

Docosahexaenoic Acid (DHA), a Primary Tumor Suppressive Omega-3 Fatty Acid, Inhibits Growth of Colorectal Cancer Independent of p53 Mutational Status

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Abstract: Human colon carcinoma COLO 205, carrying wild type p53, grown subcutaneously in athymic mice was inhibited 80% by a high fat menhaden oil diet containing a mixture of omega-3 fatty acids compared to the low fat corn oil diet containing omega-6 fatty acids. Feeding a high fat diet of golden algae oil containing docosahexaenoic acid (DHA) as the sole long chain omega-3 fatty acid resulted in 93% growth inhibition. Similar findings were previously reported for WiDr colon carcinoma containing mutated p53 (His237). In vitro, 125 μ M DHA inhibited COLO 205 growth by 81%, WiDr by 42%, while eicosapentaenoic acid (EPA) marginally inhibited growth of both lines by approximately 30%. DHA inhibited cell proliferation by 41% in WiDr but did not significantly inhibit proliferation in COLO 205. Cell cycle analysis revealed that DHA arrested cell cycle at Resting/Gap 1 (G0/G1 phase) in WiDr and at Gap 2/Mitosis (G2/M) phase in COLO 205. DHA induced apoptosis in COLO 205 but not in WiDr, and EPA did not induce apoptosis in either line. Taken together, these findings suggest DHA is the primary tumor suppressive ω -3 fatty acid in vivo and in vitro and inhibits cancer growth by p53 dependent and independent pathways, while the marginal inhibition by EPA is p53 independent.

Introduction

Colorectal carcinoma is the third most frequent cause of cancer death in the United States, with 106,680 new cases and 55,170 deaths estimated for 2006 (1). Mounting evidence suggests a relationship between high-level fat intake and colorectal cancer risk (2), with epidemiological and experimental evidence supporting a protective role for omega-3 (ω -3) polyunsaturated fatty acids (PUFAs) against the development of colon cancer. Cultures with higher consumptions of fish oil containing ω -3 PUFAs, such as Alaskan and Greenland Eskimos, when compared with North Americans, subsequently have a lower rate of colon cancer (3,4). In more recent studies, Caygill et al. (5) have reported an inverse relationship between fish and fish oil consumption and the

risk of colorectal cancer in 24 European populations. In a case-controlled study of fat consumption in women, Willet et al. (6) reported that fish oil consumption offered protection against colorectal cancer.

Studies with fish oil, which contains eicosapentaenoic acid (EPA; C20:5, ω -3) and docosahexaenoic acid (DHA; C22:6, ω -3), have shown a protective role against the induction and progression of experimentally produced colon cancer in laboratory animals (7). Connolly et al. (8), recently reported that dietary DHA, found in golden algae oil, depressed the growth of mammary carcinoma in athymic mice. Dietary DHA significantly depressed the growth of human colon carcinoma WiDr in athymic mice containing mutated p53 (9). We now extend those studies to evaluate the effects of dietary algae oil containing DHA and fish oil containing a mixture of ω -3 PUFAs on the growth of COLO205 containing wild type p53 in athymic mice.

Transcription factor p53 is a protein that functions in tumor suppression by inducing various genes and is known to play an important role in the regulation of cell cycle arrest and apoptosis. Its mutational status leading to loss of function is a commonly observed event in various types of cell transformation (10), with high mutational frequencies found in almost every location and subtype of tumor (11). Of cancers, 30–70% contain p53 mutations, which have been characterized anywhere from a point mutation in 1 or 2 alleles to the loss of the entire gene (12). Colon cancers exhibit higher percentages of mutation in contrast to breast cancers, which exhibit lower mutation incidences (13,14).

In this study, the effects of dietary oils containing ω -3 PUFA's and ω -6 UFAs on colon carcinomas with differing p53 mutational status were compared both in vivo and in vitro to evaluate the role of the p53 protein in the observed growth inhibition from ω -3 PUFAs on colon cancer cells. The effects of ω -3 PUFAs on both human cancer cell lines, WiDr and COLO 205, were examined and compared both in vivo and in vitro. WiDr, reported to have been derived from a primary adenocarcinoma of the rectosigmoid colon established from a 78-yr-old female (15), was revealed later by

Table 1. Composition of Experimental Diets^a

Ingredient	8% corn oil group (%)	16% menhaden oil group (%)	16% DHASCO oil group (%)	24% corn oil (%)
Corn oil	8.00	8.00	8.00	24.00
Menhaden oil	0.00	16.00	0.00	0.00
DHASCO oil	0.00	0.00	16.00	0.00
AIN-93 mineral mix	3.69	4.45	4.45	4.45
L-cystein	0.19	0.23	0.23	0.23
Choline	0.26	0.32	0.32	0.32
AIN-93 vitamin mix	1.05	1.27	1.27	1.27
Casein	14.76	17.80	17.80	17.80
Cellulose	5.27	6.36	6.36	6.36
Corn starch	43.15	29.45	29.45	29.45
Dyetrose	14.36	9.80	9.80	9.80
Sucrose	9.26	6.32	6.32	6.23

^aAbbreviation is as follows: DHASCO, docosahexaenoic acid-rich single-cell oil.

karyotypic analysis to be a derivative of HT-29 colon adenocarcinoma (16), which was initiated from a moderately well differentiated, grade II adenocarcinoma from a 44-yr-old female in 1964 (17). It is the faster growing cell line (doubling time of 14 h) and carries a mutated p53 at codon (His273) (18). COLO 205 is 1 of 3 colorectal adenocarcinoma cell lines (COLO 201 and 206) isolated in 1975 from the ascites fluid of a 70-yr-old Caucasian male diagnosed with colon carcinoma. The patient had been treated with 5-fluorouracil 4–6 wk prior to removal of the ascites fluid for establishment of the new cell line. Although chromosomal markers were identical for COLO 201 and 205, autosomal polysomy analysis indicated that there may be a cytogenetic basis for 3 types of cellular morphology (19). COLO 205 is the slower growing cell line (doubling time of 23 h) (11) and carries wild-type p53. Within the present study, both cell lines were utilized to elucidate the involvement of p53 in the growth inhibition provoked by ω -3 PUFAs.

Changes in cell number in vitro and tumor size in vivo results from a shift in the balance between cell proliferation and apoptosis, and in cancerous tissues, the rate of proliferation becomes higher than that of cell death, resulting in an increase in cell number and tumor size. Because of this unique characteristic of cancer cells, the tumor inhibiting effects of ω -3 PUFAs were evaluated by monitoring both proliferation and programmed cell death in the present study. Necrosis was not monitored, but rather the occurrence of necrosis was deduced through the elimination of proliferation and death.

Materials and Methods

Animals and Diets

Twenty-four adult athymic mice (homozygous BALB/c nu/–) in this study. Mice were housed under aseptic conditions (germ-free laminar-flow hood, sterilized cages, bedding, and water) at 27°C. Autoclaved laboratory mouse chow (Dyets Inc., Bethlehem, PA) was fed until implementation of the experimental diet formulation, based on purified AIN-

93M with some modifications in fats (Table 1). The animals were assigned randomly to 4 varying diets: (1) 8% Mazola corn oil, representing a low-fat diet sufficient to prevent linoleic acid (LA) deficiency, with an average group body mass of 22.4 g; (2) 24% Mazola corn oil, representing a high-fat diet rich in ω -6 (20) with an average group body mass of 22.2 g; (3) 8% Mazola corn plus 16% menhaden oil, representing a high-fat diet enriched with various ω -3 fatty acids, with an average group body mass of 24.4 g; and (4) 8% Mazola corn plus 16% DHA-rich single-cell oil (DHASCO[®]) (Martek Bioscience, Columbia, MD) representing a high-fat diet rich in 1 particular ω -3 fatty acid, DHA, with an average group body mass of 24.2 g. Experimental oil fatty acid compositions are shown in Table 2. Mice were distributed 8 to 9 mice per diet group and maintained on the experimental diets for 55 days. Food intake was monitored daily to ensure total energy consumption consumed by each mouse.

Tumor Implantation

Mice were implanted subcutaneously with COLO 205 human colon adenocarcinoma cell lines purchased from American Type Culture Collection (Manassas, VA) and maintained in our laboratory. Mice were fed respective experimental diets immediately after tumor inoculation. Tumor weights were recorded every 3–4 days and estimated by a calculation based on the formula: tumor weight (mg) = $A \times B \times C/2$, where A, B, and C represent the 3 perpendicular diameters of the tumor in millimeters. Animal body weights were measured and recorded every 3–4 days.

Cell Culture and Fatty Acid Treatments

WiDr cells were maintained in MEM Eagle (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1% streptomycin, 1.5 g/l sodium bicarbonate, and 1.0 mM sodium pyruvate. COLO 205 cells were maintained in RPMI 1640 supplemented

Table 2. Fatty Acid Composition for Oils Used in Feeding^a

Fatty acid	Menhaden oil %	DHASCO oil %	Corn oil %
8:0	—	<0.1	—
9:0	—	<0.1	—
10:0	—	0.4	—
11:0	—	<0.1	—
12:0	—	2.21	—
13:0	—	<0.1	—
14:0	9.0	13.2	—
14:1	—	0.12	—
15:0	0.7	—	—
16:0	17.1	13.1	11.1
16:1	12.5	1.8	0.1
16:2	1.7	—	—
16:3	1.7	—	—
16:4	1.8	—	—
17:0	0.9	<0.1	—
17:1	—	<0.1	—
18:0	2.8	0.7	2.0
18:1n-9	11.4	21.8	25.8
18:1n-7	—	<0.1	—
18:2n-6	1.5	1.6	58.9
18:3n-3	1.6	<0.1	1.6
18:4	3.5	—	—
20:0	0.2	0.10	—
20:1n-9	1.6	<0.1	—
20:2n-6	—	<0.1	—
20:4n-6	2.3	<0.1	—
20:5n-3	15.5	<0.1	—
21:5	0.8	—	—
22:0	—	0.21	—
22:1	0.5	—	—
22:5n-3	2.4	0.3	—
22:6n-3	9.1	44.3	—
24:0	—	<0.1	—
24:1	0.1	<0.1	—
Others	1.3	0.2	0.03
Total ω -3	28.6	44.6	1.6
Total ω -6	3.8	1.6	58.9
LA: DHA	0.16	0.04	—

^aAbbreviation is as follows: DHASCO, docosahexaenoic acid-rich single-cell oil.

with 10% FBS, 1% streptomycin, 1.5 g/l sodium bicarbonate, 0.15% D (+)glucose, 0.3 mM sodium pyruvate, and 5.0 mM 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid mixture. Both cell lines were grown in flasks as monolayers at 37°C in a saturated humidified environment consisting of 5% CO₂. Fatty acid methyl esters (LA, EPA, and DHA) were purchased from Sigma (St. Louis, MO) and diluted in ethanol to a final concentration of 10 mg/ml stock solution. Fatty acid stock solutions were flushed with nitrogen gas and stored in the dark at -20°C. Cultured cells were plated at 1.5 × 10⁶ cells per 100-mm² diameter Petri dish. Following 24 h incubation, cells were treated with 125 μM LA, EPA, and DHA, respectively. Cells treated with ethanol were used as controls for each experiment. At the end of incubation, the numbers of viable cells were counted by a trypan blue dye exclusion method.

p53 cDNA Sequencing

Total RNA was isolated from COLO 205 cells using an RNA Purification kit (Epicenter, Madison, WI) according to manufacturers' procedure. The purified RNA served as a template for cDNA synthesis with an oligo T primer and reverse transcriptase. A 1.0 kb fragment of the p53 gene (exon 1–10) was amplified by polymerase chain reaction (PCR) with the synthesized cDNA as a template and a pair of primers, 5'-ACACTTTGCGTTCGGGCT-3' and 5'-AGACCCAAAACCCAAAATGG-3'. Four PCR primers (5'-GTTGGCTCTGACTGTACC-3', 5'-TGGAGT GAGCCCTGCTCC-3', 5'-CCCCTCCTCAGCATCTTA-3', and 5'-CTGAAGGGTGAAATATTC-3'), specific for the region of high mutation within p53, were used bidirectionally to sequence the cDNA. The DNA was sequenced with an Applied Biosystems Prism 3730 DNA Analyzer at the Nevada Genomic Center (Reno, NV).

Cell Proliferation Assay

Cells were plated on a 12-well culture dish at a concentration of 1.5 × 10⁵ per well. After 24 h incubation, the cells were treated with 125 μM fatty acids (LA, EPA, DHA) for 72 h. At the end of the treatment, cells were trypsinized and counted with a hemocytometer. The cells were then replated on a 96-well culture dish at 1.5 × 10⁴/100 μL per well in quadruplicate. After 6 h incubation, cells were again treated with fatty acids for 9–12 h. Cells were incubated with bromodeoxyuridine (BrdU) for 2 h and then assayed according to kit instructions (Calbiochem, San Diego, CA).

Cell Cycle Analysis

Cells were plated on 100-mm Petri dishes at a concentration of 1.5 × 10⁶ per dish. After 24 h incubation, media was changed to 0.04% FBS media and incubated for 36 h to synchronize the cells. The cells were then treated with 125 μM fatty acids for 12 h with fresh complete media and then collected and fixed in 70% ethanol overnight at 4°C. Cells were stained in 50 μg/ml propidium iodide staining solution and their DNA was analyzed using the FACScan laser flow cytometer analysis system (BD Biosciences, Franklin Lakes, NJ). Time course studies were performed at 6, 12, 24, and 48 h to choose an optimum time point (data not shown).

Mitochondrial Membrane Potential Analysis

Cells were plated on 100-mm Petri dishes at 1.5 × 10⁶ cells and treated with 125 μM fatty acids for 72 h. Mitochondrial membranes were probed with JC-1 (BioCarta, San Diego, CA), and potential was measured using the FACScan laser flow cytometer analysis system.

Caspase-3 Analysis

Caspase-3 activity was measured with Caspase-3 Cellular Activity assay kits (Calbiochem, San Diego, CA). Cells treated with 125 μM fatty acids for 72 h were used for this assay. The harvested cells were lysed and centrifuged to obtain supernatant. The enzyme activity was determined with colorimetric measurement at 405 nm.

Statistical Analysis

Data graphically illustrated the compared average tumor mass of each test group at successive time intervals, presented as means \pm standard error of the mean. The tumor mass of each group in the animal studies was compared against the 24% corn oil group, and the cell numbers of each group in cell culture were compared against the LA-treated group using a Student's *t*-test.

Results

Monitoring of COLO 205 Tumor Growth Rates in Athymic Mice

The growth of human adenocarcinoma COLO 205 in nude mice was significantly suppressed by a diet supplemented by golden algae oil compared with a diet composed of 8% corn oil (Fig. 1). At the end of the feeding study (Day 55), the growth of COLO 205 was inhibited 93% and 88.7% by supplementation of golden algae oil compared with the 8% and 24% corn oil groups, respectively. Final mean tu-

mor weight of the golden algae supplemented group was 73.8 mg. Consumption of a diet supplemented with menhaden oil decreased tumor growth by 68.6% and 67.7% compared with the 8% and 24% corn oil fed groups, respectively. At Day 55, mean tumor weights of the menhaden oil group was 211.9 mg. Interestingly, the final mean tumor weight of the 8% corn oil fed group, representing a low-fat fed group, was 30.8% larger than that of 24% corn oil fed group representing a high-fat fed group, even though the difference between the 2 groups was not statistically significant. Final mean tumor weights of the 2 groups corn oil fed groups were 947.6 mg and 655.9 mg for the 8% and 24% corn oil diets, respectively. Final group body weights were statistically insignificant between groups and varied little from initial starting weights. DHASCO-fed mice ended at an average weight of 24.13 g, menhaden at 26.33 g, 8% corn oil were 23.78 g, and 24% corn oil were 22.16 g, respectively (data not shown).

Monitoring Effect of PUFAs on Tumor Cell Growth In Vitro

Treatment of WiDr and COLO 205 adenocarcinoma cells with 125 μM EPA and DHA decreased cell number significantly compared to LA after 72-h incubation (Figs. 2A and 2B). EPA inhibited cell number by 29.2% for WiDr and 36.6% for COLO 205 compared to LA-treated cells. DHA resulted in a 42% and 81% growth inhibition with WiDr and COLO 205, respectively, compared to the LA groups; 50 μM EPA and DHA did not result in cell number reduction with statistical significance in WiDr (data not shown).

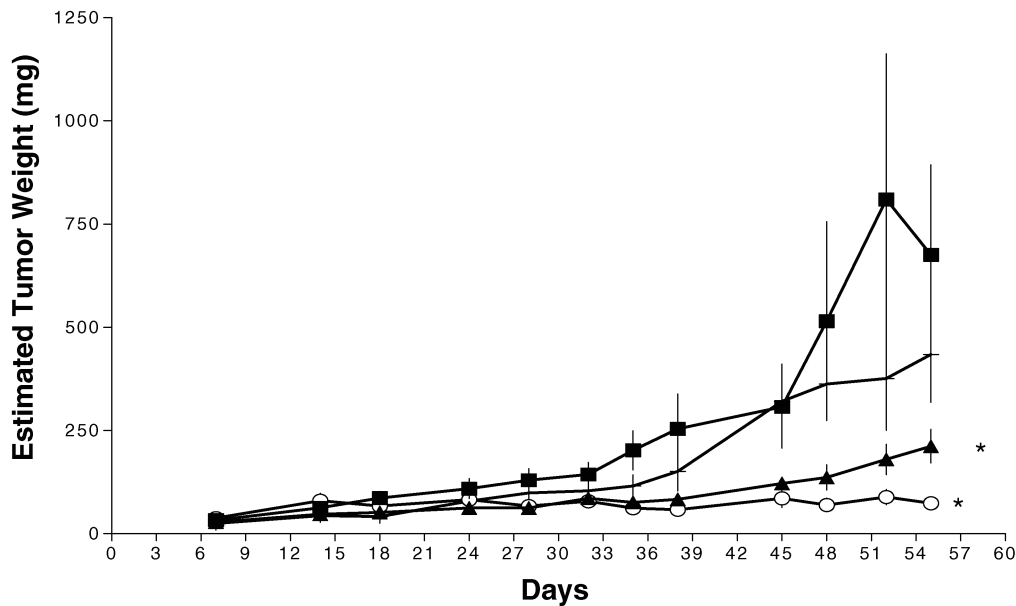


Figure 1. Effects of dietary fatty acids (corn oil, menhaden oil, golden algae oil) on the growth of COLO 205 tumor in athymic mice. Mouse weights across experimental diets were not statistically significant (data not shown). Tumor weights were estimated using the formula (tumor weight in mg = $A \times B \times C/2$), where A, B, and C represent the 3 perpendicular diameters of the tumor in millimeters. Each data point represents the mean \pm standard error of the mean from 5–6 animals. *P* values were compared to 24% corn oil diet. *, *P* < 0.05; **, *P* < 0.01 at Day 53—, 24% corn oil treated; ■, 8% corn oil treated; ▲, 8% corn oil and 16% menhaden oil; ○, 8% corn oil and 16% golden algae oil.

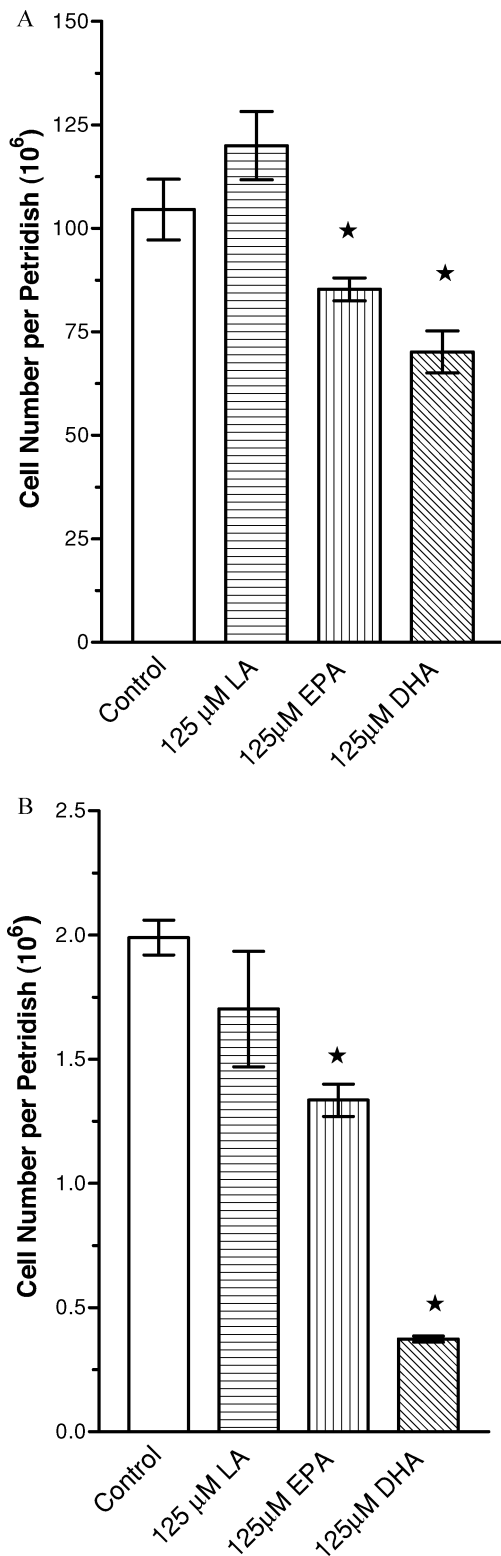


Figure 2. Effects of purified ω -3 fatty acid methyl esters on the cell numbers of WiDr (A) and COLO205 (B) in cell culture. Cultured cells were plated and treated with indicated fatty acid methyl esters for 72 h. A control group was treated with ethanol. At the end of the treatment, cells were harvested using trypsin, and cell numbers were counted using a hemocytometer. Cell viability was determined with a trypan blue dye exclusion method. Data represent the mean \pm standard error of the mean from 3 separate experiments. *P* values were calculated against the linoleic acid (LA) treated group. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. *, *P* < 0.05.

Sequence of the p53 Tumor Suppressor Protein in COLO 205

The p53 tumor suppressor gene of COLO 205 was sequenced because of conflicting data concerning the status of p53 carried by the cell line. Ho et al. (21,22) reported that the p53 gene carried by COLO 205 is wild type, while O'Conner et al. (11) reported COLO 205 carries a point mutation (base change from G to A, which caused an amino acid change from G to E) in p53 at codon 266. Four overlapping primer pairs specific for p53 were used bidirectionally to sequence the point of mutation at codon 266. Sequences from 4 primers revealed that codon 266 of p53 in COLO 205 was not mutated.

DHA Inhibited Cell Proliferation with WiDr

The BrdU incorporation assay revealed that 125 μ M of DHA caused significant inhibition of cell proliferation with WiDr but not with COLO 205 (Fig. 3). A decrease in BrdU incorporation was not observed with 50 μ M DHA in WiDr, a concentration that does not inhibit cell number (data not shown). The level of BrdU incorporation with 125 μ M DHA was decreased by 41% in WiDr compared to the LA control, which is consistent with the level of growth inhibition (42%). EPA did not significantly suppress BrdU incorporation in WiDr or COLO 205, although a nonsignificant decrease in proliferation was observed in EPA treated WiDr cells. In a similar manner, a slight decrease in BrdU incorporation was observed in the DHA treated COLO 205 compared against the LA treated group, although not statistically significant.

DHA Arrested Cell Cycle at Different Phases in Different Cell Lines

Flow cytometric analysis of cell cycle indicated that DHA arrested cells at the G0/G1 phase in WiDr and at the G2/M phase in COLO 205 (Table 3). EPA also inhibited cell cycle progression at the G1/G0 phase in WiDr but not in COLO 205. Cells in G0/G1 phase were always significantly higher in the starved control group than in LA treated cells.

DHA Induced Apoptosis with COLO 205

Mitochondrial membrane potential analysis showed that DHA induced mitochondrial membrane potential in support of apoptosis in COLO 205 but not WiDr, and cells treated with EPA did not induce apoptosis in either colon cancer cell line (Fig. 4). These findings are summarized in Table 4. This finding was further confirmed by the measurement of caspase-3 activity employing enzyme-linked immunosorbent assay (ELISA) analysis. DHA-treated COLO 205 cells showed significantly higher activity of caspase-3 after 72 h incubation (Fig. 5) EPA did not induce caspase-3 activity in COLO 205. Increases in caspase-3 activity were not observed in either EPA- or DHA-treated WiDr cells.

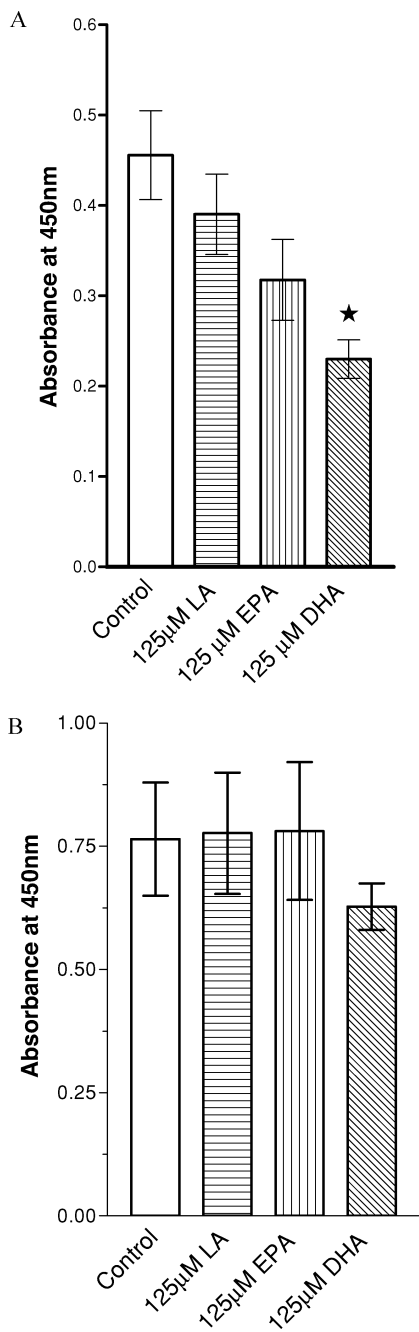


Figure 3. Effects of purified ω -3 fatty acid methyl esters on the DNA synthesis of WiDr (A) and COLO 205 (B). Incorporation of bromo-deoxyuridine (BrdU) into DNA was quantified by enzyme-linked immunosorbent assay in accordance with manufacturer's instruction. Cells were treated with purified ω -3 fatty acid methyl esters for 72 h, replated, and incubated for 12 h prior to the BrdU addition. Values are means \pm standard error of the mean of triplicates within a single experiment. The experiment was repeated three times. LA, linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. *, $P < 0.01$.

Discussion

ω -3 PUFAs Inhibit Growth of Colon Adenocarcinomas Independent of p53 Status

The influence of dietary ω -3 fatty acid supplementation on COLO 205, which carries wild type p53, in athymic nude

Table 3. The Effects of Purified ω -3 Fatty Acid Methyl Esters on Cell Cycle Progression Within Both WiDr (A) and COLO 205 (B) Cell Lines^a

Sample	G1	S	G2
A			
WiDr Control	74.24	10.91	11.63
WiDr LA	61.25	21.82	13.7
WiDr EPA	70.96	13.8	12.67
WiDr DHA	69.6	13.5	14.54
B			
COLO 205 Control	75.61	11.38	12.49
COLO 205 LA	70.28	16.43	11.79
COLO 205 EPA	71.57	15.96	11.33
COLO 205 DHA	68.75	13.49	17.14

^aAbbreviations are as follows: G1, Gap 1 phase; S, synthesis phase; G2, Gap 2 phase; LA, linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values represent relative percentages of cells in particular cell cycles; 20,000 gated cells were accounted for per sample. Values are mean standard error of the mean of the 3 independent experiments. Bold numbers indicate statistical significance compared to LA treated value.

mice was examined in this study and compared with dietary ω -3 fatty acid influence on WiDr (9), which carries mutated p53. In addition to in vivo studies, the influence of ω -3 PUFAs was examined in cell culture to elucidate the role of p53 in the tumor growth inhibition exerted by individual ω -3 PUFA supplementation. p53, a DNA housekeeping gene and tumor suppressing protein, has been shown to play a crucial role in protecting organisms from developing cancer (23) through the regulation of programmed cell death and the activation of cell cycle arrest, while in various cancers (especially colon cancer), the incidence of p53 mutation is high.

Feeding menhaden oil, containing a mixture of omega-3 fatty acids (EPA and DHA) to athymic mice implanted with WiDr and COLO-205 adenocarcinoma resulted in significant tumor growth suppression of both colon tumor types by 66% (9) and 80%, respectively, when compared to corn oil fed controls (Fig. 1). Moreover, consumption of golden algae oil, which contains a single ω -3 fatty acid (DHA), resulted in greater suppression of tumor growth compared to menhaden oil with both tumors. The effects of dietary supplementation on the growth of COLO-205 in vivo were similar to WiDr at 93% and 90% inhibition as compared to corn oil fed controls, respectively. Conflicting data regarding the mutational status of p53 in COLO 205 (11,21,22) prompted a sequencing (data not shown) of the p53 gene, revealing it to be wild type. COLO 205 growth was significantly inhibited by supplementation with golden algae oil compared to the 24% corn oil group. Both COLO 205 and WiDr feeding studies were designed employing the same parameters and resulted in final mean tumor sizes for COLO 205 as follows for each of the diets: 948 mg 8% corn oil, 656 mg 24% corn oil, 212 mg 8% corn and 16% menhaden oils, and 74 mg for 8% corn and 16% golden algae oils (Fig. 1). The COLO 205 carcinoma grew slower than WiDr, with the final tumor weights being approximately one-fourth of WiDr for the respective diets as follows: 2,303 mg 24% corn oil, 1,681 mg

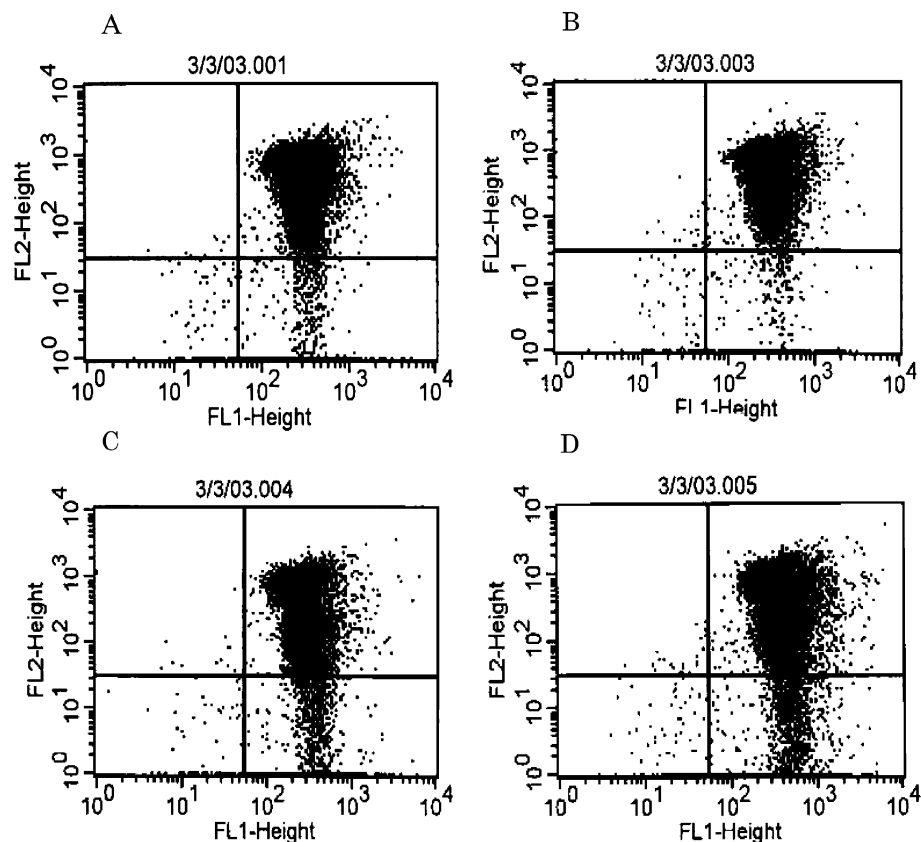


Figure 4. Effects of ω -3 fatty acid methyl esters on mitochondrial transmembrane potential in COLO 205. Cells were stained with JC-1 as manufacturer's instruction after 72-h incubation with fatty acids; 10,000 gated cells were counted per sample. Quadrant was set to have more than 90% of nontreated cells in Quadrant II. A: Nontreated group. B: Linoleic acid treated group. C: Eicosapentaenoic acid treated group. D: Docosahexaenoic acid treated group. This figure represents 1 out of 3 experiments.

8% corn oil, 782 mg 8% corn and 16% menhaden oils, and 223 mg 8% corn and 16% golden algae oils. Although the growth rate of colon carcinoma COLO 205 is approximately 25% the growth rate of WiDr, the relative extent of inhibition by fish oil and algae oil compared to the 24% corn oil fed groups is approximately the same in both the fast and slow

growing tumor lines WiDr and COLO 205, respectively. The slower growth rate observed for COLO 205 may be due to the fact that it contains a wild type p53 gene, presumably with full activity, whereas the faster growing WiDr contains a mutated p53 gene. Taken together, these in vivo studies indicate DHA as the dietary tumor suppressive long chain ω -3 fatty acid and that DHA responsiveness is independent of the p53 status within tumor lines. It is possible that the depressed growth of COLO 205 by DHASCO oil was due to the higher content of ω -3 fatty acids, the lower ratio of ω -6/ ω -3 fatty acids, or the lower LA/DHA ratio (Table 2). The ω -6/ ω -3 ratio was 0.04, 0.13, and 36.8 for the DHASCO, menhaden oil, and Mazola corn oils employed in this study, respectively. The total ω -3 fatty acid content of the diets was 44.6%, 28.6%, and 1.6% for the DHASCO, menhaden, and Mazola corn oils, respectively (Table 2).

Table 4. Percentage of Intact and Apoptotic Cells With Various Purified Fatty Acids Methyl Ester Treatments in WiDr and COLO 205^a

Groups	Intact healthy cells (% total gated cells)	Apoptotic cells (% total gated cells)
WiDr Nontreated	91	8.91
WiDr LA	88.8	10.7
WiDr EPA	96.1	3.45
WiDr DHA	83.5	16.2
COLO 205 Nontreated	91.7	8.29
COLO 205 LA	94.8	5.11
COLO 205 EPA	91.7	8.28
COLO 205 DHA	86.6	13.3

^aAbbreviations are as follows: LA, linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values are mean standard error of the mean of the 3 separate experiments. Bold numbers indicate statistical significance as compared to LA treated samples.

DHA Is the Primary Growth-Inhibiting ω -3 PUFA

To differentiate the individual effects of each of the ω -3 PUFAs on tumor growth inhibition, COLO 205 and WiDr tumor lines were cultured in vitro and individually treated with each ω -3 PUFAs. At a concentration of 125 μ M, EPA and DHA treatment resulted in significant decreases in final

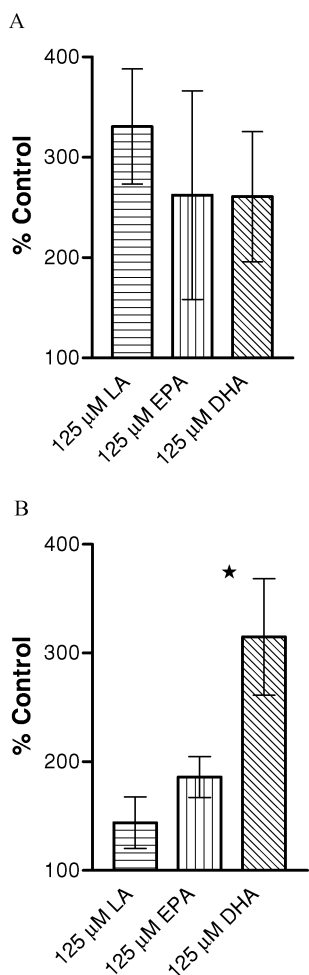


Figure 5. Effects of ω -3 fatty acid methyl esters on caspase-3 activity in WiDr (A) and COLO 205 (B). Activity of caspase-3 was measured by colorimetric assay as per manufacturer's instruction. Cells were treated with fatty acids for 72 h before the harvest. Data are shown in % control and represent mean \pm standard error of the mean of 3 independent experiments. Each sample was assayed in duplicate in each experiment. LA, linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. *, $P < 0.01$.

viable cell counts of 30% and 81% of the LA cell count, respectively, for COLO 205 and 30% and 45% of the LA control, respectively, for WiDr (Fig. 2). DHA treatment resulted in a higher percent inhibition than EPA in both cell lines, implicating DHA as the more potent ω -3 PUFA (Fig. 2) and correlating with studies in vivo (Fig. 1). Interestingly, EPA reduced cell number by approximately 30% in both cell lines, while the percent inhibition exerted by DHA was twofold higher in COLO 205 (81%) compared against WiDr (42%) (Fig. 2), suggesting that the effects of EPA may be independent of p53, while the effects of DHA may be dependent on p53 in COLO 205. The efficacy of EPA and DHA on tumor growth inhibition is highly cell-line dependent (24). For colon cancer, our data demonstrate that DHA is the primary tumor inhibiting ω -3 PUFA, while the effect of EPA remains unconvincing (25). Further studies are required to clarify the distinctive roles of EPA and DHA on the growth inhibition of colon cancers. It is interesting to note that dis-

tinctive DHA effects between WiDr and COLO 205 were not observed in vivo, suggesting that ω -3 PUFAs may inhibit cancer growth systemically by other mechanisms such as inhibiting the cyclooxygenase-2 activity, which in turn has been reported to depress angiogenesis (26).

DHA Induces Distinctive Cellular Events Depending on p53 Mutational Status, Whereas the Growth Inhibition Exerted by EPA Is p53 Independent

Neither ω -3 PUFAs induced apoptosis in WiDr; instead, DHA caused inhibition of cell proliferation in WiDr, while EPA did not inhibit cell proliferation significantly (Fig. 3). Cell cycle analysis revealed, however, that EPA increased the percentage of WiDr cells in G1 phase (Table 3), a discrepancy that may be owed to the differences in sample preparation: one requiring fixed samples and the other live cells.

Our interpretation is such that EPA delays cell cycle but does not arrest it, resulting in no significant difference in cell proliferation when compared to LA-treated cells. These results suggest that mutated p53 in WiDr may lose its function to induce apoptosis but retains its ability to delay cell cycle, a selective loss of p53 function, previously reported (27). DHA induced apoptosis within COLO 205 (Table 3), while EPA did not, and neither of the ω -3 PUFAs significantly inhibited COLO 205 cell proliferation (Fig. 3). Cell cycle analysis revealed that DHA caused an accumulation of COLO 205 cells in G2/M phase, suggesting that DHA delayed cells at G2/M phase before their route to programmed cell death (Table 3). Again, EPA did not induce apoptosis or inhibit cell proliferation in COLO 205 or cause cell cycle arrest at G2/M. The moderate inhibition of tumorigenesis by EPA may be attributed to the induction of necrosis. This conclusion is supported by the observation that EPA caused similar levels of growth inhibition in both cell lines and did not induce any specific tumor suppressing cellular events. This in turn may be the reason for the observed similar growth inhibition in 2 cell lines with differential p53 status.

The induction of apoptosis and inhibition of cellular progression observed in treatments with ω -3 PUFAs have been assessed with various types of cancer cells. In pancreatic cancer cells, both EPA and DHA induced apoptosis (28–30). In leukemia/lymphoma, the cellular process observed for the growth inhibition by EPA was apoptosis (31,32). Cell cycle arrest, induced by ω -3 PUFAs, was reported for leukemia K-562 cells (33). Apoptosis and cell cycle arrest was observed in the growth inhibition by EPA and DHA in colon cancer cell lines (34–36). In larynx cancer, the mechanism of EPA-induced growth inhibition was determined to be apoptosis (37). In hepatomas, both EPA and DHA inhibit cellular growth (38). Interestingly, mechanisms of inhibition were due to reduction in cell proliferation for EPA and induction of apoptosis for DHA similar to what we observed in this study. In melanoma, DHA induced apoptosis along with cell cycle arrest (39). The doses of the ω -3 PUFAs used in the studies described above ranged from 50 μ M to 150 μ M, which is a

similar range to what was used in this study. These studies are consistent with the growth inhibitory effect of EPA and DHA shown in this study and support the conclusion that different mechanisms of action of tumor growth inhibition by EPA and DHA exist; and furthermore, the mechanism of the inhibition of tumor growth by ω -3 PUFAs are cell-line specific, even though both ω -3 PUFAs inhibit tumor growth.

In conclusion, we have demonstrated that DHA is the primary anticancer ω -3 PUFA both in vivo and in vitro. DHA inhibits cell growth through the induction of p53-dependant apoptosis in colon tumor cells possessing wild type p53, whereas p53 independent growth inhibition was observed in colon tumor cells possessing mutated p53. While EPA also exhibits an anticancer function against both human colorectal cancer cell lines, its efficacy was lower than that of DHA. Interestingly, EPA did not trigger any specific cellular events in either cell line but caused similar percent inhibitions in both. These findings indicate that the primary tumor suppressing fatty acid, DHA, depresses colon tumor growth by multiple mechanisms depending on the p53 status, which potentially broadens the spectrum of tumors that could be impacted by ω -3 fatty acid intervention.

Acknowledgments and Notes

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