

## Effect of Dietary $\alpha$ -Linolenic Acid on Growth, Metastasis, Fatty Acid Profile and Prostaglandin Production of Two Murine Mammary Adenocarcinomas<sup>1-3</sup>

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**ABSTRACT** The purpose of this study was to determine whether dietary (*n*-3) fatty acids would affect mammary tumor growth and metastasis. Weanling female BALB/c mice were fed diets that contained 10% corn oil (CO), linseed oil (LO) or a fish oil-corn oil mix (FO) for 3–8 wk prior to receiving subcutaneous injections of one of two syngeneic mammary tumor cell types (410 and 410.4). Tumor growth was assessed by monitoring mean tumor diameter and tumor weight upon removal. Feeding LO, but not FO, reduced the growth ( $p < 0.05$ ) of 410.4 mammary tumors compared with growth in those fed CO. Metastasis data paralleled the tumor growth rate. Feeding LO and FO enhanced ( $p < 0.005$ ) incorporation of (*n*-3) fatty acids into tumors. Tumor prostaglandin E (PGE) production was reduced ( $p < 0.005$ ) by LO and FO, compared with CO. FO feeding reduced 410.4 tumor PGE synthesis more ( $p < 0.05$ ) than LO feeding, yet tumor growth was only inhibited by LO. These data suggest an inhibitory effect of dietary linolenic acid [i.e., 18:3(*n*-3)] on mammary tumor growth and metastasis. However, this effect did not directly correlate with diet-induced changes in PGE synthesis. *J. Nutr.* 120: 1601–1609, 1990.

### INDEXING KEY WORDS:

- murine mammary tumors • (*n*-3) fatty acids
- prostaglandin E • linseed oil
- fish (menhaden) oil

Recent studies have demonstrated beneficial effects of feeding fish oils in animal tumor models (1–3). Fish oils are rich in dietary (*n*-3) polyunsaturated fatty acids, particularly eicosapentaenoic acid [20:5(*n*-3)] and docosahexaenoic acid [22:6(*n*-3)]. Conversely, fats high in polyunsaturated fatty acids of the (*n*-6) family appear to enhance tumorigenesis and tumor cell growth. Explanations for this dichotomy have focused on the relative differences in the conversion of these two families of

fatty acids to the biologically active eicosanoids, particularly prostaglandins (PG)<sup>5</sup> of the E series. It was previously shown that feeding a diet rich in linolenic acid, the parent of the (*n*-3) family of fatty acids, reduces the PG synthesizing capacity of various immune tissues in rats and mice (4–7). This occurs because of reduced levels of arachidonic acid [20:4(*n*-6)] in these tissues and an elevation in (*n*-3) fatty acids, particularly 20:5(*n*-3), which directly competes for the enzymes in the eicosanoid-synthesizing pathways. Some production of prostaglandins of the 3-series and leukotrienes of the 5-series from 20:5(*n*-3) may occur. Little is known, however, about the effects of eicosanoids derived from (*n*-3) fatty acids on tumors or the immune system.

Although numerous investigators have demonstrated that mammary tumorigenesis can be significantly reduced by feeding a fish oil-containing diet, evidence that linolenic acid inhibits tumors is limited and somewhat equivocal. Tinsley et al. (8) did not observe any significant effect of linseed oil on mammary

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<sup>5</sup>Abbreviations: CMC, cell-mediated cytotoxic; CO, corn oil; FAME, fatty acid methyl esters; FO, fish oil; LO, linseed oil; LT, leukotrienes; NK, natural killer; MEM, Eagle's minimal essential medium; PG, prostaglandin.

tumor incidence in C3H mice. They concluded that linolenic acid had little effect on tumor incidence, but increasing levels of linoleic acid in the diet increased tumor incidence and decreased tumor latency. Conversely, Cameron et al. (9) recently reported that both fish oil and linseed oil feeding reduced DMBA-enhanced mammary tumorigenesis in C3H mice. The effect depended on the carcinogen dose and the level of fat in the diet. Abraham and Hillyard (10) demonstrated that linolenic acid, unlike linoleic acid, failed to stimulate the growth of a transplanted mammary tumor. In their study, mice were fed fat-free diets to which pure oleic, linoleic or linolenic acid had been added. It is uncertain, therefore, whether linolenic acid directly inhibited tumor growth or simply failed to support the tumor's essential fatty acid requirement for growth. In this study, we proposed to examine the effect of linolenic acid on mammary tumor growth in the presence of adequate dietary essential fatty acids.

It has been suggested that the production of PG is required for the tumor enhancement of dietary polyunsaturated fatty acids (10). PGE<sub>2</sub> has been described as a potent suppressor of various immune cell functions. It is proposed that through excessive production of PGE<sub>2</sub>, tumor cells escape the immunosurveillance system, allowing them to proliferate and metastasize. Evidence supporting such a mechanism has come primarily from experiments using PG synthesis inhibitors, such as aspirin and indomethacin (11–13). PGE<sub>2</sub> has been shown to suppress the cytotoxic activity of immune cells thought to play a role in immunosurveillance and tumor rejection, including natural killer cells (14), activated macrophages (15) and cytotoxic T lymphocytes (16). We recently demonstrated that feeding a diet rich in 18:3(*n*-3) enhanced cell-mediated cytotoxic (CMC) activity after a viral challenge, whereas fish oil tended to depress it (7).

The 410 and 410.4 mammary tumor cell lines have been well characterized with regard to immunogenicity, metastatic ability and PGE-synthesizing capacity, among other properties (17–19). Fulton (20) showed that continuous oral administration of indomethacin, beginning the day of tumor transplantation, led to complete regression of 410 tumors in 11 of 12 mice. In the same study, growth of 4501 and 4526 tumors (cloned cell lines from the uncloned 410.4) was reduced by indomethacin treatment. Inhibition of PG synthesis by indomethacin has also been shown to reduce the metastasis of 410.4 tumors and to increase their sensitivity to killing by natural killer cells (21). More recently, Hubbard et al. (22) demonstrated that dietary linoleate stimulated the growth and metastasis of 4526 tumors. These observations suggest that these two tumor cell lines are reasonable models for examining the interaction between dietary linolenic acid and mammary tumor growth. Tumor fatty acid composition, tumor PG synthesis and CMC activity of immune cells from tumor-bearing mice

TABLE 1  
Fatty acid composition of diets

Fatty acid <sup>2</sup>	Diet <sup>1</sup>		
	CO	LO	FO <sup>3</sup>
% of total fatty acids			
14:0	tr <sup>4</sup>	tr	8.8
16:0	14.0	5.7	11.8
16:1( <i>n</i> -7)	tr	tr	11.1
18:0	1.5	3.1	2.1
18:1( <i>n</i> -9)	30.4	20.5	12.1
18:2( <i>n</i> -6)	52.8	17.9	14.1 <sup>5</sup>
18:3( <i>n</i> -3)	2.1	55.9	0.8
20:5( <i>n</i> -3)	nd <sup>6</sup>	nd	16.2
22:5( <i>n</i> -3)	nd	nd	1.9
22:6( <i>n</i> -3)	nd	nd	5.3

<sup>1</sup>CO, corn oil; LO, linseed oil; FO, fish oil.

<sup>2</sup>Fatty acids are identified by the number of carbon atoms and the number of double bonds, followed by the position of the first double bond from the methyl end ("*n*-") of the fatty acid.

<sup>3</sup>Mixture of 4 parts menhaden oil to 1 part corn oil.

<sup>4</sup>Trace (< 0.5%).

<sup>5</sup>Provides ~ 3% of the dietary energy.

<sup>6</sup>Not detectable.

were determined in order to correlate differences in tumor growth with diet-induced changes in these parameters.

## MATERIALS AND METHODS

**Animals and diets.** Weanling female BALB/c mice (Harlan Industries, Indianapolis, IN) were used for all tumor studies. Upon receipt, mice were fed one of three purified diets and provided with tap water ad libitum. The diets were formulated, using the AIN-76 diet as a model (23), with the following dry ingredients (g/100 g): casein, 20.0; methionine, 0.03; cornstarch, 30.5; dextrose, 29.6;  $\alpha$ -cellulose, 5.0; AIN-76 mineral mix, 3.5; AIN-76 vitamin mix, 1.0; and choline chloride, 0.1. Added to these dry ingredients was either corn oil (CO), linseed oil (LO) or a mixture (4:1) of fish (menhaden) oil (FO) and corn oil, such that the final diets contained 10% fat by weight. The fatty acid profile of the diets is shown in Table 1. Fish oil was supplemented with corn oil (25%) to provide sufficient linolenic acid, an essential fatty acid. Autoxidation of the oils was prevented by the addition of a synthetic antioxidant (0.02% *tert*-butylhydroquinone, Kodak Chemical, Rochester, NY) to each oil upon receipt and by following guidelines previously described (24). Fresh diet was provided every other day with any remaining diet being discarded. Peroxide content, as measured by peroxide value (25), of diets stored at -20°C or kept at room temperature for 48 h did not significantly increase.

**Tumor cell lines.** Both 410 and 410.4 tumor cell lines were generously donated by Gloria Heppner and Amy Fulton of the Michigan Cancer Foundation. These tumor cell lines were derived from a single spontaneously arising mammary adenocarcinoma of a BALB/cfC3H mouse as described by Miller and Heppner (17). Line 410 is highly immunogenic and has a relatively low metastatic potential (< 30%). Line 410.4 came from a lung metastasis of a mouse bearing a subcutaneous implant of 410. Line 410.4 is a high PGE producer, has moderate to high immunogenicity, and metastasizes spontaneously from subcutaneous implants at a frequency of > 80%. Tumor cells were grown in Waymouth's medium (GIBCO, Grand Island, NY) supplemented with 10% Nu-serum, a serum substitute (Collaborative Research, Boston, MA); glutamine (2 mmol/L); and gentamycin sulfate (5 µg/mL). Prior to injection, tumor cells were removed from their flasks by a brief treatment with a 0.25% trypsin-EDTA solution (Sigma Chemical, St. Louis, MO). Cells were not used unless viability was > 95% as determined by trypan blue exclusion.

**Tumor fatty acid analysis.** Tumor samples were quickly thawed and then homogenized in 5 mL of a phosphate-buffered saline solution (pH 7.2) in a Potter-Elvehjem glass homogenizer. Total lipids were extracted with 10 mL chloroform and methanol (2:1, v/v) containing 0.005% butylated hydroxytoluene. The organic phase containing the lipid extract was collected and reduced in volume under N<sub>2</sub>. Tumor lipids were transmethylated in 4% sulfuric acid in methanol for 1 h in a 60°C water bath. The mixture was saponified according to the procedure of Kates (26) by the addition of 33% potassium hydroxide and subsequent heating at 60°C for 1 h. Hexane was used to remove nonsaponifiable material, and the solution was acidified by addition of 6 mol/L hydrochloric acid; the free fatty acids were extracted with hexane. The free fatty acids were again transmethylated, and fatty acid methyl esters (FAME) were analyzed using a Packard gas-liquid chromatograph, Model 428 (Packard Instruments, Downers Grove, IL) with a 30 m × 0.25 mm i.d. fused silica capillary column (SUPELCOWAX 10, Supelco, Bellefonte, PA). Helium was the carrier gas (flow rate = 1 mL/min). Oven temperature was programmed as follows: 190°C (10 min) followed by a 5 °C/min rise to a final temperature of 220°C (14 min). FAME were identified by comparing relative retention times of commercially available standards (PUFA-1 and PUFA-2; Supelco). The flame ionization detector response was assumed to be 1.000 for all FAME. Results, expressed as the percentage of total fatty acids, were determined using a Hewlett-Packard 3380A integrator (Sunnyvale, CA).

**Tumor protein and prostaglandin E determinations.** PGE production by tumor homogenates was determined as follows. Immediately following removal, a portion

(0.2–0.5 g) of a tumor was minced and quickly homogenized in Eagle's minimum essential medium (MEM, GIBCO, Grand Island, NY) without serum. The ratio of medium to tumor tissue was kept constant (2 mL/0.1 g). The homogenate was filtered through three layers of cheesecloth, then incubated at 37°C for 1 h. Following incubation, 1-mL aliquots of the homogenates were placed into microcentrifuge tubes and immediately frozen. Samples were stored at –80°C until analysis. Protein content of the homogenates was determined in duplicate by the Bradford method (27) using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Homogenates were diluted 1:10 and 1:100 with MEM, and each dilution was analyzed in duplicate for PGE content. PGE levels were determined by radioimmunoassay using rabbit antiprostaglandin E-BSA serum (Miles Laboratories, Elkhart, IN). This antiserum is reported by the manufacturer to have minimal cross-reactivity (less than 3%), with PGA<sub>1</sub>, A<sub>2</sub>, F<sub>1</sub>, B<sub>1</sub> and B<sub>2</sub> and 10% with F<sub>2</sub>. Cross reactivity with PGE<sub>3</sub> was not determined and was relatively high with PGE<sub>1</sub> (53%); therefore, results are expressed as nanograms of PGE per milligram of tumor protein.

**Experiment 1.** Thirty mice were fed diets containing CO, LO or FO for 10 wk prior to receiving tumor cell transplants. The cells were resuspended in MEM without serum at  $9 \times 10^5$  410 cells/mL, from which 0.1 mL was injected subcutaneously into the inguinal area of each mouse. Mice were weighed and tumors measured with a Vernier caliber (VWR, St. Louis, MO) at weekly intervals postinjection. Mean tumor diameters were calculated by dividing the product of the tumor length and width by 2. Variations in tumor thickness made the use of tumor volume equations problematic. Thirteen weeks after tumor cell injections, all tumor-bearing mice were killed. Tumors were removed, freed from skin and dried blood on the surface, and then weighed. A portion of each tumor was stored at –80°C for lipid analysis. Another portion was homogenized in MEM to determine PGE-synthesizing capacity, as described previously.

**Experiment 2.** Twenty-four mice were fed CO or LO diets for 8 wk, then injected with 410.4 tumor cells, as described for 410 cells. Mice were weighed and tumors measured weekly. Surgical removal of primary tumors was conducted to assess the influence of diet on metastasis to the lung. In order to maximize metastatic expression, tumors at the site of the injection (i.e., primary tumors) were surgically removed upon reaching a mean tumor diameter of 12 mm (A. M. Fulton, Michigan Cancer Foundation, personal communication). Surgery was performed aseptically using a ketamine-acepromazine solution as a general anesthetic. Wounds were closed with three or four stainless steel clips. Twenty-one days following surgery, or upon death, lungs were removed and fixed in a 10% formaldehyde solution, and metastatic foci were enumerated. Approximately 95% of all

TABLE 2  
Growth, weight and metastasis of the 410 mammary tumor in BALB/c mice fed three different sources of fat<sup>1</sup>

	Dietary fat					
	CO		LO		FO	
# Mice/# tumor-bearing mice	10/7		10/5		10/6	
Mean tumor diameter, <sup>2</sup> mm						
Week 5	6.8 ±	0.5(4) <sup>3</sup>	4.8 ±	0.9(4)	6.3 ±	0.3(3)
Week 8	9.8 ±	1.5(7)	8.4 ±	1.5(4)	8.1 ±	0.6(5)
Week 10	11.9 ±	1.7(7)	10.1 ±	1.3(5)	9.5 ±	1.1(6)
Week 13	13.6 ±	1.5(7)	11.5 ±	1.2(5)	10.8 ±	0.7(6)
Tumor weight, mg	989 ± 224 (6)		828 ± 314 (4)		476 ± 126 (6)	
Lung metastasis, # foci <sup>4</sup>	0, 0, 1, 2, 3, 7		0, 3, 6, 8, >50		0, 0, 0, 1, 3, 48	
# Mice with extrapulmonary tumors	2		1		0	

<sup>1</sup>Mice were fed diets containing 10% by weight corn oil (CO), linseed oil (LO) or a fish (menhaden) oil-corn oil mix (FO) for 10 wk, prior to subcutaneous injection of  $4 \times 10^5$  410 cells.

<sup>2</sup>Length  $\times$  width divided by 2.

<sup>3</sup>All values are expressed as mean  $\pm$  SEM with the # of tumor-bearing mice in parentheses. There was no significant difference in tumor and metastases incidence as analyzed by Chi-square. Tumor diameter and weights were not significantly different ( $p > 0.10$ ) as determined by one-way ANOVA.

<sup>4</sup>The lung from one CO tumor-bearing mouse was not recovered.

lung metastases could be observed on the surface of the lung with the aid of a stereoscopic microscope. The location and number of extrapulmonary metastases was also noted.

**Experiment 3.** Forty-five mice were fed CO, LO or FO diets for 3 wk, then injected with 410.4 tumor cells as described previously. Mice were weighed and tumors measured weekly. On 39, 42 and 45 d postinjection, five mice per dietary treatment were killed by ether inhalation. The spleen and tumor from each mouse were removed, weighed and placed in ice-cold MEM. A portion of each tumor was used for PGE synthesis determination. Splenocytes were isolated from spleens and CMC activity determined as described in detail elsewhere (7). Cytotoxic T-lymphocyte activity was assessed using 410.4 cells as targets in a 4- and 16-h <sup>51</sup>Cr release assay. Pulmonary and extrapulmonary metastases were enumerated as previously described.

**Experiment 4.** Fifteen mice from each treatment (CO, LO, FO) were injected with tumor cells (410.4) as described previously. Primary tumors were allowed to grow unimpeded until death of the mouse. Survival, in days postinjection, was recorded.

**Statistical analysis.** Data concerning tumor growth, PGE production and fatty acid profile were analyzed by one-way ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC). Incidence data (i.e., tumor and metastases) were analyzed by Chi-square (28). When significant differences occurred ( $p < 0.05$ ), treatment mean differences were identified by Fisher's least significant difference (LSD).

## RESULTS

In experiment 1, 18 of 30 mice developed tumors after receiving injection of 410 cells. Tumor incidence was not significantly different between diet treatments (Table 2). Tumors from CO-fed mice were larger in diameter than those from FO-fed mice, with LO tumors being intermediate in size. Tumor weights followed the same trend as mean tumor diameters, with CO > LO > FO. The incidence of pulmonary and extrapulmonary metastases was higher than expected. Differences between diet treatment groups were not significant ( $p > 0.10$ ). However, the overall trends suggest that a lower tumor burden in FO-fed mice compared with CO-fed mice, with LO-fed mice being intermediate.

In experiment 2, in which 410.4 tumor cells were used, all mice developed tumors at the site of injection (Table 3). Tumors grew faster and after 8 wk were larger in diameter ( $p < 0.05$ ) in the CO-fed mice than in the LO-fed mice. The average weight of the tumors on CO-fed mice was greater ( $p < 0.005$ ) than on the LO-fed mice. At the time of surgery, the mean tumor diameter of the CO-fed mice was slightly greater ( $p < 0.05$ ) than that of the LO-fed mice. Excluding the three CO and the two LO-fed mice that died within 24 h after surgery, length of survival after removal of the primary tumor was shorter for the CO-fed mice than for the LO-fed mice. CO-fed mice on the average had threefold more metastatic foci in their lungs than did LO-fed mice. However, the number of foci varied greatly and not all lungs could be examined.

TABLE 3

*Growth, weight and metastasis of 410.4 mammary tumors in BALB/c mice fed a corn oil (CO) or linseed oil (LO)-containing diet with surgical removal of the primary tumor<sup>1</sup>*

	Dietary fat		<i>p</i> value <sup>2</sup>
	CO	LO	
# Mice/# tumor-bearing mice	12/12	12/12	
Mean tumor diameter, <sup>3</sup> mm			
Week 2	3.6 ± 0.3 <sup>4</sup>	3.7 ± 0.4	ns
Week 3	5.0 ± 0.6	5.1 ± 0.5	ns
Week 5	8.4 ± 0.5	7.9 ± 0.5	ns
Week 6	10.3 ± 0.5	8.9 ± 0.5	0.06
Week 8	12.6 ± 0.4	10.9 ± 0.6	0.04
Surgery time, d postinjection <sup>5</sup>	54.5 ± 1.6	63.5 ± 2.5	0.006
Tumor weight, mg	445 ± 53	239 ± 35	0.005
Survival time, d <sup>6</sup>	12.8 ± 2 (n = 9)	17.8 ± 2 (n = 9)	0.07
Mean no. lung foci	66 ± 34 (n = 5)	20 ± 7 (n = 10)	ns

<sup>1</sup>Mice were fed CO or LO diets for 8 wk postweaning, prior to receiving subcutaneous injections of  $9 \times 10^5$  410.4 tumor cells.

<sup>2</sup>Length × width divided by 2.

<sup>3</sup>As determined by ANOVA; ns = not significant,  $p > 0.10$ .

<sup>4</sup>All values represent mean ± SEM, (n = 12).

<sup>5</sup>Tumors were surgically removed upon reaching a width of 10 mm.

<sup>6</sup>Number of days after surgery mice survived, excluding mice who died within 24 h of surgery.

The results from experiment 3 are shown in Table 4. Growth, weight and metastasis of the 410.4 tumors were significantly affected by both sources of (n-3) fatty acids. Four weeks after transplantation, tumors were

significantly smaller ( $p < 0.05$ ) in the LO-fed mice than in the CO- and FO-fed mice. At the time of killing (d 39–45), mean tumor diameters of LO-fed mice remained significantly smaller than those of CO-fed mice, with

TABLE 4

*Growth, weight and metastasis of 410.4 mammary tumors in mice fed three different sources of fat<sup>1</sup>*

	Dietary fat		
	CO	LO	FO
# Mice/# tumor-bearing mice	15/15	15/15	15/15
Mean tumor diameters, mm			
d 28 postinjection	7.5 ± 0.4 <sup>a</sup>	5.6 ± 0.8 <sup>b</sup>	7.4 ± 0.5 <sup>a</sup>
d 39 postinjection	10.1 ± 0.4 <sup>a</sup>	8.1 ± 0.8 <sup>b</sup>	9.1 ± 0.5 <sup>ab</sup>
Body weight, g	22.5 ± 0.5	23.5 ± 0.6	23.4 ± 0.6
Tumor weight, mg	400 ± 40 <sup>ab</sup>	239 ± 44 <sup>b</sup>	467 ± 99 <sup>a</sup>
Spleen weight, mg	517 ± 34	492 ± 71	629 ± 104
Splenocyte, yield, $\times 10^{-7}$ cells	8.0 ± 1.2	7.3 ± 1.6	8.6 ± 1.4
Lung metastasis			
Incidence, % <sup>2</sup>	40% (6/15)	7% (1/15)	27% (4/15)
Foci, #	1, 2, 3, 9, 11, 11	5	1, 6, 7, >50

<sup>1</sup>Mice were fed diets containing corn oil (CO), linseed oil (LO) or fish (menhaden) oil (FO) for 3 wk postweaning, prior to 410.4 tumor cell injections ( $9 \times 10^5$  cells/mouse). Values represent mean ± SEM; different letters denote statistical significance (ANOVA;  $p < 0.05$ ). When significant differences occurred ( $p < 0.05$ ), treatment mean differences were identified by Fisher's LSD.

<sup>2</sup>Incidence not statistically different among diet groups (Chi-square w/2 df = 2.595,  $p > 0.10$ ).

TABLE 5  
Fatty acid composition of tumors removed from BALB/c mice fed two different sources of (n-3) fatty acids<sup>1</sup>

	410			SEM	410.4			SEM
	CO (n = 4)	LO (n = 3)	FO (n = 4)		CO (n = 4)	LO (n = 3)	FO (n = 4)	
% of total fatty acids								
14:0	1.4 <sup>b</sup>	1.2 <sup>b</sup>	3.1 <sup>a</sup>	0.2	1.3 <sup>b</sup>	1.4 <sup>b</sup>	3.5 <sup>a</sup>	0.3
16:0	13.9 <sup>ab</sup>	11.7 <sup>b</sup>	15.4 <sup>a</sup>	0.02	12.7 <sup>b</sup>	11.3 <sup>b</sup>	16.0 <sup>a</sup>	0.9
18:0	5.3	5.2	4.0	0.08	2.2	1.8	2.1	0.2
20:0	0.3	0.2	0.2	0.04	0.1	nd	0.2	0.07
16:1	6.5 <sup>b</sup>	6.3 <sup>a</sup>	12.0 <sup>b</sup>	0.08	13.2 <sup>b</sup>	15.8 <sup>b</sup>	24.4 <sup>a</sup>	0.9
18:1	29.6 <sup>b</sup>	40.6 <sup>a</sup>	36.1 <sup>ab</sup>	3.4	31.0	35.0	28.4	2.0
20:1	0.9 <sup>b</sup>	1.2 <sup>ab</sup>	0.8 <sup>b</sup>	0.01	0.6 <sup>a</sup>	0.6 <sup>a</sup>	0.4 <sup>b</sup>	0.05
18:2(n-6)	25.1 <sup>a</sup>	14.4 <sup>b</sup>	12.2 <sup>b</sup>	0.6	30.6 <sup>a</sup>	12.9 <sup>b</sup>	11.9 <sup>b</sup>	0.8
18:3(n-6)	1.4	0.6	1.5	0.07	0.1 <sup>a</sup>	0.04 <sup>b</sup>	nd	0.03
20:2(n-6)	0.6 <sup>a</sup>	0.3 <sup>ab</sup>	0.2 <sup>b</sup>	0.1	0.3	0.2	0.2	0.05
20:3(n-6)	0.7	0.6	0.5	0.1	0.3	0.2	0.3	0.05
20:4(n-6)	7.2 <sup>a</sup>	3.4 <sup>ab</sup>	2.2 <sup>b</sup>	1.3	2.5 <sup>a</sup>	0.9 <sup>b</sup>	1.1 <sup>b</sup>	0.3
22:4(n-6)	1.0	nd	nd	0.02	tr	nd	nd	
22:5(n-6)	0.7	nd	nd	0.02	0.4	nd	nd	0.01
Total (n-6)	36.6 <sup>a</sup>	19.4 <sup>a</sup>	16.5 <sup>b</sup>	1.4	34.2 <sup>a</sup>	14.2 <sup>b</sup>	13.5 <sup>b</sup>	0.8
18:3(n-3)	0.2 <sup>b</sup>	5.8 <sup>a</sup>	0.4 <sup>b</sup>	0.05	0.3 <sup>b</sup>	14.0 <sup>a</sup>	0.6 <sup>b</sup>	0.2
20:5(n-3)	0.3 <sup>b</sup>	1.2 <sup>ab</sup>	1.9 <sup>a</sup>	0.03	0.04 <sup>c</sup>	0.7 <sup>b</sup>	2.1 <sup>a</sup>	0.1
22:5(n-3)	0.4 <sup>b</sup>	1.3 <sup>a</sup>	1.8 <sup>a</sup>	0.02	0.2 <sup>b</sup>	0.6 <sup>ab</sup>	0.9 <sup>a</sup>	0.1
22:6(n-3)	1.4	2.8	3.3	0.6	0.5 <sup>b</sup>	1.0 <sup>b</sup>	1.9 <sup>a</sup>	0.2
Total (n-3)	2.3 <sup>c</sup>	11.2 <sup>a</sup>	7.3 <sup>b</sup>	1.0	1.0 <sup>c</sup>	16.3 <sup>a</sup>	5.4 <sup>b</sup>	0.4
(n-6)/(n-3)	15.9 <sup>a</sup>	1.7 <sup>b</sup>	2.3 <sup>b</sup>	2.4	34.2 <sup>a</sup>	0.9 <sup>c</sup>	2.5 <sup>b</sup>	1.1

<sup>1</sup>Mice were fed diets containing 10% by weight corn oil (CO), linseed oil (LO) or a fish (menhaden) oil-corn oil mix (FO) for 10 wk prior to subcutaneous transplantation of  $9 \times 10^5$  410 or 410.4 tumor cells. Values are expressed as means; values in a row for a particular tumor cell line with different letters are significantly different ( $p < 0.05$ ) with  $a > b > c$  as determined by ANOVA and Fisher's LSD comparisons; nd = not detectable; tr = trace.

tumors on FO-fed mice being intermediate in size. The extent of pulmonary metastases paralleled the tumor growth data, with CO > FO > LO.

The results of experiment 4 were as follows: Mean survival, in days (range), posttransplantation was 75.3 (35–97), 75.8 (56–101) and 75.1 (60–95) for the CO-, LO- and FO-fed mice, respectively.

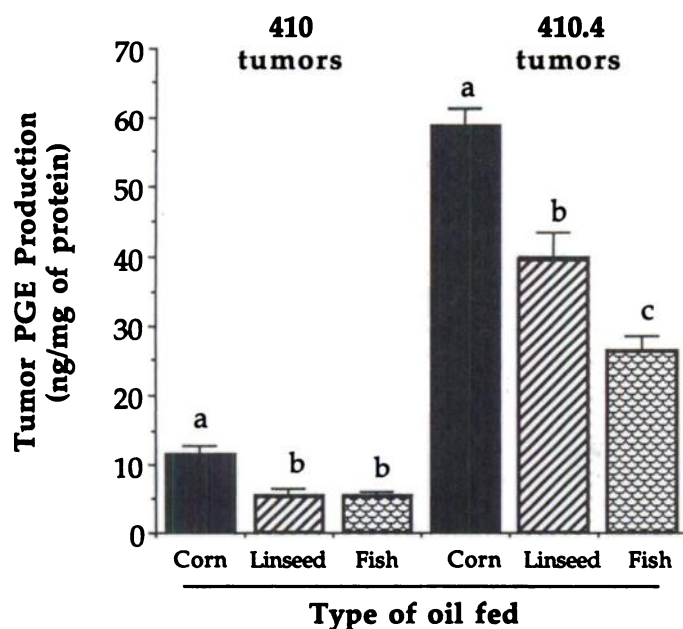
Diet treatments significantly altered the composition of both mammary tumors, with the most notable changes occurring in (n-6) and (n-3) fatty acids (Table 5). The proportion of 18:2(n-6) in both tumor types was twofold greater in CO-fed compared with LO- and FO-fed mice. The 18:3(n-3) levels were elevated only in tumors from LO-fed mice. The relative content of 20:4(n-6) in 410 tumors was decreased by 70% for FO-fed and 53% for LO-fed compared with the CO-fed mice. Furthermore, the proportion of 20:5(n-3) in 410 tumors was 6.3-fold higher in the FO group and fourfold higher in LO-fed compared with CO-fed mice. Similarly, the proportion of 20:5(n-3) in 410.4 tumors was highest in FO-fed mice, threefold lower in LO- vs. FO-fed mice, and nearly undetectable in CO-fed mice. There was a two-

fold increase in the proportion of 22:6(n-3) in 410.4 tumors between the CO- and LO-fed mice as well as another twofold increase between the LO- and FO-fed mice.

PGE synthesis by 410 and 410.4 tumors was significantly lower in LO- and FO-fed mice than in CO-fed mice ( $p < 0.005$ ). After 1 h of incubation at 37°C, tumor homogenates contained 19.5, 10.1 and 9.7 ng of PGE/g of 410 tumor (n = 4) and 129.1, 61.7 and 56.3 ng of PGE/g of 410.4 tumor (n = 9) from CO-, LO- and FO-fed mice, respectively. When these results are expressed as nanograms of PGE per milligram of tumor protein, they indicate that FO feeding reduced PGE production by 410.4 tumors almost twice as much as LO feeding, and PGE production by 410 tumors was reduced similarly in both LO- and FO-fed mice (Fig. 1).

Natural killer (NK) activity in the mice was relatively low, ranging from 1–4% in tumor-free mice to undetectable levels in tumor-bearing mice (data not shown). This reduction in splenic natural killer activity by the presence of tumors was statistically significantly ( $p < 0.001$ ), but differences between diet treatments were not. We





**FIGURE 1** PGE production by tumor homogenates. Immediately following removal a portion (0.2–0.5 g) of each tumor was minced and quickly homogenized in medium without serum. The ratio of medium to tumor tissue was kept constant (2 mL/0.1 g). The homogenate was incubated at 37°C for 1 h. Protein and PGE content of the homogenate were determined as described. Values are expressed as the mean  $\pm$  SEM ( $n = 4$  for 410;  $n = 9$  for 410.4). Values for a particular tumor type with different letters are significantly different ( $p < 0.05$ ) as determined by ANOVA with means separated by Fisher's LSD.

were unable to detect any significant cytotoxic activity against 410.4 tumors cells by splenocytes from 410.4 tumor-bearing mice.

## DISCUSSION

Unlike many previous investigations, our study assessed the effect of linolenic acid, as well as 20:5(*n*-3) and 22:6(*n*-3), on mammary tumor growth in the presence of adequate essential fatty acids. We have demonstrated that feeding mice a diet rich in linolenic acid reduced the growth of both mammary tumors (410 and 410.4) studied. Although LO- and FO-fed mice showed fewer lung and extrapulmonary metastases, these data are equivocal. Others have observed that metastasis of these tumors is related to tumor size (A. M. Fulton, Michigan Cancer Foundation, personal communication). Because the metastasis data parallel the tumor size data, it is difficult to separate the effects of diet treatments on metastasis from tumor growth. In experiment 2, we attempted to examine metastasis while controlling for differences in tumor growth. Despite allowing tumors to reach the same mean tumor diameter, tumors from LO-fed mice weighed significantly less than those from CO-fed mice. This lack of correlation

between tumor diameter and tumor weight was observed throughout these studies. This was primarily a consequence of differences in tumor thickness. For this reason, we believe the effect of our dietary treatments on tumor growth is best assessed by tumor weight rather than tumor diameter.

The mechanism(s) by which (*n*-3) fatty acids reduce tumor growth is still unknown. It is generally thought that (*n*-3) fatty acids exert their physiological effects primarily through changes in arachidonic acid metabolism, specifically eicosanoid production. It has been shown that there is a strong negative correlation between the extent of PG inhibition and tumor growth (29, 30). It is of interest that Hubbard et al. (20) recently demonstrated that linoleic acid enhanced the growth and metastasis of a mammary tumor cell line cloned from the 410.4 tumor line. They also showed that blocking PG synthesis with indomethacin counteracted the linoleate-enhanced metastasis of these tumors. Conversely, growth was not affected by the inhibition of PG synthesis. In our studies with 410.4 tumor cells, primary tumor growth and metastasis was lowest in the LO-fed mice, despite a greater reduction in tumor PGE synthesis in FO- vs. LO-fed mice. Our results, as well as those of Hubbard et al. (20), suggested that the growth-retarding effect of dietary linolenic acid on this tumor is only partly related to PG inhibition. Dietary linolenic acid may have a tumor growth-retarding activity independent of its elongation and desaturation products [i.e., 20:5(*n*-3) and 22:6(*n*-3)]. Such a possibility would best be tested in an animal with minimal desaturase activity.

Both mammary tumors studied demonstrated that their fatty acid composition reflected the differences in the dietary fats. Interestingly, 20:4(*n*-6) content of tumors was similarly reduced by feeding tumor-bearing mice LO or FO compared with CO diets. LO feeding resulted in 20:5(*n*-3) and 22:6(*n*-3) incorporation in 410 tumors at levels similar to FO feeding. However, 410.4 tumors from LO-fed mice incorporated significantly less 20:5(*n*-3) and 22:6(*n*-3) than the same tumor from FO-fed mice. It is not clear why these two tumors should differ in their incorporation of (*n*-3) fatty acids. It is possible that the desaturase activity of these tumors differs or that they differentially affect liver desaturase activity. Recently, Chapkin et al. (31) reported differences in  $\Delta$ -5 desaturase activity between two mammary tumor cell lines, 4526 and 168, which are related to the cell lines used in these studies. In any case, there is a strong correlation between tumor 20:5(*n*-3) and 22:6(*n*-3) content and PGE production. This is in agreement with the findings of Culp et al. (32) and Corey et al. (33), who have shown 20:5(*n*-3) and 22:6(*n*-3) to be potent inhibitors of PG biosynthesis.

Another objective of these experiments was to determine whether differences in tumor growth or metastasis were related to diet-induced alterations of CMC activity. We previously reported that CMC activity after a

viral challenge was greater in LO-fed mice than in CO- or FO-fed mice (7). The observed splenomegaly in tumor-bearing mice provided evidence for the involvement of the immune system and the inflammatory nature of these tumors. Natural killer activity in 410.4 tumor-bearing mice was suppressed compared with that in tumor-free control mice. This is in agreement with previous reports that in mice bearing a variety of tumors there are significantly reduced levels of NK activity (34). That the administration of indomethacin to tumor-bearing mice prevents this suppression of NK activity has been documented (10, 35). Our results indicate that despite large differences in tumor PGE-synthesizing capacity, immunosuppression of NK reactivity was similar in all diet treatment groups. However, our inability to detect any differences in NK activity may, in part, be related to the relatively low level of NK activity that is normally present in mice after 5–8 wk of age (34). Also, we were unable to detect any significant cytolytic activity against 410.4 cells in 410.4 tumor-bearing mice. It is our experience that 410.4 cells are poor targets in CMC assays, which is in agreement with others (19). Because immunosuppression in tumor-bearing mice is so overwhelming, it would have been of interest to examine the recovery of CMC activity after the removal of the primary tumor or upon stimulation of NK or CMC activity above basal levels. It is possible that under such circumstances diet-related differences in CMC activity may have been apparent.

Changes in tumor cell susceptibility to cytolysis may be of equal importance in determining diet-induced modifications in tumor growth. Fulton and Heppner (19) demonstrated that inhibition of tumor cell PG synthesis by indomethacin increased the sensitivity of 410 and 410.4 tumor cells to cell-mediated cytolysis. In addition, Gabor and Abraham (3) showed that the reduction in mammary tumor growth by fish oil feeding was associated with an increase in tumor cell loss. This raises the possibility that dietary (*n*-3) fatty acids may influence tumor growth through changes in target cell properties (e.g., increased susceptibility to lytic damage) as well as through effector cell function (e.g., relieving PGE-induced immunosuppression). Additional studies, in which tumor cells are grown *in vitro* in the presence of specific fatty acids, could clarify the effect that dietary (*n*-3) fatty acids may have on lytic susceptibility, independent of host effector cell function.

Dietary (*n*-3) fatty acids may affect tumor growth by altering the synthesis of leukotrienes (LT). Recent evidence shows an essential function for one particular LT, LTB<sub>4</sub>, in the lytic processes of cytotoxic effector cells (36). Therefore, dietary (*n*-3) fatty acids may concomitantly affect inhibitory (e.g., PGE<sub>2</sub>) and stimulatory (e.g., LTB<sub>4</sub>) signals involved in the regulation and expression of CMC activity.

In summary, we have demonstrated that feeding mice a diet rich in linolenic acid reduced the growth, and to

some extent the metastasis, of two related transplantable mammary tumors. Further research is needed in this area to better understand the role that various eicosanoids play in tumor growth and metastasis and how individual dietary fatty acids may affect these processes.

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