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Effect of n-3 Fatty Acids on Estrogen Dependency and Protein Kinase C Activity of Human Breast Cancer Cell

Sung-Hee Cho†, Sun-Hee Oh and Hee-Sung Park*

Dept. of Food Science and Nutrition, and
**Dept. of Plant Breeding, Catholic University of Taegu-Hyosung, Kyoung-buk 713-702, Korea*

Abstract

To investigate the effect of long chain n-3 polyunsaturated fatty acids on breast cancer cell growth, estrogen-dependent MCF-7 human breast cancer cells were cultured in serum-free DMEM media containing 0.5μg/ml of differnet kinds of fatty acids; linoleic acid(LA), arachidonic acid(AA), eicosapentaenoic acid(EPA) and docosahexaenoic acid acid(DHA) and 0, 0.1, 0.2, 0.5 and 1.0ng/ml 17β-estradiol as well as 10μg/ml insulin and 1.25mg/ml delipidized bovine serum albumin for 3 days. Cell growth monitored by MTT assay was lower in DHA and EPA treatments as compared to LA treatment, but not with AA treatment. Estrogen concentrations at which cell growth was initially stimulated were 0.1ng/ml for LA and DHA treatments and 0.2ng/ml for EPA and AA treatments, but the degree of stimulation was 25~30% lower in DHA and EPA treatments than in LA treatment. Fatty acid analysis showed that each fatty acid in culture medium was well incoporated into celluar lipid. Protein kinase C activity of cells was most elevated in LA treatment from 2 to 8 hours of culture followed by DHA, EPA and AA treatments. It is concluded that inhibitions of n-3 DHA and EPA on breast cancer cell growth as compard with n-6 LA is mediated via changes in membrane fatty acid composition reducing estrogen sensitivity and increasing protein kinase C activity.

Key words: breast cancer cell, n-3 fatty acid, estrogen, protein kinase C

INTRODUCTION

Evidence from epidemiological studies suggests a role for dietary fat in the etiology of breast cancer(1-5). High-fat diets are thought to increase the risk of breast cancer(1,2). However, not only does the quantity of fat consumed need to be considered, but also does its fatty acid composition. Saturated fat or animal fat intake had a strong correlation with breast cancer mortality, whereas polyunsaturated fat or vegetable fat had no or a weak association(3-5). In animal or *in vitro* cell culture experiments, those fats high in linoleic acid, an n-6 unsaturated fatty acid have enhanced the growth (6,7), but fish oil, rich in n-3 fatty acids has shown inhibitory effect(8,9).

Several mechanisms have been suggested for the inhibitory effects of n-3 polyunsaturated fatty acid containing fats(10). One of them is alterations in eicosanoid pathways, which was, however challenged by report by Rice-Evans and Burdon(11) showing that prevention of prostaglandin synthesis failed to suppress tumor growth inhibitory actions of fish oils. Another possible mechanism for the effect of n-3 fatty acids is the enhanced lipid peroxidation causing cytotoxicity to tumor cell(12). This mechanism may be valid but is counterbalanced by generally accepted hypothesis that lipid peroxidation induces carcinogenesis. Third mechanism which has been studied less than the above two is modified membrane function which results from changes in fatty acid composition by n-3 fatty acids. Fatty acids are important in the maintenance of cell membrane structure and are key determinant of cell membrane activity and receptor expression. N-3 fatty acids in diet(9) or in cell culture medium(13,14) have been shown to be well incoporated into tumor cell membrane lipid and some of the membrane modifications has been coincided with increases in sensitivity to adriamycin chemotherapy(14) and membrane permeability(15).

About one third of human breast cancers require estrogen for maximal growth. Therefore, concentration of the hormone and its binding to receptor are important for tumor cell proliferation. Feeding fish oil to nude mice has been shown to lower the production of estrogen(16) although the underlying mechanism re-

†Corresponding author

mains to be elucidated. On the other hand, it is expected that alteration in membrane fatty acid composition can affect the binding of estrogen to its receptor. Furthermore, the effect of tumor growth factors including estrogen depends on cell surface signal transduction into cell in which protein kinase C is known to play a significant role(17). It has been reported that diacylglycerol released from membrane phospholipid activating protein kinase C, could have different potency depending upon its fatty acid moieties(18).

Therefore, using estrogen-dependent MCF-7 human breast cancer cell line, we investigated that the effect of n-3 fatty acids on degree of estrogen dependency of cell growth and actuvity of protein kinase C along with fatty acid composition and oncogene expression of the cell in comparison with n-6 and n-9 unsaturated fatty acids.

MATERIALS AND METHODS

Materials

Dulbecco's modified eagle medium(DMEM), trypsin-EDTA, phosphate-buffered saline(PBS), penicillin/streptomycin and insulin were purchased from GIBCO BRL(Gaithersburg, MD, USA) and fetal bovine serum (FBS) from Hyclone(Logan, Utah, USA) and delipidized bovine serum albumin(BSA), linoleic acid($C_{18:2}$n-6), arachidonic acid($C_{20:4}$n-6), eicosapentaenoic acid($C_{20:5}$n-3), docosahexaenoic acid($C_{22:6}$n-3) and 17β-estadiol from Sigma Chemical Co.(St. Louis, MO, USA) and restriction enzymes, *Pst* I , *Bam*H I , *Hind*III and *Stu* I from either Promega(Madison, WI, USA) or from GIBCO BRL and gene clean II kit from Bio 101 Inc.(La Jolla, CA, USA) and random priming kit and nick translation kit from Promega.

Cell cultrure

The estrogen-dendent MCF-7 human breast cancer cell line obtained from Korean Cell Bank(Seoul National University, Medical School) was cultured routinely at 37°C in DMEM plus penicillin and streptomycin, supplemented with 5% FBS in a 95% air/5% CO_2 incubator. For a long term storage, cells were washed after culture, treated with trypsin-EDTA and kept in DMEM with 20% FBS and 10%(v/v) dimethylsulfoxide in liquid nitrogen.

Growth experiments

Cells were plated in 96-well plates and cultured for 24 hours in 5% FBS supplemented IMDM at a plating density of 10^4×cells/100μl/well and washed with unsupplemented medium. To the wells were added serum-free IMDM containing 1.25mg/ml delipidized BSA, 10 μg/ml insulin, 0.5μg/ml fatty acid(LA, AA, EPA, and DHA) and 0~1.0ng/ml of 17β-esradiol. Fatty acid stocks were dissolved in 100% ethanol, the volumes added to culture media being such that the final concentration of ethanol was 1%(19). Cell growth during 3 days was monitored by MTT assay(20).

Fatty acid analysis

Lipids were extracted from cells after 3 days of culture according to Folch et al.(21) and methylated with 14% BF_3/methanol(22). The fatty acid compositions of methyl esters were analyzed by gas chromatography (Shimadzu GC-14B, Tokyo, Japan). Chromatograhy was performed on 30m×0.32mm capillary column(Alltech, Deerfield, IL, USA). Helium was used as carrier gas and the temperature was programmned as the initial 150°C for 2 min and increased at the rate of 6°C/min to 230°C, that lasted 5 more minutes. Individual fatty acid methyl esters were idendified by comparison with known standards of GLC-81 and GLC-96(Nu-Chek Prep Inc. Elysian. MN, USA).

Protein kinase C activity

Cells cultured in the presence of four different fatty acids and 0.2ng/ml of 17β-estradiol for 1, 2, 4, 8, 12 and 16 hours were washed with PBS and homogenized at 0°C by sonication(50 Watt) in buffer A containing 20mM Tris-HCl(pH 7.5), 2mM EDTA, 2mM ethyleneglycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic-fluoride, 10μg/ml leupeptin, and 0.25mM sucrose. Cell homogenates were centrifuged at 100,000×g for 1 h at 4°C. The pellet was solublized in buffer B(buffer A contaning 1% Triton X-100) by stirring for 1 h at 4°C. The homogenate was centrifuged at 100,000×g for 1 h at 4°C. The resulting supernatant representing the solubilized "membrane" fraction was used as enzyme source(23). Protein kinase C activity was measured using PepTag™ assay kit for non-radioactive detection of protein kinase C(Promega).

Isolation of RNA and northern blot analysis

To obtain total RNAs, cells were cultured in 60mm

plates for 6~24 hours in the presence of estradiol and various types of fatty acids, washed two times with cold PBS buffer and then extracted in 1ml of cold denaturing solution containing 4M guanidine thiocyanate, 28mM sodium citrate, 0.55% N-lauryl sarcosine and 92mM β-mercaptoethanol as described in promega protocols manual(Promega, WI, USA). Fifteen to twenty μg of total RNA per lane were electrophoresed in formaldehyde gel for blotting to the nylon membrane filter(24). For northern blot hybridization, 1.2kb *Acc*I/*Xba*I fragment from a cDNA encoding tumor protein *p53*(25) was labelled with [α-^{32}P]dCTP by random priming method according to manufacturer's manual (Promega) and hybridized to the membrane filter for 16 hours at 63°C. The filter finally washed with 0.1×SSC at 65°C for 30min was autoradiographed.

RESULTS AND DISCUSSION

Cell growth and estrogen dependency

Fig. 1 shows growth of MDF-7 human breast cancer cells cultured during 3 days with 0.5μg/ml of four different kinds of fatty acids and 0~1ng/ml of 17β-estadiol in the media. Viable cell numbers measured by MTT assay continued to increase until 2nd day under all conditions but fluctuated in considerable degrees on the 3rd day, when growth conditions appears to have been altered by some unidentified reasons such as shortage of nutrients. Total cell growth was highest in LA treatment follwed by EPA, DHA and AA treatments. The result similar to the present one except AA treatment has been observed in estrogen independent MDA-MB231 breast cancer cells(26). Low

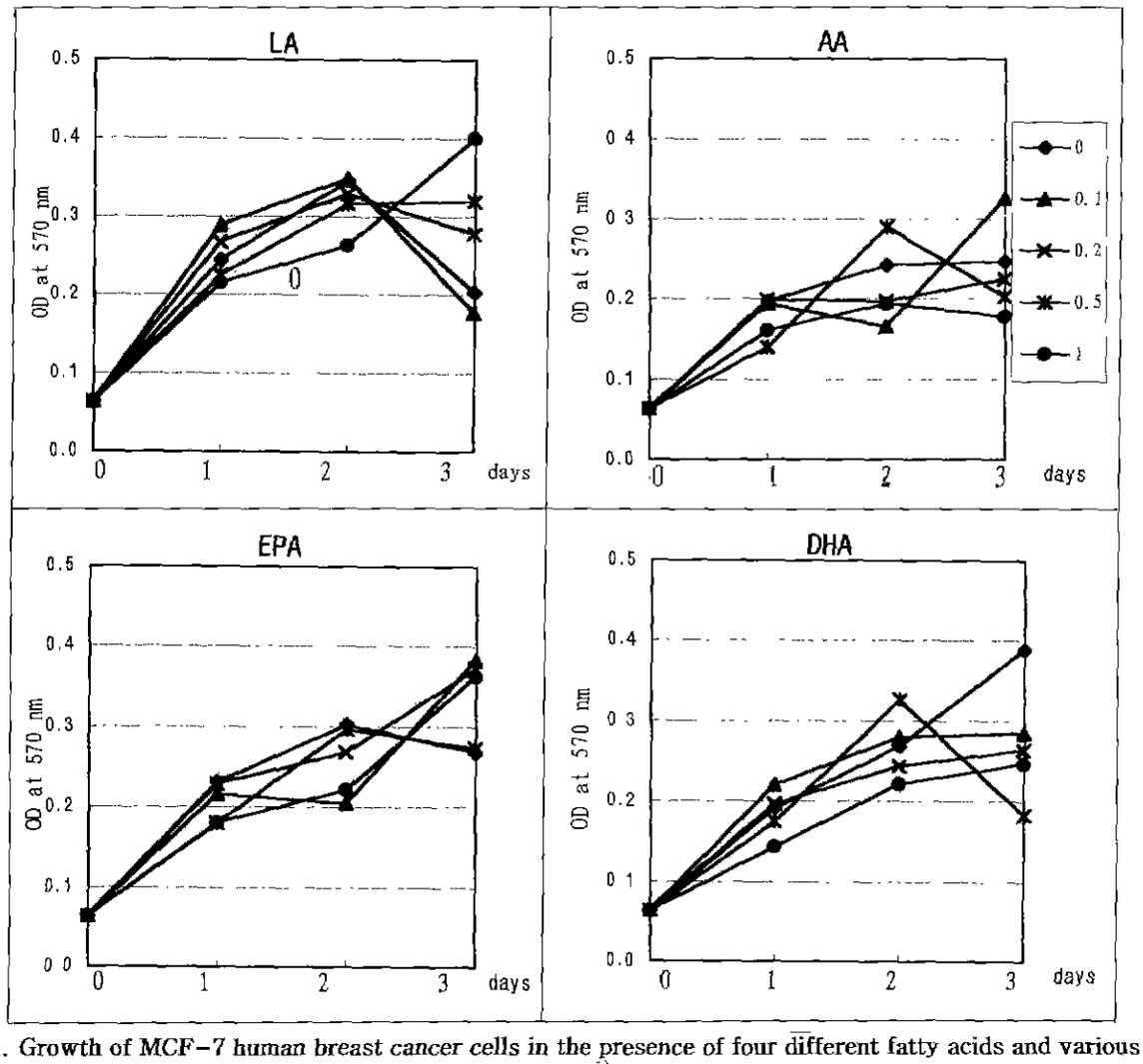

Fig. 1. Growth of MCF-7 human breast cancer cells in the presence of four different fatty acids and various concentrations of 17 beta-estradiol during three days[1)].

[1)]Concentration of each fatty acid was 0.5μg/ml and 17 beta-estradiol concentrations were 0, 0.1, 0.2, 0.5 and 1ng/ml as shown in small box.

growth in AA treatment was unexpected, since AA converted from LA was believed to be a major component in growth stimulation in LA treatment. However, the report from Wicha et al.(27) has shown that arachidonic acid reduced growth of both normal mammary epithelium and DMBA-induced tumor cells from rats. The effects of n-6 essential fatty acid in cell growth have been studied mostly using LA(6-9) instead of AA. Higher peroxidizability of AA than LA may have suppressed the cell growth(10), but further studies are needed for clarification. On the 1st day of culture, cell growth was most stimulated by estrogen at the concentration of 0.1ng/ml with LA and DHA in the media but it was highest at 0.2ng/ml with AA and EPA. Although the level of initial growth stimulation for cell growth was same in LA and DHA treatments, the degree of stimulation was 27% higher in LA than in DHA treatments. But on the second day, the hormone concentration for maximal cell growth was increased to 0.5ng/ml for AA and DHA treatments whereas it remained as 0.1ng/ml for LA. Feeding 17β-estadiol in the form of slow-release pellets to nude mice inoculated with MCF-7 breast cancer cells, Osborne et al.(28) found that tumor growth rates incresed with estrogen doses ranging from 0.01 to 0.5mg, but reduced with 10mg. Their results suggests that an optimal level of hormone is required for maximal tumor growth. The optimal hormone level in the present study appears to be 0.1~0.2ng/ml for initial stimulation of cell growth. Although data from the 3rd day varied much, cell growth in LA treatment was strictly estrogen dose-dependent as shown in first part of Fig. 1. All this observations indicate that reduced sensitivity of cells for estrogen in EPA, DHA and AA treatments, compared with LA treatment may be a part of reasons for lower cell multiflication.

Fatty acid composition of cellular lipid

Table 1 shows that four different kinds of fatty acids in culture media were well incoporated into lipid fractions of MCF-7 breast cancer cells cultured for three days, although the degrees were small. It was also noted that levels of palmitoleic acid($C_{16:1}$) and oleic acid($C_{18:1}$) were distinctively lower in LA and DHA treatments, respectively. Incorporation rates appear to vary depending on several culture conditions such as concentrations of fatty acids in the media. We used rather low concentration(0.5μg/ml) compared to 1~20 μg/ml used in other studies(7,8,13). The present result was obtained from total lipid of cultured cells and may have been different from the fatty acid composition of phospholipid fraction which is a better index for the changes in membrane sturcture. Since contents of polyunsaturated fatty acids were usually larger in phopholipid than in total lipid of cells, the incoporation rate of each polyunsaturated fatty acids used in this study are expected to be greater than seen in Table 1.

Table 1. Fatty acid composition of MCF-7 human breast cancer cells cultured in the presence of n-6 and n-3 polyunsaturated fatty acids for three days (wt %)

Fatty acid	Fatty acids added in culture medium			
	LA(n-6)	AA(n-6)	EPA(n-3)	DHA(n-3)
$C_{14:0}$	7.16	8.58	8.27	8.01
$C_{16:0}$	19.37	18.60	18.03	18.31
$C_{16:1}$	19.30	23.28	23.70	24.60
$C_{18:0}$	10.14	7.54	7.87	8.11
$C_{18:1}$ n-9	28.79	31.34	31.50	23.80
$C_{18:2}$ n-6	3.94	1.43	1.57	1.60
$C_{18:3}$ n-3				2.33
$C_{20:1}$	0.71			
$C_{20:4}$ n-6	3.87	5.46	3.70	4.18
$C_{20:5}$ n-3			0.79	0.58
$C_{22:0}$	0.65			
$C_{22:1}$				0.53
$C_{22:6}$ n-3			0.87	1.99
$C_{24:0}$	2.19	1.69	1.50	1.65
$C_{24:1}$	1.55	1.43	0.87	1.41

Protein kinase C activity

As shown in Fig. 2, protein kinase C activity as a whole appeared to increase after 2 hours of culture under the condition used in the present study. At the 2, 4 and 8 hours of culture, cells with LA treatment continued to have the higher values compared with other three fatty acid treatments and at the 8-hour culture, the value was the highest of all, although it dropped afterward. DHA treatment gave the highest enzyme activity at the 4-hour culture after which tended to be decreased, while EPA and AA treatments seemed to maintain the same level of activity from 4 to 16 hours. Among limited studies carried out for protein kinase C activity in relation to dietary fat, the report from Reddy et al.(29) is in accordance with the present result. Their result showed that feeding DHA and EPA(n-3) containing fish oil decreased colonic mucosal membrane

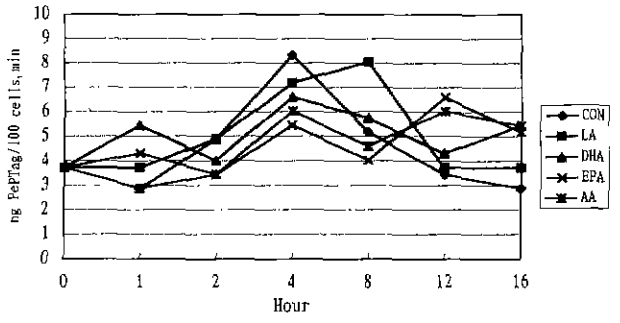

Fig. 2. Changes in protein kinase C activities in MCF-7 human breast cancer cells in the presence of four different fatty acids during 16 hours[1)].
[1)]Concentration of each fatty acid was 0.5μg/ml and 17 beta-estradiol was 0.2ng/ml.

protein kinase activity in rats treated with azoxymethane as compared with LA(n-6) containing corn oil feeding. On the other hand, Davidson et al.(30) did not see the difference in the enzyme activities between fish oil and corn oil feeding, although they studied extensively with several types of isozyme in different colonic sections. Part of this discrepancy appears to be uniqueness of the latter's experimental diet(30) lacking fibers. Since protein kinase C exists as membrane bound form and also in cytosol and partitioning of the enzyme between two compartments is reportedly influenced by dietary fat(31), it is needed to measure cytosolic enzyme activity as well in the same condition used in the present study. Recently, Jiang et al.(32) have shown the increase in mass of diacylglycerol, substrate for protein kinase C by n-6 LA. This may well be amplify the singnal transduction caused by protein kinase C activity promoted by the same n-6 LA fatty acid, resulting in increase in cell proliferation compared to other type of fatty acids.

p53 tumor suppressor gene expression

The *p53* gene containing 11 exons is located on human chromosome 17p13.1 and its transcript of 2.8 kb in size is detectable in all mammalian cells but low in normal cells(33,34). Overexpression of the *p53* is the most common genetic abnormality associated with breast cancer and emerges as a reliable, independent but provenly limited predictor for reduced survival rates in patients with breast cancer(35,36). In this study, various fatty acid treatments were examined in respect to their possible effects on transcriptional activation of the *p53* gene in a breast cancer cell line. *p53* Messenger RNA

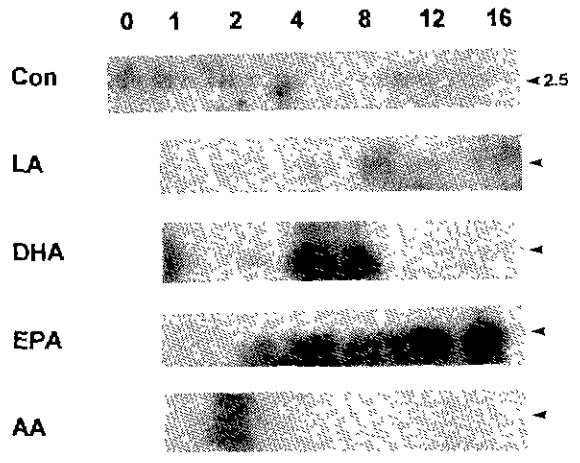

Fig. 3. Northern blot hybridization of *p53* tumor suppressor gene from MCF-7 human breast cancer cells cultured in the absence(CON)' and presence of four different kinds of fatty acids(LA, DHA, EPA, AA) during 0~16 hours.

expression was visualized using a radiolabeled 1.2 kb cDNA probe in estrogen-dependent MCF-7 breast cancer cells. The cells, supplemented with estradiol(0.2ng/ml), were grown in the absence or presence of fatty acids such as LA, DHA, EPA and AA, each of them at the concentration of 0.5μg/ml, and their *p53* mRNA levels from 0 to 16 hours of culture were determined by northern blot hybridization and autoradiography. The results are shown in Fig. 3. From the cells with no fatty acid treated as a control, the *p53* mRNA was not detectable but became visible at 8 hour of culture. Since then, its level gradually increased until 16 hours of growth period(Fig. 3, Con). When LA was added to the culture medium, the expression of the *p53* mRNA was accumulated in a transient manner. That is, it detectably emerged from 1 hour of treatment and its appearance reached at highest level in 2 hours but became gradually weakened until its absence at 12 hours(Fig. 3, LA). In the cells treated with EPA, the *p53* mRNA expression was detected in a very compatible mode of its accumulation with that of the control cells. But the treatment of EPA resulted in earlier emergence (from 4-hour) of the *p53* mRNA with comparably higher level throughout the culture period(Fig. 3, EPA). In the cells treated with either DHA or AA, the *p53* mRNA was rarely accumulated at all after 16 hours of treatment. The results that the expression of the *p53* mRNA in MCF-7 cells was likely suppressed by fatty acids such as AA, DHA and LA and more activated by the

presence of EPA seemed to suggest complex mode of interactions beween a variety of n-3 and n-6 fatty acids and *p53* gene expression. DHA and EPA as n-3 fatty acids showed apparently opposing effect, while LA and AA similarly suppresed *p53* gene expression even though LA transiently activated it. In MCF-7 cells, dietary fish oil rich in n-3 fatty acids was reported to decrease c-*ras* and c-*myc* expression(16). However, little has been reported how n-3 fatty acids regulates *p53* gene expression thereby modulating breast cancer growth. Although AA or DHA treatment inhibited the expression of the *p53* in this experiment, further detailed studies should be performed for elucidating mechanisms by which polyunsaturated fatty acids, n-3 or n-6 or both regulates *p53* overexpression *in vitro* as well as *in vivo*.

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