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Effects of Fatty Acids and Inhibitors of Eicosanoid Synthesis on the Growth of a Human Breast Cancer Cell Line in Culture[1]

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ABSTRACT

Dietary lipids may influence breast cancer progression and prognosis. The MDA-MB-231 human breast cancer cell line was used to examine the direct effects of the various classes of free fatty acids (FAs) on growth in serum-free medium and the involvement of eicosanoid biosynthesis. Linoleic acid, an ω6 FA, stimulated MDA-MB-231 cell growth with an optimal effect at a concentration of 0.75 μg/ml, whereas oleic acid, an ω9 FA, produced growth stimulation at 0.25 μg/ml but was inhibitory at higher concentrations. Docosahexaenoic acid exhibited a dose-related inhibition of cell growth at concentrations ranging from 0.5 to 2.5 μg/ml; eicosapentaenoic acid, also an ω3 FA, was less effective. Similar inhibitory effects occurred with saturated FAs. Indomethacin, which at high concentrations is an inhibitor of both the cyclooxygenase- and lipoxygenase-catalyzed pathways of eicosanoid synthesis, suppressed cell growth stimulation by an otherwise optimal dose of linoleic acid when present at 40 μg/ml. Experiments with piroxicam, nordihydroguaiaretic acid, and esculetin, other inhibitors of eicosanoid biosynthesis with varying selectivity for enzymes of the prostaglandin and leukotriene pathways, indicated that MDA-MB-231 cell growth was dependent on leukotriene rather than prostaglandin production.

INTRODUCTION

In animal models, the level of dietary fat intake has been shown to influence both the development of chemically induced mammary tumors (1, 2) and the growth and metastatic behavior of transplantable mammary carcinomas (3, 4). However, not only does the quantity of fat consumed need to be considered but so also does its FA[3] composition; those fats high in LA, an *n*-6 unsaturated FA, enhance the growth (1, 2) and metastasis (3–6) of murine mammary carcinomas, whereas fish oil, rich in *n*-3 FAs, has an inhibitory effect (7, 8).

The relevance of these experimental studies to human breast cancer risk and disease progression is unclear. Most epidemiological investigations that support an association between dietary fats and breast cancer implicate those of animal origin and, hence, saturated rather than unsaturated FAs (1, 9–12). However, both Kakar and Henderson (13) and Wynder *et al.* (14) have suggested that the increasing breast cancer incidence in the United States may be related to the sustained increase in vegetable oil consumption (principally *n*-6 unsaturated FAs) at the expense of animal fats since the early 1900s. Moreover, a recent ecological analysis based on international data showed an inverse relationship between breast cancer incidence and the level of fish consumption, suggesting a protective role for *n*-3 FAs in human breast cancer (15).

As one approach to seeking an understanding of the influence of the various dietary FAs on human breast cancer, we have studied their effects on *in vitro* cultured human breast cancer cell lines. In a previous report, we showed that LA does stimulate growth of the MDA-MB-231 and to a lesser extent the MCF-7 cell lines (16). This earlier work with MDA-MB-231 cells has now been extended to include two *n*-3 FAs (DHA and EPA), an *n*-9 unsaturated FA (OA), and two saturated FAs (PA and SA). Additional experiments to investigate the mechanism for the growth stimulation by LA and its inhibition by the *n*-3 FAs focussed on the eicosanoids; studies in animal models have indicated a role for these compounds, notably PGs, in experimental mammary carcinogenesis (17, 18) and their production by rat mammary carcinoma cells in culture (19).

MATERIALS AND METHODS

Materials. Insulin, 17β-estradiol, transferrin, OA, PA, SA, DHA, EPA, LA, INDO, and piroxicam were purchased from Sigma Chemical Co. (St. Louis, MO), human epidermal growth factor from Boehringer Mannheim (Indianapolis, IN), LA-BSA conjugate (1 μg of LA/200 μg of complex) and delipidized BSA from Collaborative Research (Lexington, MA), and NDGA and esculetin (6,7-dihydroxycoumarin) from Biomol Research Laboratories (Plymouth Meeting, PA).

Cell Culture. The estrogen-unresponsive MDA-MB-231 human breast cancer cell line (20) was obtained from the American Type Culture Association (Rockville, MD) and was cultured routinely in IMDM (Gibco, Grand Island, NY) plus penicillin and streptomycin, supplemented with 5% FBS, in a 95% air/5% CO_2 incubator. For the growth experiments, the cells were cultured in a serum-free 1:1 (v/v) mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium without phenol red indicator (Sigma) containing 10 μg/ml insulin, 20 ng/ml human epidermal growth factor, 10 μg/ml transferrin, and 1.25 mg/ml delipidized BSA (21) or in serum-free IMDM plus 1.25 mg/ml delipidized BSA and 10 μg/ml insulin.

Growth Experiments. These were performed in 24-well plates (Costar, Cambridge, MA). Initially, the cells were cultured for 24 h in 5% FBS-supplemented medium at a plating density of 1.5×10^4 cells/ml/well. They were then washed with unsupplemented medium, and the experimental culture medium was then added to the wells, which were set up in triplicate. Incubation was continued for 3 days or for 6 days with refeeding after 3 days. The cells were then harvested for evaluation of thymidine incorporation or cell number. For thymidine incorporation, 0.25 μCi of [^{3}H]thymidine (specific activity, 67 Ci/mmol; New England Nuclear, Boston, MA) in 0.1 ml of PBS was added to each well, the cells were incubated for 6 h, washed, trypsinized, and harvested with a PHD Cell Harvester (Cambridge Technologies, Inc., Cambridge, MA), and the incorporated [^{3}H]thymidine was counted for 4 min in a liquid scintillation counter. Cell numbers were determined with an electronic particle counter (Model F; Coulter Electronics, Hialeah, FL). In the initial experiments, LA was incorporated into the culture medium as LA-BSA complex with additions over the range 1–150 μg/ml (5–750 ng/ml of LA) but with the final BSA concentration always maintained at 1.25 mg/ml. Later, the FAs were dissolved in 100% ethanol, the volumes added being such that the final concentration of ethanol was 1%. Ethanol alone at the same final concentration was added to the control wells. As before, the BSA concentration throughout was

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[3] The abbreviations used are: FA, fatty acid; LA, linoleic acid (18:2, *n*-6); DHA, docosahexaenoic acid (22:6, *n*-3); EPA, eicosapentaenoic acid (20:5, *n*-3); OA, oleic acid (18:1, *n*-9); PA, palmitic acid (16:0); SA, stearic acid (18:0); INDO, indomethacin; BSA, bovine serum albumin; IMDM, Iscove's modified Dulbecco's medium; FBS, fetal bovine serum; PG, prostaglandin; DMBA, dimethylbenz(*a*)anthracene; NDGA, nordihydroguaiaretic acid; AA, arachidonic acid.

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1.25 mg/ml. Ethanol (final concentration 1%), or in the case of esculetin, dimethyl sulfoxide (final concentration 0.25%), was used for dissolving eicosanoid synthesis inhibitors.

PG Radioimmunoassays. The medium was aspirated from the monolayer cultures and immediately centrifuged at 4°C to remove cellular debris prior to protein precipitation with acetone at −20°C. Neutral lipids were first extracted with petroleum ether, and then PGs were extracted with diethylether after acidification to pH 3–4 (22). After evaporation to dryness, the PG-containing residues were reconstituted in 500 μl of assay buffer, and 2.5-μl volumes were taken for radioimmunoassay. In our hands, as determined using $[^3H]PGE_2$, the mean extraction efficiency was 69%. All extractions, sample handling, and the radioimmunoassays were performed in polypropylene plasticware. The PGE_2 concentrations were assayed using reagents purchased from Advanced Magnetics, Inc. (Cambridge, MA) and the final results corrected for the extraction efficiency and normalized to protein content. The antibody was stated by the supplier to be 100% reactive with PGE_2, with cross-reactivities of 50, 6, and 1.9% with PGE_1, PGA_1, and PGA_2, respectively. Assay sensitivity is <2 pg/assay tube, and the average intraassay coefficient of variation is 5.1%.

Statistical Comparisons. These were made using Student's unpaired *t* test; values of $P < 0.05$ were regarded as significant.

RESULTS

Effects of FAs on MDA-MB-231 Breast Cancer Cell Growth. Fig. 1 shows the influence of LA and OA, dissolved in ethanol and added to the serum-free F-12/Dulbecco's modified Eagle's medium, on the growth of the MDA-MB-231 cell line and is representative of results from experiments performed on three separate occasions. The two FAs were evaluated in different experiments and each had its own set of control wells, the cell numbers for which after 6 days of incubation were not significantly different. Stimulation by LA over a 6-day growth period was optimal with a concentration of 0.75 μg/ml; higher levels showed a progressive loss of the stimulatory effect. In contrast, OA caused enhanced growth only at the lowest level tested in this experiment (0.25 μg/ml), followed by a concentration-related inhibition. A second experiment with OA concentrations of 0.065, 0.125, 0.25, 0.5, 0.75, and 1.0 μg/ml gave an identical pattern of growth response, with 0.125 μg/ml producing a stimulation which was 129% of the control, and 0.25 μg/ml again exhibiting the peak stimulatory effect (130% of control). When the *n*-3 FAs were evaluated in a similar manner (Fig. 2), DHA was shown to be extremely effective in producing a growth suppression, a significant reduction in cell number occurring at a concentration of 1.5 μg/ml ($P < 0.01$). In contrast, while showing a similar trend, EPA produced a significant growth inhibition only at the highest level tested, 2.5 μg/ml ($P < 0.01$). Trypan blue exclusion indicated that >90% of the cells present were still viable. The greater efficacy of DHA in inhibiting MDA-MB-231 cell growth was confirmed in a second experiment performed on a separate occasion (data not shown).

When SA or PA were added at concentrations which in the case of the *n*-6 and *n*-9 FAs were stimulatory, neither of the two saturated FAs enhanced MDA-MB-231 cell growth; indeed, both produced inhibitions which increased in degree over the entire dose ranges.

Effects of Eicosanoid Synthesis Inhibitors. Selective inhibitors of PG and leukotriene synthesis were used to investigate the mechanism for the stimulation of MDA-MB-231 cell growth by LA. In Fig. 3 the cells were cultured in serum-free IMDM with 10 μg/ml insulin plus BSA at a final concentration of 1.25 mg/ml. Additional triplicate wells contained 250 or 625 ng/ml of LA added as an LA-BSA complex. Incubation was for 3 days in the presence of increasing concentrations of INDO to a maximum concentration of 40 μg/ml. This compound is primarily an inhibitor of the cyclooxygenase involved in PG syn-

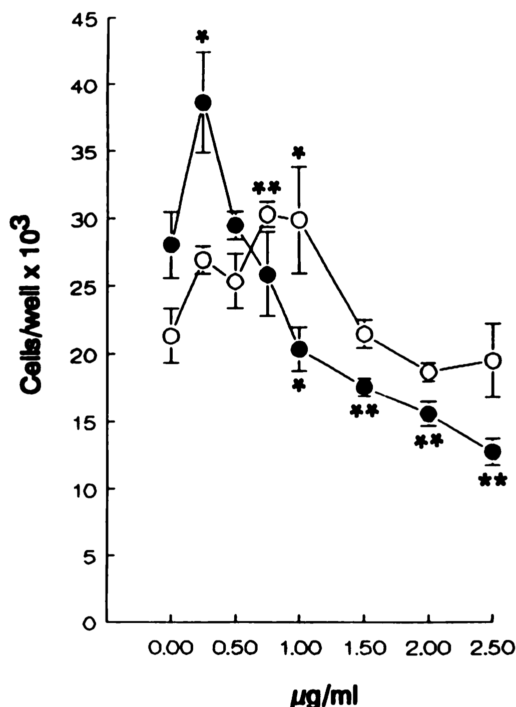

Fig. 1. The effects of LA (○) and OA (●) on growth of the MDA-MB-231 breast cancer cell line. The cells were counted after 6 days. Culture was in serum-free medium containing 1.25 mg/ml of delipidized BSA, and the FAs were added dissolved in ethanol. An equal volume of ethanol was added to the control wells. *Points*, mean values for triplicate wells; *bars*, SE. Statistically significant differences in cell numbers compared with those in the absence of FA addition are: * $P < 0.05$, ** $P < 0.01$.

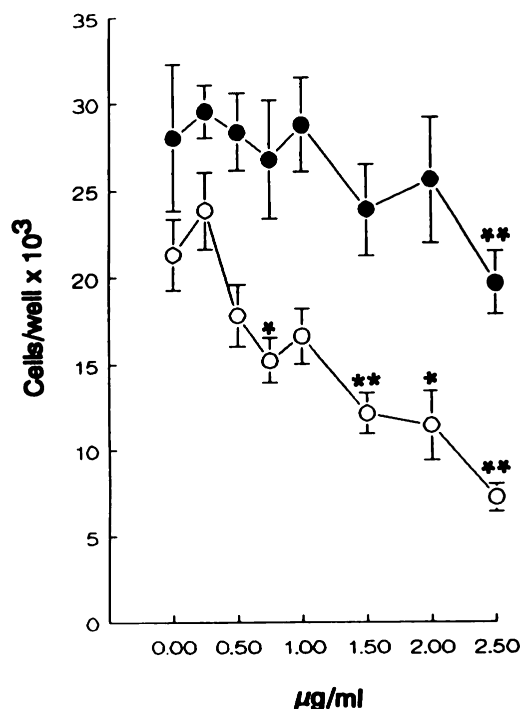

Fig. 2. Suppression of growth of the MDA-MB-231 breast cancer cell line by EPA (●) and DHA (○). *Points*, mean values for triplicate wells; *bars*, SE. Cell number reduced significantly compared with control: * $P < 0.05$, ** $P < 0.01$.

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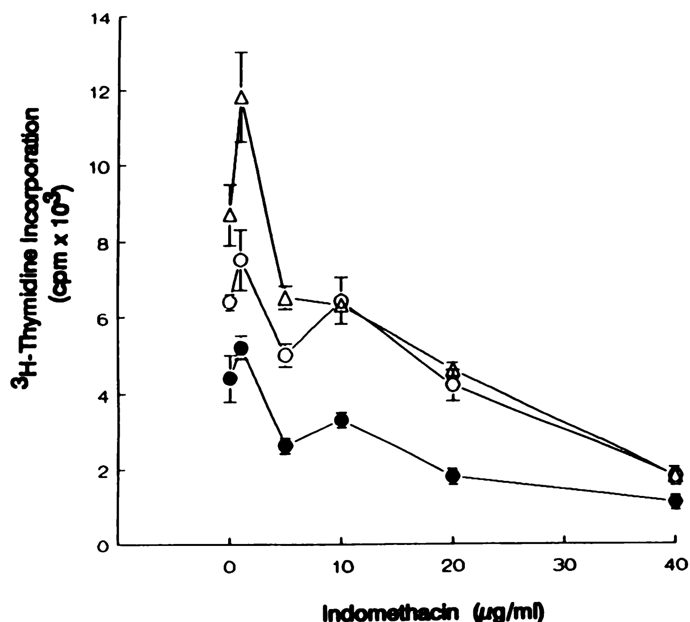

Fig. 3. Effect of increasing concentrations of indomethacin on day 3 of thymidine incorporation by MDA-MB-231 cells without the addition of LA (●) and in response to LA at levels of 250 (○) or 625 (△) ng/ml. Stimulation by the FA was suppressed in the presence of 40 μg/ml of indomethacin. *Points*, mean values for triplicate wells; *bars*, SE.

thesis, but at higher concentrations it also suppresses phospholipase A_2 (23) and 5-lipoxygenase (24) activities. Indomethacin, 20 μg/ml, eliminated the difference in the cell [^{3}H]thymidine incorporation response to 625 as compared with 250 ng/ml of LA, although the individual incorporations remained above that seen in the absence of LA. At an INDO concentration of 40 μg/ml the effect of LA on the incorporation of [^{3}H]thymidine by MDA-MB-231 cells was considerably suppressed and approached that occurring in the absence of FA. At the lowest concentration of INDO (1 μg/ml) there appeared to be a stimulation of thymidine incorporation by the cyclooxygenase inhibitor (Fig. 3). However, even with the 625 ng/ml concentration of LA, this change did not achieve statistical significance, and it was not observed in a similar, but separate, experiment performed at a later date (Table 1).

An experiment was performed to compare the effects of INDO on MDA-MB-231 cell thymidine incorporation after 3 days of culture with those of piroxicam, another nonsteroidal anti-inflammmatory drug which inhibits cyclooxygenase but not lipoxygenase or phospholipase A_2 activities (25). The cells were grown in serum-free medium without added LA and with 625 ng/ml of LA. As shown in Table 1, both INDO and piroxicam suppressed thymidine incorporation by MDA-MB-231 breast cancer cells and caused a reduced secretion of PGE_2 into the culture medium. However, the two effects were more pronounced with INDO (Table 1) and more clearly related to the concentration of the inhibitor. When the cells were refed with fresh inhibitor-containing medium on day 3, and incubated for a further 3 days, INDO produced the expected cell growth suppression, but piroxicam had no effect on cell number (Fig. 4).

Fig. 5 compares the suppressive effects of NDGA and esculetin, inhibitors of leukotriene biosynthesis (26, 27) and INDO, on MDA-MB-231 cell growth. Both of the lipoxygenase inhibitors were more active than INDO (40 μg/ml concentration, $P < 0.001$). However, at a concentration of 5 μg/ml (16 μM), NDGA caused a 74% reduction in cell number, whereas esculetin only approached this level of suppression at a concentration of 40 μg/ml (225 μM). Moreover, there was loss of cell adherence, although >75% continued to exhibit trypan blue exclusion, with NDGA concentrations of 5 μg/ml and above, which did not occur to a significant degree at any level of esculetin.

DISCUSSION

The present study was performed with the FAs added to serum-free medium supplemented with 1.25 mg/ml of delipidized BSA. There was no significant difference in the stimulatory effect of LA on MDA-MB-231 breast cancer cell growth whether it was added precomplexed with BSA, as described in our earlier communication (16), or dissolved in ethanol, as in the present study. In the latter case, binding to the albumin presumably occurred *in situ* and this prevented any cytotoxic effect which would have resulted from the presence of high concentrations of free FA. When we attempted to culture breast cancer cells in albumin-free medium, LA caused cell death over the same concentration range as that used in the present experiments.[4] In making quantitative comparisons between the effects of the various FAs on cell growth, one should be cautious; the rates of uptake between classes are likely to vary, and differences in the binding constants to BSA may exert an additional modulating effect.

The requirement for binding of FA to albumin most likely explains the cytotoxic effects of polyunsaturated FAs on human cancer cells described by Bégin *et al.* (28). These investigators reported that 20-μg/ml concentrations of several *n*-6 and *n*-3 FAs, including LA, were cytotoxic when added to serum-free, albumin-unsupplemented medium used for the culture of several human cancer cell lines, including ZR-75-1 breast cancer cells, but they observed a "protective" effect with the addition of increasing concentrations of FBS. Furthermore, in our own experiments performed in the presence of BSA, the optimal concentration of LA for stimulation of breast cancer cell growth was <1 μg/ml.

Wicha *et al.* (29) studied the effects of several FAs, added to the medium dissolved in ethanol, on the growth of a cell line derived from a DMBA-induced rat carcinoma. The cells were grown in a medium containing 5% delipidized FBS plus supplemental hormones. This FBS concentration would have given a final albumin concentration close to the 1.25 mg/ml which was added in our experiments. Also, the FAs were added dissolved in ethanol, the final concentration of which was the same as that in the present study. These investigators found that the addition of LA produced a stimulation of DMBA-induced mammary cancer cells; the optimal concentration was 1 μg/ml. Oleic acid had an even greater stimulatory effect, with an optimal concentration of 0.1–0.5 μg/ml, whereas SA, a saturated FA, produced a dose-related inhibition of cell proliferation.

The results reported here confirm and extend our preliminary report (16) that LA stimulates the growth of human breast cancer cell lines in culture. Oleic acid stimulated growth of MDA-MB-231 cells when present at concentrations below 1 μg/ml but was inhibitory at higher concentrations. A stimulation of tumor cell growth is consistent with the reported promotional effect of olive oil, rich in the *n*-9 FA, on DMBA-

[4] D. P. Rose and J. M. Connolly, unpublished observations.

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Table 1 *Comparison of effects of indomethacin and piroxicam on [³H]thymidine incorporation and PGE_2 secretion by MDA-MB-231 human breast cancer cells*

This experiment was performed in serum-free medium with or without 625 ng/ml of LA with a 3-day incubation period. The PGE_2 assays were performed only on media without added LA.

Inhibitor concentration (μg/ml)	Indomethacin			Piroxicam		
	[³H]thymidine (cpm)			[³H]thymidine (cpm)		
	Without LA	With LA	PGE_2 (pg/μg protein)	Without LA	With LA	PGE_2 (pg/μg protein)
0	2983 ± 1046[a]	6775 ± 163	235 ± 99	2983 ± 1046	6775 ± 163	235 ± 99
1	1989 ± 435	5390 ± 98[b]	46 ± 10[b,c]	1463 ± 86[d]	2253 ± 161[e]	101 ± 25
5	673 ± 12[b]	1421 ± 147[e,f]	108 ± 22[d]	680 ± 41[b]	2099 ± 77[e]	117 ± 29
10	439 ± 24[b,g]	929 ± 45[c,e]	92 ± 2[c,d]	985 ± 67[b]	2169 ± 483[e]	173 ± 69
20	252 ± 38[b,g]	1105 ± 39[c,e]	46 ± 4[d,g]	875 ± 45[b]	2510 ± 184[b]	108 ± 10
30	97 ± 14[e,g]	219 ± 47[e]	85 ± 7[d,g]	458 ± 50[b]	1178 ± 68[e]	174 ± 4
40	78 ± 11[e,f]	140 ± 35[e,f]	70 ± 24[d,f]	472 ± 155[b]	806 ± 259[e]	166 ± 16[d]

[a] Mean ± SD of triplicate wells.
[b] Significantly different from corresponding control value, $P < 0.01$.
[c] Reduction significantly greater than corresponding value with piroxicam, $P < 0.05$.
[d] Significantly different from corresponding control value, $P < 0.05$.
[e] Significantly different from corresponding control value, $P < 0.001$.
[f] Reduction significantly greater than corresponding value with piroxicam, $P < 0.01$.
[g] Reduction significantly greater than corresponding value with piroxicam, $P < 0.001$.

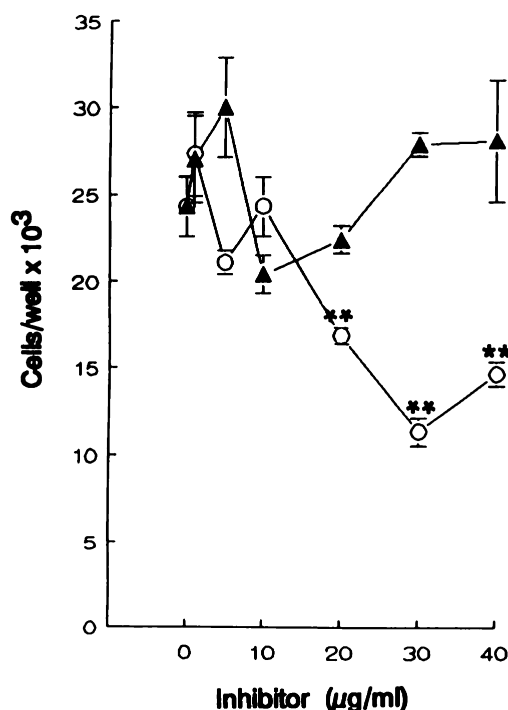

Fig. 4. The effects of indomethacin (○) or piroxicam (▲) on MDA-MB-231 breast cancer cell growth. The cells were cultured for 6 days in serum-free medium containing 625 ng/ml of LA. *Points,* mean values for triplicate wells; *bars,* SE. Cell number significantly less than control value: $^{**}P < 0.01$.

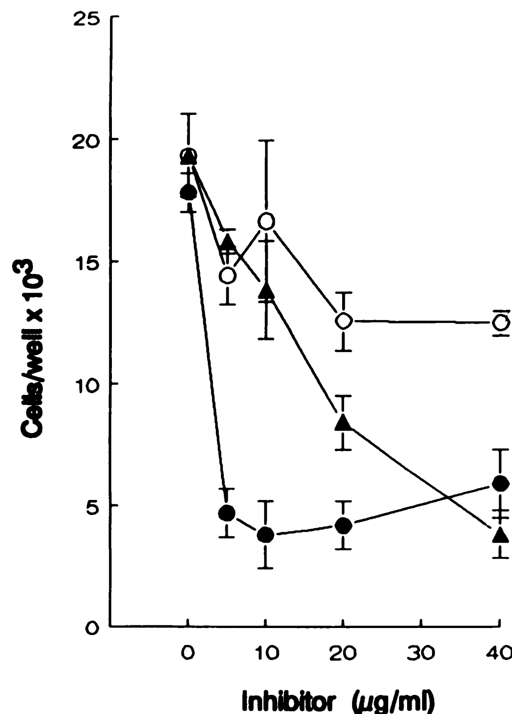

Fig. 5. Comparison of the inhibitory effects of indomethacin (○), NDGA (●), and esculetin (▲) on MDA-MB-231 cell growth. The cells were counted after 6 days of growth in serum-free medium containing 625 ng/ml of LA. *Points,* mean values for triplicate wells; *bars,* SE.

induced rat mammary tumors (30), as well as the activity of OA in stimulating growth of DMBA-induced rat mammary carcinoma cells in culture (29). However, in another study, olive oil was found to have no promotional effect on the *N*-nitrosomethylurea-induced rat mammary tumor model (31), and it did not have a significant effect on mammary tumor incidence when fed to C3H mice (32). Our observation that a given concentration of OA may be inhibitory, while the same concentration of LA is stimulatory, suggests that levels of the various FAs attained in the mammary tissue are important in determining the outcome of rat mammary carcinogenesis experiments. While the biochemical mechanisms remain to be determined, OA, LA, and α-linolenic acid, the precursor *n*-3 FA, all compete for the same $▲_6$ desaturase, but, because of their differing affinities for the enzyme, the rates of the desaturation reaction are ranked in the order α-linolenic acid > LA > OA (33). There is consequently competition between the three FA groups for the desaturation process, and at relatively high concentrations OA will inhibit the conversion of LA to γ-linolenic acid and, hence, its entry into the eicosanoid biosynthetic pathways (33, 34).

The growth inhibition by SA is also in agreement with the effect of this saturated FA on cultured rat mammary carcinoma cells (29) and the suppression of tumor development in C3H mice fed high levels of SA contained in linseed oil (32). Indirectly, the mechanism here may also involve competition for the desaturase systems since SA is converted to OA by a microsomal oxidative enzyme system (33).

The *n*-3 FAs, notably DHA, inhibited MDA-MB-231 breast cancer cell growth. This is consistent with the known inhibition of rat mammary carcinogenesis (7) and the suppression of growth of a transplantable rat mammary carcinoma (8) and of

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a human breast cancer in athymic mice (35) achieved by feeding fish oils rich in DHA and EPA. The growth of a human prostate cancer cell line which forms solid tumors in athymic nude mice was also shown to be suppressed in animals fed menhaden oil (36) or the fish oil preparation MaxEPA (37). Eicosapentaenoic acid is incorporated into phospholipids at the expense of AA and competes for the same enzymes in the eicosanoid biosynthetic pathway, thus suppressing prostanoid synthesis (38). Corey *et al.* (39) found DHA to be an inhibitor of PG synthesis and to a lesser extent of the lipoxygenase responsible for leukotriene biosynthesis from AA, and, in a dietary study of mammary carcinogenesis induced by DMBA, feeding *n*-3 FA-rich menhaden oil not only lowered tumor incidence but caused a reduction in both leukotriene B_4 and PGE levels in the tumors which did develop (40). Thus, the suppression of MDA-MB-231 cell growth observed in the presence of EPA and DHA may involve inhibition of synthesis of one or both classes of eicosanoids.

Indomethacin also inhibited MDA-MB-231 cell growth and suppressed the otherwise stimulatory effect of LA. Fulton (41) reported that INDO stimulates replication of cultured mouse mammary tumor cell lines at concentrations which, although lower than those examined in our experiments, still inhibited tumor PGE synthesis. It was suggested that higher INDO concentrations inhibit mammary tumor cell growth by affecting enzymes other than the cyclooxygenase which regulates entry into the PG- and thromboxane-synthesizing pathway. Two such enzymes are phospholipase A_2 (23), which is responsible for the release of AA from phospholipids, and the 5-lipoxygenase, which catalyzes the initial step in the metabolism of AA to the leukotrienes (34). Overall, our results obtained with the three other enzyme inhibitors tested are consistent with growth suppression of MDA-MB-231 cells being dependent on inhibition of leukotriene, rather than PG, biosynthesis. Both esculetin and NDGA were more effective than INDO in suppressing cell growth. Esculetin is a selective inhibitor of the 5- and 12-lipoxygenases; it does not inhibit PG synthesis and, indeed, is stimulatory at high (10^{-5} M) concentrations (27). The inhibitory effects of low levels of esculetin on MDA-MB-231 cell growth were less than those observed with similar concentrations of NDGA, a result which is consistent with its higher half-inhibition dose in other cell systems (27, 42). However, NDGA also antagonizes leukotriene B_4 binding to its receptor (43), which may also contribute to its efficacy when present at a low dose. These effects of the lipoxygenase inhibitors are consistent with the report that NDGA suppresses *N*-nitrosomethylurea-induced rat mammary carcinogenesis (44).

Piroxicam, which inhibits only cyclooxygenase (25), exhibited no suppressive effect on cell proliferation after 6 days of culture, although there was some inhibition of thymidine incorporation into the cells after 3 days of exposure to the drug. This absence of growth suppression again has a parallel in chemically induced rat mammary carcinogenesis. Carter *et al.* (45) found that, while INDO inhibited tumor development, the cyclooxygenase inhibitor carprofen had no such effect, although it was at least as effective in reducing mammary epithelial cell PGE_2 levels. An unresolved question, however, is whether piroxicam and INDO were taken up equally well by the MDA-MB-231 cells. Thus, INDO appeared to be more effective than piroxicam in inhibiting PGE_2 production by this cell line, whereas in experiments with transformed mouse fibroblasts (MC5-5 cells) the two drugs had similar levels of inhibitory activity with half-inhibition dose values of 1.4 and 1.2×10^{-7} M, respectively (46).

The results reported here indicate that the various classes of FAs exert effects on the growth *in vitro* of at least one human breast cancer cell line which correspond to those reported previously for rat mammary carcinoma cells and that, in general, these effects also parallel the promotional properties of these same FAs in rat mammary carcinogenesis. In addition, they draw attention to the role of the leukotriene class of eicosanoids in human breast cancer cell growth.

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Effects of Fatty Acids and Inhibitors of Eicosanoid Synthesis on the Growth of a Human Breast Cancer Cell Line in Culture

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