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Effects of linoleic acid and γ-linolenic acid on the growth and metastasis of a human breast cancer cell line in nude mice and on its growth and invasive capacity in vitro

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Effects of Linoleic Acid and γ-Linolenic Acid on the Growth and Metastasis of a Human Breast Cancer Cell Line in Nude Mice and on its Growth and Invasive Capacity *In Vitro*

David P. Rose, Jeanne M. Connolly, and Xin-Hua Liu

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Abstract

It has been reported that γ-linolenic acid (GLA)-rich diets suppress mammary carcinogenesis and transplanted tumor growth and that GLA inhibits the growth of cultured human cancer cell lines. We compared the effects of dietary GLA and linoleic acid (LA) on the growth of MDA-MB-435 human breast cancer cells and their expression of the metastatic phenotype in vivo *and* in vitro. *Athymic nude mice (30/dietary group) were fed isocaloric diets containing 20% (wt/wt) fat but providing 8% GLA or LA for 7 days, and 10^6 tumor cells were then injected into a thoracic mammary fat pad. The diets were continued for a further 11 weeks.*

The primary tumor growth rates were similar in mice from the two dietary groups; there was a nonstatistically significant trend for the incidence of macroscopic lung metastases and the total lung metastatic volumes to be higher in the GLA-fed mice (79% and 40.1 ± 13.9 mm^3) than in the LA-fed mice (64% and 15.5 ± 5.4 mm^3). The tumor cell phospholipids from the 8% GLA-fed mice contained significantly lower LA levels but higher arachidonic acid levels (both $p < 0.001$) than those from 8% LA-fed mice. Also the arachidonate-derived eicosanoids (prostaglandin E, leukotriene B_4, and 5-, 12-, and 15-hydroxyeicosatetraenoic acids) were significantly higher in tumors from the 8% GLA group. Zymography showed higher 92-kDa type IV collagenase activity in tumors from 8% GLA-fed mice. In vitro, *GLA and LA, at 0.5–2 μg/ml, stimulated MDA-MB-435 cell growth; 10 μg/ml was mildly inhibitory. Whereas LA stimulated tumor cell invasion and 92-kDa type IV collagenase production* in vitro, *GLA inhibited invasion and did not induce activity of the proteolytic enzyme.*

Our results do not support the hypothesis that supplementation with GLA would exert a beneficial effect on the progression of an existing breast cancer, perhaps because it is metabolized in vivo *to arachidonate-derived eicosanoids that are known to be involved in the metastatic process.*

(Nutr Cancer ***24****, 33–45, 1995)*

Introduction

Whereas there is continuing controversy concerning the influence of dietary fat intake on breast cancer risk (1,2), some clinical studies showed a high-fat diet to be associated with a poor outcome in breast cancer patients (3), an effect that may apply specifically to ω-6 fatty acids (n-6 FA) (4). Japanese breast cancer patients, who consume a relatively low-fat diet, are more likely than Caucasian patients to have *in situ* or localized disease without lymph node

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involvement at diagnosis, and even among those with this pathological staging, the Japanese patients have a better prognosis (5). Moreover this same study again showed that, among Caucasian Americans, high fat consumption was associated with reduced survival and high n-6 FA intakes with more advanced disease at the time of diagnosis.

In a series of studies, we found that a high-fat n-6 FA-rich diet (23% wt/wt corn oil) stimulates growth of the MDA-MB-435 human breast cancer cell line in athymic nude mice and enhances its capacity to metastasize to the regional lymph nodes and lungs (6); however, a diet supplemented with ω-3 FA exerts suppressive effects on growth and metastasis (7,8). When 23% (wt/wt) total fat isocaloric diets containing different mixtures of safflower oil [rich in linoleic acid (LA), an n-6 FA] and coconut oil (rich in saturated FAs) were fed, MDA-MB-435 cell growth and metastasis to the lungs were enhanced by high levels of dietary LA (9).

LA is metabolized to arachidonic acid (AA), the substrate for the biosynthesis of 2-series prostaglandins and a series of lipoxygenase products; γ-linolenic acid (GLA) is formed from LA under the influence of a Δ^6-desaturase and is rapidly elongated to dihomo-γ-linolenic acid (DGLA), which in turn is converted by a Δ^5-desaturase to yield AA. It has been reported that GLA suppresses the growth of human cancer cell lines *in vitro* (10,11) and that feeding a diet rich in primrose oil, which contains 9% GLA, inhibits the development of mammary adenocarcinomas in rats exposed to dimethylbenz[*a*]anthracene (DMBA) (12,13).

In the present study, we compared the effects of 20% (wt/wt) total fat diets providing 8% (wt/wt) LA or GLA on MDA-MB-435 human breast cancer cell growth and metastasis in nude mice and on the primary tumor type IV collagenase activity, phospholipid FA contents, and the levels of five eicosanoids formed from AA. Also we compared the effects of LA and GLA on the growth of this same cell line in culture, its invasive capacity (14), and the expression of type IV collagenase (15) *in vitro.*

Materials and Methods

Animals and Diets

Female athymic nude mice (NCr-*nu/nu*) were purchased from Simonsen Laboratories (Gilroy, CA) at three to four weeks of age and maintained in microisolator cages in a specialized nude mouse facility. There were 30 mice in each of the two dietary groups.

The experimental diets were prepared by BioServ (Frenchtown, NJ) and were based on the purified AIN-76A diet (16,17). Both diets contained 20% (wt/wt) fat but with saturated FA-containing coconut oil plus either LA-rich safflower oil to give 8% LA or 8% GLA. These diets were isocaloric, with 4.32 kcal/g of diet. The safflower and coconut oils were provided by BioServ; GLA was a product of the Vitamins and Fine Chemicals Division, Hoffmann-La Roche (Basel, Switzerland) and contained 3 mg/g of α-tocopherol as an antioxidant. The diets were sterilized with ^{60}Co, pelleted, and packaged in plastic bags that were flushed with N_2 gas and stored in the dark at –20°C until used (18). The mice were fed the sterile diets and provided autoclaved drinking water ad libitum. Feeding trays were emptied and replenished with fresh diet every other day. The sealed bags were opened only once. All animals were weighed and palpated for mammary fat pad tumors each week.

Cell Line

The estrogen-independent MDA-MB-435 human breast cancer cell line was originally isolated from a pleural effusion (19). Its invasive capacity (14) and type IV collagenase production (15) *in vitro* are stimulated by LA. Routine culture is performed in Iscove's modified

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Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) with 5% fetal bovine serum (FBS). For injection into the mammary fat pads of nude mice, the cells are grown to 70–90% confluence in T-150 flasks, the medium was changed 24 hours before cell harvesting, and the recovered cells were resuspended at 20×10^6/ml.

Experimental Procedure

Feeding the experimental diets was begun seven days before the tumor cell injections so that the desired lipid environment was present during the stage of initial tumor proliferation and angiogenesis (20). A right-sided thoracic mammary fat pad was then exposed surgically, and 10^6 cells were injected, as detailed elsewhere (6). When palpable, the tumors were measured, and their surface areas were calculated as described previously (6). The mice were killed by cervical dislocation 11 weeks after tumor cell injection, the body and mammary fat pad ("primary") tumor weights were recorded, macroscopic lung metastases were counted, and the total metastatic volumes were quantified using the method described by Welch and co-workers (21). The tumor volumes, calculated as for a sphere, were then summed to obtain the total volume of lung metastases per animal (6,21). Portions of the primary tumors were trimmed free of necrotic tissue and frozen immediately in liquid nitrogen until the FA and eicosanoid extractions and the type IV collagenase assays were performed. The lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy.

Tumor FAs

Tumor lipid extractions were carried out using the method of Folch and co-workers (22), the FAs were transesterified (23), and the FA methyl esters were then separated by gas chromatography on a 30 m × 0.25 mm capillary column (Supelco, Bellefonte, PA), with helium carrier gas and temperature programming from 145°C at 1°C/min to 181°C. Individual FA methyl esters were identified by comparison with known standards (Nu Chek Prep, Elysian, MN). The individual FAs are presented as a percentage of the total measured FAs.

Tumor Eicosanoid Assays

The procedure for the tumor extractions before the radioimmunoassays has been described in detail elsewhere (9). Radioimmunoassays for prostaglandin (PG) E_2, leukotriene B_4 (LTB_4), and 5-, 12-, and 15-hydroxyeicosatetraenoic acid (5-HETE, 12-HETE, and 15-HETE) were performed using ^{3}H-label kits obtained from Perseptive Diagnostics (Cambridge, MA). Whereas the cross-reactivity between the antibodies prepared against each of the lipoxygenase products was <1.0% in each instance, that between the PGE_2 antibody and PGE_1 was 50%; therefore these latter results are referred to as "PGE." The results are expressed as picograms of eicosanoid per milligram of tumor supernatant protein.

Tumor Processing and Zymography

The tissues were thawed and homogenized in 50 mM tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.5) containing 75 mM NaCl and centrifuged at 6,000 *g* for 20 minutes at 4°C. The pellet was discarded, and the supernatant was aliquoted and stored at –20°C. The various isoforms of type IV collagenase in 100 μg of tissue extracts were detected by gelatin zymography on 9% sodium dodecyl sulfate (SDS)-polyacrylamide gels, as described previously (15). After electrophoresis, the gels were incubated overnight at 37°C, stained with 0.05% Coomassie blue, and then destained with 10% isopropyl alcohol and 10% acetic acid in water.

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The gelatinolytic activity of each collagenase isoform appeared as a white zone against the blue background of stained gelatin.

The effects of LA and GLA on the expression and secretion of the 92-kDa isoform of type IV collagenase *in vitro* was examined by growing MDA-MB-435 cells in serum-free IMDM containing delipidized bovine serum albumin (1 mg/ml; Sigma Chemical, St. Louis, MO). After incubation, with or without the FA, for 40 hours, the conditioned medium was collected and centrifuged, and a 500-µl aliquot was concentrated 20-fold using an Ultrafree-30 filter (Millipore, Bedford, MA) and mixed with SDS sample buffer without β-mercaptoethanol. Electrophoresis in SDS-polyacrylamide gels was then performed as described above. Protein content was determined using a protein assay reagent (Sigma Chemical).

Northern Blot Analysis

The Northern blot analysis procedure has been detailed elsewhere (15). Briefly, the cultured cells were harvested, the total RNA was isolated, and its concentration was determined spectrophotometrically at 260 nm and then fractionated on a 1.2% agarose-formaldehyde gel. After transfer to nitrocellulose membranes, hybridization was performed with an α-^{32}P-labeled 92-kDa type IV collagenase cDNA probe (kindly provided by Dr. G. Goldberg, Washington University, St. Louis, MO), and the membranes were then washed with saline sodium citrate-SDS, air dried, and exposed to XAR-5 Kodak X-ray film. To standardize for RNA loading, the membranes were subsequently hybridized with a β-actin cDNA probe, and the amounts of specific RNA were quantitated with an Ultroscan XL laser densitometer (LKB, Uppsala, Sweden).

Growth and Invasion Studies In Vitro

The cell proliferation experiment was carried out in 24-well plates (Costar) and phenol red-free RPMI-1640 (GIBCO) plus 0.1% delipidized bovine serum albumin. The GLA, without added antioxidant, was from Hoffmann-La Roche; LA and docosahexaenoic acid were purchased from Sigma Chemical. The FAs were incorporated into the medium dissolved in 100% ethanol, the volumes added resulting in a final concentration of <0.05%. Ethanol alone (0.05%) was added to the control wells. Initially, the cells were cultured for 24 hours in 5% FBS-supplemented IMDM at a plating density of 10^4 cells/ml/well. They were then washed with unsupplemented medium, and the experimental medium was added to the wells, which were set up in triplicate. Incubation was continued for six days, with refeeding after three days. The cells were then harvested, and cell numbers were determined with an electronic particle counter (Coulter Electronics, Hialeah, FL).

The invasion assays were performed according to the method of Hendrix and colleagues (24) with Matrigel (Collaborative Research, Bedford, MA)-coated polycarbonate membrane filters. Tumor cells were routinely maintained in 5% FBS-supplemented IMDM and harvested for the invasion assay by exposure to nonenzymatic cell dissociation medium (Sigma Chemical). The cells were then plated into membrane invasion culture system plates (24) at 10^5 cells/well in phenol red-free RPMI-1640 plus 1% FBS and 0.1% delipidized bovine serum albumin, with or without the FA under evaluation. After a 72-hour incubation, cells that had invaded through the Matrigel into the lower chambers were harvested and counted as described previously (14,24); cells remaining in the upper wells were also harvested, and their number was determined by counting on a Coulter Counter so that any required correction could be made for cell growth during the 72-hour incubation. In fact, neither LA nor GLA had any significant effect on MDA-MB-435 cell proliferation under the conditions of the assay. The assays were set up in triplicate, and the results are expressed as the total number of cells recovered in the lower chamber divided by the number of cells seeded per well and then referenced to the average result (%) for the control medium wells (14).

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Statistical Analyses

The data are presented as means ± SE. The incidence of lung metastases in the different dietary groups were compared by the χ^2 test and the differences in the total volumes of lung metastases and tumor eicosanoid concentrations by the nonparametric Mann-Whitney *U* test. All other comparisons utilized the unpaired Student's *t* test, with $p < 0.05$ in a two-tailed test considered to be significant.

Results

One mouse in the 8% LA dietary group died of unknown cause early in the experiment, and another developed acute weight loss due to an intestinal volvulus; both were excluded from the study.

Body Weights, Primary Tumor Growth, and Metastasis

The body weight gains and the growth rates of the mammary fat pad tumors over the 10-week observation period were similar in mice fed the 8% LA diet and those fed the 8% GLA diet (data not shown), and the body weights and the primary tumor weights determined at necropsy were not significantly different between groups (Table 1). Table 1 also summarizes the occurrence and extent of lung metastases from mammary fat pad primary tumors and shows a clear trend but one that did not achieve statistical significance for enhanced metastasis in the mice fed the 8% GLA diet compared with those that consumed the 8% LA diet.

Tumor Phospholipid FAs and Tissue Eicosanoid Concentrations

Table 2 provides the phospholipid FA profiles for eight tumors taken at random from each of the two dietary groups. Compared with the 8% LA group, the cell membrane phospholipids of tumors from mice fed the 8% GLA diet contained a reduced percentage of the total FAs assayed as LA ($p < 0.001$), an increase in DGLA ($p < 0.001$), and an almost twofold greater proportion of AA ($p < 0.001$). In addition, the tumors from GLA-fed mice contained higher levels of palmitic acid ($p < 0.05$) and stearic acid ($p < 0.001$) and reductions in oleic acid ($p < 0.001$). GLA was not detected in tumors from either dietary group.

The increased availability of AA substrate for the biosynthesis of PGs of the 2-series, 4-series LTs, and HETEs by mammary fat pad tumors from mice fed 8% GLA may have been responsible for the differences in the tissue eicosanoid concentrations between the dietary groups shown in Table 3. Assays were performed on seven tumors taken at random from each dietary group, and those from 8% GLA-fed mice showed significantly higher levels of LTB_4

Table 1. Body Weights, Weights of Primary Tumors, and Macroscopic and Microscopic Lung Metastases in Nude Mice Fed 8% LA- or 8% GLA-Containing Diets[a,b]

			Lung Metastases in Primary Tumor-Bearing Mice		
Diet Group	Body Wt,[c] g	Primary Tumor Wt,[c] g	Total incidence[d]	Macroscopic incidence[d]	Total macroscopic lung metastatic volume/mouse,[c] mm^3
8% LA	28.5 ± 0.6	3.8 ± 0.3	22/28 (79)	18/28 (64)	15.5 ± 5.5
8% GLA	27.2 ± 0.6	3.3 ± 0.3	27/29 (93)	23/29 (79)	40.1 ± 13.9

a: LA, linoleic acid; GLA, γ-linolenic acid.
b: None of the differences between groups was statistically significant.
c: Values are means ± SE.
d: Values in parentheses are percentages.

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Table 2. Principal Fatty Acids in Tumor Cell Phospholipid Fractions[a,b]

Fatty Acid[c]	Diet Group 8% LA	Diet Group 8% GLA
Myristic (14:0)	2.37 ± 0.18	2.12 ± 0.06
Palmitic (16:0)	23.32 ± 0.32	24.71 ± 0.37†
Palmioleic (16:1)	2.55 ± 0.12	2.17 ± 0.09†
Stearic (18:0)	21.36 ± 0.55	25.07 ± 0.49*
Oleic (18:1)	19.19 ± 0.70	13.86 ± 0.19*
Linoleic (18.2)	18.45 ± 0.31	7.72 ± 0.23*
Dihomo-γ-linolenic (20:3)	0.36 ± 0.05	4.55 ± 0.13*
Arachidonic (20:4)	11.52 ± 0.53	19.14 ± 0.47*
Eicosapentaenoic (20:5)	0.03 ± 0.01	ND
Docosahexaenoic (22:6)	0.46 ± 0.02	0.50 ± 0.02

a: Values are means ± SE of 8 tumors from each dietary group expressed as percentage of total measured fatty acid content. ND, not detected.
b: Statistical significance is as follows: significantly different from 8% LA group: *, $p < 0.001$; †, $p < 0.05$.
c: GLA (18:3) was not detected on chromatograms.

Table 3. Eicosanoid Concentrations in Mammary Fat Pad Tumor Tissues[a,b]

Eicosanoid[c]	Diet Group 8% LA	Diet Group 8% GLA
PGE	847 ± 105	1,385 ± 136‡
LTB_4	135 ± 12	214 ± 18‡
5-HETE	1,266 ± 101	2,958 ± 393*
12-HETE	17,546 ± 1231	42,468 ± 7421*
15-HETE	1,800 ± 259	3,409 ± 566†

a: Values are means ± SE in pg/mg protein; radioimmunoassays were performed on 7 tumors taken at random from each dietary group.
b: Statistical significance is as follows: significantly different from 8% LA group: *, $p = 0.002$; †, $p < 0.05$; ‡, $p < 0.01$.
c: PGE, prostaglandin E; LTB_4, leukotriene B_4; 5-, 12-, and 15-HETE, 5-, 12-, and 15-hydroxyeicosatetraenoic acid.

($p < 0.01$) and 5-HETE ($p = 0.002$), both products of 5-lipoxygenase activity, and also of 12-HETE ($p = 0.002$) and 15-HETE ($p < 0.05$), formed from AA under the influence of the corresponding lipoxygenases, than tumors from mice fed the 8% LA diet. These tumors also contained higher concentrations of eicosanoid assayed as PGE ($p < 0.01$); because of the cross-reactivity of the "PGE_2" antibody with PGE_1, it cannot be concluded with certainty that this increase was due solely to enhanced PGE_2 biosynthesis from AA.

Tumor Tissue Type IV Collagenase (Gelatinase)

Figure 1 shows that the supernatants prepared from MDA-MB-435 cell mammary fat pad solid tumors contained two major gelatinase isoforms, matrix metalloproteinase-9 (MMP-9, mol mass 92 kDa) and MMP-2 (mol mass 72 kDa). We previously described the 92-kDa isoform in medium conditioned by MDA-MB-435 cells *in vitro* and demonstrated its induction by LA (15); the 72-kDa isoform was not secreted by the cultured cells, and in the solid tumors it may have been produced by host stromal tissue. Tumors from the LA and GLA dietary

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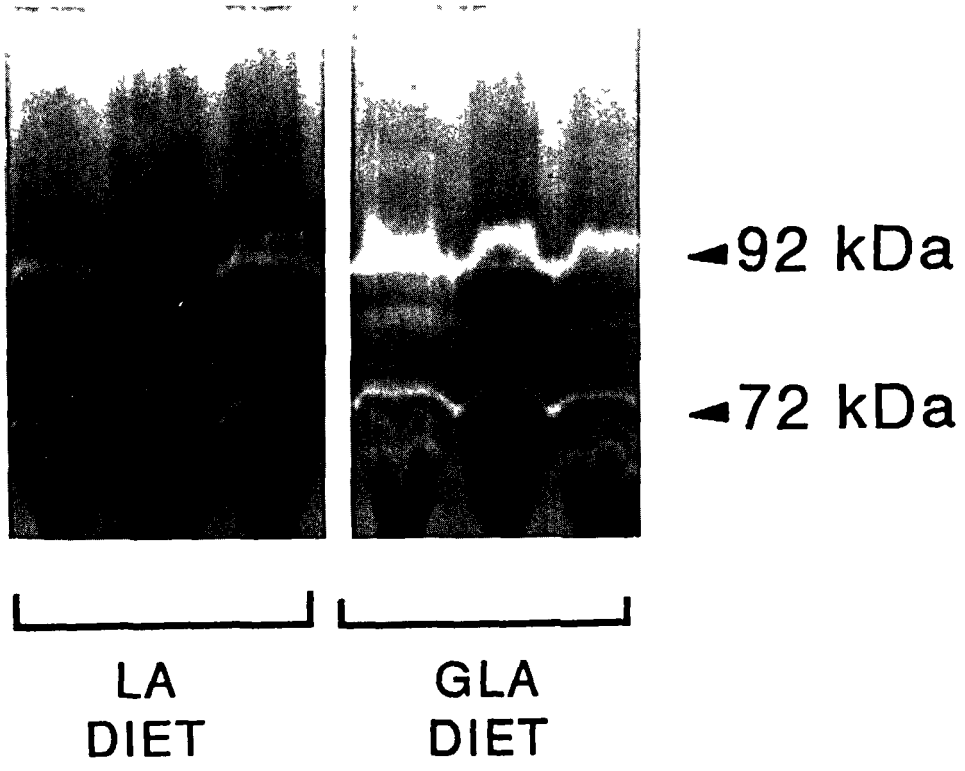

Figure 1. Zymographic analysis of gelatinase (type IV collagenase) activity in supernatants prepared from primary tumors of 3 mice fed 8% linoleic acid (LA) and 3 mice fed 8% γ-linolenic acid (GLA). Molecular masses were estimated in relation to migrations of molecular weight standards.

groups contained the 92-kDa isoform, but, as indicated in Figure 1, its gelatinolytic activity appeared to be higher in tumors from mice fed the 8% GLA-containing diet. This observation was confirmed by a quantitative scanning analysis of a total of six zymograms from each dietary group, which showed significantly denser bands of 92-kDa activity in tumor preparations from the GLA-fed group ($p < 0.005$). No differences were detected in the 72-kDa bands.

The first of the GLA zymograms in Figure 1 shows an additional band of gelatinolytic activity with an apparent molecular mass of 84 kDa, which is consistent with the activated form of MMP-9, together with a 59/62-kDa doublet, which corresponds to the active form of MMP-2.

Tumor Cell Growth, Invasion, and Type IV Collagenase Expression In Vitro

Figure 2 compares the influence of LA and GLA on the growth of the MDA-MB-435 cell line. Both FAs exerted a stimulatory effect on cell growth, which was maximal at 0.5 μg/ml (LA: 190%, $p < 0.001$; GLA: 145%, $p = 0.003$) over the six-day incubation period. With FA at 10 μg/ml, LA and GLA produced a mild degree of inhibition.

The effects of LA and GLA on MDA-MB-435 cell invasion in the *in vitro* assay system are illustrated in Figure 3. LA (0.5 μg/ml) increased invasion through the Matrigel-reconstituted basement membrane by an average of 37% compared with the non-FA-containing control medium ($p = 0.03$). In contrast, GLA at 0.25 or 0.5 μg/ml inhibited MDA-MB-435 cell invasion by 27% and 41%, respectively (both $p < 0.001$), an inhibitory effect similar to that obtained with docosahexaenoic acid (33%, $p < 0.001$). Docosahexaenoic acid is one of the principal

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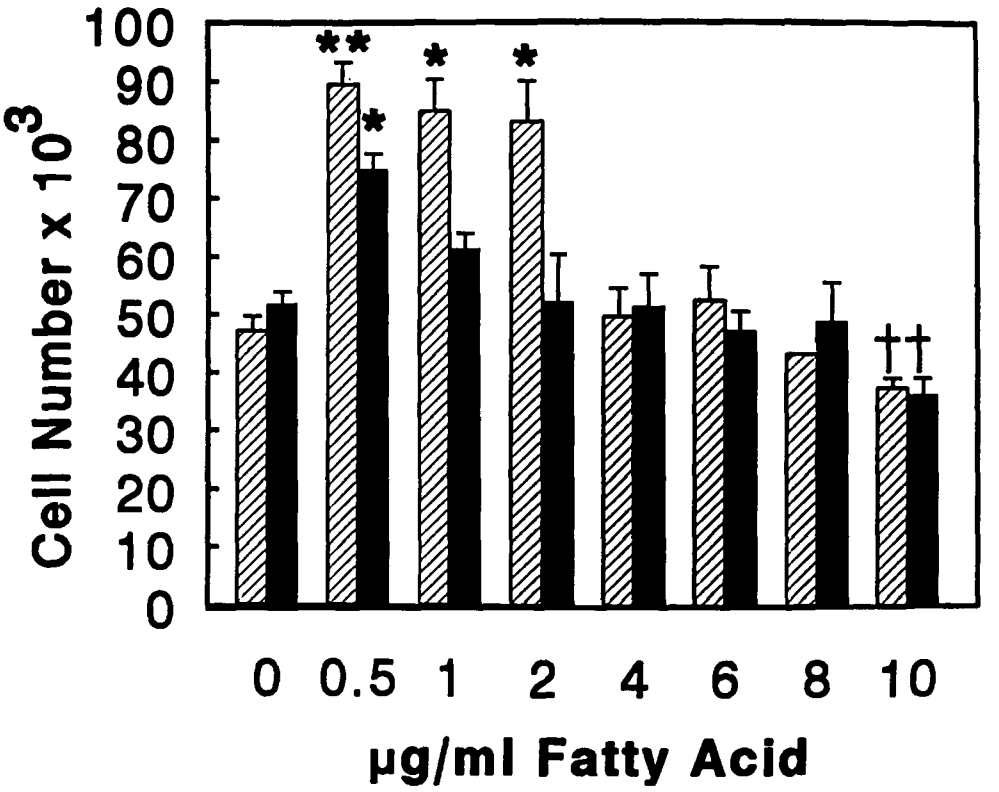

Figure 2. Effects of LA *(hatched columns)* and GLA *(filled columns)* on MDA-MB-435 cell growth in serum-free medium. Values are means ± SE for triplicate wells. Statistically significant differences in cell number after 6 days of incubation compared with those in absence of fatty acid addition: **, $p < 0.001$; *, $p < 0.01$; †, $p < 0.05$.

long-chain ω-3 FAs in fish oils and suppresses MDA-MB-435 cell growth and metastasis in nude mice (8).

Figure 4 shows the expected stimulatory effect of LA on the secretion of the 92-kDa isoform of type IV collagenase by MDA-MB-435 cells. A second band of gelatinolytic activity, with a molecular mass of approximately 120 kDa, is probably 92-kDa protein, which migrated bound to tissue inhibitor of metalloproteinase-1 and was subsequently activated by exposure to SDS. The zymogram of medium conditioned by cells growing in the presence of 0.75 µg/ml LA also shows the 84-kDa activated MMP-9 band. In contrast to LA, GLA had no effect on gelatinolytic activity, which was not detectable on the zymogram (Figure 4). Similarly, although we previously demonstrated that the level of mRNA for the 92-kDa isoform in MDA-MB-435 cells is increased by including 0.5–0.75 µg/ml of LA in the culture medium (15), Figure 5 shows that GLA was without effect.

Discussion

Despite reports that GLA inhibits human breast cancer cell growth *in vitro* (10,11), we observed no suppressive effect of dietary GLA on the growth and metastasis of a human breast cancer cell line in nude mice. There have, however, been clinical reports that evening primrose oil, administered as a dietary supplement, exerts a beneficial effect in patients with benign breast disease (25,26). Moreover, in animal studies utilizing the DMBA-induced rat mammary carcinoma model (12,13), a diet rich in primrose oil (75% LA, but also 9% GLA) was associated with reduced tumor development compared with a diet rich in corn oil (60% LA and only 0.5% GLA).

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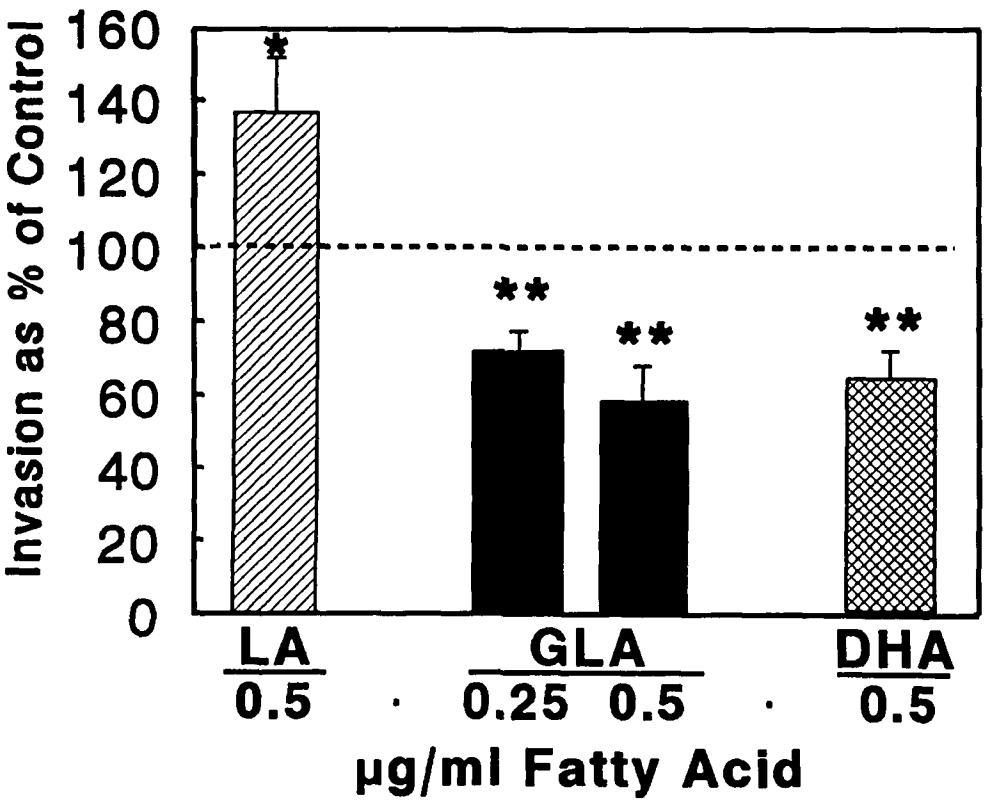

Figure 3. Effects of LA, GLA, and docosahexaenoic acid (DHA), an ω-3 fatty acid, on invasion of MDA-MB-435 cells in an *in vitro* assay. Statistically significant differences compared with invasive capacity in medium without a fatty acid addition: **, $p < 0.001$; *, $p = 0.03$.

Further work is indicated to determine why feeding GLA-containing primrose oil suppresses DMBA-induced mammary carcinogenesis, whereas dietary GLA supplementation had no effect in the present study. It should be stressed, however, that whereas dietary experiments in conjunction with a chemical carcinogen examine the induction and/or promotion stages of tumor development, this study evaluated dietary effects on human breast cancer cell growth and progression to metastatic disease. In this context, it is of interest that Karmali and colleagues (27), while confirming an earlier report (28) that dietary primrose oil suppresses the growth of a transplantable rat mammary carcinoma, found no such effect when pure GLA was administered by gastric lavage. One interpretation of these various results is that a component other than GLA may be responsible for the antitumorigenic effects of primrose oil.

Taken overall, the results obtained from our tumor phospholipid FA and tissue eicosanoid assays would have predicted enhancement of breast cancer growth and metastasis in the nude mouse model by a GLA-rich diet as well as a diet containing high levels of LA. Thus tumors from the GLA dietary group, despite a considerable reduction in phospholipid LA content, contained high levels of AA compared with tumors from mice fed 8% LA; the absence of detectable GLA, but an increase in DGLA, is consistent with the Δ^6-desaturase responsible for the conversion of LA to GLA being rate limiting in the net metabolism of LA to AA (29). Furthermore the mammary fat pad tumors from GLA-fed mice were clearly capable of metabolizing the readily available AA substrate to eicosanoids, including the lipoxygenase products LTB_4 and 12-HETE, which we previously proposed have a critical role in LA-stimulated breast cancer cell growth and metastasis (8,30). The special involvement of 12-HETE in

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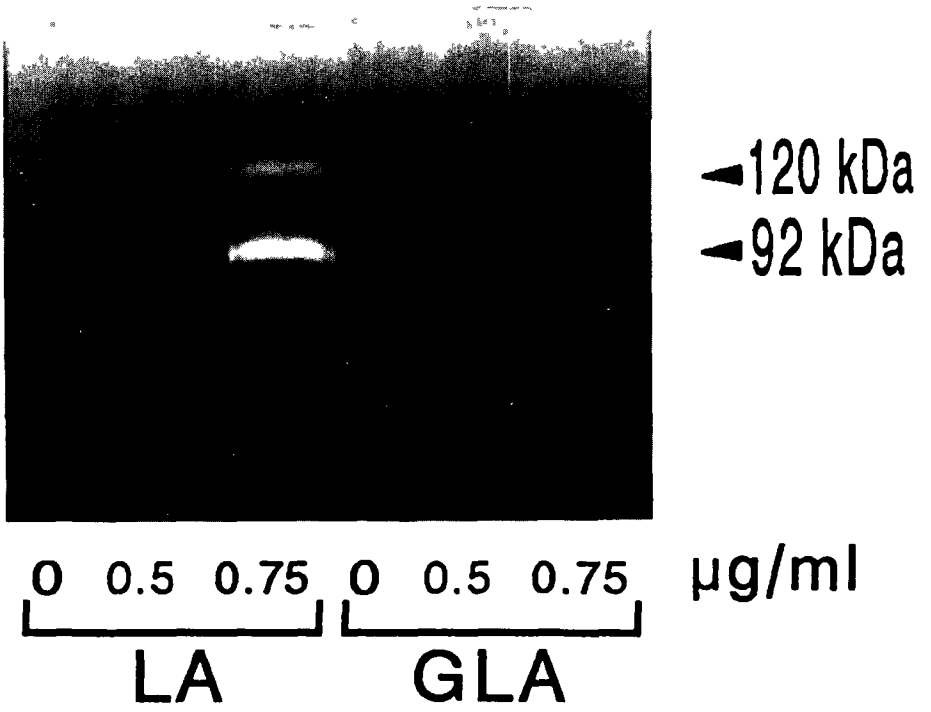

Figure 4. Zymographic analysis of gelatinase activity in medium conditioned by MDA-MB-435 cells; 92-kDa gelatinase was inducible by LA, at 0.75 µg/ml, but not by GLA.

the metastatic process has also been emphasized by the work of Liu and colleagues (32). These investigators showed that 12-HETE, the concentration of which was increased approximately twofold in MDA-MB-435 cell solid tumors from 8% GLA-fed compared with 8% LA-fed mice, enhances tumor cell progression through several steps of the metastatic cascade (31) and stimulates invasive capacity in an *in vitro* assay (32).

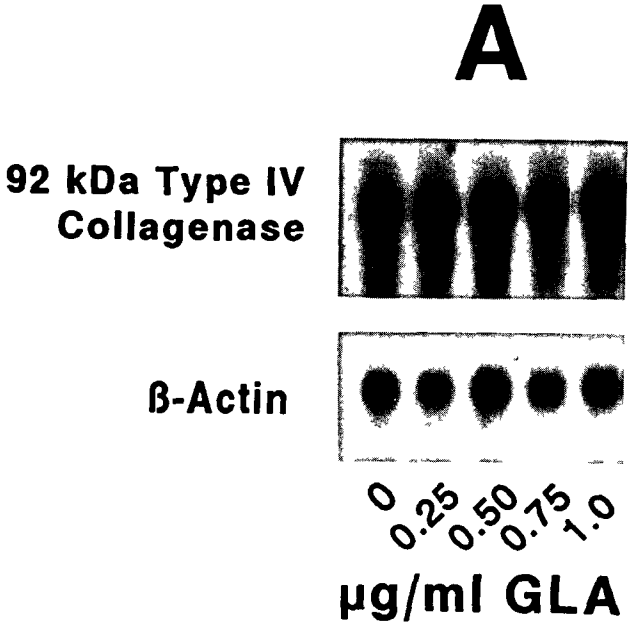

Figure 5. A: Northern blot analysis of 92-kDa (2.8-kb) type IV collagenase (gelatinase) mRNA. Total RNA was isolated from MDA-MB-435 cells after 3 days of incubation in absence or presence of GLA. Each lane contains 20 µg of total RNA. Uniformity of loading was monitored by β-actin. B: amount of specific mRNA was quantitated by densitometry, and results are calculated as ratio of 92-kDa type IV collagenase mRNA to β-actin RNA.

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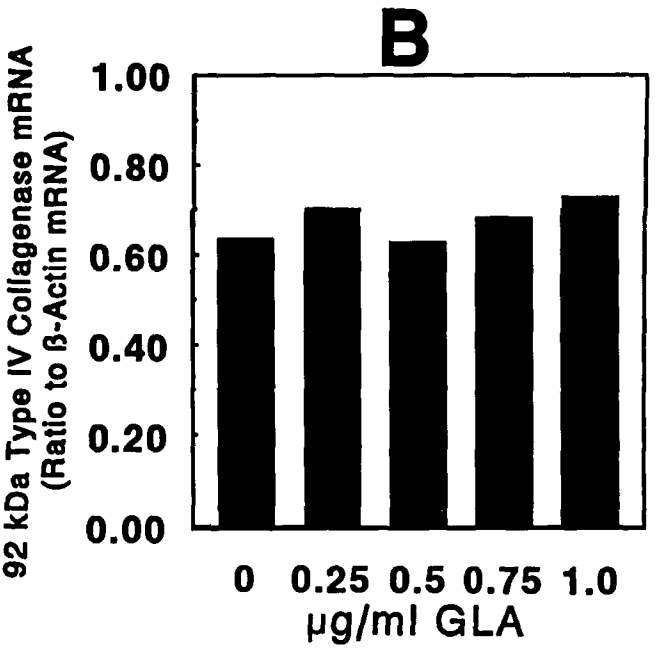

A key element of the invasive process is the secretion of proteolytic enzymes by tumor cells, among which the 92-kDa isoform of type IV collagenase, in particular, has been associated with the metastatic phenotype (30). Recently, we showed that MDA-MB-435 cell tumors from mice fed eicosapentaenoic acid, which suppresses the development of metastases, contain reduced levels of the 92-kDa isoform (33). Moreover, in cell culture, this ω-3 FA produces a downregulation of the 92-kDa protein mRNA expression. Thus it is particularly noteworthy that neither the activity of this enzyme nor its mRNA expression was reduced in cells exposed to GLA.

Several groups of investigators reported that GLA inhibits cancer cell growth *in vitro* (10,11,34–36) and suggested that the observed cytotoxic effects may have clinical applicability. However, the concentrations employed were generally extremely high, with a 50% inhibitory effect requiring 73 μg/ml in the case of the MCF-7 human breast cancer cell line (36) and 41, 43, and 100 μg/ml for three colon cancer lines (35). Bégin and co-workers (34) used 20 μg/ml to demonstrate the cytotoxic effect of GLA on the ZR-75-1 estrogen-dependent human breast cancer cell line; significantly, the extent of cell death decreased as the serum concentration increased, presumably because of enhanced binding to serum albumin. Thus it becomes difficult to translate the FA concentrations used in such *in vitro* experiments to the situation in the whole animal.

In the present study, we found that GLA and LA stimulated MDA-MB-435 breast cancer growth *in vitro* at 0.5–1.0 μg/ml; GLA produced significant inhibition only at 10.0 μg/ml, at which LA was also inhibitory. However, in contrast to LA, GLA suppressed tumor cell invasion *in vitro* and did so at a concentration that was stimulatory in the growth assay. Consistent with its failure to enhance invasion, GLA, unlike LA, did not induce tumor cell type IV collagenase activity *in vitro,* which contrasts with our finding from an analysis of mammary fat pad tumor tissues and the associated trend for a greater metastatic burden. Clearly, these results require further investigation, such as specific assays for eicosanoids of the PG_1 series (13,37) and 15-hydroxyeicosatrienoic acid (37), which are formed from DGLA, and analyses for lipid peroxidation products (34), to determine the biochemical mechanisms involved. In the meantime, they stress again that *in vitro* studies with these FAs are not necessarily applicable to the whole animal, in which host tissues contribute to their metabolism.

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