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Fatty acid modulation of MCF-7 human breast cancer cell proliferation, apoptosis and differentiation

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Abstract

Epidemiological studies suggest that dietary polyunsaturated fatty acids (PUFA) may influence breast cancer progression and prognosis. In order to study potential mechanisms of action of fatty acid modulation of tumor growth, we studied, *in vitro*, the influence of n-3 and n-6 fatty acids on proliferation, cell cycle, differentiation and apoptosis of MCF-7 human breast cancer cells. Both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibited the MCF-7 cell growth by 30% and 54%, respectively, while linoleic acid (LA) had no effect and arachidonic acid (AA) inhibited the cell growth by 30% ($p < 0.05$). The addition of vitamin E (10uM) to cancer cells slightly restored cell growth. The incubation of MCF-7 cells with PUFAs did not alter the cell cycle parameters or induce cell apoptosis. However, the growth inhibitory effects of EPA, DHA and AA were associated with cell differentiation as indicated by positive Oil-Red-O staining of the cells. Lipid droplet accumulation was increased by 65%, 30% and 15% in the presence of DHA, EPA and AA, respectively; ($p < 0.05$). These observations suggest that fatty acids may influence cellular processes at a molecular level, capable of modulating breast cancer cell growth.

Keywords: Breast cancer; MCF-7 cells; PUFA; Cell differentiation

1. Introduction

Differences in the rates of breast cancer incidence among women in different countries and corresponding changes in the incidence of breast cancer for women who migrate from an area of lower incidence to one of higher incidence suggest that environmental factors may play a role in this disease [1]. Epidemiological and experimental studies have revealed an association between dietary fat and the incidence of breast cancer [2–3]. While many factors are clearly involved, the consumption of high fat diets which are also rich in polyunsaturated fats may be an important factor in the high incidence of breast cancer observed in Western countries. Moreover, specific fatty acids may exert opposing effects so that the net result is dependent on their relative concentration in the diet. N-6 polyunsaturated fatty acids (PUFA) appear to enhance tumor growth and proliferation [4–5], while n-3 PUFAs seems to have an inhibitory influence on this process [6–7].

The exact mechanisms by which dietary PUFA influence mammary carcinogenesis are not known. While many theories have been suggested, very few researchers have studied the effect of fatty acids on breast cancer cells at the cellular and molecular level. This study was performed to determine whether the known growth inhibitory and stimulatory effect of fatty acids are accompanied by changes in proliferation, cell cycle, apoptosis and differentiation of MCF-7 human breast cancer cells.

2. Materials and methods

2.1. Cell line and chemicals

The human breast cancer MCF-7 cell line was obtained from American Type Culture Collection (Rockville, MD). Fatty acids, in methyl ester form, were purchased from Nu Check Prep, Inc. (Elysian, MN, USA). All fatty acids were used as methyl esters. Vitamin E (dl-α-tocopherol) was

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provided by Sigma Chemicals Company (St. Louis, MO, USA).

2.2. Monolayer growth assay of MCF-7 cells

MCF-7 cells were seeded at 10^5 cells in 2 ml of growth medium (DMEM with 5% FBS, 100 units/ml penicillin, 100 mcg/ml streptomycin and 4 mM glutamine) per well in a 6-well tissue culture plate. The cells were grown at 37°C in a 5% CO_2-humidified incubator. 24 hr after seeding, one set of cultures was supplemented with 100 μM fatty acids in growth medium and the control cultures received only fresh medium. Unsupplemented or fatty acid supplemented cultures were incubated in parallel with 10 μM of Vitamin E. At day 5, cells were harvested and counted by using Coulter automated cell counter.

2.3. Anchorage-independent growth assay of MCF-7 cells

The agar base for the cell colony growth assay was prepared in two layers to keep the cancer cells in suspension in the upper layer. The bottom layer was more dense than the upper layer and was composed of the following: 400 μl of 1% agar, 400 μl of double concentrated DMEM, 100 μl of single concentration DMEM, and 100 ul of fetal bovine serum (FBS). The top layer was composed of 350 μl of 1% agar, 350 μl double concentrated DMEM, 100 μl of FBS, and 200 μl cell suspension of 5×10^3 breast cancer cells. Six wells were tested for each treatment condition and each experiment was repeated 3 times. All colony counts were made after 2 weeks in culture at 37°C in a humidified 5% CO_2 incubator. Groups of cells greater than 50 micron diameter were counted as colonies. For the fatty acid experiments the 100 μl of 1× DMEM in the bottom layer was replaced by 100 μl of fatty acid.

2.4. Assessment of differentiation

Differentiated breast cancer cells display properties that are associated with lactation and include the generation of fat deposits within the cytoplasm. Cells were cultured on chamber slides at a density of 10^5 in 3 ml medium. At 24 hr after seeding, at a time when cell adhesion was complete fatty acids were added at 100uM for 5 days. Differentiation of MCF-7 cells grown in the presence or the absence of fatty acids, with and without Vitamin E, were assessed by detecting intracellular lipid droplets using the lipid-specific Oil-Red-O staining technique.

Lipid droplets were visualized by Oil-Red-O staining and the percent of lipid positive cells was determined. The breast cancer cell lines contain cells that exhibit morphological maturation and differentiation markers associated with milk production; they contain large lipid droplets.

2.5. Apoptosis

Apoptosis of MCF-7 cells was assessed by analyzing changes in cell morphology and DNA fragmentation after cell incubation with fatty acids in both the presence and absence of Vitamin E. Morphologically, cells undergoing apoptosis were identified by the following parameters: intense staining, highly condensed chromatin, fragmented nuclear chromatin, a general decrease in overall cell size, and cellular fragmentation into apoptotic bodies. These changes were readily observed in cytospin preparations stained with Diff-Quik Stain Set (Baxter Healthcare Corp. Miami, FL). Enumeration of apoptotic cells was performed by evaluating approximatly 300 cells using light microscopy.

2.6. Cell cycle analysis

Cell cycle analysis was performed on breast cancer cells incubated 5 days with or without fatty acids and Vitamin E. The method used [8] was a modification of Krishan's technique [9]. Cells were incubated for 30 min at 4°C with a hypotonic DNA staining solution and analysis was performed immediately after staining using the CELL Quest program (Becton Dickinson), whereby the S-phase was calculated with an ModFit LT software (Verity Software House, Topsham, ME).

2.7. Statistical analysis

Statistical analysis was carried out using Student's unpaired t-test; an alpha of <0.05 was regarded as significant.

3. Results

3.1. Effect of fatty acids on MCF-7 cell growth

The monolayer growth of MCF-7 cells was examined in the presence of 100μM of different fatty acids. As shown in Fig. 1, EPA and DHA, from the n-3 fatty acids family, inhibited the MCF-7 cells growth by 30% and 54% as compared to controls, respectively. From the n-6 fatty acids family, LA did not have any effect while AA decreased cell growth by about 30%. The presence of vitamin E (10 μM) did not change the response of cancer cells to either n-6 or n-3 fatty acids. Vitamin E (10 μM) alone displayed no effect on cell proliferation.

3.2. Cytotoxicity of PUFA

In an attempt to determine whether the reduction in cell number mediated by PUFAs was a result of a cytotoxic effect from fatty acids, we monitored the viability of cells during the course of incubation. After 5 days of incubation with fatty acids, the viability of cancer cells was more than 90%. Thus the growth arrest observed in breast cancer cells

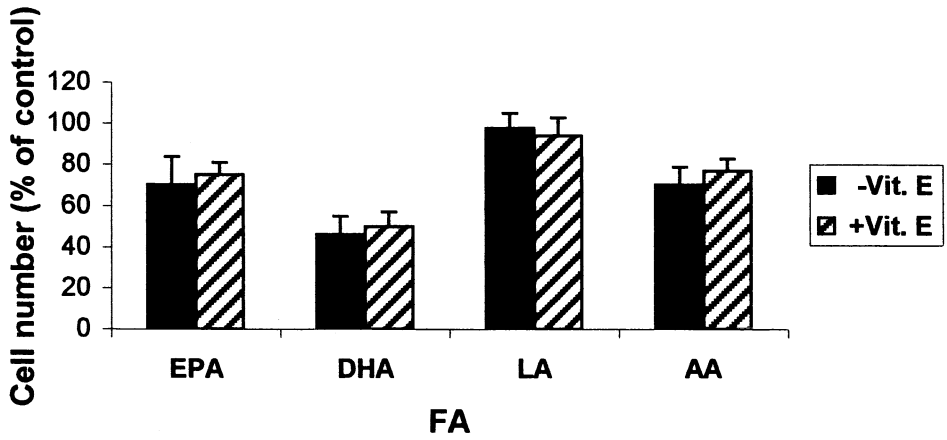

Fig. 1. Effect of fatty acids on the growth of MCF-7 cells in the presence or absence of 10 μM vitamin E. Results are expressed as percentage of control plates. Each point represents a mean of three experiments with each experimental point in triplicate. The bars represent standard deviations, $p < 0.05$.

during the incubation with PUFAs was either not, or only to a small degree, due to the effect of toxicity.

3.3. Effect of fatty acids on MCF-7 colony growth

The effect of PUFAs on MCF-7 colony growth in soft agar is shown in Fig. 2. Due to the higher sensitivity of the clonogenic assay in comparison to the monolayer growth technique, cells were exposed to 1, 10 or 100 μM of fatty acids. MCF-7 colony growth was inhibited by 34% and 38% in the presence of 10μM of EPA and DHA, respectively. At a concentration of 100 μM fatty acid, the growth inhibition was almost at maximum as compared to controls. 72% and 82% decrease in colony numbers was observed in the presence of EPA and DHA, respectively. LA did not have any significant effect on colony growth while AA at 100 μM inhibited the growth by 53%.

3.4. Apoptosis

In order to determine if the antiproliferative effect of fatty acids is caused by the induction of apoptosis, morphology and DNA fragmentation of MCF-7 cells were analyzed by light microscopy. No significant differences were observed in the morphology of MCF-7 cells, and the number of apoptotic cells was not increased compared to normal cells. The presence of apoptotic cells was also examined by flow cytometry. No significant changes were observed in the percent of apoptotic cells after incubation with fatty acids in the presence or the absence of vitamin E.

3.5. Cell cycle analysis of MCF-7 cells

Cell cycle analysis of MCF-7 cells was performed by flow cytometry, after exposure of the cells to either LA or

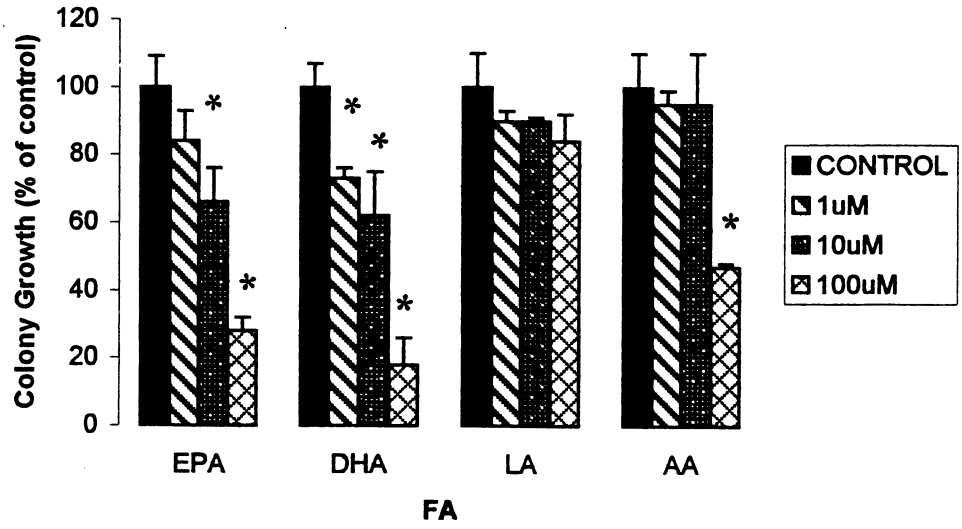

Fig. 2. Effect of fatty acids on MCF-7 colony growth in soft agar. Results are expressed as percentage of control plates. Each point represents a mean of three experiments with each experimental point in triplicate. The bars represent standard deviations, $p < 0.05$.

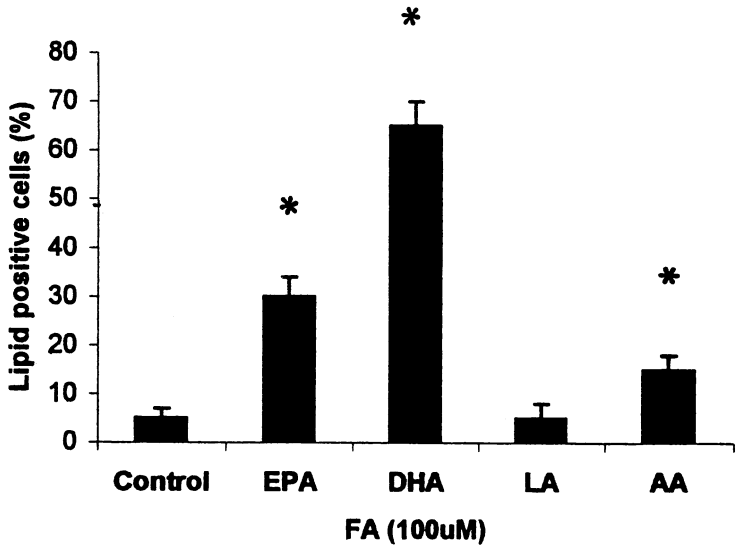

Fig. 3. Induction of differentiation in MCF-7 cell line by fatty acids. Results are expressed as a percentage of breast cancer cells that were lipid positive. Data represents mean of three independent experiments. The bars represent standard deviations, $p < 0.05$.

DHA (50 or 100 μM). The presence of fatty acids in the medium did not affect the cell-cycle profile of MCF-7 cells and no significant changes were observed in the percentage of cells in G0/G1, G2-M or S phase.

3.6. The effect of fatty acids on MCF-7 cells differentiation

The analysis of differentiation of breast cancer cells was determined by the expression of lipid in these cells after exposure to fatty acids. Approximately 5% of control cells accumulated lipid. Lipid expression was increased in the presence of fatty acids. From the n-3 fatty acids, DHA was a more potent inducer of differentiation (65%) than EPA (30%). Interestingly AA, which had an antiproliferative effect on MCF-7 cells, only slightly induced the lipid expression (15%). LA did not induce cell differentiation as compared to control (Fig. 3). Vitamin E at 10 μM did not have any effect on the differentiation induced by fatty acids.

4. Discussion

In the present study, we have demonstrated that from the omega 3 fatty acid family, EPA and DHA inhibited estrogen receptor-positive human breast cancer MCF-7 cell growth. From the omega 6 family LA did not have any effect, while AA inhibited the growth. Similar results were obtained from the anchorage-independent growth of MCF-7 colonies in soft agar. The decrease in cell growth was not due to the toxicity of fatty acids. Assay for lipid oxidation markers indicated no significant difference between treatments (data not shown). In that respect, the addition of 10 μM Vitamin E, a potent antioxidant, did not restore completely the fatty acid-induced cell growth inhibition in monolayer cultures. Our approach to study the effect of fatty acids at the cellular level revealed no effect of fatty acids on programmed cell death (apoptosis) or cell cycle; however the inhibitory effect of EPA, DHA and AA was associated with induction of differentiation of MCF-7 cells.

Inhibition of breast cancer cell growth by omega 3 but not by omega 6 fatty acids, has been reported in several *in vitro* and *in vivo* studies. Rose and Connolly [10–11] demonstrated that DHA, and to a lesser extent EPA, inhibited the estrogen-independent MDA-MB-231 cell growth in a dose-dependent manner. However, LA was found to stimulate the growth of MDA-MB-231 and, to lesser extent MCF-7 cells. Later, they reported that the particular MCF-7 cells used in these experiments were of questionable origin and hormone dependence [12] and the authentic MCF-7 cells did not show any mitogenic response to LA. The same authors found that the growth of another estrogen-dependent human breast cancer cell line ZR-75, was also unaffected by LA *in vitro*. Our results are in agreement with Grammatikos et al. [13] who found that LA had no effect on MCF-7 cell growth. Also, inhibition of MCF-7 cell growth by n-3 PUFAs is consistent with different effects of fish oil compared with corn oil in rat models of mammary carcinogenesis [14–18].

Long chain n-3 fatty acids competitively inhibit the $\Delta 5$ and $\Delta 6$ desaturase pathways necessary for conversion of 18:2n-6 to 20:4n-6. The metabolites of n-3 fatty acids produce eicosanoids that differ or oppose those produced via the n-6 fatty acid-ecosanoid pathway. Thus eicosanoid-regulated growth and metabolism may be reduced, favoring suppression of tumor cell growth. However, our results as well as previous studies have shown no stimulation of estrogen-dependent MCF-7 cell growth by LA [19]. This

suggests that a pathway other than the one interfering with the conversion of LA to AA is responsible for the inhibitory effect of n-3 FAs, or at least, in estrogen-dependent breast cancer cells. This hypothesis is further strengthened by the study of Carroll and Hopkins who demonstrated that a small amount of n-6 FA at the expense of saturated fat, completely eliminated the ability of high saturated fat diet to diminish tumor development [20]. In addition, many but not all transformed malignant cell lines *in vitro*, have a reduced capacity for Δ6 desaturation [21–25]. Grammatikos et al reported that the loss of desaturation activity in the MCF-7 mammary tumor cell line was associated with differential growth effects of PUFA's [19]. The relationship between Δ6 desaturation and cancer remains unclear, but Horrobin has suggested that the loss of desaturation ability is relevant to the process of malignant transformation [26]. Consistent with this hypothesis, it has been reported that the expression of the activated ras oncogene in normal human mammary epithelial cell line resulted in the loss of Δ6 desaturation activity [19].

The growth-inhibitory and sometimes cytotoxic effect of PUFAs on cancer cells are often explained in terms of the intracellular fatty acid susceptibility to oxidation [26–28]. It has recently been reported that breast cancer cells are more susceptible to oxidation than normal cells [29]**.** We minimized oxidation of PUFAs during incubation in the extracellular milieu by supplying 10 μM vitamin E. Chages et al have demonstrated that the addition of PUFAs to breast cancer cell causes a significant increase in the formation of conjugated dienes and lipid hydroperoxides in the cellular lipids; the addition of Vitamin E almost completely restored cell growth [30]. In our study, the presence of 10 μM Vitamin E in the incubation medium did not completely restore the cell growth inhibition and did not change the overall response of MCF-7 cells to PUFAs. In addition we did not detect any significant increase in the congugated dienes formation of breast cancer cells. Abdi Dezfuli et al. reported that tetradecylthioacetic acid, a non-beta-oxidizable fatty acid, inhibits the MCF-7 cell growth by a mechanism independent of oxidative status [31]. However, in the present study, the analysis of differentiation in breast cancer cell lines, as measured by the accumulation of lipid droplets, showed that EPA, DHA and AA induced differentiation of MCF-7 cells. To our knowledge this is the first report on the fatty acid-induced differentiation of MCF-7 cells.

The peroxisome proliferator-activator receptor γ (PPARγ) has an important role in the differentiation of adipocytes and in fat metabolism [32,33]. Recent data have shown that human breast cancer cell lines, as well as primary and metastatic breast adenocarcinomas expressed PPARγ and ligand activation of PPARγ caused inhibition of proliferation and extensive lipid accumulation in cultured breast cancer cell lines [34,35]. Therefore we analyzed the expression of PPARγ in breast cancer cells exposed to different fatty acids, by using the Northern blot technique. Our data indicated a slight but not significant increase of PPARγ expression when cells were incubated with DHA and AA (data not shown).

Dunbar and Bailey [21] found that in all the cell systems studied, the activity of the enzyme Δ-6-desaturase is lost when cells are transformed by viruses, radiation or chemical carcinogens. This suggests that although carcinogenic initiating factors are multifactorial and innumerable, it is possible that secondary carcinogenic 'promoters' are partly responsible for the initial transformation and that they may be acting through metabolic pathways, which are common to many different cells and many different carcinogens.

In conclusion, the present study suggests that fatty acids may influence cellular processes at a molecular level, capable of modulating breast cancer cell growth. Although many different mechanisms are involved in the regulation of breast cancer cells by fatty acids, the induction of differentiation associated with the expression of lipids is the main pathway of growth inhibition proposed by the current study.

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