Three Percent Dietary Fish Oil Concentrate Increased Efficacy of Doxorubicin Against MDA-MB 231 Breast Cancer Xenografts[1]

W. Elaine Hardman,[2] C. P. Reddy Avula, Gabriel Fernandes, and Ivan L. Cameron

Departments of Cellular and Structural Biology [W. E. H., I. L. C.] and Medicine [C. P. R. A., G. F.], University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229-3900

ABSTRACT

Omega 3 polyunsaturated fatty acids (the type of fat found in fish oil) have been used to kill or slow the growth of cancer cells in culture and in animal models and to increase the effectiveness of cancer chemotherapeutic drugs. An AIN-76 diet containing 5% corn oil (CO) was modified to contain 3% w/w fish oil concentrate (FOC) and 2% CO to test whether a clinically applicable amount of FOC is beneficial during doxorubicin (DOX) treatment of cancer xenografts in mice. Compared with the diet containing 5% CO, consumption of FOC increased omega 3 polyunsaturated fatty acids and lipid peroxidation in tumor and liver, significantly decreased the ratio of glutathione peroxidase activity to superoxide dismutase activity (a putative indicator of increased oxidative stress) in tumor but not in the liver, and significantly decreased the tumor-growth rate. The decreased glutathione peroxidase:superoxide dismutase ratio, indicating an altered redox state, in the tumor of FOC-fed mice was significantly correlated with decreased tumor-growth rate. Assay of the body weight change, blood cell counts, and number of micronuclei in peripheral erythrocytes indicated that the toxicity of DOX to the host mouse was not increased in mice fed FOC. Thus, a small amount of FOC increased the effectiveness of DOX but did not increase the toxicity of DOX to the host mouse. These positive results justify clinical testing of FOC in conjunction with cancer chemotherapy.

INTRODUCTION

There is increasing interest in the use of n-3[3] PUFAs of the type found in fish oil as an agent to retard the growth of tumorigenic cells or xenografts (reviewed in Refs. 1–3). Other reported benefits of n-3 PUFA dietary supplements given before or during cancer therapy include reversing tumor cell drug resistance (4); reducing the gastrointestinal, hematological, or cardiac side effects of various chemotherapeutic treatments (5–7); decreasing cancer cachexia (8–10); and protecting from alopecia (11). The formation of cytostatic and cytotoxic compounds after peroxidation of long chain PUFAs has been proposed as the primary mechanism for the activity of n-3 PUFAs against cancers (12–14), but other mechanisms have also been proposed including the alteration in prostaglandin synthesis (15), alteration in gene transcription (16), suppression of n-6 fatty acid transport (17), and modulation of AOEs and of apoptosis (18). The addition of fish oil to the diet of nude mice bearing human tumor xenografts increased the efficacy of the cancer chemotherapy drugs including edelfosine against MDA-MB 231 human breast cancer tumors (19), irinotecan (CPT-11) against MCF-7 human breast tumors (5), DOX against A-549 human lung tumors (20), epirubicin against rat mammary tumors (6), and cyclophosphamide or mitomycin against MX-1 human mammary tumors (Refs. 7 and 21, respectively). The results of *in vitro* studies have shown that a small amount of either EPA or of DHA (the major long chain PUFAs found in fish oil) added to cell culture medium can cause tumor cell death but not kill cultured normal cells (22–25). Thus, it is thought that one or both of these n-3 fatty acids is responsible for the beneficial effects of fish oil against tumor growth.

This report deals with the effects of incorporating a relatively low level (3% w/w) of a FOC in the diet of nude mice bearing an MDA-MB 231 human breast cancer. FOC containing 34% EPA, 24% DHA, and 10% other n-3 fatty acids, mostly α-linolenic acid, was used in this study instead of fish oil because supplementing the diet with the FOC allowed incorporation of a larger amount of EPA and DHA in a smaller volume than if fish oil was added to the diet. In most of the rodent studies cited above, the volume of fish oil incorporated in the diet was 10–23% w/w of the diet. It has been reported that humans can readily consume from 12 (26) to 21 g (27) of fish oil per day. Incorporation of 3% w/w FOC in the diet was selected because in a 3% FOC diet, 7% of the calories consumed

Received 2/7/01; revised 4/13/01; accepted 4/18/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the American Institute for Cancer Research and the Susan G. Komen Breast Cancer Foundation. Gas chromatograph (Grant 1 RO1 AG 14541) was provided by Dr. Gabriel Fernandes. Clinical trials with a FOC product (INCELL AAFA) will be initiated in collaboration with INCELL Corp., LLC, San Antonio, TX.

[2] To whom requests for reprints should be addressed, at University of Texas Health Science Center at San Antonio, Department of Cellular and Structural Biology, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. E-mail: hardman@uthscsa.edu.

[3] The abbreviations used are: n-3, omega 3; PUFA, polyunsaturated fatty acid; AA, arachidonic acid (20:4n-6); AOE, antioxidant enzyme; CAT, catalase; CO, corn oil; DHA, docosahexaenoic acid (22:6n-3); DOX, doxorubicin; EPA, eicosapentaenoic acid (20:5n-3); FOC, fish oil concentrate; LYM, lymphocyte; GPX, glutathione peroxidase; SNK, Student-Newman-Keuls multiple range test; MN, micronuclei; MCV, mean corpuscular volume; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

by the mice came from the FOC, which equates to 14 g FOC/day for a human consuming 1800 calories/day.

The aims of our continuing studies on the effects of FOC are to determine: (*a*) if a small amount (3% w/w of the diet) of FOC can be an effective adjuvant for chemotherapy; (*b*) if consumption of FOC to increase the toxicity of the drug against the tumor does or does not also increase the toxicity of the drug to the host (or patient); and (*c*) to increase understanding of the mechanisms of action of FOC in tumor and normal tissues.

MATERIALS AND METHODS

Preparation of Cells. MDA-MB 231 cancer cells were used in this study because past studies (19, 28) have demonstrated that their growth is suppressed by high doses of n-3 PUFA, and it has been reported that n-3 PUFAs increased the efficacy of DOX against MDA-MB 231 cells in culture (29). The effect of DOX and clinically relevant doses of n-3 PUFAs on the growth of MDA-MB 231 xenografts *in vivo* needed to be tested. MDA-MB 231 cells are estrogen independent and, in nude mice, form solid tumors that grow at a moderate rate. Cultured MDA-MB 231 human breast cancer cells (American Type Culture Collection, Rockville, MD) were trypsinized, harvested, rinsed, then suspended in serum-free M3D base culture medium (INCELL Corporation, LLC, San Antonio, TX). Cells in suspension were counted using a hemocytometer, and the cell count was adjusted to 20×10^6/ml. The suspension was kept well mixed during the time of injection. MDA-MB 231 cells (1×10^6 cells in 0.05 ml of serum-free medium) were injected s.c. between the scapulae of each mouse.

Dietary Fatty Acids. CO contains ~50% linoleic acid, 23% oleic acid, and 10% C16 fatty acids and contains <0.6% n-3 PUFAs. The n-3 ethyl ester FOC contained 34% EPA, 24% DHA, and ~10% of other n-3 fatty acids (mostly α-linolenic acid) and was supplied antioxidant-free (Pronova, Lysaker, Norway). This oil is made in accordance with Good Manufacturing Practice and is approved as a food additive for humans. A Certificate of Analyses is supplied with each batch of concentrate. The oil is saturated with nitrogen to prevent oxidation during shipping and storage.

Animals and Diet. One hundred twenty 3-month-old female athymic *nu/nu* mice (Harlan Sprague Dawley Inc., Madison, WI) received tumor cells. The mice were housed under aseptic conditions in a temperature- (24°C) and light-controlled (12 h/day) room. All of the animal use and handling was approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee.

The AIN-76 semipurified diet (30) containing 5% CO was used as the basal diet or was modified to contain 2% CO plus 3% FOC.

Diet Components and Chemicals. Purified high nitrogen casein, pure corn starch, Alphacel (non-nutritive bulk cellulose), AIN-76 vitamin mixture, AIN-76 mineral mixture, and choline bitartrate (99% pure) were obtained from ICN Nutritional Biochemicals, Cleveland, Ohio. Imperial brand (Sugarland, TX) extra fine pure cane sugar and 100% pure CO (Wesson) were purchased locally. DL-methionine (cell culture, MW 149.2) was obtained from Sigma Chemical Co. (St. Louis, MO). Antioxidant-free FOC (>33% EPA and >24% DHA) was purchased from Pronova.

The tumor cell-bearing mice were fed the AIN-76 semipurified diet containing 5% CO from receipt until 3 weeks after injection of cells to allow the tumor to become established. Nude mice bearing growing MDA-MB-231 human breast carcinoma xenografts were then divided into two dietary groups such that the mean tumor size was not different between groups. One group received the standard AIN-76 diet containing 5% CO (the CO diet) and the other received the AIN-76 diet modified to contain 3% FOC and 2% CO (the FOC diet). Diets were prepared weekly, and daily portions were individually packaged and stored at −20°C in sealed containers with nitrogen gas atmosphere to prevent lipid peroxidation. Mice were fed a measured amount of fresh food each day, and food remaining in the cage was discarded.

Tumor and Body Weight Measurements. Lengths and widths of tumors and body weights were measured three times weekly. Tumor sizes were calculated using the formula for the volume of a prolate spheroid: volume = $4/3 \times 3.14 \times$ (length/2) $\times$ (width/2) $\times$ (depth/2). The width measurement was used as the depth of the tumor.

DOX Therapy. After 2 weeks of consumption of the diets, mice from each dietary group were randomly selected for initiation of DOX therapy, 5 mg/kg body weight every 4 days, i.v. in a lateral tail vein, as used in a past study (31). Mice in this past study (31) lost weight suggesting that this dose is at or slightly more than the maximum tolerated dose. Some mice were killed after one dose of DOX to obtain sufficient tumor tissue for lipid analyses and for determination of the acute effects of DOX on induction of lipid peroxidation. The remainder of the mice received DOX treatment for a total of 5 weeks to allow time to generate tumor-growth curves and to determine the longer-term effects of FOC consumption and/or DOX treatment. After 2 weeks of DOX treatment every 4 days, the weight loss of most CO-fed mice was approaching 10% of their initial (about 25 g) body weight indicating that the maximum tolerated dose of DOX was met or exceeded. Therefore, to decrease the dosage of DOX, the spacing of injections was increased to every 7 days for the next 3 weeks, and mouse weight stabilized. Thus, if day 0 was the day of the first injection, mice received DOX injections on days 0, 4, 8, 12, 19, 26, and 33 and were sacrificed on day 34. Groups of mice will be referred to as: CO=CO diet, no DOX; CO/DOX=CO diet, DOX treated; FOC=FOC diet, no DOX; FOC/DOX=FOC diet, DOX treated.

Necropsy and Tissue Processing. Mice were deeply anesthetized using a ketamine/Rompun solution prepared by the University of Texas Health Science Center at San Antonio veterinarian. Blood samples for serum analyses were collected via cardiac puncture into anticoagulant (EDTA)-containing tubes. The tumor and liver were removed at necropsy. Portions of each tissue were placed in individually labeled vials and flash frozen in liquid nitrogen. At a later date, frozen tissues were thawed and homogenized individually in 280 mM of mannitol with 10 mM HEPES buffer with 0.01% butylated hydroxytoluene at 4°C using a Polytron homogenizer then divided into aliquots and frozen at −70°C until subsequent analyses.

Products of Lipid Peroxidation in Tumor and Liver. An aliquot of each whole tissue homogenate was assayed for lipid peroxidation. The total protein content of each specimen was analyzed by the method of Bradford (32) using the Bio-Rad protein assay (micro-method). The TBARS assay was used to estimate lipid peroxidation (33) on the remainder of the homogenate. The absorbance at 535 nm was compared against a standard curve of known concentrations of malondialdehyde and normalized to the protein content of the specimen. The results were reported as nmol of TBARS per mg of protein.

Gas Chromatography. The incorporation of diet-specific fatty acids into the mitochondria and microsomes of liver and tumor was determined in mice that consumed each diet. An aliquot of each homogenized specimen was centrifuged at 600 $\times$ *g* for 10 min to remove large debris and nuclei. Supernatants were successively centrifuged at 15,000 $\times$ *g* for 5 min to pellet the mitochondrial fraction then at 100,000 for 1 h to pellet the microsomal fraction. Chloroform:methanol was used for extraction of lipids from each fraction, and lipids were esterified in acetyl chloride-methanol as described (34). Gas chromatography was performed using a Hewlett Packard 5890 Series 11 Gas Chromatograph (Palo Alto, CA) with a fused silica capillary column (DB-225; 30 m $\times$ 0.25 mm; J & W Scientific, Folsom, CA) and oven conditions from 170°C at 5°C/min gradient to 220°C, injector temperature at 225°C, flame-ionization detector temperature at 250°C, and helium-carrier gas at 400 Kpa. Fatty acid methyl ester standards (Nu-Chek-Prep; Elysian, MN) were used for peak identification. The EPA and DHA content are reported as the percentage of the total fatty acids (area under the curve).

Enzyme Assays. All of the assays were prepared in triplicate. Standards with known enzyme activity (CAT, C-3515; Sigma Chemical Co.; GPX control, 27617; Oxis; Portland, Oregon; and SOD standard, 27619; Oxis) were used to prepare standard curves for determining specific enzyme activities of the specimens. A Perkin-Elmer Corp. HTS 7000 bioassay reader was used for all of the assays. Flat areas at the beginning (indicating a lag in initiation of the reaction) or end (indicating that all of the substrate was consumed) of all of the reaction curves were removed before linear regression analyses to determine the rate of the reaction (slope of the linear regression) of each well. The activity of each unknown was determined from the standard curve. CAT activity in the tissue homogenates was assayed using a microplate adaptation of the method of Aebi (35). SOD activity of tissue homogenates was determined using a microplate adaptation of the ferricytochrome C reduction assay of Flohé and Ötting (36). GPX activity of tissue homogenates was determined using a microplate adaptation of the GPX assay of Paglia and Valentine (37).

Blood Counts. Our Laboratory Animal Resources division performed complete blood counts using a Cell-Dyn 3500R blood analyzer with veterinary pack.

MN. MN were identified in acridine orange-stained smears of peripheral blood as described previously (38). Fields containing a single layer of erythrocytes were identified, and the number of erythrocytes and MN were counted and recorded.

Statistical Analyses. SAS and PRISM (GraphPad Software; San Diego, CA) software were used for statistical analyses. Tests for normality (basic statistics) were used on each data set. Two-way ANOVA was used to determine whether, across all of the groups, either consumption of FOC or DOX treatment significantly changed the parameter. One-way ANOVA followed by SNK multiple-range tests were used to determine statistically significant ($P \leq 0.05$) differences between the four groups. The mean tumor size of each group at each time was used to calculate the linear regression of tumor size over time. The slope ($\pm$ SE) of this linear regression is the tumor-growth rate. ANOVA was used to test for differences between the tumor-growth rates.

Multiple linear regression analysis with forward selection was used to test for correlations in the data between the independent variables (SOD, CAT, and GPX activity or TBARS) and the dependent variable [the tumor-growth rate expressed as the logarithm (tumor-growth rate + 1)]. An independent variable was entered into the equation if the *P* for the correlation was $\leq$0.10 and if adding the variable to the equation added to the explanation of the variation in the dependent variable. The logarithmic transformation of the tumor-growth rate + 1 was used because: (*a*) logarithmic transformations are frequently useful for problems concerning growth (39); (*b*) adding 1 to the tumor-growth rate was necessary and appropriate to correct the technical problem of finding the logarithm of a negative growth rate (39); and (*c*) a plot of tumor-growth rate *versus* TBARS indicated the relationship was logarithmic rather than linear.

Fisher's exact test was used to compare the proportions of mice with tumors <3.5 mm diameter in CO/DOX and FOC/DOX groups.

RESULTS

A Relatively Low Level (3% w/w) of FOC in the Diet Increased the Fraction of EPA in Tumor and in Host Liver. To determine that EPA was incorporated into tumor and host cellular membranes, the fatty acid compositions of the mitochondrial and microsomal fractions of the liver and tumor of mice fed the CO or the FOC diet for 2 weeks before sacrifices were analyzed by gas chromatography. There were no significant differences in the lipid compositions of mitochondrial and microsomal fractions of each specimen; thus, only the results from the microsomal fractions are reported (percentage of total fatty acids):

Liver: (*a*) CO diet: *EPA*, 0.07 $\pm$ 0.4 and *DHA*, 0.8 $\pm$ 0.5; and (*b*) FOC diet: *EPA*, 2.4 $\pm$ 0.9 and *DHA*, 2.5 $\pm$ 1.2

Tumor: (*a*) CO diet: *EPA*, 0.0 $\pm$ 0.0 and *DHA*, 0.3 $\pm$ 0.2; and (*b*) FOC diet: *EPA*, 1.3 $\pm$ 0.5 and *DHA*, 0.02 $\pm$ 0.02

The EPA was significantly higher in the liver and tumor, and DHA was higher in the liver of the FOC mice than in CO mice, demonstrating that even this low level of FOC in the diet was sufficient to increase EPA in the phospholipids of liver and tumor cell membranes.

Effect of FOC and DOX on Lipid Peroxidation in the Tumor and Liver. Assay of TBARS was used as an indicator of lipid peroxidation. The results of two-way and one-way ANOVA of TBARS in the human breast cancer xenograft and in the liver of mice killed 24 h after one injection of DOX (to assess the effects of DOX on lipid peroxidation) are presented in Fig. 1.

Two-way ANOVA revealed that either the 3% FOC diet or

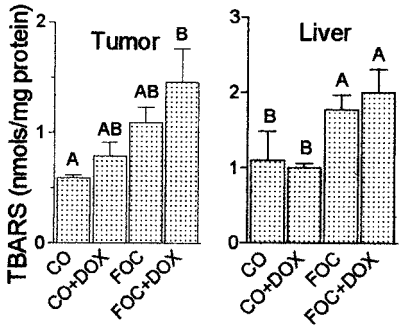

Fig. 1 TBARS (nmols/mg protein) in the tumor and liver in groups of mice fed diets containing either 5% CO or 3% FOC and 2% CO and that were or were not treated 24 h previously with DOX at 5 mg/kg body weight. One-way ANOVA + SNK indicated that TBARS of groups on each graph that do not share a superscript letter were significantly ($P < 0.05$) different; *bars*, SD.

treatment with DOX significantly increased TBARS in the tumor. One-way ANOVA followed by an SNK test revealed that TBARS in the tumors of the FOC/DOX mice were significantly greater than TBARS in the tumors of the CO mice.

Two-way ANOVA revealed that TBARS in the livers of FOC mice were significantly higher than TBARS in the livers of CO mice (significant main effect); however, DOX treatment did not significantly increase TBARS in the liver. One-way ANOVA followed by SNK revealed that TBARS in the liver of both FOC and FOC/DOX mice were significantly higher than in the CO and CO/DOX mice.

Alteration in the Activity of AOEs in Tumor and Host Liver and Host Liver following FOC in the Diet and/or Treatment with DOX. The activities of SOD, CAT, and GPX in the tumor and liver were assayed to determine whether the activities of these AOEs were altered by the consumption of FOC or by 5 weeks of DOX treatment. The results are summarized in Fig. 2. Because of tumor-growth suppression and tumor regression, there was not sufficient tumor tissue remaining in the FOC/DOX mice for enzyme analyses.

Oxidative Stress in the Tumor and Liver after Consumption of the FOC Diet or DOX Treatment. A decrease in the GPX:SOD ratio has been used as a putative index of a relative increase in oxidative stress in tissues (40). As illustrated in Fig. 3, either treatment with DOX or FOC in the diet decreased the GPX:SOD ratio in the tumor. However, the GPX:SOD ratio in the liver of FOC mice was increased indicating that the tumor and liver responded differently to dietary FOC.

Tumor Growth after Dietary FOC and/or DOX Treatment. To determine the effects of FOC consumption and DOX treatment on tumor growth, the mean volume of the tumors of each group of mice, as calculated at each measurement during the 5 weeks of DOX treatment, was subjected to linear regression analyses. A plot of tumor growth and the mean tumor-growth rate (slope ± SE of the linear regression analysis) for each group of mice is presented in Fig. 4.

Comparison of the tumor size after 5 weeks of DOX treatment revealed that none of the 13 mice fed the 5% CO diet and treated with DOX had tumors <3.5 mm in diameter (most were >8 mm), whereas 8 of 10 mice fed the 3% FOC diet and

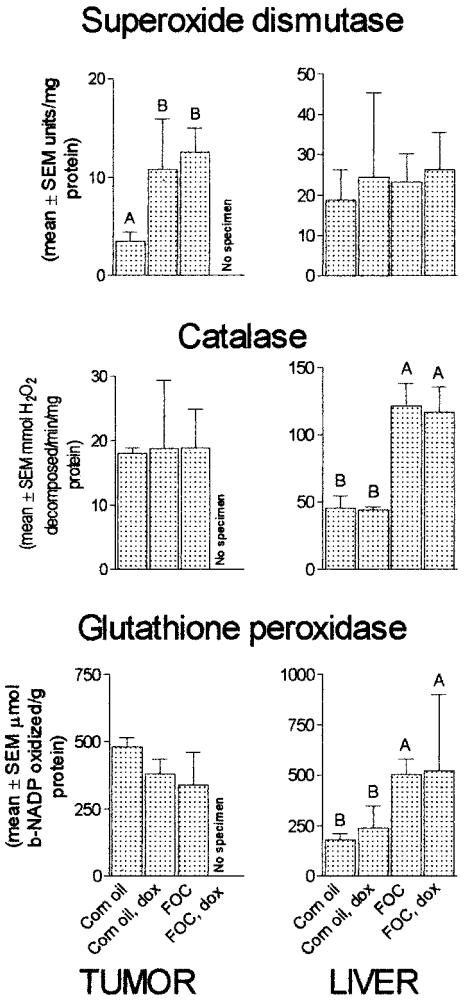

Fig. 2 Activity of SOD (units/mg protein), CAT (µmol H_2O_2 decomposed/min/mg protein), and GPX (µmol β-NADP oxidized/g protein) in tumors and livers in groups of mice fed diets containing either 5% CO or 3% FOC and 2% CO and that were or were not treated with DOX for 5 weeks (see "Materials and Methods" for dose). Two-way ANOVA could not be performed for tumor tissues because there were no results for the FOC/DOX mice. One-way ANOVA before SNK showed that either DOX treatment in CO mice or FOC alone significantly increased SOD in the tumor but that GPX and CAT activities in the tumors were not significantly different between groups. However, two-way ANOVA revealed that SOD activity in the liver was not significantly altered by dietary fat or by DOX treatment but that consumption of the FOC diet significantly increased CAT and GPX activity. DOX treatment did not significantly increase GPX or CAT activity in the liver. Groups on each graph that do not share a superscript are significantly different; ± SD.

treated with DOX had tumors <3.5 mm in diameter (significantly different by Fisher's exact test, $P < 0.01$). This demonstrates that DOX treatment was much more effective in inhibiting tumor growth in mice fed the 3% FOC diet than in mice fed the 5% CO diet.

Relationship between Lipid Peroxidation and AOE Activities in the Tumor and the Tumor-growth Rate after Treatment with FOC and DOX. To help determine the quantitative relationship between changes in AOE activity or

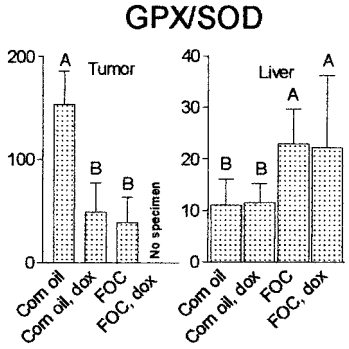

Fig. 3 Ratio of GPX activity to SOD activity in the tumors and livers in the groups of mice fed diets containing either 5% CO or 3% FOC and 2% CO and that were or were not treated with DOX for 5 weeks (see "Materials and Methods" for dose). There was not sufficient tumor tissue from the FOC/DOX mice for enzyme analyses. One-way ANOVA + SNK showed that in the tumor either DOX treatment to CO-fed mice or consumption of FOC significantly decreased the GPX:SOD ratio indicating that oxidative stress was significantly increased. However, in the liver, two-way ANOVA indicated that FOC significantly increased the GPX:SOD ratio, indicating that oxidative stress was significantly less than in the mice fed CO. DOX treatment did not significantly change the GPX:SOD ratio in the liver. Groups that do not share a superscript are significantly different; *bars*, SD.

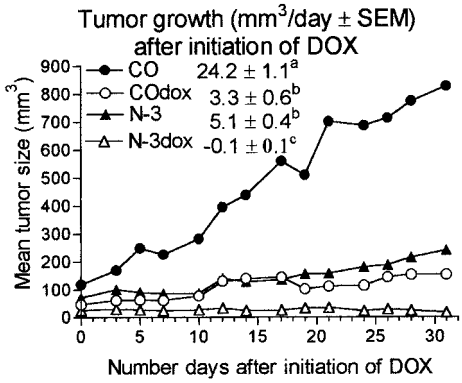

Fig. 4 Tumor growth after initiation of DOX treatment. Mice were divided into groups such that the mean tumor size at the beginning of the diet change was not different. The small difference in mean tumor size at initiation of DOX treatment (day 0) indicates that FOC consumption during the 2-week run-in period was already decreasing the tumor-growth rate. Mean of the tumor-growth rate of each group (n = 9 or 10 per group) is indicated, and rates that do not share a superscript are significantly different; *bars*, SE. One-way ANOVA before SNK of the mean tumor-growth rates revealed that: (*a*) the mean tumor-growth rate of the group of mice fed 5% CO but not treated with DOX was significantly higher than the tumor-growth rate of the other three groups; (*b*) the mean tumor-growth rates of the CO/DOX mice and the FOC mice were not significantly different from each other indicating that FOC consumption was as effective as DOX at slowing tumor growth; and (*c*) the tumor-growth rate of the group of mice fed FOC and treated with DOX was significantly less than the tumor-growth rates of all of the other groups.

lipid peroxidation and the variation in tumor-growth rate, multiple regression analyses were performed. The results of these regression analyses are presented in Table 1. The correlation coefficients between the log (tumor-growth rate + 1) and each of the independent variables are listed at the bottom of Table 1. SOD activity was the first variable entered into the multiple regression equation and "explains" 61% of the variation in tumor-growth rate. Next, CAT activity was added for an additional 29% explanation, and GPX activity added an additional 6% explanation for a total cumulative explanation of 96% of the variation in tumor growth. These results indicate that the variation in AOE activity provides an excellent correlation with the variation in tumor growth.

Regression analyses between the GPX:SOD ratio in the tumor and the log (tumor-growth rate + 1) revealed that the GPX:SOD ratio was positively correlated with tumor-growth rate and explained 85.5% of the tumor-growth rate. Therefore, the decreased GPX:SOD ratio, an indicator of altered redox status in cells (40), significantly correlated with decreased tumor-growth rate and provided a better explanation for the variation in tumor-growth rate than TBARS (which assays only the products of lipid peroxidation) in the tumor.

FOC in the Diet and the Toxicity of DOX to the Host Mouse: Hematology. Complete blood counts of peripheral blood specimens were used to assay the effects of FOC and of DOX treatment. Two-way ANOVA of the hematology data presented in Table 2 revealed significant main effects attributable both to DOX treatment and to FOC consumption.

The number of peripheral erythrocytes containing one or more MN was scored as an assay for chromosomal breakage because of dietary FOC or DOX treatment (38). As summarized in Table 2, two-way ANOVA revealed that DOX treatment for 5 weeks caused a significant increase in the number of MN found in peripheral erythrocytes. In other words, DOX treatment cause a significant increase in chromosomal breakage. However, the number of MN was not significantly altered by dietary fat. One-way ANOVA revealed no significant differences between the four individual group means.

Body Weight Change. Results of two-way ANOVA revealed a significant main effect attributable to DOX treatment, *i.e.*, 5 weeks of DOX treatment caused a significant decrease in body weight (*i.e.*, mean body weight change without DOX = 0.12 g *versus* with DOX = −1.82 g). There was not a significant main effect attributable to type of dietary fat (FOC = −0.47 g *versus* 5% CO = −1.04 g); however, FOC/DOX mice lost less weight than CO/DOX mice (FOC/DOX, −1.56 ± 0.43; CO/DOX, −2.08 ± 0.56). There was not a significant interaction between the effects of dietary fat and of DOX treatment.

Overall, these toxicity data indicate that, compared with consumption of the CO diet, consumption of the FOC diet before and during DOX treatment did not increase DOX-induced toxicity to the host mouse although the efficacy of DOX against the tumor was increased.

DISCUSSION

The experimental results demonstrate that 3% w/w FOC in the diet was sufficient to: (*a*) increase the amount of n-3 PUFA in the cellular membranes of tumor and liver; and (*b*) increase the efficacy of DOX therapy against the tumor-growth rate. Dietary FOC did not increase the toxicity of DOX to the host mouse. The mean tumor-growth rate of FOC mice was not

Table 1 Summaries of regression analyses between biochemical assays of individual tumor variables and the logarithm [tumor growth rate (mm^3/day) + 1][a]

A. Multiple regression analysis between the measured independent variables: SOD, CAT, and GPX activities and TBARS and the dependent variable, logarithm[b]

Variable entered into equation	Parameter estimate	Partial r^2 contribution each variable	Cumulative r^2 cumulative contribution
Intercept	1.89		
SOD	−0.12	0.61	0.61
CAT	0.05	0.29	0.90
GPX	−1.71	0.06	0.96[c]

B. Results of regression analysis between GPX:SOD (independent variable) and the dependent variable, logarithm[d]

Variable entered into equation	Parameter estimate	r^2 (contribution %)
Intercept	0.56	
GPX:SOD	6.53	0.855[e]

C. Correlation coefficients (r)[f]

	SOD	CAT	GPX	TBARS	GPX:SOD
Logarithm	−0.78	0.29	0.74	0.18	0.92

[a] Values for each available tumor ($n = 10$) were entered into the regression analyses.
[b] $P \leq 0.10$ was used for entry of a variable into the calculation.
[c] 96% of the variation in tumor-growth rate is explained by the three variables entered into the equation. Thus, the equation for tumor-growth rate with a correlation of 0.98 is: logarithm = 1.89 − 0.12(SOD) + 0.05(CAT) − 1.71(GPX).
[d] Since GPX:SOD is not independent of GPX or SOD activity, this variable cannot be included in the same regression with GPX and SOD.
[e] 85.5% of variation in tumor-growth rate is explained by the ratio of GPX:SOD (an indicator of oxidative stress).
[f] Correlation coefficients $> \pm 0.63$ are significant at $P < 0.05$.

significantly different from the mean tumor-growth rate of the CO/DOX mice; thus, consumption of the 3% FOC alone was as effective against the growth of the MDA-MB 231 human breast carcinoma as was DOX chemotherapy in conjunction with the standard AIN-76 diet containing 5% CO.

In the experimental design, the CO was decreased as FOC was added so that the total fat in the diet remained constant. Diets containing high levels of CO have been shown to increase tumor growth (12, 28). Although 5% CO is generally considered to be a low level of fat, there is the possibility that decreasing the CO from 5% to 2% of the diet contributed to the reduction in the tumor-growth rate.

Hypothesis to Explain the Differential Effects of Dietary n-3 PUFA on the Tumor and on Normal Host Tissues. Incorporation of FOC in the diet resulted in increased EPA in the membrane phospholipids of the tumor and liver. Because EPA was increased in membrane phospholipids of FOC-fed mice and because EPA is very susceptible to spontaneous lipid peroxidation, the increase in the products of lipid peroxidation in the tissues of FOC-fed mice was expected. In addition, consumption of FOC altered AOE balance in the tumor. SOD was increased in the tumor, but the tumor did not (or could not) increase the activity of GPX or CAT following consumption of FOC. Because GPX and/or CAT neutralize the H_2O_2 produced by SOD, increased SOD in the absence of increased GPX and/or CAT could result in an accumulation of H_2O_2 and an increase in oxidative stress in tumor cells. Thus, the decreased GPX:SOD ratio and increased lipid peroxidation in the tumor provides evidence that the redox status within the tumor cells was altered by consumption of FOC in a manner that would be expected to increase oxidative stress. The loss of the ability to produce protective AOEs seems to be a characteristic of tumor cells (41) and in the present study was associated with the decreased tumor-growth rate. However, the normal liver seems to be able to adapt to the FOC diet by increasing the production of GPX and CAT resulting in an increased GPX:SOD ratio (indicating decreased oxidative stress).

Oxidative stress in the tumors of FOC-fed mice was additionally increased by DOX treatment, as evidenced by the increased TBARS and by the decreased GPX:SOD ratio, resulting in a significantly decreased tumor-growth rate. This decreased tumor-growth rate could have been attributable to decreased proliferation and/or to increased apoptosis induced by oxidative damage (18, 42, 43). The combined effect of FOC/DOX would be expected to result in the lowest GPX:SOD ratio and did result in the highest tumor lipid peroxidation and the lowest tumor-growth rate in the group of FOC/DOX mice.

Vankatraman *et al.* (44) reported that CAT, GPX, and SOD activities were significantly increased in the livers of mice fed 19% w/w fish oil for 6 months. Our results demonstrate that a smaller amount of FOC (3% w/w) fed for 7 weeks was sufficient to significantly increase CAT and GPX in the liver. Thus, the results presented here demonstrate that consumption of smaller amounts of FOC for a shorter time than tested previously is adequate to alter AOE activities in the liver in a manner that would be expected to protect the liver from oxidative damage.

Consumption of FOC before and during DOX Treatment on Side Effects. Analyses of blood counts and body weight changes indicated that consumption of FOC before and during DOX treatment did not increase DOX toxicity compared with the DOX toxicity in mice that consumed the CO diet. RBC counts and LYM counts were higher in the peripheral blood of

Table 2 Statistical analyses of peripheral blood cell counts of nude mice bearing an MDA-MB 231 human breast carcinoma following consumption of a diet containing either 5% CO or 3% FOC with 2% CO for 7 weeks and treatment with or without DOX for the last 5 weeks.

Diet (Mean ± SE)[a]	*n*	RBC (m/ml)	MCV (f/l)	LYM (k/ml)	RBC with MN[b] (*n*)
5% CO					
without DOX	7	8.61 ± 0.10b	46.7 ± 0.75c	0.48 ± 0.24b	12.8 ± 2.1 (5)
with DOX	12	7.78 ± 0.13c	50.3 ± 0.48a	0.29 ± 0.08b	17.5 ± 2.7 (6)
3% FOC					
without DOX	11	9.43 ± 0.16a	46.3 ± 0.51c	1.81 ± 0.50a	11.0 ± 1.4 (7)
with DOX	9	8.23 ± 0.12b	48.6 ± 0.41b	0.52 ± 0.13b	20.0 ± 3.8 (6)
Results of two-way ANOVA[c]					
Main Effects					
Dietary fat					
CO	19	8.08 ± 0.13b	49.0 ± 0.57b	0.36 ± 0.10b	15.4 ± 2.4a
FOC	20	8.93 ± 0.17a	47.3 ± 0.43a	1.23 ± 0.31a	15.2 ± 2.5a
DOX					
without DOX	18	9.11 ± 0.14a	46.7 ± 0.41a	1.30 ± 0.35a	11.8 ± 1.7a
with DOX	21	7.96 ± 0.10b	49.6 ± 0.37b	0.39 ± 0.07b	18.8 ± 3.3b
Interactions Fat X DOX		ns[d]	ns	ns	ns

[a] Means (± SE) with different letters are significantly different ($P < 0.05$).

[b] MN are expressed as total number of MN identified in 2000 RBC.

[c] The results of two-way ANOVAs revealed that: RBC and LYM counts were significantly higher and MCV was significantly lower in mice that consumed FOC than in mice that consumed CO, and treatment with DOX significantly decreased RBC and LYM counts but significantly increased MCV and MN.

[d] ns, not significant. Significant differences between individual groups were determined by one-way ANOVA followed by SNK multiple comparison tests.

FOC mice than in mice that consumed the CO diet. Atkinson *et al.* (45) reported that the cellularity and the number of granulocyte-macrophage colony-forming units were higher in bone marrow of rats fed DHA than in rats fed a CO diet and that analysis of fatty acid composition of the liver indicated that some of the DHA had been retroconverted to EPA (45). It seems likely that the EPA and DHA in the FOC consumed by mice in the present study should have a similar effect to purified DHA on the bone marrow and that increased bone marrow cellularity would result in higher peripheral blood counts as was observed (Table 2).

Decreased osmotic fragility of the RBCs because of incorporation of n-3 fatty acids in the RBC membrane could be another explanation for the higher RBC counts and the smaller MCV. Fischer and Black (46) reported that the erythrocytes of mice fed 12% fish oil had higher levels of n-3 fatty acids and were less osmotically fragile than the erythrocytes of mice fed CO. Frenoux *et al.* (47) report that a diet rich in γ-linolenic acid (18:3n-6), EPA, and DHA increased the antioxidant status of rats, defined as the ability of RBC to withstand free radical-induced hemolysis. This could result in a longer life span for individual erythrocytes, a reduced need for new erythrocytes, and a reduced need for proliferation of erythroblasts to maintain RBC counts in the peripheral blood. Release of fewer new, larger, immature erythrocytes from the bone marrow would result in a smaller MCV in the peripheral blood. Thus, one possibility for the beneficial effect of FOC on RBC counts is a longer mean life span attributable to decreased fragility of the erythrocyte membrane and reduced hemolysis upon exposure to the oxidative stress of DOX treatment.

In this study, the FOC/DOX mice lost less weight than CO/DOX mice. Sauer *et al.* (17) have reported that the release of fatty acids from inguinal fat pads was suppressed when rats were fed EPA, which may explain the reduced weight loss in FOC fed mice. It has also been reported that body weight loss was stopped, lean body mass was preserved, and the performance status was improved in pancreatic cancer patients who consumed a nutritional supplement containing ~600 additional calories and 3 g of n-3 fatty acids/day (9, 10). Burns *et al.* (27) have reported use of an n-3 PUFA supplement in a Phase-I (dose-finding) clinical study in cancer patients with cachexia but have not completed the cachexia-reducing portion of the study.[4]

Supplementing the diet with a FOC that contains a high level of EPA and DHA has proved a safe diet supplement for humans (27), and dietary n-3 PUFAs have been shown to increase the efficacy of various chemotherapeutic agents against different tumor types in preclinical studies (5–7, 19–21). The small volume of FOC demonstrated to be beneficial against MDA-MB 231 xenografts in the athymic mouse model (this report) was equivalent to an amount that humans can easily and safely consume (26). Although the mechanisms of action of FOC are yet to be established, clinical trials with cancer patients seem justified at this time based on current and past reports that dietary supplementation with FOC is a safe and beneficial adjuvant to cancer chemotherapy. A clinical trial to determine the ability of FOC dietary supplements to reduce the side effects of commonly used cancer chemotherapy drugs (such as DOX, 5-FU, or CPT-11) in humans could be performed in <1 year. Determining the effect of dietary FOC on tumor growth in humans will require a longer-term trial using matched cases and control patients.

In the present study, incorporation of 3% FOC in the diet, even without DOX chemotherapy, was as effective at suppress-

[4] W. E. Hardman, personal communication.

ing tumor growth as was a course of DOX chemotherapy in mice that were consuming the 5% CO diet. Thus, continued consumption of nontoxic FOC between cycles of toxic chemotherapy may slow growth of residual tumor. The higher blood cell counts and the reduced weight loss of the mice that consumed FOC provide support for the idea that FOC supplements will aid patient recovery between cycles of chemotherapy.

REFERENCES

1. Rose, D. P. Dietary fatty acids and cancer. Am. J. Clin. Nutr., *66:* 998S–1003S, 1997.

2. Bougnoux, P. n-3 Polyunsaturated fatty acids and cancer. Curr. Opin. Clin. Nutr. Metab. Care, *2:* 121–126, 1999.

3. Das, U. N. Essential fatty acids and their metabolites and cancer. Nutrition, *15:* 239–240, 1999.

4. Das, U. N., Madhavi, G., Kumar, G. S., Padma, M., and Sangeetha, P. Can tumour cell drug resistance be reversed by essential fatty acids and their metabolites? Prostaglandins Leukot. Essent. Fatty Acids, *58:* 39–54, 1998.

5. Hardman, W. E., Moyer, M. P., and Cameron, I. L. Fish oil supplementation enhanced CPT-11 (Irinotecan) efficacy against MCF7 breast carcinoma xenografts and ameliorated intestinal side effects. Br. J. Cancer, *81:* 440–448, 1999.

6. Germain, E., Lavandier, F., Chajès, V., Schubnel, V., Bonnet, P., Lhuillery, C., and Bougnoux, P. Dietary n-3 polyunsaturated fatty acids and oxidants increase rat mammary tumor sensitivity to epirubicin without change in cardiac toxicity. Lipids, *34:* S203, 1999.

7. Shao, Y., Pardini, L., and Pardini, R. S. Intervention of transplantable human mammary carcinoma MX-1 chemotherapy with dietary menhaden oil in athymic mice: increased therapeutic effects and decreased toxicity of cyclophosphamide. Nutr. Cancer, *28:* 63–73, 1997.

8. Karmali, R. A. Historical perspective and potential use of n-3 fatty acids in therapy of cancer cachexia. Nutrition, *12:* S2–S4, 1996.

9. Tisdale, M. J. Mechanism of lipid mobilization associated with cancer cachexia: interaction between polyunsaturated fatty acid, eicosapentaenoic acid and inhibitory guanine nucleotide-regulatory protein. Prostaglandins Leukot. Essent. Fatty Acids, *48:* 105–109, 1993.

10. Barber, M. D., Ross, J. A., Voss, A. C., Tisdale, M. J., and Fearon, K. C. H. The effect of an oral nutritional supplement enriched with fish oil on weight-loss in patients with pancreatic cancer. Br. J. Cancer, *81:* 80–86, 1999.

11. Takahata, K., Tada, M., Yazawa, K., and Tamaki, T. Protection from chemotherapy-induced alopecia by docosahexaenoic acid. Lipids, *34:* S105, 1999.

12. Gonzalez, M. J., Schemmel, R. A., Gray, J. I., Dugan, L. J., Sheffield, L. G., and Welsch, C. W. Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in athymic nude mice: relationship between carcinoma growth and lipid peroxidation product levels. Carcinogenesis (Lond.), *12:* 1231–1235, 1991.

13. Gonzalez, M. J. Fish oil, lipid peroxidation and mammary tumor growth. J. Am. Coll. Nutr., *14:* 325–335, 1995.

14. Das, U. N. γ-linolenic acid, arachidonic acid, and eicosapentaenoic acid as potential anticancer drugs. Nutrition, *6:* 429–434, 1990.

15. Rose, D. P., Rayburn, J., Hatala, M. A., and Connolly, J. M. Effects of dietary fish oil on fatty acids and eicosanoids in metastasizing human breast cancer cells. Nutr. Cancer, *22:* 131–141, 1994.

16. Jump, D. B., and Clarke, S. D. Regulation of gene expression by dietary fat. Annu. Rev. Nutr., *19:* 63–90, 1999.

17. Sauer, L. A., Dauchy, R. T., and Blask, D. E. Mechanism for the antitumor and anticachectic effects of n-3 fatty acids. Cancer Res., *60:* 5289–5295, 2000.

18. Fernandes, G., Chandrasekar, B., Luan, X., and Troyer, D. A. Modulation of antioxidant enzymes and programmed cell death by n-3 fatty acids. Lipids, *31:* S91–S96, 1996.

19. Hardman, W. E., Barnes, C. J., Knight, C. W., and Cameron, I. L. Effects of iron supplementation and ET-18-OCH_3 on MDA-MB 231 breast carcinomas in nude mice consuming a fish oil diet. Br. J. Cancer, *76:* 347–354, 1997.

20. Hardman, W. E., Moyer, M. P., and Cameron, I. L. Dietary fish oil sensitizes A549 lung xenografts to doxorubicin chemotherapy. Cancer Lett., *151:* 145–151, 2000.

21. Shao, Y., Pardini, L., and Pardini, R. S. Dietary menhaden oil enhances mitomycin C antitumor activity toward human mammary carcinoma MX-1. Lipids, *30:* 1035–1045, 1995.

22. de Vries, C. E. E., and Van Noorden, C. J. F. Effects of dietary fatty acid composition on tumor growth and metastasis. Anticancer Res., *12:* 1513–1522, 1992.

23. Begin, M. E., Ells, G., Das, U. N., and Horrobin, D. F. Differential killing of human carcinoma cells supplemented with n-3 and n-6 polyunsaturated fatty acids. J. Natl. Cancer Inst., *77:* 1053–1062, 1986.

24. Price, S. A., and Tisdale, M. J. Mechanism of inhibition of a tumor lipid-mobilizing factor by eicosapentaenoic acid. Cancer Res., *58:* 4827–4831, 1998.

25. de Salis, H. M., and Meckling-Gill, K. A. EPA and DHA alter nucleoside drug and doxorubicin toxicity in L1210 cells but not in normal murine S1 macrophages. Cell. Pharmacol., *2:* 69–74, 1995.

26. Anti, M., Marra, G., Armelao, F., Bartoli, G. M., Ficarelli, R., Percesepe, A., DeVitis, I., Maria, G., Sofo, L., Rapaccini, G. L., Gentiloni, N., Piccioni, E., and Miggiano, G. Effect of ω-3 fatty acids on rectal mucosal cell proliferation in subjects at risk for colon cancer. Gastroenterology, *103:* 883–891, 1992.

27. Burns, C. P., Halabi, S., Clamon, G. H., Hars, V., Wagner, B. A., Hohl, R. J., Lester, E., Kirshner, J. J., Vinciguerra, V., and Paskett, E. Phase I clinical study of fish oil fatty acid capsules for patients with cancer cachexia: cancer and leukemia group B study 9473. Clin. Cancer Res., *5:* 3842–3947, 1999.

28. Rose, D. P., Connolly, J. M., and Liu, X-H. Effect of linoleic acid on the growth and metastasis of two human breast cancer cell lines in nude mice and the invasive capacity of these cell lines *in vitro*. Cancer Res., *54:* 6557–6562, 1994.

29. Germain, E., Chajes, V., Cognault, S., Lhuillery, C., and Bougnoux, P. Enhancement of doxorubicin cytotoxicity by polyunsaturated fatty acids in the human breast tumor cell line MDA-MB-231: relationship to lipid peroxidation. Int. J. Cancer, *75:* 578–583, 1998.

30. American Institute of Nutrition Report of the American Institute of Nutrition *ad hoc* committee on standards for nutritional studies. J. Nutr., *107:* 1340–1348, 1977.

31. Hardman, W. E., Moyer, M. P., and Cameron, I. L. Efficacy of treatment of colon, lung and breast human carcinoma xenografts with: doxorubicin, cisplatin, irinotecan or topotecan. Anticancer Res., *19:* 2269–2274, 1999.

32. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., *72:* 248–254, 1976.

33. Esterbauer, H., and Zollner, H. Methods for determination of aldehydic lipid peroxidation products. Free Radic. Biol. Med., *7:* 197–203, 1989.

34. Ackman, R. G. Simplification of analyses of fatty acid in fish lipids and related lipid samples. Acta Med. Scand., *222:* 99–103, 1987.

35. Aebi, H. Catalase *in vitro*. Methods Enzymol., *105:* 121–125, 1984.

36. Flohe, L., and Otting, F. Superoxide dismutase assays. Methods Enzymol., *105:* 93–104, 1984.

37. Paglia, D. E., and Valentine, W. N. Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med., *70:* 158–169, 1967.

38. Vijayalaxmi, Frei, M. R., Dusch, S. J., Guel, V., Meltz, M. L., and Jauchem, J. R. Frequency of micronuclei in the peripheral blood and bone marrow of cancer-prone mice chronically exposed to 2450 MHz radiofrequency radiation. Radiat. Res., *147:* 495–500, 1997.

39. Sokal, R. R., and Rohlf, F. J. Assumptions of analysis of variance. *In:* Biometry, pp. 400–453. New York: Freeman and Co., 1981.

40. Somani, S. M., Husain, K., and Schlorff, E. C. Response of antioxidant system to physical and chemical stress. *In:* S. I. Baskin and H. Salem (eds.), Oxidants, Antioxidants and Free Radicals, pp. 125–141. Washington, D.C.: Taylor & Francis, 1997.

41. Masotti, L., Casali, E., and Galeotti, T. Lipid peroxidation in tumor cells. Free Radic. Biol. Med., *4:* 377–386, 1988.

42. Calviello, G., Paola, P., Maggiano, N., Franceschelli, P., Di Nicuolo, F., Marcocci, M. E., and Bartoli, G. M. Effects of eicosapentaenoic and docosahexaenoic acids dietary supplementation on cell proliferation and apoptosis in rat colonic mucosa. Lipids, *34:* S111, 1999.

43. Bartram, H., Gostner, A., Scheppach, W., Reddy, B. S., Rao, C. V., Dusel, G., Richter, F., Richter, A., and Kasper, H. Effects of fish oil on rectal cell proliferation, mucosal fatty acids, and prostaglandin E2, release in healthy subjects. Gastroenterology, *105:* 1317–1322, 1993.

44. Vankatraman, J. T., Chandrasekar, B., Kim, J. D., and Fernandes, G. Effects of n-3 and n-6 fatty acids on the activities and expression of hepatic antioxidant enzymes in autoimmune-prone NZB X NZW F_1 mice. Lipids, *29:* 561–568, 1994.

45. Atkinson, T. G., Barker, H. J., and Meckling-Gill, K. A. Incorporation of long-chain n-3 fatty acids in tissues and enhanced bone marrow cellularity with docosahexaenoic acid feeding in post-weaning Fischer 344 rats. Lipids, *32:* 293–302, 1997.

46. Fischer, M. A., and Black, H. S. Modification of membrane composition, eicosanoid metabolism, and immunoresponsiveness by dietary omega-3 and omega-6 fatty acid sources, modulators of ultraviolet-carcinogenesis. Photochem. Photobiol., *54:* 381–387, 1991.

47. Frenoux, J. M., Prost, E. D., Belleville, J. L., and Prost, J. L. A polyunsaturated fatty acid diet lowers blood pressure and improves antioxidant status in spontaneously hypertensive rats. J. Nutr., *131:* 39–45, 2001.