

PROSTAGLANDINS LEUKOTRIENES AND ESSENTIAL FATTY ACIDS

Prostaglandins Leukotrienes and Essential Fatty Acids (1993) 48, 305-308
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Ingestion of Marine Oil Reduces Excretion of 11-Dehydrothromboxane B₂, an Index of Intravascular Production of Thromboxane A₂

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ABSTRACT. We evaluated the effect of anchovy oil supplementation on the endogenous production of thromboxane A₂ by measuring the excretion of its stable metabolite, 11-dehydrothromboxane B₂ (11-DTXB₂), in 24-h urine. In a longitudinal study, 35 male volunteers consumed a controlled basal diet for two experimental periods lasting a total of 20 weeks. During period 1 (10 weeks) the diet was supplemented with placebo (PO) capsules (15 × 1 g/d) consisting of a blend of fats approaching the fatty acid profile of the basal diet. During period 2 the subjects received 15 × 1 g/d capsules of fish oil concentrate (FOC). PO and FOC capsules contained 1 mg alpha-tocopherol per gram of fat as antioxidant. A 38% reduction of 11-DTXB₂ excretion was observed after 10 weeks of FOC supplementation (period 2, n-6/n-3 PUFA ratio = 2.3), compared to an identical period of PO supplementation (period 1, n-6/n-3 = 12.5), $p = 0.0001$. The 11-DTXB₂ excretion reduction (Δ) fits the quadratic equation $\Delta = 136.0038 - 0.3178(\text{tx}1) - 0.0002(\text{tx}1)^2$, ($R^2 = 0.8944$), where tx1 is the excretion rate at the end of period 1. This finding supports the hypothesis that the antithrombotic effect of marine oil is mediated, at least in part, by diet-induced shifts in the eicosanoid system.

INTRODUCTION

The ability of lipid components of diets to modulate the eicosanoid system has been an issue of intense interest among nutritional biochemists and clinicians during the last 15 years. This interest is justified by the demonstrated roles of several members of the arachidonic acid cascade in atherosclerosis and thrombotic events (1-3), in immunoregulation (2, 4, 5), and in tumorigenesis (6, 7). Thromboxane A₂ (TXA₂) and prostacyclin (PGI₂) are of special importance in reference to cardiovascular biology: TXA₂, which is largely (about 80%) synthesized by platelets, is one of the most powerful vasoconstrictor and platelet agonist while PGI₂, synthesized mainly by vascular endothelium, is a potent vasodilator and inhibitor of platelet aggregation. The antagonistic activity of these two eicosanoids prompted Vane et al (8) to hypothesize that a chronic imbalance in the formation of TXA₂ and PGI₂ might have profound effects in thrombogenesis and atherosclerosis. It seems reasonable to assume that any dietary component that reduces the synthesis of TX is likely to reduce the incidence of fatal cardiovascular events (9).

We at Lipid Nutrition Laboratory have focused in the past several years on the effect of dietary manipulations on the production of prostaglandin (PG) E at sites easily accessible to systemic clearance (10-14). In the present study we determined the effect of an anchovy oil supplementation on the excretion of 11-dehydrothromboxane B₂ (11-DTXB₂), an index of endogenous production of TXA₂ in human circulation (15).

MATERIALS AND METHODS

Subjects

The 35 subjects that completed this study were recruited, screened and selected as previously described (13). They were required not to ingest any drug formulation containing aspirin or other antiinflammatory agents and to report any antibiotic and other medications prescribed by a physician during the study for evaluation for possible effects on TX production. All procedures were approved by the Human Studies Committee of the National Institutes of Health and the Georgetown University School of Medicine.

Controlled diets

The basal diet used during the dietary intervention

periods was designed to reflect the typical dietary pattern of Beltsville area volunteers as determined in earlier studies, except that the fat level of the diet was reduced to allow isoenergetic supplementation with 15 g/day placebo oil (PO) or fish oil concentrate (FOC). The PO consisted of commonly available fats, and FOC was an anchovy oil derivative. They were prepared and administered as described earlier (13). Constant body weights were maintained by adjusting caloric intakes, and all nutrients were provided by the diets in amounts to meet the Recommended Dietary Allowances. The estimated intake of nutrients for both periods was (in percent of energy): fat, 40%; carbohydrate, 45%; protein, 15%. The fatty acid composition of placebo and fish oil supplements, and the estimated daily fatty acid intake on controlled diets were published previously (13).

Experimental protocol

The controlled diets described above were provided for a total of 20 weeks divided in two periods according to the supplement given: period 1 (10 weeks) 15 g/day PO, and period 2, 15 g/day FOC.

Urine collection

Three consecutive 24-h urines were collected during the last week of each period. Samples were collected in silanized glass bottles and kept on ice during the collection period. After the 24-h collections were completed, the volumes were measured, 2% portions of each 24-h collection were pooled and stored at -22°C until analyzed.

Measurement of 11-DTXB₂

Quantitative analyses of 11-DTXB₂ were done on 10-ml portions of the 72-h pools obtained as described above. This enabled us to assess the mean daily excretion of 11-DTXB₂ during the 72-h periods. Instruments and detailed procedures have been described elsewhere (16). In short, 10 ml of pooled urine, after addition of 9 ng of tetradeutero-11-DTXB₂ as internal standard, was acidified (pH 2.7) and extracted with a SepPak C₁₈ cartridge. The analyte (along with the internal standard) recovered from the cartridge, was methylated with diazomethane and, after several purification steps including water/ethyl acetate partitions and TLC, was delactonized and converted to the 11-pentafluorobenzyl ester 9,12,15-tris(trimethylsilyl) ether derivative by treatment with pentafluorobenzyl bromide followed by *N,O*-bis(trimethylsilyl) trifluoroacetamide. Quantification was achieved by capillary gas chromatography-tandem mass spectrometry with a Varian 3400 gas chromatograph interfaced with a Finnigan-MAT TSQ-70B triple stage mass spectrometer operated in the negative ion detection mode with methane used as ionization gas. For MS-MS

analysis we used the pair of daughter fragments at *m/z* 345 and *m/z* 349 (P^- (3×90)) of the parent ions (P^-) at *m/z* 615 and *m/z* 619 ($[M-C_6F_5CH_2]^-$).

Statistical analysis

We analyzed the results using paired observations, i.e. the differences between 11-DTXB₂ excretion rate at the end of period 2 and the rate at the end of period 1. The mean difference was evaluated by the *t*-test and the non-parametric signed rank test. Pearson and Spearman correlations between the paired difference, age, weight and body mass index (BMI) were determined.

RESULTS

The 11-DTXB₂ excretion rate at the end of period 2 (FOC supplementation) was lower than the rate at the end of period 1 (PO supplementation) in 32 out of 35 subjects (Table). The mean 11-DTXB₂ excretion (ng/24 h) during the last week of periods 1 and 2 were 826 ± 404 (SD) ($N = 35$) and 515 ± 155 (SD) ($N = 35$),

Table 1 11-DTXB₂ excretion rates (ng/24 h) during the last week of the indicated diet period^a

Subject	Diet period		% Reduction ^b
	1	2	
1	792	537	32.2
2	968	608	37.2
3	938	622	33.7
4	1174	890	24.2
5	625	451	27.8
6	822	465	43.4
7	702	679	3.3
8	388	730	-88.1
9	743	348	53.2
10	1056	716	32.2
11	930	663	28.7
12	68	113	-66.2
13	994	650	34.6
14	392	311	20.7
15	1415	549	61.2
16	778	388	50.1
17	649	384	40.8
18	1213	630	48.1
19	584	308	47.3
20	820	633	22.8
21	1109	605	45.4
22	2508	473	81.1
23	1056	506	52.1
24	750	457	39.1
25	882	549	37.8
26	898	470	47.7
27	797	543	31.9
28	618	471	23.8
29	480	586	-22.1
30	395	323	18.2
31	1056	545	48.4
32	656	475	27.6
33	661	489	26.0
34	641	623	2.8
35	335	228	31.9

^a Mean daily excretion during a 72-h period (for details, see Materials and Methods).

^b Percent reduction calculated as ((period 1 - period 2)/period 1) \times 100

respectively. The mean reduction (311 ± 63 (SEM) ng/24 h) was evaluated with the t-test and the signed rank test. Both tests indicated that the reduction was highly significant ($p = 0.0001$).

Pearson and Spearman correlations between the paired difference, age, weight and BMI showed no linear relationship. Quadratic polynomial regression models of age, weight and BMI did not explain the observed variability in the paired difference. The 11-DTXB₂ excretion reduction $\Delta = tx_2 - tx_1$ fits the quadratic equation $\Delta = 136.0038 - 0.3178(tx_1) - 0.0002(tx_1)^2$, ($R^2 = 0.8944$), where tx_1 and tx_2 are the excretion rates at the end of periods 1 and 2, respectively. Thus, the drop in 11-DTXB₂ excretion is greater in subjects with higher initial values.

DISCUSSION

This *in vivo* human diet study is part of a larger study conducted with healthy subjects, and is unique in terms of number of participating volunteers, design and duration. Its purpose was to determine if a shift in the n-6/n-3 dietary PUFA ratio from 12.5 to 2.3 brings about a significant modulation of TXA₂ synthesis. Originally we planned to determine the effect of fish oil supplementation on the TXA₂/PGI₂ synthetic ratio by measuring both 11-DTXB₂ and 2,3-dinor-6-oxo-prostaglandin F_{1 α} (PGI₂-M) excretion. However, the objective of assessing PGI₂-M could not be realized because we were unable to develop an assay system of sufficient precision. We found that an anchovy oil concentrate, administered at the rate of 15 g/day for 10 weeks sharply reduces TXA₂ production as measured by the excretion of its major metabolite, 11-DTXB₂. The mean reduction (38%) is comparable to that which is obtained by administering about 10 mg of aspirin daily (17). These results are supportive of the hypothesis that the antithrombotic effect of marine oil is mediated, at least in part, by diet-induced shifts in the eicosanoid system. Results of previous investigations from other laboratories also indicate that long-chain PUFA of the n-3 series tend to depress both endogenous and *ex-vivo* synthesis of TXA₂ as determined by the excretion of 2,3-dinor-thromboxane B₂ (another TX metabolite) or by direct measurement of TXB₂ after platelet stimulation (18–24).

Subjects with atherosclerosis produce more TXA₂ than healthy ones (25). On the other hand, our study indicates that TX reduction was greater in subjects with high initial TXA₂ production according to the quadratic relationship reported under Results. Thus, it appears that subjects at higher risk are likely to benefit more from fish oil ingestion. One might speculate that the results observed in this study were caused by some unknown component present in the FOC or by absence of dietary components replaced by FOC. This possibility is, in our view, remote because the n-3 fatty acids used by us and by previous investigators were obtained from a wide

variety of dietary sources, and their ingestion invariably resulted in TXA₂ reduction.

The magnitude of TX reduction observed in this study (mean, 38%) is likely to be physiologically significant. Indeed, based on its effect on TX, marine oil can be a dietary tool for lowering blood clotting tendency without the disadvantages associated with use of aspirin and other drugs. However, a more definitive judgement will be possible when present uncertainty on the effect of n-3 PUFA on prostacyclin production will be resolved. We isolated and identified 11-DTXB₃ in urine of volunteers at the end of period 2. Procedural details are described elsewhere (26). The presence of this metabolite of TXA₃ in human urine was first reported by Ishibashi et al (27) after administration of eicosapentaenoic acid.

Acknowledgments

We are indebted to Cmdr W. Campbell, USPHS, and M. Sunkin for keeping medical records, for recruiting, instructing and supervising the subjects, and for blood drawing; to E.J. Maida for competent assistance in conducting the analyses; and to Mary Camp for the statistical analysis. This study was supported in part by a Grant-in-aid from Hoffmann-La Roche Inc, Nutley, NJ.

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Prostaglandins Leukotrienes and Essential Fatty Acids (1993) 48, 309-314
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Plant and Marine n-3 Fatty Acids Inhibit Experimental Metastasis of Rat Mammary Adenocarcinoma Cells

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ABSTRACT. The effectiveness of dietary n-3 plant and marine fatty acids and n-6 gamma-linolenic acid (GLA) was tested as an antimetastatic modality in the experimental model of metastasis of 13762MAT:B mammary adenocarcinoma cells. Weanling female Fischer 344 rats were placed on one of the following diets: I—23.52% blackcurrant oil (BCO), II—23.52% corn oil (CO), III—15.52% BCO + 8% fish oil (FO), IV—20.52% FO + 3% CO, and V—5% CO. After 8 weeks, 15 rats per group were injected i.v. with 10^5 cells and diets were continued until sacrifice. In the 23.52% CO group (II), the number of small (<2 mm) and large (>2 mm) lung metastatic foci and their total volume were significantly greater than the BCO- and/or FO-fed groups (I, II and IV). Although the number of small metastatic foci was comparable in the 5% and 23.52% CO groups, the number of large foci and the total tumor volume were reduced in the 5% CO group. These results suggest that, compared to a low-corn oil diet or a high-fat diet containing a mixture of marine and plant n-3 fatty acids plus n-6 GLA, a 23.52% corn oil diet can enhance experimental metastasis of mammary adenocarcinoma cells. Total number of metastatic foci and tumor volume were the smallest in group III, receiving a combination of plant and marine n-3 fatty acids.

INTRODUCTION

The role of dietary fat in the development of some cancers has been investigated in controlled studies in humans and animal tumor models (1, 2). While significant advances have been made in understanding the effects of different amounts and types of fat on tumor development and growth, few experiments have investigated these effects on development of metastatic disease (3).

When metastatic cancer cells have disseminated to other parts of the body, then surgical treatment is often not curative. The response to adjuvant chemotherapy and hormonal therapy, although beneficial, remains limited (4). Recently, it has been reported by several investigators that n-3 polyunsaturated fatty acids (PUFA) in fish oil inhibit development and progression of a number of tumor types in animals. This topic has been reviewed (5). Data reported to date indicate that marine n-3 PUFA displace linoleic acid (LA) and arachidonic acid (AA) in tumor cell membranes and inhibit the synthesis of prostanoid metabolites of AA in normal and

cancer cells. It has been proposed that increased ratio of eicosapentaenoic acid (EPA) to AA in platelet membrane phospholipid after fish oil consumption is responsible for decreased platelet aggregation. These antiaggregatory effects are mediated by alterations in the balance of prostacyclin (PGI_2), thromboxanes (TX), prostaglandins (PG), and leukotrienes (LT) (6). These changes would be expected to alter properties of cancer cell and vascular membranes as well as platelet activity. EPA decreases release of AA from phospholipids and competes with AA at the level of cyclooxygenase and lipoxygenase steps to inhibit production of proaggregatory TXA_2 , immunosuppressive PGE_2 , and chemotactic LTB_4 .

There is evidence that platelets play an important role in metastasis formation. Tumor cells display specific properties towards platelets and the vascular endothelium (7, 8). Platelet aggregation is induced by tumor cells, and aggregating platelets elaborate growth factors that promote tumorigenesis (9). Therefore, inhibition of TXA_2 synthesis by EPA would be expected to exert antimetastatic effects. A number of experimental and

Date received 10 September 1992
Date accepted 3 December 1992

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clinical studies have been conducted to study the relationship of TXA₂, PGI₂ and PGE₂ in progression of metastatic disease (10–12). Kort et al (13) reported that, when a diet containing 7% EPA + docosahexaenoic acid (DHA) and 3% LA was fed to BN/Bi inbred female rats, growth of the transplanted BN472 primary tumor was inhibited, but there was no effect on spontaneously occurring lung metastasis compared with remaining groups maintained on low n-3 PUFA. In these studies, the primary tumors were removed when they reached a diameter of 20 mm, and the experiment was terminated 2 weeks thereafter. Boylan & Cohen employed a similar procedure to induce metastasis from 13762 mammary adenocarcinoma, but the primary tumors were left intact during the experiment. They reported large variations, and low rates of metastasis formation within groups (14). Therefore, even though spontaneous models of metastasis are closer to the clinical situation, in order to avoid variations in the flow of cancer cells from primary tumors, we employed the artificial model involving intravenous administration of cancer cells into the bloodstream.

In the present study, we chose to study two sources of n-3 PUFA:

- blackcurrant oil (BCO), a source of plant n-3 PUFA, α -linolenic acid (α -LN, 18:3)
- fish oil (FO), a source of marine n-3 PUFA, EPA (20:5) and DHA (22:6). BCO also contains 18% gamma-linolenic acid (GLA, 18:3), a desaturated derivative of LA (18:2, n-6) (15). The reason for studying GLA is because it is the precursor PUFA for dihomo-gamma-linolenic acid (DGLA, 20:3, n-6) which, like EPA, is a competitive inhibitor of AA and substrate for PGE₁, an antiaggregatory prostanoid for platelets (16).

Five different diets containing BCO, corn oil (CO), or FO were tested to determine:

1. if there was a difference in response at 5% and 23.52% CO by weight
2. the effect of different ratios of n-3 and n-6 PUFA at 23.52% fat level on metastasis formation.

MATERIALS AND METHODS

Experimental protocol

Weanling female Fischer 344 rats (Charles River Breeding Laboratory, Kingston, NJ) were housed 5 per cage and kept in a temperature- and humidity-controlled facility with a 12 h light and dark cycle. Rats were sorted into 5 groups to ensure even weight distribution. 20 rats in each group were given water and one of the test diets ad libitum for the duration of the experiment. After 8 weeks on the test diets, 15 rats in each group were injected with 10⁵ cells i.v. in the tail vein.

Body weight was recorded every 2 weeks, and all rats

were checked daily after inoculation of tumor cells. The remaining 5 rats in each diet group were maintained on the same dietary regimens.

Diets

The semi-synthetic fat-free diet mixes for 5% and 23.52% (w/w) fat levels were obtained from ICN Biochemicals, Cleveland, OH. These diets were formulated to ensure that the rats were fed minerals, vitamins and fiber based on the recommendations of the Committee on Laboratory Animal Diets of the National Academy of Sciences (17). Corn oil was provided by Best Foods, Union, NJ, fish oil by Seven Seas Health Care, Marfleet, UK and blackcurrant oil by Nestle Ltd, Lausanne, Switzerland. Diet composition and fatty acid composition of the oils tested are given in Tables 1 and 2, respectively.

Five dietary fats were tested:

- Group I — 23.52% BCO
- Group II — 23.52% CO
- Group III — 15.52% BCO + 8% FO
- Group IV — 20.52% FO + 3% CO
- Group V — 5% CO.

The diets were prepared at intervals of 3 days and stored in air-tight containers lined with heavy aluminum foil at 5 °C, since tissues have been found to incorporate

Table 1 Composition of diets containing 5% and 23.52% fat (w/w)

Nutrient	5%	23.52%
Casein	20	23.5
Corn starch	52	32.9
Dextrose	13	8.30
DL-methionine	0.3	0.35
Choline bitartrate	0.2	0.24
Alphacel	5	5.9
Mineral mix (AIN-76)	3.5	4.11
Vitamin mix (AIN-76)	1.0	1.18
Fat	5	23.52
Total	100	100
Energy value (Kcal/g)	3.89	4.73

Table 2 Fatty acid composition (%) of four dietary oil mixtures tested

	BCO I	CO II & V	15% BCO+8% FO III	20.52% FO+3% CO IV
14:0	—	—	2.36	5.24
14:1	—	—	—	—
16:0	6.88	11.84	9.97	13.17
16:1	—	—	2.89	6.69
18:0	1.30	1.35	1.83	3.14
18:1	9.20	25.14	9.64	13.61
18:2n-6	42.79	61.04	31.66	11.68
18:3n-6	17.54	1.28	12.48	—
18:3n-3	15.55	—	11.43	3.56
18:4n-3	5.2	—	5.21	6.06
20:3n-6	—	—	—	—
20:4n-6	—	—	—	1.84
20:5n-3	—	—	6.12	14.10
22:5n-3	—	—	0.72	1.63
22:6n-3	—	—	3.39	8.31

plasticers when stored in plastic bags (18). Fresh diets were fed at the same time each day, and residues left over were discarded.

Tumor cell line

The subline 13762MAT:B used for the experimental metastasis studies was obtained from Dr A. Bogden, Mason Research Institute, Worcester, MA. This line is carried as an ascites tumor that replicates rapidly and requires *in vivo* passage every 7–10 days. The line is derived from a 7,12 dimethylbenz (a) anthracene (DMBA)-induced tumor, which often retains hormone responsiveness to both estrogens and prolactin and which readily metastasizes to lungs (19). A pool of cells from four rats was washed in phosphate-buffered saline, and 10^5 cells in 0.2 ml saline were injected *i.v.* via the lateral tail vein for the experimental metastasis model.

Since survival time was one of the end-points, the animals were left in the experiment until their state became moribund, when they were sacrificed by exsanguination under anesthesia with metophane. The lungs were fixed *in situ* and processed by a lung clearing technique that allows the enumeration and sizing of sub-surface as well as surface tumor foci (20). Metastatic frequency was determined by counting tumor foci in the right superior lobe of each lung, and the volume of the nodules was calculated from their radii by the formula $\text{volume} = 4/3r^3$. Those rats surviving at 21 days after tumor inoculation were sacrificed to terminate the experiment, and the lungs were processed as described above. The remaining 5 rats in each group, which did not receive the tumor cell inoculum, were sacrificed, and spleen, heart and thymus weights were recorded. Red blood cell pellets were collected from heparinized blood samples, washed with 0.9% saline, and stored at -70°C for fatty acid measurement. Since test diets were fed for more than 8 weeks, fatty acid profiles of red blood cells were recorded. Red blood cells have a longer life span than platelets. Platelet fatty acid profiles are more useful in studying short-term changes (2 weeks) in membrane fatty acids caused by dietary fats.

Fatty acid analysis

Cold distilled water (2 ml) was added to red blood cell pellets, vortexed, and transferred to extraction tubes. Lipid extraction, separation of phospholipids by TLC, methylation of fatty acids, and measurements of FAME has been described previously (18).

Statistical analysis

End-point analyses of nine different measurements were carried out using SAS programs for ANOVA, Duncan's test, and the general linear models procedure. The nine parameters analyzed include:

1. body weight
2. organ weight
3. organ/body weight
4. total tumor foci
5. small tumor foci
6. large tumor foci
7. total tumor volume
8. survival time
9. red blood cell phospholipid fatty acids.

RESULTS

Body weight

Body weights were recorded biweekly during the 12-week feeding period for 5 normal rats and 15 rats challenged with 13762MAT:B.

Analysis of data from normal rats by ANOVA and Duncan's test indicated significant differences between dietary groups during the 12-week period. Overall, there were interesting differences in rate of weight gain between the 23.52% CO group (II) and 20.52% FO + 3% CO (IV) or 15.52% BCO + 8% FO groups (III). Weight gain in groups III and IV was significantly greater at 4 weeks compared to group II (Duncan's test: $p = 0.0001$) (Table 3).

There was no significant difference in thymus weight, but mean spleen weight for group I was significantly

Table 3 Mean body and organ weights (g, $n = 5$, \pm SD)

	Group				
	I	II	III	IV	V
Body weight at (weeks)					
0	66.2 \pm 5.2	66.6 \pm 7.0	70.0 \pm 2.4	65.5 \pm 8.7	69.1 \pm 2.7
2 ^a	105.8 \pm 3.7	108.6 \pm 1.3	112.4 \pm 2.7	110.2 \pm 5.3	105.4 \pm 2.7
4 ^b	134.0 \pm 3.5	135.2 \pm 3.4	141.0 \pm 2.7	147.0 \pm 2.9	135.8 \pm 2.6
6 ^c	139.6 \pm 1.8	139.2 \pm 2.4	142.8 \pm 3.4	150.4 \pm 2.7	142.6 \pm 3.1
8	166.0 \pm 3.7	158.7 \pm 1.5	170.4 \pm 8.3	166.7 \pm 6.1	165.6 \pm 4.6
10	169.8 \pm 1.9	164.0 \pm 5.3	173.6 \pm 7.9	170.0 \pm 4.0	169.2 \pm 6.0
12	184.8 \pm 6.2	174.4 \pm 6.1	175.1 \pm 17.3	184.6 \pm 8.2	167.1 \pm 4.7
Organ weight					
Spleen ^d	0.52 \pm 0.06	0.41 \pm 0.01	0.50 \pm 0.06	0.51 \pm 0.06	0.42 \pm 0.04
Heart ^e	0.57 \pm 0.08	0.59 \pm 0.01	0.52 \pm 0.03	0.52 \pm 0.03	0.50 \pm 0.04
Thymus	0.26 \pm 0.07	0.26 \pm 0.02	0.26 \pm 0.04	0.29 \pm 0.03	0.27 \pm 0.05

ANOVA: a) $p = 0.02$ (III from I); b) $p = 0.0001$ (III & IV from I, II, & V); and c) $p = 0.0001$ (IV from all remaining); d) $p = 0.02$ (I from II) and e) $p = 0.007$ (IV from V).

higher than that of group II and mean heart weight for group IV was significantly greater than that of group V (Table 3).

Trends in body weight gain in 15 rats used for tumor studies (tumor implantation at 8 weeks postfeeding test diets) were similar to those in 5 normal control rats of comparable age and maintained on similar diets.

Pulmonary metastases

At necropsy, 14 out of 15 rats in groups I, III and V, and 13 out of 15 rats in groups II and IV contained visible metastases. The total number of metastatic foci were significantly higher in group II receiving 23.52% CO diet ($p = 0.0026$) (Table 4). When metastatic foci < 2 mm were analyzed, two groups receiving CO diets (II and V) had significantly higher numbers of foci compared with groups receiving FO (III and IV). The number of large metastatic foci was highest in group II (23.52% CO) ($p = 0.0219$).

Total volume of metastatic foci was the largest in group II (23.52% CO) compared with remaining groups ($p = 0.0073$). There was no evidence of extra pulmonary metastasis with the exception of one enlarged lumbar

lymph node in a rat from group II. Survival time after i.v. injection of tumor cells was the shortest in group V (5% CO) ($p = 0.04$), but was similar in the remaining groups.

Comparing the low (group V) and high (group III) CO diets, the number of large metastatic foci and total tumor volume were greater in the high-fat group, even though the numbers of small metastatic foci were comparable in the two groups.

Fatty acid composition of red blood cell phospholipids

Changes in fatty acid composition of red blood cells were monitored because they reflect changes in platelets and other tissues to a certain extent.

AA content of phospholipids was lower in rats fed the high FO diet (group IV) than in those fed CO diets (Table 5). The FO diet lowered the proportion of AA but there was no change with feeding the BCO diet. LA levels were in the order (highest \rightarrow lower): II \rightarrow I \rightarrow III \rightarrow V \rightarrow IV. The percentage of EPA, docosapentaenoic acid (DPA), and DHA in phospholipids was higher in rats fed the FO or BCO diets, the change being signifi-

Table 4 Survival time, number of metastatic foci and total metastatic tumor volume (Mean \pm SD, $n = 15$).

Group	Time ^a (days)	Total No. of foci ^b	No. of foci < 2 mm ^c	No. of foci < 2 mm ^d	Tumor volume ^e (mm ³)
I - 23.52% BCO	20.9 \pm 0.1	6.3 \pm 2.7	3.4 \pm 2.4	2.9 \pm 2.1	16.9 \pm 15.3
II - 23.52% CO	20.2 \pm 0.2	10.3 \pm 4.8	4.5 \pm 2.5	5.8 \pm 3.4	31.4 \pm 21.4
III - 15% BCO + 8% FO	20.8 \pm 0.1	4.6 \pm 2.0	1.9 \pm 1.6	2.7 \pm 1.9	8.9 \pm 7.2
IV - 20% FO + 3% CO	20.1 \pm 0.3	5.9 \pm 3.1	2.0 \pm 2.2	3.9 \pm 3.2	16.7 \pm 14.8
V - 5% CO	18.9 \pm 1.5	7.9 \pm 5.1	5.1 \pm 4.8	2.8 \pm 2.8	16.5 \pm 14.5

^a Wilcoxon test: $p = 0.04$.

^b ANOVA: $p = 0.003$ (II significantly different from III).

^c ANOVA: $p = 0.02$ (II and V from III and IV).

^d ANOVA: $p = 0.02$ (II from I, III, and V).

^e ANOVA: $p = 0.007$ (III from I, III, IV, and V).

Table 5 Percent fatty acid composition of red blood cell phospholipid (Mean \pm SEM, $n = 3$)

Fatty acid	I	II	III	IV	V
14:0	4.31 \pm 1.61	5.39 \pm 0.60	4.03 \pm 0.34	5.54 \pm 0.72	5.37 \pm 0.85
14:1	0.16 \pm 0.05	0.01 \pm 0.01	0.19 \pm 0.04	0.14 \pm 0.06	0.25 \pm 1.17
16:0	28.07 \pm 1.64	30.08 \pm 1.05	31.19 \pm 1.07	28.88 \pm 1.33	30.25 \pm 2.22
16:1 ^a	1.96 \pm 0.65	2.47 \pm 2.73	1.05 \pm 0.42	4.08 \pm 0.31	7.00 \pm 1.98
18:0	20.15 \pm 1.53	21.92 \pm 3.11	19.76 \pm 1.56	16.39 \pm 3.83	16.47 \pm 0.69
18:1 ^b	14.88 \pm 0.99	18.35 \pm 2.62	22.11 \pm 2.66	19.73 \pm 0.42	22.87 \pm 2.14
18:2n-6 ^c	15.89 \pm 2.49	17.50 \pm 2.36	11.45 \pm 0.44	7.40 \pm 0.87	9.71 \pm 1.68
18:3n-6 ^d	2.67 \pm 0.70	0.10 \pm 0.16	0.72 \pm 0.18	0.01 \pm 0.01	0.19 \pm 0.15
18:3n-3	2.48 \pm 0.49	0.93 \pm 0.21	1.1 \pm 0.10	0.71 \pm 0.42	2.01 \pm 1.87
18:4n-3/20:1 ^e	0.77 \pm 0.68	0.01 \pm 0.01	0.14 \pm 0.01	3.59 \pm 1.04	0.01 \pm 0.01
20:3n-6 ^f	0.75 \pm 0.16	0.01 \pm 0.01	0.64 \pm 0.11	0.01 \pm 0.01	0.01 \pm 0.01
20:4n-6 ^g	5.8 \pm 2.09	3.11 \pm 1.01	3.23 \pm 0.41	1.35 \pm 0.42	5.37 \pm 1.32
20:5n-3 ^h	0.15 \pm 0.24	0.01 \pm 0.01	1.22 \pm 0.27	6.66 \pm 1.19	0.01 \pm 0.01
22:5n-3 ⁱ	1.00 \pm 0.36	0.01 \pm 0.01	0.83 \pm 0.13	1.36 \pm 0.44	0.01 \pm 0.01
22:6n-3 ^j	0.95 \pm 0.18	0.15 \pm 0.12	1.82 \pm 0.45	4.16 \pm 0.88	0.50 \pm 0.08

ANOVA, Duncan's Test: ^a $p = 0.02$. (V significantly different from I, II, and III); ^b $p = 0.005$ (V from I); ^c $p = 0.0004$ (I and II from III, IV, and V; III from IV); ^d $p = 0.0001$ (I from II, III, IV, and V); ^e $p = 0.0005$ (IV from I, II, III, and V); ^f $p = 0.0001$ (I and III from II, IV, and V); ^g $p = 0.01$ (I from IV); ^h $p = 0.0001$ (IV from III; III from I, II, and V); ⁱ $p = 0.0005$ (I, III, and IV from II and V); ^j $p = 0.0001$ (IV from I, II, III, and V; III from II and V).

cantly higher with the FO diet (IV). Alpha-linolenic acid (ALNA 18:3n-3), GLA (18:3 n-6), and DGLA were higher in BCO-fed groups than in those fed CO or FO diets.

DISCUSSION

This study was conducted to determine the influence of dietary plant and marine n-3 fatty acids, n-6 LA and n-6 GLA, on experimental metastasis. Dietary n-3 fatty acids and n-6 GLA significantly influence the development and size of pulmonary metastases. The total number of metastatic lung foci was lower in the BCO-fed group (I) than the CO-fed group (II), and, when BCO and FO were combined (III), the difference between III and II was significant. Metastatic tumor burden, measured as total volume of metastatic nodules in the lung, was significantly lower in groups fed FO and/or BCO than CO. The largest decrease was in group III receiving a combination of BCO and FO. This mixture of fat contained α -LN, GLA, EPA, DPA and DHA (Table 2) and fatty acid profiles of rbc indicate that these fatty acids were incorporated into the phospholipid fraction.

Two of the possible explanations for the marked difference in total number and volume of lung metastatic foci between the CO- and BCO/FO-fed groups are: 1) a lower content of LA in BCO and FO, and 2) biochemical interactions and competitions between the various n-3 and n-6 fatty acids. To test the first possibility, correlation analyses for dietary LA (4 diets at the level of 23.52% fat) and total number of foci and tumor volume were carried out. The relationship was not significant (Pearson correlation coefficient = 0.76 and 0.68, respectively). Similarly, correlation coefficients for GLA = -0.49 and -0.54; α -LN + 18:4 n-3 = -0.81 and -0.82; and EPA + DPA + DHA = -0.52 and -0.44, respectively. However, the inverse correlation with plant + marine n-3 fatty acids (α -LN + 18:4 + EPA + DPA + DHA) was significant = -0.91 and -0.85, respectively ($p = 0.001$). These results may explain in part why group III receiving a combination of plant and marine n-3 fatty acids had the smallest number of metastatic foci and tumor volume.

Secondly, both DGLA and EPA competitively inhibit AA metabolism and, thus, would be expected to inhibit synthesis of TXA₂ and PGE₂. The dienoic eicosanoids (TXA₂ and PGE₂) have been associated with development and/or migration of metastatic mammary tumor cells (10-12, 21). Whether the dietary fatty acids affect tumor cell dissemination, lodging, or proliferation after implantation in the lung has not been determined by this study. Whether the dietary fatty acids act directly on 13762MAT:B cells or by altering the eicosanoid pools is difficult to ascertain. However, these results support earlier reports that inhibition of TXA₂ and PGE₂ synthesis decreases metastasis formation (10, 22).

Both the number of total metastatic lung foci (NS) and

total tumor burden ($p = 0.0073$) were lower in the 5% CO (V)-fed group than the 23.52% CO (II)-fed group. These results suggest that higher levels of LA in the diet promote metastasis formation. These effects may be mediated partly by dienoic eicosanoids synthesized in the absence of competition by DHLA and/or EPA. These mechanisms yet to be investigated may provide partial explanation for the observation that BCO and/or FO were effective in inhibiting blood-borne lodgement and growth of 13762MAT:B mammary tumor cells in the lung.

Acknowledgements

The authors thank Alicia Hernandez for typing the manuscript. This study was supported in part by Research Grants from Nestle Ltd, Lausanne, Switzerland and the New Jersey Commission on Cancer Research.

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