JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Inhibition Of Tumorigenesis in *Apc*$^{Min/+}$ Mice by a Combination of (–)-Epigallocatechin-3-gallate and Fish Oil

MOUSUMI BOSE, XINGPEI HAO, JIHYEUNG JU, ALI HUSAIN, SUNNY PARK, JOSHUA D. LAMBERT, AND CHUNG S. YANG*

Susan L. Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, the State University of New Jersey, 164 Frelinghuysen Road, Piscataway, New Jersey 08854-8020

The effect of a combination of (–)-epigallocatechin-3-gallate (EGCG) with fish oil on intestinal tumorigenesis in *Apc*$^{Min/+}$ mice fed a high-fat diet was investigated in the present study. The combined treatment of EGCG and fish oil for 9 weeks reduced the tumor number by 53% as compared to controls while neither agent alone had a significant effect. Apoptosis was significantly increased in all treatment groups. β-Catenin nuclear positivity in adenomas from the combination group was lower than control mice, implicating the modulation of *Wnt* signaling by the combination. Fish oil and the combination significantly reduced prostaglandin E_2 (PGE_2) levels in small intestinal tumors as compared to controls, suggesting modulation of aberrant arachidonic acid metabolism by fish oil. Akt phosphorylation in adenomas was significantly reduced in all treatment groups, which may have contributed to the observed increase in apoptosis. The results indicate that a combination of low doses of EGCG and fish oil can inhibit tumor multiplicity in *Apc*$^{Min/+}$ mice.

KEYWORDS: (–)-Epigallocatechin gallate; fish oil; *Apc*$^{Min/+}$ mouse; intestinal cancer

INTRODUCTION

Colon cancer is the second leading cause of death in the United States (*1*). Epidemiological studies have suggested that the consumption of certain dietary constituents, including green tea polyphenols and ω-3 polyunsaturated fatty acids (ω-3 PUFAs), may decrease colon cancer risk (*2–4*). Animal studies using these dietary constituents have been performed to evaluate the potential chemopreventive activities of these agents. Many studies have suggested that (–)-epigallocatechin-3-gallate (EGCG), the most abundant and biologically active polyphenol in green tea, is responsible for the majority of the chemopreventive activities of green tea (*4–6*).

Several studies in mice have shown that green tea can reduce intestinal and colon cancer risk. Orner et al. (*7*) showed that green tea extract reduced tumor multiplicity in the *Apc*$^{Min/+}$ mouse, a widely used model for intestinal tumorigenesis. An earlier study demonstrated that green tea extract significantly reduced the occurrence of aberrant crypt foci in azoxymethane (AOM)-treated mice on a high-fat diet (*8*). We also found that 0.1% EGCG in the drinking fluid inhibited colon tumor incidence in AOM-treated mice (Bose, Chin, Park, Husain, Liao, Vittal, Kopelovich, Huang, and Yang, unpublished results). Recent studies in our laboratory showed that EGCG in the drinking fluid dose-dependently decreased intestinal tumor multiplicity in the *Apc*$^{Min/+}$ mouse; with 0.32% EGCG, the inhibition was ~40% (*9*). EGCG has been shown to inhibit aberrant *Wnt* signaling by inhibiting the translocation of the *Wnt* mediator β-catenin to the nucleus (*9*). This inhibition may prevent β-catenin from interacting with the transcription factor TCF-4, which suppresses the transcription of genes associated with the cell cycle and cell proliferation (as reviewed in ref *10*).

ω-3 PUFAs, found predominantly in fish oils, have been shown in different animal models to inhibit intestinal tumorigenesis (*11–13*). A study using *Apc*$^{Min/+}$ mice showed that a fish oil concentrate of ω-3 PUFA (2.5% w/w in the diet) reduced tumor multiplicity by 66% in comparison to untreated mice; a similar reduction in tumor growth was found by a fish oil diet (16% w/w in the diet) in nude mice bearing human colon carcinoma xenografts (*11, 14*). Rao et al. (*12*) observed a 50% decrease in colon tumors in AOM-treated rats on a high-fat diet composed of mainly fish oil as compared to similarly treated rats on a high-fat diet consisting of mixed lipids. Studies in colon cancer cell lines have shown that the inhibition of arachidonic acid metabolism by treatment with ω-3 PUFA reduces the proliferation and tumorigenicity of these cells, suggesting that the reduction of inflammatory prostaglandins, such as prostaglandin E_2 (PGE_2), produced by arachidonic acid may be one way by which ω-3 PUFAs mediate their chemopreventive effect (*15*).

Although these and other compounds have shown promise as chemopreventive agents, there are limitations in using a single agent for effective cancer prevention in practice (*13, 16–18*).

* To whom correspondence should be addressed. E-mail: csyang@rci.rutgers.edu.

10.1021/jf071004r CCC: $37.00 © 2007 American Chemical Society
Published on Web 08/16/2007

This appears to be the case for both drugs and dietary agents. Cyclooxygenase-2 inhibitors initially showed promise as effective chemopreventive agents against colon cancer, but it was later revealed that long-term use of these compounds has undesirable side effects (*19*, *20*). Preclinical trials with dietary chemopreventive agents demonstrate less toxicity than the drugs but often have poor bioavailability and lower potency than drugs (*21*–*23*). These findings may imply that consumption of any one dietary nutrient may not be a practical means of effective cancer chemoprevention. The combination of two or more pharmacological agents has been shown to be effective for cancer chemoprevention, maximizing efficacy by affecting different molecular targets and minimizing toxicity by lowering doses of the individual drugs (*24*, *25*). The combination of two or more dietary compounds may be a practical and effective approach to cancer chemoprevention for those same reasons.

The *Apc*$^{Min/+}$ mouse is a transgenic model of human intestinal tumorigenesis that bears a germline mutation at codon 850 of the mouse homologue of the human *Adenomatous Polyposis Coli* (APC) gene, which is frequently mutated in human colon cancer. By age 4–5 months, these mice bear numerous visible tumors in the intestinal tract (~40–50) and usually die due to intestinal blockage, bleeding, and severe anemia (*26*). Because of its genetic similarity to human colon cancer and the quick development of the tumor phenotype, this model is widely used in cancer chemoprevention studies. In the present study, we sought to determine whether EGCG in combination with fish oil could decrease intestinal tumor multiplicity much more effectively than EGCG or fish oil alone in the *Apc*$^{Min/+}$ mouse model.

MATERIALS AND METHODS

Chemicals and Diets. EGCG (Mitsui Norin, Tokyo, Japan) solution was prepared in deionized H_2O containing 0.5% citric acid and used as drinking fluid. Fish oil was a gift from the menhaden oil refinery of Omega Protein, Inc. (Reedville, VA). Menhaden oil contains about 1.7% α-linolenic acid, 13% eicosapentaenoic acid (EPA), and 12% docosahexaenoic acid (DHA) (*27*). The control high-fat diet (**Table 1**) consisted of a 20% fat content (w/w), formulated using a minor modification of the American blend fat developed by the Institute of Shortening and Edible Oils [beef fat (16%), lard (10%), butter fat (12%), hydrogenated soybean oil (30%), peanut oil (5%), and corn oil (27%)] (*28*). This mixed lipid formulation was aimed to represent the fat composition of the average American diet (*28*). This formulation has also been used as a control diet in previous studies that have observed the effects of fish oil on colon carcinogenesis (*12*). The fish oil diet (**Table 1**) also had a 20% fat content (w/w); however, it consisted of 12% fish oil and 8% mixed lipids. Previous studies reported that 20% fish oil significantly inhibited colon tumorigenesis in the AOM-induced rat model (*12*). This concentration of 12% fish oil was used for the present study so as not saturate the dose of fish oil in the combination group. Diets were purchased from Research Diets, Inc. (New Brunswick, NJ).

Breeding and Genotyping of *Apc*$^{Min/+}$ Mice. Male C57BL/6J-*Apc*$^{Min/+}$ and female wild-type littermate mice were initially purchased from The Jackson Laboratory (Bar Harbor, ME) as founders, and a breeding colony was established in the animal facility of the Susan Lehman Cullman Laboratory for Cancer Research (Rutgers, The State University of New Jersey, Piscataway, NJ). Pups were produced from the colony and weaned at 3 weeks of age. Genotyping was performed as previously described (*9*).

Diet Treatment and Tissue Harvesting. Experiments with mice were carried out according to a protocol approved by the Institutional Review Board for the Animal Care and Facilities Committee at Rutgers University. After genotyping, female C57BL/6J-*Apc*$^{Min/+}$ mice (5–6 weeks old, 15 mice per group) were treated as follows: (i) high-fat diet, (ii) high-fat diet plus 0.16% EGCG (as sole source of drinking fluid), (iii) high-fat fish oil diet, or (iv) high-fat fish oil diet plus 0.16% EGCG (as a sole source of drinking fluid). Body weight, food consumption, and fluid consumption were measured weekly. After 9 weeks of treatment, mice were euthanized by CO_2 asphyxiation. The entire intestinal tract was harvested, flushed thoroughly with cold 0.9% saline, cut open longitudinally, and flattened on filter paper to expose tumors in the lumen. The flattened tissues on filter paper were placed on dry ice briefly to score the visible tumors. All tumors were excised from five females per group and snap frozen on dry ice for biochemical analyses. A 150 mm segment from the jejunal portion of the small intestine from another five females in each group was fixed in a 10% formalin solution for 24 h and then transferred to 1× phosphate-buffered saline (PBS) for further tissue processing and immunohistochemical analyses.

Table 1. Diet Composition of High-Fat Mixed Lipid and High-Fat Fish Oil Diets

	high fat		high fat fish oil	
macronutrient	gram %	kcal %	gram %	kcal %
protein	23.9	20.8	23.9	20.8
carbohydrate	45.9	40.0	45.9	40.0
fat	20.0	39.2	20.0	39.2
total		100.0		100.0
kcal/g	4.6		4.6	
ingredient	gram	kcal	gram	kcal
casein	200.0	800.0	200.0	800.0
DL-methionine	3.0	12.0	3.0	12.0
corn starch	203.0	812.0	203.0	812.0
maltodextrin	100.0	400.0	100.0	400.0
dextrose	77.0	308.0	77.0	308.0
cellulose	50.0	0.0	50.0	0.0
corn oil	46.0	414.0	18.5	166.5
fish oil	0.0	0.0	102.0	918.0
beef fat	27.2	244.8	11.0	99.0
lard	17.0	153.0	6.8	61.2
butter fat, anhydrous	20.4	183.6	8.0	72.0
soybean oil	51.0	459.0	20.4	183.6
peanut oil	8.5	76.5	3.4	30.6
mineral mix[a]	35.0	0.0	35.0	0.0
vitamin mix[a]	10.0	40.0	10.0	40.0
choline bitartrate	2.0	0.0	2.0	0.0
total	850.2	3903.0	850.2	3903.0

[a] AIN-76A mineral and vitamin mixtures (American Institute of Nutrition 1977 and 1980).

In a separate experiment, 12 week old female C57BL/6J-*Apc*$^{Min/+}$ mice were administered the same treatments for 3 weeks (n = 7–10/group). After this short-term treatment, mice were euthanized and the intestinal tract was harvested as described before to score intestinal tumors.

Immunohistochemistry. Embedded tissue blocks were cut serially for at least 30 slides and labeled numerically. Slides 1, 10, 20, and 30 were stained for H&E for histopathological evaluation, and the remaining slides were used for immunohistochemistry. A standard ABC method was used for immunohistochemistry as we previously described (*29*). Briefly, tissue sections were deparaffinized in xylene and rehydrated to distilled water, and the endogenous peroxidase was quenched in 0.3% hydrogen peroxide in methanol for 30 min. Subsequently, sections were subjected to antigen retrieval by heating the slides in sodium citrate buffer (0.01 M, pH 6.0) in a pressure cooker for 3 min after reaching full pressure. Nonspecific staining was blocked with either 10% normal horse or goat serum. Antibodies diluted to appropriate concentrations were applied to tissue sections, and the slides were incubated in a humidified chamber overnight at room temperature. Following rinsing in 1× PBS, the sections were incubated with the appropriate biotinylated antibody and then stained using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) for 30 min. 3-3′-Diaminobenzidine (Vector Laboratories) was used as the chromogen.

Proliferative cells were identified by staining with antibodies against Ki-67 (TEC-3, Dako, Carpinteria, CA). Quantification of the number of total cells and Ki-67 positive cells in adenomas was performed by using the Image-Pro Plus system (Silver Spring, MD). The color image

Table 2. Effect of EGCG and Fish Oil on Tumor Multiplicity and Size in *Apc*$^{Min/+}$ Mice after Treatment for 9 Weeks[a]

	small intestine tumor number						
	region			size			
diet (*n*)	proximal	middle	distal	<1 mm	1–2 mm	>2 mm	total
control (15)	7.13 ± 1.2	15.60 ± 3.0	23.93 ± 3.4 [b]	18.00 ± 2.4	20.73 ± 4.1	7.93 ± 1.5 [b]	46.67 ± 6.5 [b]
control + 0.16% EGCG (15)	6.20 ± 1.1	13.73 ± 2.5	18.07 ± 3.0 [bc]	21.33 ± 3.3	12.53 ± 2.4	4.13 ± 0.9 [bc]	38.00 ± 5.9 [bc]
fish oil (15)	5.73 ± 0.9	16.13 ± 3.11	26.27 ± 4.26 [b]	27.07 ± 3.91	17.47 ± 3.4	3.60 ± 1.2 [c]	48.13 ± 7.7 [b]
fish oil + 0.16% EGCG (15)	3.60 ± 0.7	8.20 ± 2.8	10.13 ± 2.4 [cd]	13.13 ± 2.2	8.33 ± 3.9	0.47 ± 0.2 [c]	21.93 ± 5.2 [c]

[a] Age 5 weeks at beginning of treatment. Data are presented as averages ± SE. Different letters ([b–d]) indicate statistical significance ($p < 0.05$, one-way ANOVA).

Table 3. Effect of EGCG and Fish Oil on Tumor Multiplicity and Size in *Apc*$^{Min/+}$ Mice after Treatment for 3 Weeks[a]

	small intestine tumor number						
	region			size			
diet (*n*)	proximal	middle	distal	<1 mm	1–2 mm	>2 mm	total
control (9)	9.56 ± 1.7	11.11 ± 0.8	17.89 ± 1.6	13.22 ± 0.8	19.22 ± 1.1	6.11 ± 1.2 [b]	38.56 ± 1.7
control + 0.16% EGCG (7)	9.29 ± 1.4	9.14 ± 2.1	16.71 ± 3.2	15.43 ± 3.2	17.29 ± 3.7	2.43 ± 0.2 [bc]	35.14 ± 4.8
fish oil (9)	5.78 ± 1.3	12.00 ± 2.9	18.67 ± 3.6	14.78 ± 3.7	18.56 ± 4.7	3.11 ± 1.0 [bc]	36.44 ± 7.1
fish oil + 0.16% EGCG (10)	7.22 ± 1.5	18.33 ± 3.3	19.67 ± 4.2	23.44 ± 3.7	19.44 ± 2.8	2.33 ± 0.7 [c]	45.22 ± 6.3

[a] Aged 12 weeks. Data are presented as average ± SE. Different letters ([b–c]) indicate statistical significance ($p < 0.05$, one-way ANOVA).

containing tumor cells was converted into a black and white image. The area of tumor cells was selected manually and circled with green on the black and white image. The selected cells were highlighted with red and adjusted according to cell intensity to ensure that the highlighted cells were matched well with that in the color image. The number of Ki-67 positive cells and the total number of tumor cells were counted automatically. The proliferation index was defined as the percentage of the number of Ki-67 positive cells in the total number of tumor cells. Apoptotic cells were stained with an antibody against cleaved-caspase 3 (rabbit polyclonal Asp175, Cell Signaling Technology, Danvers, MA) and were quantified similarly. The positivity of nuclear staining for β-catenin and phospho-Akt (Antibodies purchased from Cell Signaling, Danvers, MA) was counted manually and expressed as the percentage of positive-staining cells in the total number of tumor cells. All of the tumor cells in the adenomas were counted.

PGE_2 Levels in Small Intestinal Tumors. Small intestinal tumors were homogenized in tissue lysis buffer containing protease inhibitors and 10 μM indomethacin (a cyclooxygenase inhibitor). Indomethacin was added to maintain the steady state levels of PGE_2. Homogenates were acidified with 0.1 N HCl and extracted with 1 mL of ethyl acetate. The ethyl acetate fraction was dried under vacuum and redissolved in 500 μL of enzyme immunoassay (EIA) buffer (Cayman Chemical, Ann Arbor MI). The levels of PGE_2 were measured using an EIA kit (Cayman Chemical) according to the manufacturer's protocol.

Statistical Analyses. One-way analysis of variance (ANOVA) with appropriate posthoc tests were used for statistical analysis of tumor multiplicity, PGE_2 levels (GraphPad software, San Diego, CA), and immunohistochemistry results (*30*) for comparison among multiple groups. Two-way ANOVA was used to determine interaction of treatments on tumorigenesis. For simple comparisons between groups, a two-tailed Students' *t* test was used. Significance was assigned at $p < 0.05$.

RESULTS

Effect of EGCG in Combination with Fish Oil on Intestinal Tumorigenesis in *Apc*$^{Min/+}$ Mice. In this experiment, we investigated the effects of EGCG in combination with fish oil on intestinal tumorigenesis in *Apc*$^{Min/+}$ mice fed a high-fat diet. The average fluid intake in the EGCG groups was significantly lower (about ~50%) than the groups without EGCG (data not shown). As a consequence, the average food consumption and body weight in the EGCG groups was lower than the other groups (on average, by 19 and 8%, respectively, data not shown), although the differences were not statistically significant.

The combination of fish oil and 0.16% EGCG significantly reduced total tumor multiplicity in female *Apc*$^{Min/+}$ mice (53% decrease as compared to control group, $p < 0.05$ by one-way ANOVA) (**Table 2**). There appeared to be no effect of fish oil on total tumor multiplicity. The inhibition by the combination of agents was greater than that observed in mice treated with EGCG alone (18.5% decrease), although analysis by two-way ANOVA showed no significant interaction of the two agents on inhibition of tumorigenesis. The inhibition of tumor multiplicity by the combination was largely attributable to the decrease in tumor number in the distal portion of the small intestine (58% decrease as compared to control group, $p < 0.05$ by one-way ANOVA). The combination treatment significantly decreased the number of large-sized tumors (94%, $p < 0.05$ by one-way ANOVA), as did the fish oil treatment (55%, $p < 0.05$). Students' *t* test indicated that the combination treatment significantly decreased the number of large-sized tumors in comparison to that of either individual agent.

In the second study, we treated 12 week old female *Apc* $^{Min/+}$ mice with these agents for a period of 3 weeks. As expected, we found that there was no effect of the treatments on total tumor multiplicity; however, the combination did significantly reduce the number of large-sized tumors (**Table 3**, 66% decrease, $p < 0.05$ by one-way ANOVA) in comparison to the control group. Two-way ANOVA analysis showed no significant interaction of the two agents on the inhibition of large-sized tumors.

Effects of 9 Weeks of Treatment on Cell Proliferation and Apoptosis. Antibodies against Ki-67 and cleaved caspase-3 were used to immunohistochemically determine the treatment effect on cell proliferation and apoptosis. Both antibodies showed positive staining in the nucleus (**Figure 1**). The Ki-67 staining was decreased in tumors of all treatment groups, but not in the normal crypts, in comparison to the control group. There was a significant decrease in the proliferation index in adenomas from the EGCG group (by 53%) and the combination group (by 27%) in comparison to that of the control group (**Table 4**). The apoptotic index was significantly higher in the tumors of all treatment groups (about four-fold) in comparison to the control group (**Figure 1** and **Table 4**). The apoptotic index was low in the normal epithelia, and no appreciable change was observed as a result of the treatment.

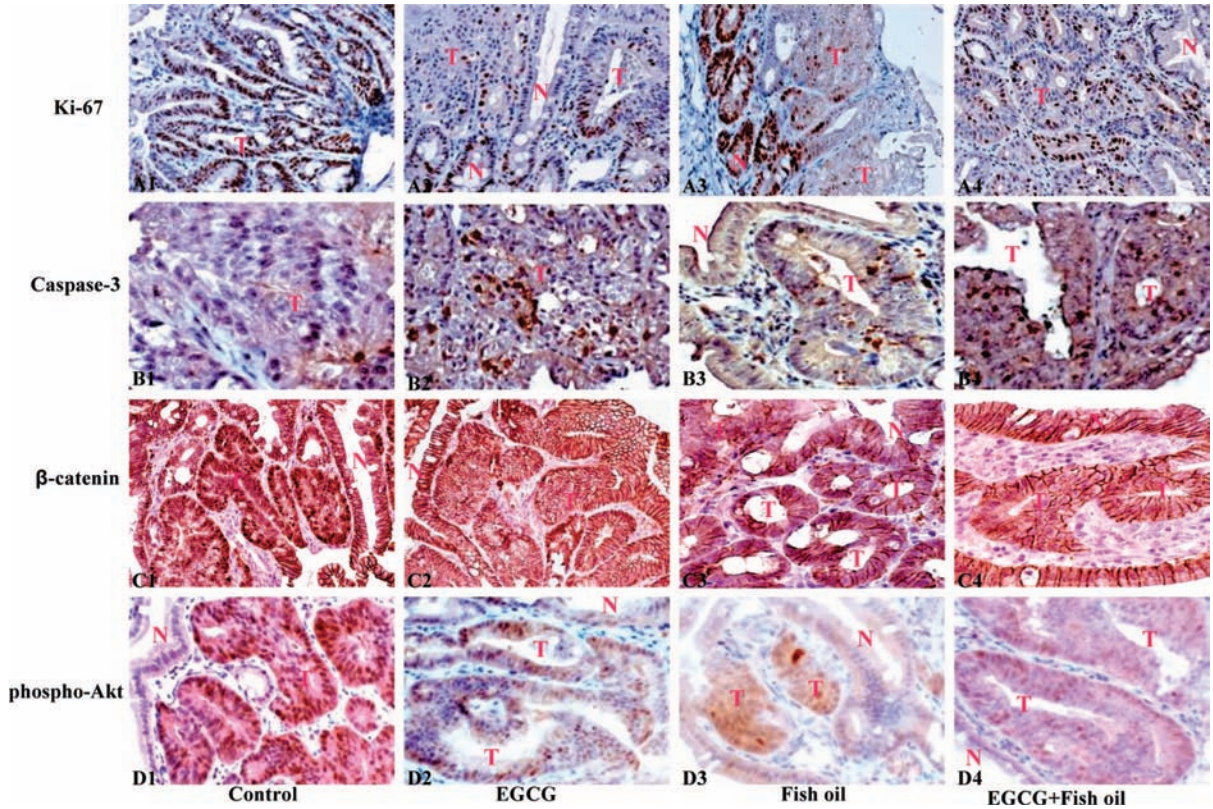

Figure 1. Effect of EGCG and fish oil on the proliferation (**A**), apoptosis (**B**), β-catenin (**C**), and phospho-Akt levels (**D**) in $Apc^{Min/+}$ mice. Strong K_i-67 nuclear staining is observed at the base of the normal crypts from control (A1) and treated mice (A2 and A3), and reduced K_i-67 staining is observed in adenomas from treated mice with EGCG (A2), fish oil (A3), and their combination (A4), in comparison to the control mice. Apoptosis is visualized by detecting cleaved caspase-3. Treatment with EGCG (B2), fish oil (B3), and the combination (B4) increased the number of apoptotic cells in the adenomas in comparison to the control adenomas (B1). Strong β-catenin membrane staining is observed in normal mucosa from both control (C1) and treated mice (C2, C3, and C4). Adenomas from untreated mice showed strong nuclear and cytoplasmic β-catenin staining (C1). Treatment with EGCG (C2), fish oil (C3), and the combination (C3) reduced nuclear staining and increased membrane staining. Treatment with EGCG (D2), fish oil (D3), and the combination (D4) reduced phospho-Akt staining in adenomas in comparison to adenomas from the control group (D1). "N" and "T" indicate normal epithelium and tumor tissue, respectively.

Table 4. Effect of EGCG and Fish Oil (9 Week Treatment) on Cell Proliferation, Apoptosis, PGE_2 Levels, and Nuclear Staining Positivity of β-Catenin and Phospho-Akt in Small Intestinal Tumors[a]

					nuclear positivity (%)	
treatment	adenomas analyzed	proliferation index (%)	apoptotic index (%)	PGE_2 levels pg/μg protein	β-catenin	phospho-Akt
control	20	65.1 ± 3.5^{b}	2.2 ± 0.4^{b}	28.29 ± 3.97^{b}	53.4 ± 5.1^{b}	78.7 ± 1.9^{b}
control + 0.16% EGCG	16	24.2 ± 3.1^{d}	8.3 ± 2.8^{c}	20.98 ± 3.81^{bc}	29.5 ± 5.0^{c}	35.5 ± 5.9^{c}
fish oil	15	52.3 ± 5.5^{bc}	8.3 ± 1.1^{c}	2.90 ± 0.49^{c}	20.0 ± 3.1^{c}	32.6 ± 6.4^{c}
fish oil + 0.16% EGCG	15	39.1 ± 6.0^{cd}	9.7 ± 1.8^{c}	5.53 ± 2.03^{c}	19.9 ± 4.4^{c}	41.9 ± 4.8^{c}

[a] The proliferation activity was evaluated by immunohistochemical analysis using an antibody against Ki-67, and the apoptotic activity was determined by immunohistochemistry using an antibody against cleaved caspase-3. The proliferation index, apoptotic index, and positivity of β-catenin and phospho-Akt are presented as the number of positively stained cells expressed as a percentage in the total number of tumor cells counted. Different letters ($^{b-d}$) indicate statistical significance ($p < 0.05$, one-way ANOVA).

Effects of 9 Weeks of Treatment on β-Catenin Expression and Levels of Phosphorylated Akt. All adenomas from the control group had enhanced nuclear and cytoplasmic β-catenin staining but reduced membranous staining (**Figure 1**). Significantly reduced nuclear staining of β-catenin, as expressed in nuclear staining positivity, was observed in the adenomas from the groups treated with EGCG (by 44.8%), fish oil (by 62.5%), and the combination of the two agents (by 62.7%) in comparison to the control group (**Table 4**). In adenomas from the treated groups, the intensity of cytoplasmic staining was reduced to moderate expression levels while membranous staining was increased or even restored totally (**Figure 1**). In the adenomas from untreated $Apc^{Min/+}$ mice, phospho-Akt staining was observed in both the nucleus and the cytoplasm (**Figure 1**). All treatment groups showed reduced nuclear staining of phospho-Akt in the adenomas, both in terms of staining intensity and in number of positive-staining cells. The percent of phospho-Akt nuclear positive-staining cells in the adenomas was significantly decreased in the treatment groups as compared to those of the control group (by 47–59%, **Table 4**).

Effects of 9 Weeks of Treatment on PGE_2 Levels in Tumors. In the long-term study, we examined the effects of the different treatments on PGE_2 levels in the small intestinal tumors. Treatment with fish oil alone and in combination with EGCG significantly decreased PGE_2 levels (89 or 81%, respectively, **Table 4**).

DISCUSSION

In the present study, we found that a combination of EGCG and fish oil inhibits tumor formation and size in high-fat fed

Apc$^{Min/+}$ mice, whereas the single agents did not produce a significant effect. The effects on decreasing tumor size can also be observed after only 3 weeks of treatment. To our knowledge, this is the first report to investigate the combination effects of EGCG and fish oil in an animal model of cancer.

The dose of EGCG used in these studies corresponds to about 5–6 cups of green tea a day in terms of human consumption. These calculations are based on allometric scaling conversions of caloric requirements for mice and humans (*31*). Assuming 12 kcal as average daily energy requirements for an adult mouse (*32*), 0.16% EGCG would equal 0.3 mg EGCG per kcal consumed, if mice consume ~2 mL fluid per day. Assuming the energy requirements for the average human as 2000 kcal per day, human EGCG consumption at this dose would be 600 mg per day. This dose corresponds to about five tea green tea bags a day, assuming that one bag contains 2 g of green tea. Under these same caloric requirements, 12% fish oil would equal to 7.9 mg ω-3 PUFA per kcal consumed, which corresponds to about 16 g ω-3 PUFA per day in terms of human consumption. Although this dose of fish oil is rather high for human consumption, the dose of fish oil used in the present study is the lowest reported dose that has been shown to inhibit intestinal tumorigenesis in animals. Future studies will determine whether lower doses that may be more applicable to human consumption are also effective against tumorigenesis.

Although previous studies have shown that both EGCG and fish oil alone can inhibit tumor multiplicity (*9, 14, 33, 34*), under our experimental conditions, neither single agent had a significant effect on tumor multiplicity. This may be partially due to the large standard deviation in tumor multiplicity in each group and the variability between experiments often associated with this model (*9*). The former factor may have also affected our analysis on the interaction of EGCG and fish oil on tumor multiplicity.

β-Catenin translocation from the cell membrane to the nucleus is a key event in colon carcinogenesis that results in increased transcription of genes involved with cell proliferation. Our results show that β-catenin translocation from the cell membrane to nucleus is significantly reduced in adenomas of all treatment groups as compared to the control group. Our findings with the effects of EGCG on β-catenin localization are consistent with previous findings from our laboratory that show reduced nuclear protein expression of β-catenin in adenomas from *Apc*$^{Min/+}$ mice treated with 0.32% EGCG in the drinking fluid (*9*). To our knowledge, this is the first study to report decreased nuclear β-catenin localization in tumors from an animal model of cancer by treatment with fish oil.

We found that adenomas of EGCG- and fish oil-treated *Apc*$^{Min/+}$ mice show decreased Akt phosphorylation in comparison to adenomas of control mice. This is the first study to show the effect of fish oil on Akt activation in an in vivo model for colon cancer. Recent studies by Schley et al. (*35*) have shown that breast cancer cells incubated with EPA and DHA showed decreased Akt phosphorylation. Other studies in colon cancer cell lines and in vivo models have also shown the inhibitory effect of EGCG on Akt phosphorylation (*9, 36, 37*).

The observed decrease in phospho-Akt levels found in adenomas of mice treated with fish oil and the combination may be related our observed decrease in PGE_2 levels in the tumors of these mice. Previously, Moran et al. (*38*) have shown that PGE_2 can activate EGFR in *Apc*$^{Min/+}$ small intestinal tissues, which can activate PI3-kinase and subsequently Akt. It is possible that fish oil decreased the availability of arachidonic acid and PGE_2 levels in tumors, and this led to lower levels of Akt phosphorylation. Previous studies have shown that EGCG inhibits EGFR phosphorylation, which results in reduced levels of Akt phosphorylation (*37, 39*). EGCG has also been shown to inhibit Akt phosphorylation by a non-EGFR-mediated pathway (*40*). This decrease in Akt phosphorylation by EGCG, fish oil, and the combination may contribute to the observed increase in caspase-3 activation, since Akt phosphorylation has been shown to inhibit apoptosis (*41, 42*).

The present study shows that a combination of EGCG and fish oil is effective at inhibiting intestinal tumorigenesis in the *Apc*$^{Min/+}$ mouse model. The combination treatment also decreased cell proliferation, PGE_2 formation, nuclear localization of β-catenin, and the level of phosphorylated Akt in nucleus of the small intestinal tumors, as well as enhanced apoptosis. Most of the changes in these parameters were also caused by individual agents, except that cell proliferation was not significantly reduced by fish oil and PGE_2 levels were not significantly reduced by EGCG. All of these changes should contribute to the inhibition of tumorigenesis, but the present observed changes could not be quantitatively correlated with the tumor yield due to the high standard deviation in the tumor multiplicity.

ABBREVIATIONS USED

EGCG, (–)-epigallocatechin-3-gallate; ω-3 PUFA, ω-3 polyunsaturated fatty acids; AOM, azoxymethane; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PGE_2, prostaglandin E_2.

ACKNOWLEDGMENT

We thank Dr. Y. Hara (Mitsui Norin Co. Ltd., Tokyo, Japan) for providing tea polyphenols for the study. We also thank Dr. B. Reddy for providing the fish oil for this study.

LITERATURE CITED

(1) Cancer Facts and Figures 2007. http://www.cancer.org/downloads/STT/CAFF2007PWSecured.pdf (April 15, 2007).

(2) Kono, S.; Shinchi, K.; Ikeda, N.; Yanai, F.; Imanishi, K. Physical activity, dietary habits and adenomatous polyps of the sigmoid colon: A study of self-defense officials in Japan. *J. Clin. Epidemiol.* **1991**, *44*, 1255–1261.

(3) Tavani, A.; Franceschi, S.; Levi, F.; La Vecchia, C. Fish, omega-3 polyunsaturated fat intake and cancer at selected sites. *World Rev. Nutr. Diet* **2005**, *94*, 166–175.

(4) Yang, C. S.; Maliakal, P.; Meng, X. Inhibition of carcinogenesis by tea. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 25–54.

(5) Lambert, J. D.; Hong, J.; Yang, G. Y.; Liao, J.; Yang, C. S. Inhibition of carcinogenesis by polyphenols: Evidence from laboratory investigations. *Am. J. Clin. Nutr.* **2005**, *81*, 284S–291S.

(6) Higdon, J. V.; Frei, B. Tea catechins and polyphenols: Health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 89–143.

(7) Orner, G. A.; Dashwood, W. M.; Blum, C. A.; Diaz, G. D.; Li, Q.; Al-Fageeh, M.; Tebbutt, N.; Heath, J. K.; Ernst, M.; Dashwood, R. H. Response of Apc(min) and A33 (delta N beta-cat) mutant mice to treatment with tea, sulindac, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Mutat. Res.* **2002**, *506–507*, 121–127.

(8) Ju, J.; Liu, Y.; Hong, J.; Huang, M. T.; Conney, A. H.; Yang, C. S. Effects of green tea and high-fat diet on arachidonic acid metabolism and aberrant crypt foci formation in an azoxymethane-induced colon carcinogenesis mouse model. *Nutr. Cancer* **2003**, *46*, 172–178.

(9) Ju, J.; Hong, J.; Zhou, J. N.; Pan, Z.; Bose, M.; Liao, J.; Yang, G. Y.; Liu, Y. Y.; Hou, Z.; Lin, Y.; Ma, J.; Shih, W. J.; Carothers, A. M.; Yang, C. S. Inhibition of intestinal tumorigenesis in

Apcmin/+ mice by (–)-epigallocatechin-3-gallate, the major catechin in green tea. *Cancer Res.* **2005**, *65*, 10623–10631.
(10) Clapper, M. L.; Coudry, J.; Chang, W. C. Beta-catenin-mediated signaling: a molecular target for early chemopreventive intervention. *Mutat. Res.* **2004**, *555*, 97–105.
(11) Kato, T.; Hancock, R. L.; Mohammadpour, H.; McGregor, B.; Manalo, P.; Khaiboullina, S.; Hall, M. R.; Pardini, L.; Pardini, R. S. Influence of omega-3 fatty acids on the growth of human colon carcinoma in nude mice. *Cancer Lett.* **2002**, *187*, 169–177.
(12) Rao, C. V.; Hirose, Y.; Indranie, C.; Reddy, B. S. Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. *Cancer Res.* **2001**, *61*, 1927–1933.
(13) Reddy, B. S. Studies with the azoxymethane-rat preclinical model for assessing colon tumor development and chemoprevention. *Environ. Mol. Mutagen.* **2004**, *44*, 26–35.
(14) Paulsen, J. E.; Elvsaas, I. K.; Steffensen, I. L.; Alexander, J. A fish oil derived concentrate enriched in eicosapentaenoic and docosahexaenoic acid as ethyl ester suppresses the formation and growth of intestinal polyps in the Min mouse. *Carcinogenesis* **1997**, *18*, 1905–1910.
(15) Calviello, G.; Di Nicuolo, F.; Gragnoli, S.; Piccioni, E.; Serini, S.; Maggiano, N.; Tringali, G.; Navarra, P.; Ranelletti, F. O.; Palozza, P. n-3 PUFAs reduce VEGF expression in human colon cancer cells modulating the COX-2/PGE2 induced ERK-1 and -2 and HIF-1alpha induction pathway. *Carcinogenesis* **2004**, *25*, 2303–2310.
(16) Schwartz, B.; Birk, Y.; Raz, A.; Madar, Z. Nutritional-pharmacological combinations—A novel approach to reducing colon cancer incidence. *Eur. J. Nutr.* **2004**, *43*, 221–229.
(17) Orner, G. A.; Dashwood, W. M.; Blum, C. A.; Diaz, G. D.; Li, Q.; Dashwood, R. H. Suppression of tumorigenesis in the Apc(min) mouse: down-regulation of beta-catenin signaling by a combination of tea plus sulindac. *Carcinogenesis* **2003**, *24*, 263–267.
(18) Serrano, D.; Lazzeroni, M.; Decensi, A. Chemoprevention of colorectal cancer: An update. *Technol. Coloproctol.* **2004**, *8* (Suppl. 2), s248–s252.
(19) Bresalier, R. S.; Sandler, R. S.; Quan, H.; Bolognese, J. A.; Oxenius, B.; Horgan, K.; Lines, C.; Riddell, R.; Morton, D.; Lanas, A.; Konstam, M. A.; Baron, J. A. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N. Engl. J. Med.* **2005**, *352*, 1092–1102.
(20) Solomon, S. D.; McMurray, J. J.; Pfeffer, M. A.; Wittes, J.; Fowler, R.; Finn, P.; Anderson, W. F.; Zauber, A.; Hawk, E.; Bertagnolli, M. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N. Engl. J. Med.* **2005**, *352*, 1071–1080.
(21) Ireson, C. R.; Jones, D. J.; Orr, S.; Coughtrie, M. W.; Boocock, D. J.; Williams, M. L.; Farmer, P. B.; Steward, W. P.; Gescher, A. J. Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. *Cancer Epidemiol. Biomarkers Prev.* **2002**, *11*, 105–111.
(22) Lambert, J. D.; Yang, C. S. Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. *Mutat. Res.* **2003**, *523–524*, 201–208.
(23) Lambert, J. D.; Hong, J.; Kim, D. H.; Mishin, V. M.; Yang, C. S. Piperine enhances the bioavailability of the tea polyphenol (–)-epigallocatechin-3-gallate in mice. *J. Nutr.* **2004**, *134*, 1948–1952.
(24) Sporn, M. B.; Suh, N. Chemoprevention of cancer. *Carcinogenesis* **2000**, *21*, 525–530.
(25) Jalving, M.; Koornstra, J. J.; De Jong, S.; De Vries, E. G.; Kleibeuker, J. H. Review article: The potential of combinational regimen with non-steroidal anti-inflammatory drugs in the chemoprevention of colorectal cancer. *Aliment Pharmacol. Ther.* **2005**, *21*, 321–339.
(26) Moser, A. R.; Dove, W. F.; Roth, K. A.; Gordon, J. I. The Min (multiple intestinal neoplasia) mutation: Its effect on gut epithelial cell differentiation and interaction with a modifier system. *J. Cell. Biol.* **1992**, *116*, 1517–1526.
(27) Omega Protein, I. Virginia Prime Silver Menhaden Oil Typical Fatty Acid Composition. http://www.omegaproteininc.com/pdf/VPs-spec.pdf (July 2, 2007).
(28) Yang, K.; Fan, K.; Newmark, H.; Leung, D.; Lipkin, M.; Steele, V. E.; Kelloff, G. J. Cytokeratin, lectin, and acidic mucin modulation in differentiating colonic epithelial cells of mice after feeding Western-style diets. *Cancer Res.* **1996**, *56*, 4644–4648.
(29) Hao, X. P.; Pretlow, T. G.; Rao, J. S.; Pretlow, T. P. Beta-catenin expression is altered in human colonic aberrant crypt foci. *Cancer Res.* **2001**, *61*, 8085–8088.
(30) Lowry, R. VassarStats: Statistical Computation Website. http://faculty.vassar.edu/lowry/VassarStats.html (March 31, 2006).
(31) Schneider, K.; Oltmanns, J.; Hassauer, M. Allometric principles for interspecies extrapolation in toxicological risk assessment--empirical investigations. *Regul. Toxicol. Pharmacol.* **2004**, *39*, 334–347.
(32) Subcommitee on Laboratory Animal Nutrition, C. o. A. N., Board on Agriculture, National Research Council. *Nutrient Requirements of Laboratory Animals*, 4th revised ed.; National Academies Press: Washington, DC, 1995.
(33) Petrik, M. B.; McEntee, M. F.; Chiu, C. H.; Whelan, J. Antagonism of arachidonic acid is linked to the antitumorigenic effect of dietary eicosapentaenoic acid in Apc(Min/+) mice. *J. Nutr.* **2000**, *130*, 1153–1158.
(34) Petrik, M. B.; McEntee, M. F.; Johnson, B. T.; Obukowicz, M. G.; Whelan, J. Highly unsaturated (n-3) fatty acids, but not alpha-linolenic, conjugated linoleic or gamma-linolenic acids, reduce tumorigenesis in Apc(Min/+) mice. *J. Nutr.* **2000**, *130*, 2434–2443.
(35) Schley, P. D.; Jijon, H. B.; Robinson, L. E.; Field, C. J. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. *Breast Cancer Res. Treat.* **2005**, *92*, 187–195.
(36) Peng, G.; Dixon, D. A.; Muga, S. J.; Smith, T. J.; Wargovich, M. J. Green tea polyphenol (–)-epigallocatechin-3-gallate inhibits cyclooxygenase-2 expression in colon carcinogenesis. *Mol. Carcinog.* **2006**, *45*, 309–319.
(37) Shimizu, M.; Deguchi, A.; Lim, J. T.; Moriwaki, H.; Kopelovich, L.; Weinstein, I. B. (–)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin. Cancer Res.* **2005**, *11*, 2735–2746.
(38) Moran, A. E.; Hunt, D. H.; Javid, S. H.; Redston, M.; Carothers, A. M.; Bertagnolli, M. M. Apc deficiency is associated with increased Egfr activity in the intestinal enterocytes and adenomas of C57BL/6J-Min/+ mice. *J. Biol. Chem.* **2004**, *279*, 43261–43272.
(39) Shimizu, M.; Deguchi, A.; Joe, A. K.; McKoy, J. F.; Moriwaki, H.; Weinstein, I. B. EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells. *J. Exp. Ther. Oncol.* **2005**, *5*, 69–78.
(40) Sah, J. F.; Balasubramanian, S.; Eckert, R. L.; Rorke, E. A. Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases. *J. Biol. Chem.* **2004**, *279*, 12755–12762.
(41) Datta, S. R.; Dudek, H.; Tao, X.; Masters, S.; Fu, H.; Gotoh, Y.; Greenberg, M. E. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **1997**, *91*, 231–241.
(42) Cardone, M. H.; Roy, N.; Stennicke, H. R.; Salvesen, G. S.; Franke, T. F.; Stanbridge, E.; Frisch, S.; Reed, J. C. Regulation of cell death protease caspase-9 by phosphorylation. *Science* **1998**, *282*, 1318–1321.

Received for review April 5, 2007. Revised manuscript received July 5, 2007. Accepted July 9, 2007. This work was supported by NIH Grant CA88961 (to C.S.Y.) and a fellowship from the New Jersey Commission for Cancer Research (to M.B.).

JF071004R