

Fish Oil Blocks Azoxymethane-Induced Rat Colon Tumorigenesis by Increasing Cell Differentiation and Apoptosis Rather Than Decreasing Cell Proliferation^{1,2}

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ABSTRACT The purpose of this study was to determine whether the protective effect of fish oil against colon carcinogenesis is due to decreased proliferation, increased differentiation and/or increased apoptosis. Male Sprague Dawley rats ($n = 260$) were fed one of two oils (corn or fish) and two fibers (pectin or cellulose), plus or minus the carcinogen azoxymethane (AOM). Rats were killed at wk 18 ($n = 80$) or 36 ($n = 180$) for cytokinetic measurements. In vivo cell proliferation was measured by incorporation of bromodeoxyuridine into DNA, differentiation by binding of *Dolichos biflorus* agglutinin and apoptosis by immunoperoxidase detection of digoxigenin labeled genomic DNA. Fish oil resulted in a lower adenocarcinoma incidence (56.1 vs. 70.3%) compared with corn oil. There was no effect of fat or fiber on number of proliferative cells/crypt column in either the proximal or distal colon. In contrast, fish oil resulted in a greater degree of differentiation compared with corn oil in both colonic sites. In addition, fish oil resulted in a higher number of apoptotic cells/crypt column in both the proximal and distal colon as compared with corn oil. AOM treatment increased the ratio of proliferative cells/crypt column to apoptotic cells/crypt column in both the proximal and distal colon compared with saline controls. Fish oil, however, resulted in a lower ratio in both sites in the colon as compared with corn oil. These results suggest that an increase in apoptosis and differentiation, rather than a decrease in proliferation, accounts for the protective effect of fish oil against experimentally induced colon tumorigenesis. *J. Nutr.* 128: 491-497, 1998.

KEY WORDS: • rats • fish oil • proliferation • apoptosis • azoxymethane

Both epidemiological and experimental studies support a protective role for (n-3) polyunsaturated fatty acids [i.e., cosapentaenoic acid, 20:5(n-3) and docosahexaenoic acid, 22:6(n-3)] against colon cancer. Alaskan and Greenland Eskimos have lower rates of colon cancer and a higher consumption of 20:5(n-3) and 22:6(n-3) fatty acids than other North Americans (Bang et al. 1976, Blot et al. 1975). Recent reports (Caygill et al. 1996) from 24 European populations showed an inverse relation between fish and fish oil consumption and colorectal cancer risk. Case-control studies of fat consumption also have shown protective effects of fish oil against colorectal cancer (Willett et al. 1990). Fish oil, high in (n-3) fatty acids, has been shown to be protective against experimentally induced colon cancer in a large number of studies (Chang et al. 1997b, Deschner et al. 1990, Reddy et al. 1991). However, the mechanism(s) behind this protective effect is not known.

Fearon and Vogelstein (1990) have elucidated the multistep nature of colon cancer development, and there are a number of stages at which fish oil theoretically could act to interfere with tumor development. Reddy et al. (1991) have shown an effect of dietary fish oil at both the initiation and promotion

stages of colon carcinogenesis and have explored the initiation stage effects in some detail. After cells have been initiated, multiple mutations still are required for tumor development.

In a recent study with two fats (fish oil and corn oil) and two fibers (pectin and cellulose) and the experimental colon carcinogen, azoxymethane (Jiang et al. 1995), we reported that dietary fish oil blunts ras mutations and influences ras membrane localization at a point approximately half-way through the tumor-development process. This is important because prolonged ras activation could result in a stimulation of cell proliferation (Maher et al. 1994). Another documented effect of prolonged ras activation is a reduced susceptibility to apoptosis (Chen and Faller 1995). Although increased cell division, which drives the accumulation of genetic errors, is necessary for neoplastic transformation (Preston-Martin et al. 1990), the number of studies that do not show a relationship between changes in cell proliferation and subsequent tumor development are increasing (Klurfeld et al. 1987, Paganelli et al. 1991). In addition, it should be noted that the relationship between diet and cell proliferation depends on the mode of the nutritional agent. In contrast, inhibition of apoptosis is known to be an integral component of the genesis of colorectal adenomas and carcinomas (Bedi et al. 1995). Tomlinson and Bodmer (1995) also used mathematical models to prove that failure of apoptosis can cause tumor development even though apoptosis is numerically small. It is still unclear which event (growth inhibition or apoptosis induction) is the major consequence after fish oil diminishes ras activation.

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In a previous study (Chang et al. 1997b), we reported the predictive value of proliferation, differentiation and apoptosis during the early stages (wk 18) of colon tumorigenesis. We found that measurements of apoptosis revealed the synergistic effect of the fish oil/pectin combination as compared with fish oil/cellulose. In another study from our laboratory with three fats (fish oil, corn oil and beef tallow) and two fibers (pectin and cellulose) (Lee et al. 1993a), we reported that the effect of fat on cell proliferation was highly dependent on the source of fiber in the diet. We therefore hypothesized that fish oil might be protective against experimentally induced colon carcinogenesis by enhancing apoptosis (compared with corn oil diets) depending on the dietary fiber source. Because maintenance of the colonic epithelium and tumor growth are dependent upon a delicate balance between cell proliferation, differentiation and apoptosis, we determined these variables of cell kinetics at two different time points (wk 18 and 36), and data from the two time points were combined for statistical analyses.

MATERIALS AND METHODS

Animals and study design. The animal use protocol was approved by the University Animal Care Committee of Texas A&M University and conformed to National Institutes of Health guidelines. Male weanling (21-d-old) Sprague-Dawley rats (260; Harlan Sprague-Dawley, Houston, TX) were housed individually in cages and maintained in a temperature- and humidity-controlled animal facility with a daily photoperiod of 12-h light and dark. This study was a $2 \times 2 \times 2 \times 2$ factorial design with two types of fat (corn oil or fish oil), two types of fiber (cellulose or pectin), two injected subgroups (with or without carcinogen) and two time points (18 and 36 wk). There were 10 rats in each group at the first time point (2 fats \times 2 fibers \times carcinogen or saline = 80 rats). At the final time point (wk 36, $n = 180$) there were 10 rats in the saline-injected groups and 33 in each of the carcinogen-injected groups, except there were 8 extra rats ($n = 41$ rats) in the corn oil/cellulose/azoxymethane (AOM)⁴ group. The extra rats in this group were to be used for another set of analyses, but they were included for determination of tumor development so as not to potentially bias this observation. Animals were stratified by body weight so that mean initial body weights of the groups did not differ. Body weights were recorded weekly throughout the study.

Diets. After a 1-wk acclimation period of consuming standard rat nonpurified diet, animals were assigned by weight to one of four diets, which have been described in detail previously (Chang et al. 1997b). The diets differed only in the type of fat (corn oil or fish oil) and in the type of dietary fiber (cellulose or pectin). The fats were chosen because of their different fatty acid compositions. The major differences between the fatty acid compositions of the two lipid sources are significantly higher amounts of 14:0, 16:1($n-7$), 20:5($n-3$) and 22:6($n-3$) in the fish oil diets and higher amounts of 18:1($n-9$) and 18:2($n-6$) in the corn oil diets. The amount of antioxidants in fish oil was adjusted to equal that in corn oil. The fibers were chosen because of their different degrees of fermentability, with pectin being highly fermentable in the colon and cellulose poorly fermented. Dietary fat was provided at the 15% level by weight (30% of energy) to simulate the current recommendations for humans (U.S. Public Health Service 1991). Dietary fiber was provided at the 6% level by weight, corresponding to the recommended level of 30 g fiber/day for humans (U.S. Public Health Service 1991). Food and water were freely available at all times. Forty-eight hour food intakes and fecal outputs were measured 1 wk after the second injection of either carcinogen or saline and again 1 wk before each killing time point.

Carcinogen treatment and tumor typing. Rats were injected with saline (controls) or azoxymethane at wk 2 and 3 as previously de-

scribed (Chang et al. 1997b). AOM (Sigma Chemical, St. Louis, MO) was injected subcutaneously at a dose of 15 mg/kg body wt. Control rats received an equal volume of saline. For cell kinetics measurements, 80 rats (10 rats/group) were terminated at wk 18. For final tumor typing, 180 rats were terminated at wk 36. All macroscopic tumors were taken, fixed in 4% paraformaldehyde and examined under light microscopy. Tumors were classified as adenomas or adenocarcinomas as previously described (Chang et al. 1997b). A randomly selected subset of these 180 rats (10 rats/group) was used for the cell kinetics measurements at the final time point.

Tissues for in vivo measurements of cell kinetics. Exactly 1 h before being killed, each rat was injected intraperitoneally with bromodeoxyuridine (BrdU) (5 mg/kg body wt) in phosphate-buffered saline (PBS), pH 7.4, to measure in vivo cell proliferation (Chang et al. 1997b). The length of the large bowel was recorded immediately, and the intestine was opened longitudinally. The rectum was resected, and the remainder of the colon was divided equally lengthwise for proximal and distal segments, which were flushed clean with ice-cold PBS (pH 7.4). A 1-cm length of colon was taken from the cecal-proximal colon junction for proximal colon, and the most distal end of the distal colon was taken for distal colon samples. Each 1-cm length of colon was further divided in half longitudinally. One half was fixed in 70% ethanol, and the other half was fixed in a 4% paraformaldehyde solution for 4 h before processing for histology.

In vivo measurements of cell proliferation, differentiation and apoptosis. The ethanol fixed tissues were used for in vivo measurements of cell proliferation. Incorporation of BrdU into DNA was localized using a monoclonal anti-BrdU antibody, and detection of bound antibody was achieved using peroxidase-conjugated antibody to mouse immunoglobulin. Slides were scored as previously described (Chang et al. 1997b). Cell differentiation was measured by lectin histochemistry with a procedure originally developed by Boland (Boland et al. 1982). Biotinylated *Dolichos biflorus* agglutinin (DBA) was used to detect the specific carbohydrate α -N-acetylgalactosamine residues, which are thought to increase with normal differentiation of colonic epithelial cells. Each crypt was equally divided by thirds (bottom third, middle third and top third of the crypt). Scoring of DBA positive cells for each third of the crypt was performed using a technique developed in our laboratory (Chang et al. 1997b). The measurement of in situ apoptosis was based on the deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling of fragmented DNA (Gavrieli et al. 1992). This method relies on the specific binding of TdT to exposed 3'-OH ends of DNA followed by the synthesis of a labeled polydeoxynucleotide molecule. The apoptotic cells were scored based on a combination of positive staining and morphological criteria as described by Kerr et al. (1995).

Statistical analyses. Data were analyzed using four-way analysis of variance (ANOVA) to determine the effect of dietary fat, fiber, carcinogen, time and any potential interactions. When P -values for the interactions were <0.05 , means of interactions were separated using Duncan's multiple range test (SAS Institute Inc. 1985). When P -values were <0.05 for the effects of fat, fiber, carcinogen or time but not for the interactions, the main effect means were separated by Duncan's multiple range test. Tumor incidence data were analyzed by χ^2 analysis and reported as percentage of rats bearing tumors (SAS Institute Inc. 1985).

RESULTS

Food intake and weight gain. These data have been described in detail in a previous report (Chang et al. 1997b). There were no differences in food intake among groups at either time point. However, there was a significant difference in weight gain between the two fat treatments, beginning at wk 6, with fish oil-fed animals weighing more than their corn oil-fed counterparts. Because of the potential for differences in weight gain (between fish oil and corn oil animals) to affect outcome variables, data were reanalyzed using weight as a covariate. There was no effect ($P > 0.05$) of weight on any of the outcome measurements.

Colon adenocarcinoma incidence. At the final time point (wk 36), there was no evidence of adenoma or carcinoma

⁴ Abbreviations used: AOM, azoxymethane; BrdU, bromodeoxyuridine; COX-2, cyclooxygenase-2; DAG, diacylglycerol; DBA, *Dolichos biflorus* agglutinin; PBS, phosphate-buffered saline; PG, prostaglandin; PGE₂, prostaglandin E₂; PKC, protein kinase C; SCFA, short chain fatty acids; TdT, terminal deoxynucleotidyl transferase.

TABLE 1

Effect of dietary fat on apoptosis, differentiation and proliferation of colonocytes in rats¹

	Proximal colon				Distal colon			
	Corn oil	Fish oil	Pooled SEM	P-value	Corn oil	Fish oil	Pooled SEM	P-value
Apoptosis and proliferation								
Cells per crypt column, <i>n</i>	26.53	26.76	0.32	0.6168	33.19	32.51	0.23	0.0555
Apoptotic cells/crypt column, <i>n</i>	0.58	0.90	0.05	0.0001	0.85	1.13	0.07	0.0054
Proliferative cells/crypt column, <i>n</i>	2.14	2.06	0.11	0.5974	2.25	2.20	0.08	0.6117
Differentiation-lectin score ²								
Bottom third of crypt	1.25	1.78	0.07	0.0001	1.02	1.53	0.06	0.0001
Middle third of crypt	1.65	1.91	0.06	0.0015	1.40	1.87	0.06	0.0001
Top third of crypt	2.13	2.43	0.05	0.0001	2.09	2.43	0.05	0.0001

¹ Values given are means, *n* = 80.² Lectin score, as described by Chang et al. (1997b), is a combination of staining intensity (rated from 0 to 3) and the proportion of the cells that are stained.

found in any rat injected with saline as previously reported (Chang et al. 1997b). For those injected with AOM (140 rats), only three rats had adenomas without an adenocarcinoma. There was no difference ($P > 0.05$) in the number of tumors per tumor-bearing rat across the carcinogen treatments, and no main effect of fiber ($P > 0.05$) was found on tumor incidence. However, there was a significant main effect of fat ($P < 0.05$) with fish oil resulting in a lower tumor incidence (56.1%) than corn oil (70.3%). When all four treatment values were compared, rats fed corn oil/cellulose (75.6%) had a higher tumor incidence than rats fed the combination of fish oil/pectin (51.5%) ($P < 0.05$).

Effects of dietary fat and fiber on apoptosis, differentiation and proliferation. In addition to the fish oil diet being more protective against colon tumorigenesis than the corn oil diet, it also resulted in 55% more apoptotic cells per crypt column in the proximal colon ($P = 0.0001$) and 33% more apoptotic cells per crypt column in the distal colon ($P = 0.0054$) compared with the corn oil treatment (Table 1). At the same time, fish oil promoted differentiation, as shown by a significantly higher lectin-binding stain intensity in all three compartments of the crypt in both the proximal and distal colon compared with the corn oil treatment ($P \leq 0.0015$). There was a minimal effect of dietary fat on cell proliferation. Fish oil resulted in a lower number of cells/crypt column in the distal colon compared with corn oil ($P = 0.0555$).

Dietary fiber source had a greater effect on apoptosis proximally, than distally (Table 2). The number of apoptotic cells

per crypt column in the proximal colon was 48.3% higher with the pectin diet compared with the cellulose diet ($P = 0.0002$). For cell differentiation, rats provided with the pectin diets had a more differentiated phenotype in the bottom third of the crypt in the distal colon compared with the cellulose treatment ($P = 0.0407$); with respect to the effect of fiber on cell proliferation, pectin resulted in a reduced ($P = 0.0011$) number of cells per crypt column in the distal colon compared with cellulose (Table 2).

Effects of dietary fat and fiber interactions on apoptosis, differentiation and proliferation. There was a significant interaction between dietary fat and fiber on apoptosis but not on differentiation or proliferation. As shown in Figure 1, rats fed the fish oil/pectin diet had the highest number of apoptotic cells per crypt column both in the proximal ($P = 0.0173$) and distal colon ($P = 0.0135$) compared with animals fed corn oil/cellulose, corn oil/pectin, or fish oil/cellulose diets. Interestingly, the fish oil/pectin group also had the lowest number of tumors.

Effects of carcinogen on apoptosis, differentiation and proliferation. The effects of carcinogen on apoptosis, differentiation and proliferation were the opposite of the effects of fish oil. That is, rats injected with AOM had a lower number of apoptotic cells per crypt column (26% lower in the proximal colon and 23.2% in the distal colon) as compared with saline controls (Table 3). AOM treatment also resulted in lower indices of differentiation, as assessed by a lower lectin staining score in the bottom, middle, and top thirds of the crypts in

TABLE 2

Effect of dietary fiber on apoptosis, differentiation and proliferation of colonocytes in rats¹

	Proximal colon				Distal colon			
	Cellulose	Pectin	Pooled SEM	P-value	Cellulose	Pectin	Pooled SEM	P-value
Apoptosis and proliferation								
Cells per crypt column, <i>n</i>	26.53	26.76	0.32	0.6112	33.51	32.19	0.23	0.0011
Apoptotic cells/crypt column, <i>n</i>	0.60	0.89	0.05	0.0002	0.93	1.05	0.07	0.2110
Proliferative cells/crypt column, <i>n</i>	2.09	2.11	0.11	0.8627	2.19	2.26	0.08	0.4894
Differentiation-lectin score ²								
Bottom third of crypt	1.46	1.57	0.07	0.2625	1.19	1.37	0.06	0.0407
Middle third of crypt	1.74	1.82	0.06	0.2948	1.55	1.72	0.06	0.0571
Top third of crypt	2.25	2.30	0.05	0.4979	2.23	2.29	0.05	0.3777

¹ Values given are means, *n* = 80.² Lectin score, as described by Chang et al. (1997b), is a combination of staining intensity (rated from 0 to 3) and the proportion of the cells that are stained.

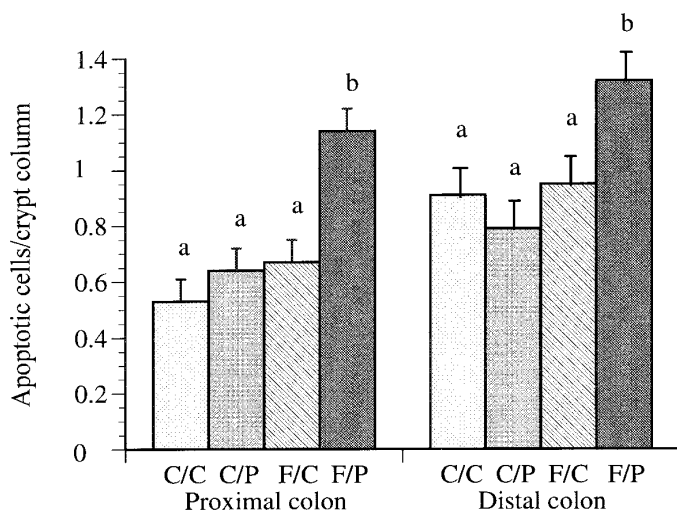


FIGURE 1 The interactive effects of fat and fiber on number of apoptotic cells per crypt column in proximal and distal colon of rats. Values are means \pm pooled SEM, $n = 40$. Bars with different letters are significantly different, $P < 0.05$. Rats fed fish oil/pectin, F/P, had more apoptotic cells per crypt column than rats fed corn oil/cellulose, C/C, corn oil/pectin, C/P, or fish oil/cellulose, F/C.

both the proximal and distal colons as compared with rats injected with saline ($P < 0.0210$). The effect of AOM on cell proliferation was less dramatic. There was no effect of AOM on any index of cell proliferation in either the proximal or distal colon (Table 3).

Effects of aging on apoptosis, differentiation and proliferation. The effect of aging on apoptosis, differentiation and proliferation was similar to the effect of carcinogen and opposite to the effect of fish oil. The rate of apoptosis decreased as a function of age in both the proximal and distal colon (Table 4). The number of apoptotic cells per crypt column declined by 36.3% in the proximal colon ($P = 0.0001$) and 29.3% in the distal colon ($P = 0.0008$) between wk 18 and 36. The aging process also resulted in a less differentiated phenotype as evaluated by lectin staining, which was significantly lower at wk 36 compared with wk 18 in the top third of the crypt in the proximal colon and in the bottom, middle and top third of the crypt in the distal colon ($P < 0.004$). In contrast to the lower numbers of apoptotic and differentiated cells over time, aging increased the number of proliferative cells per crypt column in the distal colon ($P = 0.0008$).

Effects of age, carcinogen, fat and fiber on the ratio of proliferation to apoptosis. The ratio of proliferation to apoptosis (number of proliferative cells per crypt column/number apoptotic cells per crypt column) is shown in Figure 2. Rats injected with AOM had a higher ratio of proliferation to apoptosis (58 and 51% higher) in both the proximal ($P = 0.0030$) and distal ($P = 0.0129$) colon than the saline control rats (Fig. 2A). The ratio of proliferation to apoptosis increased by 31% in the proximal colon ($P = 0.0541$) and 34% in the distal colon ($P = 0.0754$) in rats at wk 36 as compared with rats at wk 18 (Fig. 2B). In the proximal colon, the ratio of proliferation to apoptosis of the rats consuming the fish oil diet was only 55% ($P = 0.0001$) of the ratio of the rats consuming the corn oil diet (Fig. 2C). In the distal colon, the fish oil treatment also decreased the ratio of proliferation to apoptosis by 23.5% as compared with the corn oil treatment, even though the difference was not significant ($P = 0.1003$). Pectin treatment also decreased ($P = 0.0527$) the ratio of proliferation to apoptosis by 24.7% in the proximal colon as compared with the cellulose treatment (Fig. 2D). The effect of fiber on the ratio of cell proliferation to apoptosis in the distal colon was almost numerically identical to its effect in the proximal colon.

DISCUSSION

The type of dietary fat affects tumor outcome in experimental diet/colon carcinogenesis studies (Rao and Reddy 1993, Reddy et al. 1991). Our data are consistent with published reports from other laboratories showing a protective effect of fish oil against colon tumorigenesis (Deschner et al. 1990). This protective effect of fish oil has been shown to occur at both the initiation and promotion stages of colon tumorigenesis (Reddy et al. 1991) and is thought to be related to a decrease in colonic cell proliferation. An increase in cell proliferation generally is considered promotive of tumor development (Preston-Martin et al. 1990).

Fish oil has been shown to decrease colonic epithelial cell proliferation both in mice (Deschner et al. 1990) and in humans on controlled basal diets supplemented with fish oil as compared with corn oil supplements (Bartram et al. 1993). Bartoli et al. (1993) investigated the effect of fish oil supplements in a 30-d clinical trial. They found that cell proliferation was lower in the fish oil group than in the placebo group. Our study showed a marginal effect of fish oil treatment on reducing colonic cell proliferation in that there was a lower number of cells/crypt column in the distal colon compared with corn oil.

TABLE 3

Effect of azoxymethane injection on apoptosis, differentiation and proliferation of colonocytes in rats¹

	Proximal colon				Distal colon			
	AOM	Saline	Pooled SEM	P-value	AOM	Saline	Pooled SEM	P-value
Apoptosis and proliferation								
Cells per crypt column, n	26.80	26.49	0.32	0.5103	33.01	32.69	0.23	0.3395
Apoptotic cells/crypt column, n	0.63	0.85	0.05	0.0042	0.86	1.12	0.07	0.0089
Proliferative cells/crypt column, n	2.24	1.96	0.11	0.0763	2.19	2.26	0.08	0.5165
Differentiation-lectin score ²								
Bottom third of crypt	1.35	1.68	0.07	0.0018	1.17	1.38	0.06	0.0205
Middle third of crypt	1.58	1.98	0.06	0.0001	1.47	1.80	0.06	0.0005
Top third of crypt	2.06	2.50	0.05	0.0001	2.06	2.45	0.05	0.0001

¹ Values given are means, $n = 80$.

² Lectin score, as described by Chang et al. (1997b), is a combination of staining intensity (rated from 0 to 3) and the proportion of the cells that are stained.

TABLE 4

Effect of time point on apoptosis, differentiation and proliferation of colonocytes in rats¹

	Proximal colon				Distal colon			
	18 wk	36 wk	Pooled SEM	P-value	18 wk	36 wk	Pooled SEM	P-value
Apoptosis and proliferation								
Cells per crypt column, <i>n</i>	26.72	26.57	0.32	0.7392	32.54	33.16	0.23	0.0778
Apoptotic cells/crypt column, <i>n</i>	0.91	0.58	0.05	0.0001	1.16	0.82	0.07	0.0008
Proliferative cells/crypt column, <i>n</i>	2.20	2.00	0.11	0.2144	2.03	2.42	0.08	0.0008
Differentiation-lectin score ²								
Bottom third of crypt	1.52	1.51	0.07	0.9155	1.46	1.09	0.06	0.0001
Middle third of crypt	1.53	2.03	0.06	0.0001	1.77	1.50	0.06	0.0033
Top third of crypt	2.47	2.09	0.05	0.0001	2.48	2.04	0.05	0.0001

¹ Values given are means, *n* = 80.² Lectin score, as described by Chang et al. (1997b), is a combination of staining intensity (rated from 0 to 3) and the proportion of the cells that are stained.

However, tumor growth and the number of cells in the colonic mucosa are determined not only by the production of cells (cell proliferation) but also by the rate of cell loss (apoptosis). Inhibition of apoptosis now is thought to be an integral component of the genesis of colorectal adenomas and carcinomas (Bedi et al. 1995, Garewal et al. 1996). Garewal et al. (1996)

have suggested that reduced apoptotic ability may predispose an individual to an increased risk for cancer. Alternatively, agents that increase apoptosis would have the potential to decrease cancer risk. This was seen in the present study where fish oil significantly increased apoptosis in both the proximal and distal colon to a greater degree than it reduced cell prolifer-

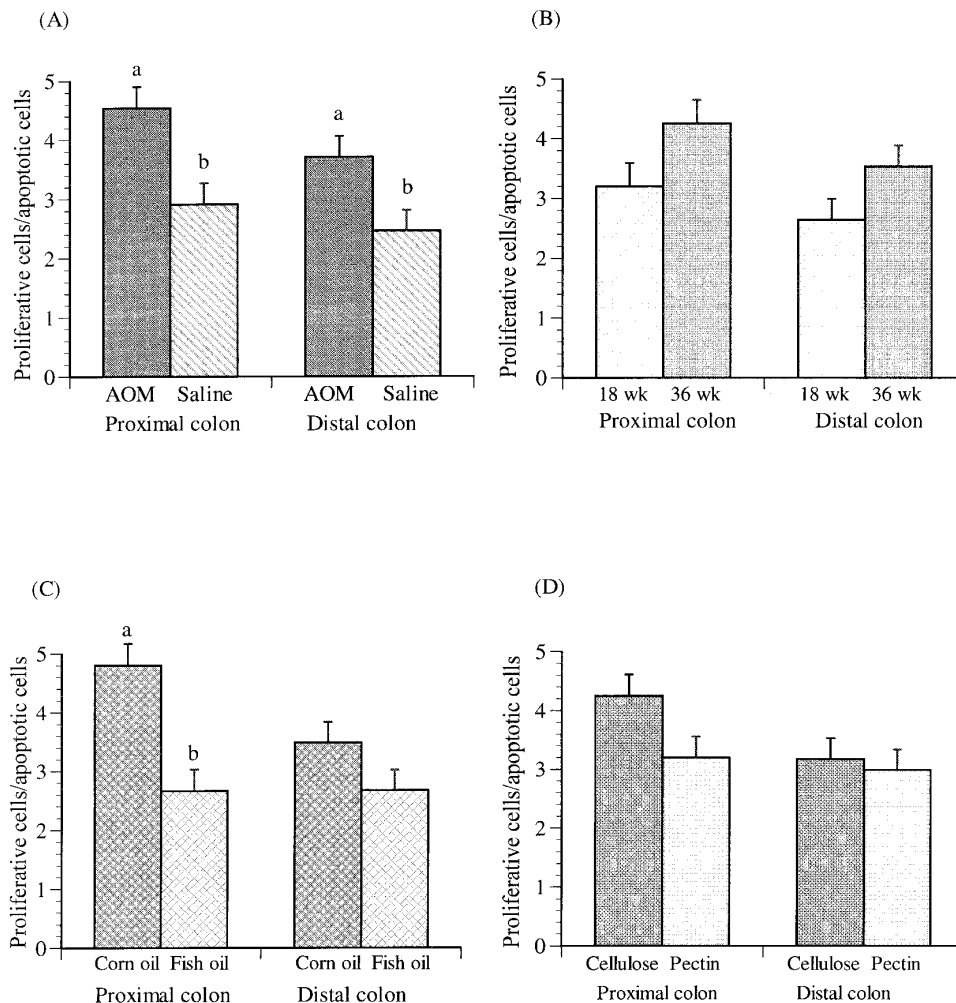


FIGURE 2 Main effects of azoxymethane (AOM, A), time point (B), fat (C) and fiber (D) on the ratio of proliferation to apoptosis (number of proliferative cells per crypt column/number of apoptotic cells per crypt column). Values are means \pm pooled SEM, *n* = 80. Bars with different letters are significantly different, *P* < 0.05.

ation. This highly significant effect of fish oil on apoptosis may be even more important, biologically, than its effect on cell proliferation.

There are no reports in the literature on the effect of fish oil on both cell proliferation and apoptosis with which to compare the present study, but it appears that the most important biological consequence with respect to tumor growth would be documentation of the relationship between proliferation and apoptosis rather than independent measurements of either of these cell kinetic changes. We have attempted to characterize this relationship by calculating a ratio of proliferation to apoptosis (proliferative cells per crypt column/apoptotic cells per crypt column). Again, fish oil supplementation decreased this ratio by 23.5% compared with the corn oil treatment. The only other study that we have found that addresses both cell proliferation and apoptosis in colon cancer (Tsujitani et al. 1996) measured apoptosis and cell proliferation in biopsy specimens from 38 cases of cancer with adenoma and 29 cases of cancer de novo and found that the more malignant phenotype had a higher ratio of proliferation to apoptosis (consistent with our findings). Collectively, the data suggest that the ratio of rates of apoptosis and cell proliferation may be key to our understanding of colon tumor development.

Although it is not known how fish oil decreases cell division and enhances apoptosis, one hypothesis is that (n-3) fatty acids (high in fish oil) inhibit prostaglandin (PG) production, which in turn decreases colonic cell proliferation and tumor formation (Bartram et al. 1993, Rao and Reddy 1993). We have shown previously that fish oil diets result in lower levels of arachidonic acid in colonic mucosal phospholipids of rats than does corn oil (Lee et al. 1993b), presumably thereby supplying less substrate for PG production. In addition, there were significantly lower levels of colonic mucosal prostaglandin E₂ (PGE₂) with fish oil supplemented diets compared with corn oil or beef tallow (Lee et al. 1993b). PGE₂ levels also have been shown to be lower in colonic biopsies from humans after fish oil consumption compared with individuals consuming corn oil (Bartram et al. 1993). Other agents that inhibit PG production, e.g., nonsteroidal anti-inflammatory agents, also appear to work by this mechanism and recent epidemiological studies show a protective effect of aspirin ingestion on colon cancer incidence (Schreinemachers and Everson 1994, Thun et al. 1991). The connection between PG synthesis and apoptosis recently was explored by Tsujii and DuBois (1995), who overexpressed cyclooxygenase-2, (COX-2, the inducible enzyme for PG synthesis) in epithelial cells and found that cells overexpressing COX-2 were resistant to apoptosis induction by butyrate. In a separate study, Shiff et al. (1995) have shown that the PG synthesis inhibitor sulindac sulfide inhibits proliferation, causes cell cycle quiescence and induces apoptosis in HT-29 colon adenocarcinoma cells.

A second hypothesized mechanism by which fish oil may decrease cell proliferation and increase apoptosis is through its modulation of ras gene mutation and expression. In a recent report from our laboratory using the same rats as in the present study, we also measured K-ras mutations as a function of diet and carcinogen administration (Jiang et al. 1995). At the same midpoint date after AOM injection, rats fed fish oil diets had significantly fewer ($P < 0.01$) ras mutations than animals fed the corn oil diets (5 vs. 20%). In addition, fish oil reduced total ras expression (compared with corn oil) at the intermediate and final time points. These results are also in agreement with the recent study of Singh et al. (1997) that showed a decreased colonic expression and membrane association of ras p21 with fish oil feeding versus corn oil feeding.

Again, the mechanism by which ras mutations and/or over-

expression of ras p21 affects changes in proliferation/apoptosis has not been clearly delineated, although this is an area of active research. What is known is that ras activation by point mutation or overexpression is associated with elevated levels of cellular diacylglycerol (DAG) (Laurenz et al. 1996) and elevations of colonic DAG levels modulate protein kinase C (PKC). Over time, high levels of DAG appear to down regulate PKC (Jiang et al. 1996). There is recent evidence that down regulation of PKC arrests the induction of apoptosis (Rusnak and Lazo 1996). We have shown, again using the same animals described in the current study, that dietary fish oil relative to corn oil blocks an increase in DAG mass (Jiang et al. 1996) and prevents the chronic down regulation of PKC isozymes (Jiang et al. 1997). Thus in theory, this may explain the higher levels of apoptosis that we observed with fish oil supplementation vs. corn oil supplemented diets.

Finally, fish oil also may affect the colonic luminal contents since we have shown recently that it results in different mixed populations of colonic microflora relative to corn oil feeding (Maciorowski et al. 1997). Because the microflora are directly responsible for the production of short chain fatty acids (SCFA) from fiber fermentation, these fish oil-induced changes in the microflora may result in different patterns of SCFA production. We recently have documented such shifts in SCFA patterns as a consequence of fish oil versus corn oil and as a function of dietary fiber (Chang et al. 1997a). This may help to explain the synergistic interaction between fat and fiber observed in the present study. We are currently exploring this possibility.

In conclusion, the present study shows that diets containing fish oil result in fewer rats with tumors than diets containing corn oil, and the balance between apoptosis and cell proliferation appears to be key to tumor development. Fish oil significantly decreases certain markers of cell proliferation, as previously reported by others, but its greatest effect is on the maintenance of higher levels of apoptosis in the presence of carcinogen. The specific mechanisms by which fish oil decreases proliferation and increases apoptosis is not known but may involve decreased prostaglandin production and/or decreased ras mutation/over expression and blockage of the carcinogen-induced down regulation of PKC.

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LITERATURE CITED

- Bang, H. O., Dyerberg, J. & Hjorne, N. (1976) The composition of food consumed by Greenland Eskimos. *Acta. Med. Scand.* 200: 69-73.
- Bartoli, G. M., Palozza, P., Marra, G., Armelao, F., Franceschelli, P., Luberto, C., Sgarlata, E., Piccioni, E. & Anti, M. (1993) N-3 PUFA and α -tocopherol control of tumor cell proliferation. *Mol. Aspects Med.* 14: 247-252.
- Bartram, H.-P., Gostner, A., Scheppach, W., Reddy, B. S., Rao, C. V., Dusel, G., Richter, F., Richter, A. & Kasper, H. (1993) Effects of fish oil on rectal cell proliferation, mucosal fatty acids, and prostaglandin E₂ release in healthy subjects. *Gastroenterology* 105: 1317-1322.
- Bedi, A., Pasricha, P. J., Akhtar, A. J., Barber, J. P., Bedi, G. C., Giardiello, F. M., Zehnbauber, B. A., Hamilton, S. R. & Jones, R. J. (1995) Inhibition of apoptosis during development of colorectal cancer. *Cancer Res.* 55: 1811-1816.
- Blot, W. J., Lanier, A., Fraumeri, J. F. & Bender T. R. (1975) Cancer mortality among Alaska natives, 1960-69. *J. Nat. Cancer Inst.* 55: 547-554.
- Boland, C. R., Montgomery, C. K. & Kim, Y. S. (1982) Alterations in colonic mucin structure occurring with cellular differentiation and malignant transformation. *Proc. Natl. Acad. Sci. USA* 79: 2051-2055.
- Caygill, C.P.-J., Charlett, A. & Hill, M. J. (1996) Fat, fish, fish oil and cancer. *Br. J. Cancer* 74: 159-164.
- Chang, W.-C.L., Chapkin, R. S. & Lupton, J. R. (1997a) Luminal butyrate concentration is positively associated with colonocyte differentiation and

- apoptosis and negatively associated with proliferation. *FASEB J.* 11: A566 (abs.).
- Chang, W.-C.L., Chapkin, R. S. & Lupton, J. R. (1997b) Predictive value of proliferation, differentiation and apoptosis as intermediate markers for colon tumorigenesis. *Carcinogenesis* 18: 721–730.
- Chen, C. Y. & Faller, D. V. (1995) Direction of p21^{ras}-generated signals towards cell growth or apoptosis is determined by protein kinase C and Bcl-2. *Oncogene* 11: 1487–1498.
- Deschner, E. E., Lytle, J. S., Wong, G., Ruperto, J. F. & Newmark, H. L. (1990) The effect of dietary omega-3 fatty acids (fish oil) on azoxymethanol-induced focal areas of dysplasia and colon tumor incidence. *Cancer* 66: 2350–2356.
- Fearon, E. R. & Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell* 61: 759–767.
- Garewal, H., Bernstein, H., Bernstein, C., Sampliner, R. & Payne, C. (1996) Reduced bile acid-induced apoptosis in "normal" colorectal mucosa: a potential biological marker for cancer risk. *Cancer Res.* 56: 1480–1483.
- Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. (1992) Identification of programmed cell death in-situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119: 493–501.
- Jiang, Y. H., Lupton, J. R., Chang, W.-C.L., Jolly, C. A., Aukema, H. M. & Chapkin, R. S. (1996) Dietary fat and fiber differentially alter intracellular second messengers during tumor development in rat colon. *Carcinogenesis* 17: 1227–1233.
- Jiang, Y. H., Lupton, J. R. & Chapkin, R. S. (1997) Dietary fish oil blocks carcinogen-induced down-regulation of colonic protein kinase C isozymes. *Carcinogenesis* 18: 351–357.
- Jiang, Y. H., Lupton, J. R. & Chapkin, R. S. (1995) Modulation of intermediate biomarkers of colon cancer by dietary fat, fiber and carcinogen in rat colonocytes. *FASEB J.* 9: A868 (abs.).
- Kerr, J.F.K., Gobe, G. C., Winterford, C. M. & Harmon, B. V. (1995) Anatomical methods in cell death. *Methods Cell Biol.* 46: 1–27.
- Klurfeld, D. M., Weber, M. M. & Kritchevsky, D. (1987) Inhibition of chemically induced mammary and colon tumor promotion by caloric restriction in rats fed increased dietary fat. *Cancer Res.* 47: 2759–2762.
- Laurenz, J. C., Gunn, J. M., Jolly, C. A. & Chapkin, R. S. (1996) Alteration of glycerolipid and sphingolipid-derived second messenger kinetics in ras transformed 3T3 cells. *Biochim. Biophys. Acta.* 1299: 146–154.
- Lee, D. Y., Chapkin, R. S. & Lupton, J. R. (1993a) Dietary fat and fiber modulate colonic cell proliferation in an interactive site-specific manner. *Nutr. Cancer* 20: 107–118.
- Lee, D. Y., Lupton, J. R., Aukema, H. M. & Chapkin, R. S. (1993b) Dietary fat and fiber alter rat colonic mucosal lipid mediators and cell proliferation. *J. Nutr.* 123: 1808–1817.
- Maciorowski, K. G., Turner, N. D., Lupton, J. R., Chapkin, R. S., Shermer, C. L., Ha, S. D. & Ricke, S. C. (1997) Diet and carcinogen alter the fecal microbial populations of rats. *J. Nutr.* 127: 449–457.
- Maher, J., Colonna, F., Baker, D., Luzzatto, L. & Roberts, I. (1994) Retroviral-mediated gene transfer of a mutant H-ras gene into normal human bone marrow alters myeloid cell proliferation and differentiation. *Exp. Hematol.* 22: 8–12.
- Paganelli, G. M., Biasco, G., Santucci, R., Brandi, G., Lalli, A. A., Miglioli, M. & Barbara, L. (1991) Rectal cell proliferation and colorectal cancer risk level in patients with non-familial adenomatous polyps of the large bowel. *Cancer* 68: 2451–2454.
- Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A. & Henderson, B. E. (1990) Increased cell division as a cause of human cancer. *Cancer Res.* 50: 7415–7421.
- Rao, C. V. & Reddy, B. S. (1993) Modulating effect of amount and types of dietary fat on ornithine decarboxylase, tyrosine protein kinase and prostaglandins production during colon carcinogenesis in male F344 rats. *Carcinogenesis* 14: 1327–1333.
- Reddy, B. S., Burill, C. & Rigotty, J. (1991) Effect of diets high in ω -3 and ω -6 fatty acids on initiation and postinitiation stages of colon carcinogenesis. *Cancer Res.* 51: 487–491.
- Rusnak, J.M. & Lazo, J. S. (1996) Downregulation of protein kinase C suppresses induction of apoptosis in human prostatic carcinoma cells. *Exp. Cell Res.* 224: 189–199.
- SAS Institute Inc. (1985) *SAS User's Guide*. SAS Institute, Cary, NC.
- Schreinemachers, D. M. & Everson, R. B. (1994) Aspirin use and lung, colon, and breast cancer incidence in a prospective study. *Epidemiology* 5: 138–146.
- Shiff, S. J., Qiao, L., Tsai, L. L. & Rigas, B. (1995) Sulindac sulfide, an aspirin-like compound, inhibits proliferation, cause cell cycle quiescence, and induces apoptosis in HT-29 colon adenocarcinoma cells. *J. Clin. Invest.* 96: 491–503.
- Singh, J., Hamid, R. & Reddy, B. S. (1997) Dietary fat and colon cancer: modulating effect of types and amount of dietary fat on ras-p21 function during promotion and progression stages of colon cancer. *Cancer Res.* 57: 253–258.
- Thun, M. J., Namboodiri, M. M. & Heath, C. W., Jr. (1991) Aspirin use and reduced risk of fatal colon cancer. *N. Engl. J. Med.* 325: 1593–1596.
- Tomlinson, I. P. & Bodmer, W. F. (1995) Failure of programmed cell death and differentiation as causes of tumors: some mathematical models. *Proc. Natl. Acad. Sci. USA* 92: 11130–11134.
- Tsuji, M. & DuBois, R. N. (1995) Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* 83: 493–501.
- Tsujitani, S., Shirai, H., Tatebe, S., Sugamura, K., Ohfuji, S., Gomyo, Y., Maeta, M., Ito, H. & Kaibara, N. (1996) Apoptotic cell death and its relationship to carcinogenesis in colorectal carcinoma. *Cancer* 77: 1711–1716.
- US Public Health Service (1991) *National Health Promotion and Disease Prevention Objectives*. US Department of Health and Human Services, Publication PHS 91-50212. Washington, D.C.
- Willett, W. C., Stampfer, M. J., Colditz, G. A., Rosner, B. A. & Speizer, F. E. (1990) Relation of meat, fat and fiber intake to the risk of colon cancer in a prospective study among women. *N. Engl. J. Med.* 323: 1664–1672.