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Effects of Vitamins E and C on Human Breast Cancer Cell Growth in the Presence of Various Fatty Acids

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Abstract

To investigate the effects of antioxidative vitamins in combination with various fatty acids on breast cancer cell proliferation, MDA-MB231 human breast cancer cells were cultured for 3 days in the serum-free Iscove's modified Dulbecco's medium(IMDM) supplemented with 1.25mg/ml delipidized bovine serum albumin and 10μg/ml insulin. Alpha-tocopherol, ascorbic acid or both vitamins were added to the medium at the concentrations of 10 and 50μM in the presence of 3μg/ml of oleic(OA), linoleic(LA) α-linoleinic(LNA) and docosahexaenoic acid(DHA). Cell growth was reduced significantly by α-tocopherol in a dose-dependent manner, but not affected by ascorbic acid. The four different fatty acids did not have significant effects on cell growth, although DHA exerted inhibitory effect on the growth after 1 day. However, the each fatty acid was well incorporated into celluar lipid as such or elongated forms. Addition of α-tocopherol remarkably increased its cellular contents and reduced cellular levels of thiobarbituric acid substances(TBARS) that were elevated notably in the presence of DHA in the culture media. But ascorbic acid addition did not change much of either cellular α-tocopherol or TBARS contents. Northern blot hybridization showed that tumor suppressor gene *p53* was most highly expressed by the combination of α-tocopherol and DHA in 8 hours of cell culture. In conclusion, the growth inhibitory effect of vitamin E suggests that breast cancer cell proliferation is reduced by the mecahnism other than cytotoxicity of lipid peroxide and it is related to expression of tumor supprosser gene *p53*, that can be increased by both vitamin E and n-3 fatty acid, DHA.

Key words: breast cancer, vitamin E, fatty acid, *p53*

INTRODUCTION

A role for dietary fat in the etiology of breast cancer has been shown in population studies and animal experiments(1). The quantity of fat consumed appears to be an important factor, since high-fat diets have been shown to increase the risk of breast cancer(1). However, fatty acid composition of diet has been recognized in many studies to be another factor to be considered. Among them, differences in effects between n-6 and n-3 polyunsaturated fatty acid(PUFA) have attracted most attention in recent years. In animal or *in vitro* cell culture experiments, n-6 PUFA has enhanced the growth(2,3), whereas n-3 PUFA has shown inhibitory effect(4,5).

One of the suggested mechanisms for the effect of n-3 fatty acids is due to increased lipid peroxidation which exerts cytotoxicity against tumor cell(6). This mechanism may be valid but is counterbalanced by generally accepted hypothesis that lipid peroxidation induces carcinogenesis. Preventive roles of antioxidant nutrients on cancer development have been repeatedly suggested(7,8). Among those nutrients, vitamins E, C and β-carotene are well known. Major mechanism of their roles is suppression of the formation of free radical and other reactive oxygen species that are likely to be involved in initiation step of carcinogenesis. Few works have been done to see the effects of antioxidants beyond the initiation step. Fat in carcinogensis is believed to be involved in promotion step, where increased levels of some reactive species from highly unsaturated fatty acids such as n-3 PUFA could lead cancer cells to death. But they can also damage normal cells as well as initiate carcinogensis. Thus, it is necessary to study the combined effects of antioxidant vitamins and various fatty acids on cancer cell proliferation to clarify their contradictory actions.

Therefore, using estrogen-independent MDA-MB231 human breast cancer cell line, we investigated the effect of vitamins E and C on cell growth in the presence of

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four different fatty acids and measured lipid peroxidation and the level of α-tocopherol in the cell as well as expression of *p53*, tumor suppressor gene.

MATERIALS AND METHODS

Materials

Iscove's modified Dulbecco's media(IMDM), 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), oleic acid(OA, C18:1n-9), linoleic acid(LA, C18:2n-6), α-linolenic acid(LNA, C18:3n-3), docosahexaenoic acid(DHA, C22:6n-3), α-tocopherol, L-ascorbic acid and other biochemical reagents from Sigma Chemical Co.(St.Louis, MO, USA). Standard fatty acid methyl esters were obtained from Nu Chek(Nu Chek Prep, Inc MN, USA).

Cell culture

The estrogen-indendent MDA-MB231 human breast cancer cell line obtained from Korean Cell Line Bank (Seoul National University, Korea) was cultured routinely at 37°C in IMDM plus penicillin(5 units/ml) and streptomycin (5μg/ml), supplemented with 5% FBS in a 95% air/5% CO_2 incubator. For a long term stroge, cells were washed after culture, treated with trypsin-EDTA and kept in IMDM with 20% FBS and 10%(v/v) dimethylsulfoxide in liquid N_2.

Growth experiments

Cells were plated in 96-well plates and cultured for 24 hr in 5% FBS supplemented with IMDM at a plating density of 10^4 cells/100μl/well and washed with unsupplemented IMDM medium. To the wells were added serum-free IMDM containing 1.25mg/ml delipidized BSA, 10μg/ml insulin, 3μg/ml fatty acid(OA, LA, LNA and DHA). Alpha-tocopherol and L-ascorbic acid were added at the concentrations of 10 and 50μM, respectively. Fatty acid and α-tocopherol stocks were dissolved in 100% ethanol, the volumes added to culture media being such that the final concentration of ethanol was 1%, the concentration of which did not have any significant effect on cell growth(9). Cell growth during 3 days were monitored by MTT(thiazoyl blue) assay(10).

Fatty acid analysis

Lipids were extracted from cells after 3 days of culture according to Folch et al.(11) and methylated with 14% BF_3/methanol(12). 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 2min 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).

Determination of α-tocopherol

Alpha-tocopherol was extracted from cultured cells for three days after a brief sonication, in the presence of α-tocopherol acetate, an internal standard(13). To 200 μl of sonicated cell homogenate was added 2.5μg tocopherol acetate/200ul ethanol. After mixing well, 400μl of hexane was added and vortexed for 1 min and centrifuged at 1500 rpm for 10 min. Upper hexane layer was taken and lower layer was extracted again with hexane. Total extract was filtered through 0.45μm nylon membrane, dried with N_2 and dissolved with 60μl of diethyl ether/methanol(1:3, v/v) for high pressure liquid chromatography(HPLC) analysis. A column for the HPLC was C_{18} microbondapak(10μm, 3.9×300 mm, Waters, Milford, MA, USA), a mobile phase was methanol/H_2O(97:3) and detection was at 292nm.

Measurement of lipid peroxide

The sonicated cell homogenates were used to measure thiobarbituric acid reacitive substances(TBARS) according to Yagi(14), using 1,1,3,3-tetraethoxypropane as a standard. To 150~250μl of the cell homogenate, 4ml of 1/12 N H_2SO_4 and 0.5ml of 10% phosphotungstic acid were added. To the resultant precipitate, 4ml of water and 1ml of 0.67% TBA were added. Reaction was carried out at 95°C for 60 min. After cooling, the reaction mixture was extracted with 5ml n-butanol and centrifuged at 4000 rpm for 15 min and fluoresence was measured at 515 nm for excitiation and 553 nm for emission.

Isolation of RNA and northern blot analysis

To obtain total RNAs, cells were cultured in 60mm plates for 8 and 16 hrs in the presence and absense of

Table 1. Number of viable MDA-MB231 human breast cancer cells cultured in the presence of various fatty acids and vitamins E and C for three days.

Day	Vit[1] \ FA[2]	OA	LA	LNA	DHA	Effect
		number of cell $\times 10^{-4}$/ml[3]				
1	None	1.00±0.01[a4]	0.83±0.15[ab]	0.76±0.06[ab]	0.66±0.03[b]	FA; p<0.05
	C10μM	0.71±0.09	0.86±0.08	0.83±0.11	0.81±0.08	
	C50μM	0.85±0.09	0.84±0.11	0.76±0.12	0.79±0.08	
	E10μM	0.74±0.09	0.86±0.10	0.71±0.05	0.77±0.07	Vit; p<0.05
	E50μM	0.63±0.11	0.79±0.08	0.66±0.07	0.67±0.08	
	E50μM+C50μM	0.71±0.08	0.72±0.09	0.49±0.07	0.55±0.06	
2	None	0.68±0.05	0.76±0.10	0.83±0.06	0.77±0.08	FA; NS[5]
	C10μM	0.70±0.07	0.81±0.07	0.72±0.11	0.74±0.09	
	C50μM	0.80±0.04	0.81±0.09	0.70±0.06	0.73±0.08	
	E10μM	0.76±0.13	0.62±0.08	0.79±0.08	0.69±0.05	Vit; <0.05
	E50μM	0.56±0.08	0.57±0.07	0.73±0.05	0.60±0.08	
	E50μM+C50μM	0.70±0.07	0.56±0.06	0.62±0.06	0.61±0.07	
3	None	0.41±0.02	0.57±0.04	0.69±0.05	0.63±0.08	FA; p<0.05
	C10μM	0.47±0.05	0.51±0.07	0.76±0.07	0.70±0.07	
	C50μM	0.59±0.08	0.65±0.05	0.86±0.08	0.70±0.04	
	E10μM	0.44±0.07	0.38±0.02	0.50±0.04	0.51±0.09	Vit; p<0.05
	E50μM	0.31±0.07	0.16±0.02	0.25±0.04	0.16±0.03	
	E50μM+C50μM	0.25±0.04	0.16±0.02	0.15±0.02	0.16±0.05	

IMDM used for cell culture contains neither fatty acid nor vitamins E and C
[1]Addition of vitamins C or(and) E at the given concentrations
[2]OA: oleic acid, LA: linoleic acid, LNA: linolenic acid, DHA: docosahexaenoic acid
[3]Initial cell number: 1×10^4 cell/ml
[4]Values are means±SE and those within a row with different superscripts are significantly different from each other at p<0.05 [5]NS: Not significant

α-tocopherol and ascorbic acid and various types of fatty acids at the concentrations used for growth experiments, 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(15). For northern blot hybridization, 1.2 kb *AccI/XbaI* fragment from a cDNA encoding tumor protein *p53*(16) was labelled with [α-^{32}P]dCTP by random priming method according to manufacturer's manual(Promega) and hybridized to the membrane filter for 1~2 days at 42°C. The filter washed with 2×SSC, 1×SSC and 0.5×SSC in 0.1% SDS consecutively was autoradiographed.

Statistical analysis

Data are expressed as means with their standard errors. Significances among groups were analyzed using one- and two-way analyses of variance and Tukey's test.

RESULTS

Cell growth

Table 1 shows growth of MDA-MB231 human breast cancer cells cultured for 3 days with or without 10 and 50μM of ascorbic acid(vitamin C) and α-tocopherol (vitamin E) in the presence of 3μg/ml of oleic(OA), linoleic (LA), α-linolenic(LNA) or docosahexaenoic acid(DHA). With no vitamin in the medium, number of viable cells of the day 1 were mostly decreased by DHA treatment followed by LNA, LA and OA treatments, but this tendency was somewhat reversed on the day 3. Except in these two conditions, the fatty acid effect was not, however, very apparent. On the other hand, vitamin E appeared to suppress cell growth consistently and in a dose-dependent manner, especially on the day 3, whereas vitamin C had no such effect. From these results, two way

Table 2. Fatty acid composition of MDA-MB231 human breast cancer cell cultured in the presence of four differnet fatty acids (wt %)

Fatty acid	OA	LA	LNA	DHA
C14:0	2.79	2.74	2.56	2.39
C16:0	25.07	25.46	23.86	24.37
C16:1	3.38[a]	3.01[a]	1.87[b]	3.33[a]
C18:0	15.32[a]	15.26[a]	12.15[b]	18.93[c]
C18:1n-9	39.79[a]	36.03[b]	28.43[c]	33.06[b]
C18:2n-6	1.42[a]	3.53[b]	2.42[a]	1.56[a]
C18:3n-3	0.62[a]	0.43[a]	1.04[b]	0.43[b]
C20:3n-9	4.27[a]	3.30[b]	3.63[ab]	2.41[c]
C20:4n-6	2.86[a]	5.32[b]	2.82[a]	2.59[a]
C20:5n-3	0.21[a]	0.07[a]	3.81[b]	0.37[c]
C22:4n-6	0.26	0.40	0.35	0.87
C22:5n-3	0.69[a]	0.73[a]	3.30[b]	0.57[a]
C22:6n-3	1.38[a]	1.32[a]	3.71[b]	6.88[c]

Values are means of 4~6 replicates amd those with differnet superscript letters are significantly different each other at $p<0.05$

analysis of variance was conducted to compare effects of fatty acid and vitamin additions more clearly. Fatty acid effect was not consistent when the results from same fatty acid treated conditions were combined regardless of addition of vitamins. But the suppressive effect of vitamin E on cell growth was seen significantly in a dose-dependent manner from day 1 and resulted in one third of cell numbers with 50μM vitamin E with or without vitamin C. At the concentrations of 10 and 50μM, vitamin C had neither independent nor additive effect.

Cellular fatty acid composition

Table 2 shows fatty acid compositions of lipid extracted from cells cultured in the presence of four different fatty acids for 3 days. Results from one type of fatty acid treatments with various vitamin E and C levels in the media were combined. Each fatty acid added in the culture media was well incorporated such as elongated forms, arachidonic acid in LA treated cells and EPA and DHA in LNA treated cells.

Cellular alpha-tocopherol contents

Table 3 shows α-tocopherol contents in the cells cultured during 3 days with four different fatty acid and vitamins E and C. Alpha-tocopherol in culture media was well incorporated into cells in a dose-dependent manner. Treatments of LA and DHA appeared to accumulate more vitamin E in the cell, compared with OA and LNA treatments, but this was not statistically significant. Vitamin C at 10 and 50μM concentrations decreased cellular α-tocopherol contents that were low levels when no additional vitamin E was added, but had no effect when large amount of vitamin E present.

Lipid peroxide contents

Lipid peroxide contents in the cell were estimated by measuring thiobarbituric acid reactive substances(TBARS). As shown in Table 4, TBARS contents were highest in DHA treated cells when no vitamin or vitamin C was added, but they were not very different from other fatty acid treatments when vitamin E was present. Vitamin E at the concentration of 10μM was sufficient to reduce cellular TBARS contents to about one third of cells cultured with no vitamin. There was no further decrease in TBARS contents as vitamin E concentration increased to 50μM. It is interesting that vitamin C had an effect on reducing cellular TBARS contents by 30~40%. At 10μM of concentration, vitamin C appeared more effective

Table 3. Alpha-tocopherol contents of MDA-MB231 human breast cancer cells cultured in the presence of various fatty acids and vitamins E and C for three days

Vit[1] \ FA[2]	OA	LA	LNA	DHA	Effect
			μg/mg protein		
None	0.70±0.25[A3)]	0.59± 0.05[A]	0.77± 0.18[A]	1.01± 0.31[A]	FA;
C10μM	0.29±0.12[A]	0.26± 0.06[B]	0.46± 0.20[B]	0.68± 0.23[AB]	NS[4)]
C50μM	0.23±0.05[B]	0.45± 0.26[AB]	0.27± 0.07[B]	0.33± 0.15[B]	
E10μM	9.00±3.56[C]	18.79± 6.41[C]	6.17± 1.46[C]	23.50±10.03[C]	Vit;
E50μM	23.53±6.40[D]	37.16± 9.85[D]	22.96±10.05[D]	45.16±21.05[C]	$p<0.05$
E50μM+C50μM	22.61±6.31[D]	46.42±21.94[D]	23.48±11.18[D]	34.25±13.33[C]	

[1)]Addition of vitamins C or(and) E at the given concentrations

[2)]OA: oleic acid, LA: linoleic acid, LNA: linolenic acid, DHA: docosahexaenoic acid

[3)]Values are means±SE and those with different small and capital letters are significantly different from each other within a row and a column, respectively at $p<0.05$

[4)]NS: Not significant

Table 4. TBARS contents of MDA-MB231 human breast cancer cells cultured in the presence of various fatty acids and vitamins E and C for three days

Vit[1] \ FA[2]	OA	LA	LNA	DHA	Effect
		MDA nmoles/mg protein			
None	$0.20\pm0.06^{a3)}$	0.94 ± 0.26^{bA}	0.59 ± 0.12^{abA}	2.44 ± 1.40^{bcA}	FA;
C10μM	0.24 ± 0.03^{a}	0.78 ± 0.32^{bA}	0.40 ± 0.04^{abB}	0.97 ± 0.28^{bcA}	$p<0.05$
C50μM	0.42 ± 0.06^{a}	0.83 ± 0.27^{bA}	0.41 ± 0.07^{aB}	1.43 ± 0.55^{bcA}	
E10μM	0.32 ± 0.04	0.37 ± 0.16^{B}	0.29 ± 0.12^{BC}	0.49 ± 0.15^{B}	Vit;
E50μM	0.30 ± 0.05	0.41 ± 0.11^{B}	0.27 ± 0.08^{BC}	0.32 ± 0.11^{B}	$p<0.05$
E50μM+C50μM	0.42 ± 0.08	0.36 ± 0.12^{B}	0.23 ± 0.05^{C}	0.39 ± 0.15^{B}	

[1)]Addition of vitamins C or(and) E at the given concentrations
[2)]OA: oleic acid, LA: linoleic acid, LNA: linolenic acid, DHA: docosahexaenoic acid
[3)]Values are means±SE and those with different small and capital letters are significantly different from each other within a row and a column, respectively at $p<0.05$

than at 50μM, although the difference was not statistically significant.

p53 tumor suppressor gene expressiom

Fig. 1 shows northern blot hybridization of *p53* mRNA from cells incubated with vitamins E and C and LNA(1) and DHA(2) for 8(a) and 16(b) hrs. The *p53* mRNA expression did not vary in LNA treated cells(1) but did in DHA treated ones. DHA alone was more effective in 8 hrs than in 16 hrs but addition of vitamin E at both 10 and 50μM of concentration to DHA treated cells increased *p53* mRNA expression significantly in 8 hours of culture. However, vitamin C had no effect either alone or with the combination of vitamin E.

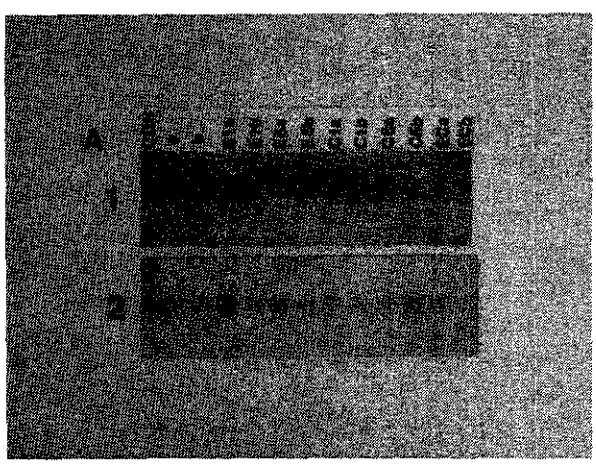

Fig. 1. Autoradiography of *p53* mRNA expression in MDA-MB231 cells determined by gel blot northern hybridization.
RNA expression after 8 hr(a) or(b) after treatment of LNA(1) or DHA(2) with or without vitamin E(E1; 10μM, E5; 50μM) or vitamin C(C1; 10μM, C5; 50μM)

DISCUSSION

The present study shows that vitamin E has suppressive effect on growth of human breast cancer cells supplemented with various types of fatty acid. Fatty acids depending on the type has been shown to have regulatory effects on cell growth. In our previous study(17) with no vitamin used, DHA at the concentration of 0.5μg/ml had a clear inhibitory effect on cell growth, compared with other types of fatty acid. But at the concentrations used in the present study(3μg/ml), the DHA effect was not prominent possibly due to common mild toxicity generally observed at higher concentrations of any type of fatty acid, although Chajes et al.(18) have shown the distinctive inhibition of n-3 PUFA on breast cancer cell at the concentration of 20μg/ml.

Under the condition used in the present study, however, vitamin E was the most effective inhibitory factor in breast cancer cell growth. Normal level of serum vitamin E is about 10μg/ml(25μM). Thus, 10μM of vitamin E that had the growth inhibitory effect in this study was not a extraordinarily high level but rather as low as suboptimal one. Other investigators have reported that vitamin E at 1~20μg/ml(2.5~50μM) exhibited the inhibitory effects on growth of various types of cancer cells(19-21). On the contrary, vitamin E has been shown in several studies(18,22-24) to counteract cytotoxic effect on cancer cells of lipid peroxide that were increased due to highly unsaturated fatty acids such as n-3 PUFA, and thereby to enhance the growth of cancer cells. These results look very plausible, in relation with the report(18,22) that cancer cells are more sensitive to peroxidation products than normal cells. In most of these studies, however, PUFA contents in culture medium were much higher

than used in the present study. Therefore, cytotoxic effects due to increased lipid peroxidation are expected to have been a dominant factor for the growth suppression of cells. The cytotoxicity of lipid peroxide can not be excluded as one of the factors for the cell growth suppression in the present study, since TBARS levels were clearly higher in DHA treatment than other fatty acids, but it certainly does not appear a major one. Moreover, Diplock et al.(25) have shown that cancer cells converted from kidney fibroblast had higher amounts of peroxidation products than unconverted original cells and effect of vitamin E as a growth enhancer was not conclusive. The possible inhibitory effect of vitamin E on cancer cell proliferation as shown in this study may be related to the new roles of vitamin E reported by a few investigators (26). The roles included regulation of activities of membrane bound enzymes, and thereby signal transduction and alterations in oncogene expressions. Prasad et al.(19) have reported that vitamin E reduced expression of c-myc and H-ras in melanoma cells and Israel et al.(20) have suggested that tocopherol may interfere with tumor cell cycle. In this regards, it is interesting in the present study that expression of tumor suppression gene, *p53* was increased by vitamin E, although the increase was not shown in the presence of additional vitamin C. In our previous studies, we have shown increases of *p53* gene expression by DHA of human breast cancer cells of estrogen-dependent type, MCF-7(27) and estrogen-independent MDA-MB231(17). Therefore, vitamin E may have played a role to amplify the same effect in combination with DHA, although the precise mechanism has to be elucidated in the future. In contrast to vitamin E, vitamin C did not influence cell growth and any of related parameters examined except a slight decrease in TBARS. Concentrations of vitamin C used in the present study were physiological ranges. Whether vitamin C has effects of any kind at the different concentrations needs further studies.

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