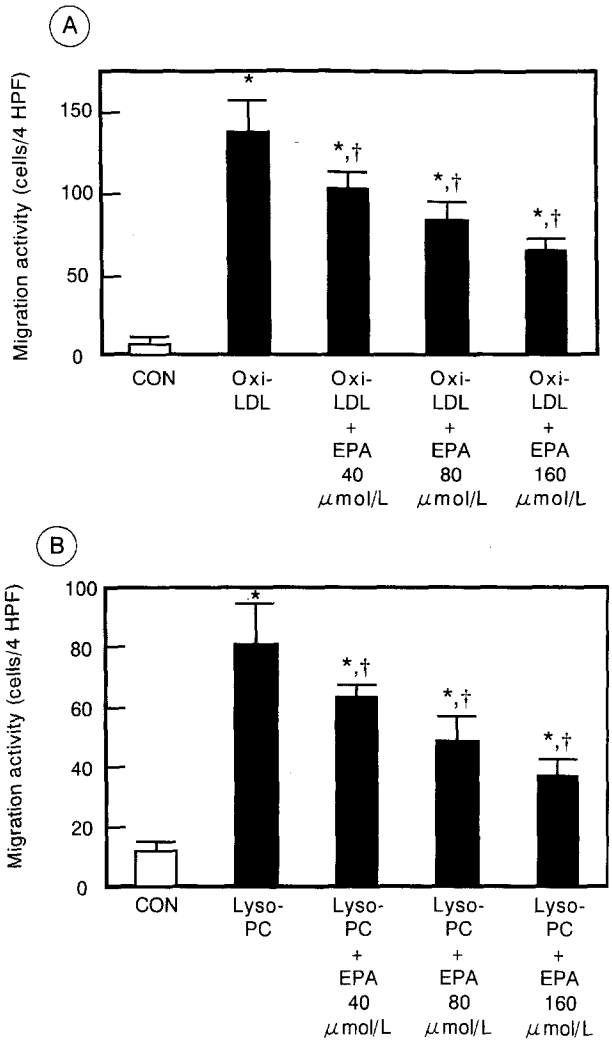

Fig 2. Effects of pretreatment with various concentrations (40, 80, and 160 μmol/L) of EPA on rat VSMC migration induced by 200 μg/mL oxidized LDL (A) or 10^{-5} mol/L lyso-PC (B). Values are the mean ± SD of 4 measurements. Migration activity is expressed as the number of cells per 4 high-power fields (4 HPF). *$P < .05$ *v* control. †$P < .05$ *v* oxidized LDL alone or lyso-PC alone. CON, control.

EPA on VSMC migration are shown in Fig 2A and B and Table 1. EPA pretreatment significantly suppressed oxidized LDL (200 μg/mL)– and lyso-PC (10^{-5} mol/L)–induced migration between 40 and 160 μmol/L. EPA pretreatment also suppressed Ang II (10^{-7} mol/L)– and PDGF BB (5 ng/mL)–induced migration at a concentration of 80 and 160 μmol/L (data not shown). However, in a trypan blue exclusion test, dead cells stained with trypan blue were not found 24 hours after EPA treatment.

In the present study, we have confirmed that oxidized LDL and its major phospholipid component, lyso-PC, induced rat VSMC migration. We have also shown that EPA pretreatment for 24 hours clearly suppressed oxidized LDL– and lyso-PC–induced VSMC migration without killing the cells. To the best of our knowledge, this is the first demonstration concerning the effects of EPA on oxidized LDL– or lyso-PC–induced VSMC

Table 1. Effects of Pretreatment With Various Concentrations (40, 80, and 160 μmol/L) of EPA on Ang II (10^{-7} mol/L)– or PDGF BB (5 ng/mL)–Induced Migration of Rat VSMCs

Pretreatment	Migration Activity (cells/4 HPF)
Control	9 ± 3
Ang II alone	74 ± 13*
Ang II +40 μmol/L EPA	67 ± 11*
Ang II +80 μmol/L EPA	51 ± 8*†
Ang II +160 μmol/L EPA	40 ± 9*†
PDGF BB alone	168 ± 21*
PDGF BB +40 μmol/L EPA	149 ± 23
PDGF BB +80 μmol/L EPA	118 ± 18*‡
PDGF BB +160 μmol/L EPA	96 ± 15*‡

NOTE. Values are the mean ± SD of 4 measurements. Migration activity is expressed as the number of cells per 4 high-power fields (4 HPF).

*$P < .05$ *v* control.

†$P < .05$ *v* Ang II alone.

‡$P < .05$ *v* PDGF BB alone.

migration. Furthermore, we have shown that EPA suppresses Ang II– and PDGF-induced VSMC migration.[3-5] However, the cellular mechanism of EPA in VSMC migration is not clear at present. Among the possibilities, the effect on EPA on prostaglandin I_2 (PGI_2) production in VSMCs is interesting. The formation of PGI_2 in rat VSMCs is increased during incubation with EPA,[11] and PGI_2 can inhibit both migration and proliferation through a cyclic adenosine monophosphate–dependent mechanism.[12,13] This possibility should be evaluated in future studies.

Previously, EPA was shown to inhibit proliferation in cultured rat VSMCs. Therefore, EPA may play a role as an antiatherosclerotic agent via the suppression of migration and proliferation induced by various types of vasoactive substances. Consequently, these findings may raise the possibility that EPA antagonizes the development of intimal thickening during the process of atherosclerosis in certain pathological conditions. However, this study was performed on cultured VSMCs. Therefore, any extrapolation of this finding to the in vivo condition should be made carefully. In addition, further studies are necessary to clarify the cellular mechanisms responsible for the action of EPA and the interaction of EPA and oxidized LDL or lyso-PC.

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