Prevention of Colon Cancer by Low Doses of Celecoxib, a Cyclooxygenase Inhibitor, Administered in Diet Rich in $\omega$-3 Polyunsaturated Fatty Acids


Introduction

Colon cancer, which is the fourth most common cancer in the world, is one of the leading causes of cancer death in both men and women in Western countries, including the United States where ~145,290 new cases of colorectal cancer are estimated for the year 2005 (1, 2). Therefore, it is a major public health problem worldwide. Epidemiologic studies suggest that dietary factors, particularly caloric intake and saturated fat, may be of importance in the etiology of colon cancer (3). A report by an expert panel assembled by the American Institute for Cancer Research and World Cancer Research Fund came to the scientific consensus that evidence for an association between the intake of saturated fat and/or animal fat and colon cancer risk is very strong (3). Continuing population studies revealed that diets particularly high in animal fat are generally associated with increased risk of developing colon cancer, whereas diets high in fish oil or fish reduces this risk (4, 5). In a phase II clinical trial of patients with colonic polyps, dietary fish oil supplements inhibited cell proliferation in the colonic mucosa (6). In support of this, several animal studies using well-established colon cancer models provided ample and consistent experimental evidence that diets containing high levels of saturated fatty acids, such as those in Western diets, promote colon carcinogenesis, whereas diets high in $\omega$-3 polyunsaturated fatty acids (PUFA) had no such promoting effect (7–11). An assay in mice showed that a high-fat diet simulating the mixed lipid composition of the average American diet induced dysplastic lesions in the colon, indicative of colon carcinogenesis (12). Existing evidence suggests that the $\omega$-3 PUFA–induced colon tumor inhibition is mediated in part through the suppression of cyclooxygenase-2 (COX-2) activity and increases in apoptosis in colon tumors (7, 13–15). Also, dietary $\omega$-3 PUFAs may protect against colon carcinogenesis by decreasing DNA adduct formation and/or enhancing DNA repair (14).

Studies using either transgenic and knockout mice have provided convincing evidence for the association between COX-2 expression and colon tumorigenesis (16, 17). The discovery that COX-2 inhibitors, such as celecoxib, inhibit development of colonic polyps in patients with familial adenomatous polyposis is a seminal advance in the chemoprevention of colon cancer (16, 18). Studies conducted by several investigators clearly show that celecoxib, rofecoxib, and other nonsteroidal anti-inflammatory drugs (NSAID) are effective for the prevention and regression of adenomas in the mouse model of adenomatous polyposis (19–25). The results from our studies have provided convincing evidence that celecoxib administered during the initiation, postinitiation, and progression stages of colon carcinogenesis inhibits colon carcinogenesis in chemically induced colon carcinogenesis (26–28).

Preclinical studies conducted in our laboratory have affirmed that colon tumor inhibition by celecoxib at 40% maximum tolerated dose is much more effective than by traditional NSAIDs, including aspirin, ibuprofen, piroxicam, and sulindac, administered at 80% maximum tolerated doses (29). Patients with familial adenomatous polypl after 6 months of twice daily treatment with 400 mg celecoxib had 31% reduction in polyposis number; whereas 200 mg celecoxib twice daily reduced polyp number to 12% (18). Regression of rectal polyps was reported in placebo-controlled studies by Giardiello...
Materials and Methods

Materials. Azoxymethane was purchased from the Midwestern Research Institute (Kansas City, MO). 14C-Arachidonic acid was purchased from Amersham (Detroit, MI). Precocital silica G plastic thin layer chromatography plates were bought from Fisher Scientific Co. (Springfield, NJ) and Amersham (Detroit, MI). Precoated silica G plastic thin layer chromatography plates were bought from Fisher Scientific Co. (Springfield, NJ) and Amersham (Detroit, MI). Precoated silica G plastic thin layer chromatography plates were bought from Fisher Scientific Co. (Springfield, NJ) and Amersham (Detroit, MI).

Animals and diets. A total of 230 male F344 rats received at weaning by the Institute of Shortening and Edible Oils (15). The mixed lipid content of the average American diet. The composition of mixed lipids is described in detail in Materials and Methods.

Experimental procedure. The experimental protocol is summarized in Fig. 1. Following quarantine, all animals were distributed by weight into various experimental groups (treatment and appropriate controls) and continued on the standard low-fat (5% corn oil), semipurified AIN-76A diet. Then, beginning at 7 weeks of age, all rats in each dietary group (n = 24), except the vehicle-treated control groups, received s.c. azoxymethane (15 mg/kg body weight) once weekly for 2 weeks (Fig. 1). Rats in the vehicle-treated groups (n = 12) received an equal volume of normal saline. One day after the last second azoxymethane or saline injection (postinitiation stage), groups of animals were transferred to their respective HFML and HFFO diets with 0, 250, 500, and 1,000 ppm celecoxib and maintained on their dietary regimen until termination of the study at 26 weeks after the second azoxymethane or normal saline treatment. The rats in each group were weighed once weekly until they were 16 weeks of age and then measured once every 4 weeks.

As scheduled, all rats were killed by CO2 asphyxiation. After laparotomy, the entire stomach, small intestine, and large intestine were resected. They were opened longitudinally and the contents were flushed with normal saline. They were examined for intestinal tumors, and the location and number of tumors were assessed with a dissection microscope and recorded. Colon tumors were laid flat on a glass plate, and the tumor-free colon mucosa was scraped off with a glass slide. Colon tumors with a diameter of >0.5 cm were cut into halves; one portion of the tumor was assayed for COX enzyme activity and expression levels, and other halves of the tumors and neoplasms <0.5 cm were used for histopathologic examination. The mucosal scrapings and portions of colon tumors were quickly frozen in liquid nitrogen and stored at −80°C until analysis. The rationale for determining COX-2 activity and expression in colon tumors has been based on the observation that COX-2 gene expression and protein expression are markedly elevated in human colon tumors and also in chemically induced colon tumors in rats compared with accompanying colon mucosa (28, 33–35). Therefore, we investigated whether the modulation of colon tumorigenesis by celecoxib administered in high-fat diets containing mixed lipids and ω-3 PUFAs is mediated through COX-2 activity in colon tumors. The mucosal scrapings from morphologic tumors were assessed by conventional light microscopy. The stained

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<tr>
<th>Table 1. Percentage composition of experimental diets</th>
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<td>Diet ingredients</td>
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<tr>
<td>Casein</td>
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<tr>
<td>ω-3-Methionine</td>
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<td>Cornstarch</td>
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<tr>
<td>Dextrose</td>
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<td>Alphacel</td>
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<tr>
<td>Fish oil</td>
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<tr>
<td>Mixed lipids*</td>
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<tr>
<td>Mineral mix</td>
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<tr>
<td>Vitamin mix</td>
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<tr>
<td>Choline bitartrate</td>
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NOTE: All diets were formulated on the basis of the AIN standard reference diet with the modification of varying sources of carbohydrate (10, 15).

Mixed lipid diet containing American blend fat developed by the Institute of Shortening and Edible Oils was formulated to simulate the lipid content of the average American diet. The composition of mixed lipids is described in detail in Materials and Methods.
sections were examined for tumor types by a pathologist according to the classification of Pozharisski (36), which is followed routinely in our laboratory (15, 28). Most of the colon tumors were invasive or noninvasive adenocarcinomas (15, 28).

**Total cyclooxygenase and cyclooxygenase-2 specific activity and Western blot analysis of cyclooxygenase-2.** Because COX enzyme is firmly bound to the luminal surface of the endoplasmic reticulum and nuclear envelope, the microsomal fractions of the colonic tumor samples from the animals fed the 250 ppm celecoxib in HFML and HFFO diets were prepared as previously described (15). COX activities in colonic tumor samples were assayed by using previously published methods (15). Western blot analysis of COX-2 in colonic mucosa and tumors was done as previously described (15).

**Statistical analysis.** Differences in colon tumor multiplicity and COX activities were compared between the animals fed HFML diet or the HFFO diet with or without different levels of celecoxib were comparable throughout the study (data not shown). In saline-treated rats, feeding of HFML and HFFO diets containing various levels of celecoxib did not produce any gross changes in several organs that would indicate toxicity or adverse side effects.

**Efficacy of different levels of celecoxib administered in high-fat mixed lipid and high-fat fish oil diets.** There is no evidence of colon tumors in saline-treated animals fed the control or experimental diets. Azoxymethane-treated animals fed the control HFFO diet had a significantly lower colon tumor incidence (percentage animals with tumors; \( P < 0.001 \); Fig. 2) and multiplicity (number of tumors/animal; \( P < 0.001 \); Fig. 3) when compared with those fed the control HFML diet. Importantly, administration of

**Results**

**General observations.** The body weights of rats treated with azoxymethane and fed the HFML diet or the HFFO diet with or without different levels of celecoxib were comparable throughout the study (data not shown). In saline-treated rats, feeding of HFML and HFFO diets containing various levels of celecoxib did not produce any gross changes in several organs that would indicate toxicity or adverse side effects.

**Efficacy of different levels of celecoxib administered in high-fat mixed lipid and high-fat fish oil diets.** There is no evidence of colon tumors in saline-treated animals fed the control or experimental diets. Azoxymethane-treated animals fed the control HFFO diet had a significantly lower colon tumor incidence (percentage animals with tumors; \( P < 0.001 \); Fig. 2) and multiplicity (number of tumors/animal; \( P < 0.001 \); Fig. 3) when compared with those fed the control HFML diet. Importantly, administration of
celecoxib to rats at 1,000, 500, and 250 ppm levels in the HFML diet dramatically suppressed the incidence of adenocarcinomas by 100%, 90%, and 84%, respectively, compared with those fed the control HFML diet without celecoxib (Fig. 2). Furthermore, celecoxib at 1,000, 500, and 250 ppm levels in HFFO diet also greatly inhibited the incidence of adenocarcinomas compared with those fed the control HFFO diet without celecoxib. It is noteworthy that administration of 250 ppm celecoxib in HFML diet significantly (P < 0.05) inhibited colon tumor multiplicity compared with those fed the HFML diet containing 250 ppm celecoxib (P < 0.05; Fig. 3), suggesting that very low dose of celecoxib administered in ω-3 PUFA diet inhibits colon carcinogenesis better than when it is administered in mixed lipid diet.

Cyclooxygenase activity and expression. We investigated whether the inhibition of colon tumorigenesis by very low dose of celecoxib (250 ppm) administered in HFML and HFFO diets is associated with the modulation of arachidonic acid metabolism by COX activity and COX-2 expression in colon tumors. Rats fed the HFFO diet had significant inhibition of total COX activity and COX-2 activity in the colon tumors as indicated by the arachidonic metabolites (eicosanoids) formed from arachidonic acid compared with rats fed the HFML diet (Table 2). Colonic tumors from rats fed the HFFO diet and very low dose (250 ppm) of celecoxib had lower COX activity (29% inhibition) and COX-2 activity (36% inhibition) compared with those fed the HFML diet and 250 ppm celecoxib. The effects of very low dose of celecoxib administered in HFML and HFFO diets on COX-2 expression were determined in colonic tumors. Figure 4 shows a representative Western blot analysis of COX-2 expression in colonic tumors and mucosa. COX-2 expression was not detected in colonic mucosa irrespective of dietary treatment. This supports our previous observations that COX-2 expression was either minimally or not detected in the colon mucosa of rats treated with colon carcinogen (15, 34). It is noteworthy that the expression of COX-2 in colon tumors of rats received azoxymethane treatment and fed 250 ppm celecoxib in HFFO diet is lower compared with those fed 250 ppm of celecoxib in HFML diet.

Discussion

The present study is a part of an ongoing preclinical investigation of the effects on types of dietary fat and combinations of chemopreventive agents against colon carcinogenesis. Here and in our previous studies, we have formulated the HFML diet so as to simulate the types of fat that are often consumed in the United States and other Western countries (15). The current study also investigated a HFFO diet that contained 10% mixed lipids and 10% fish oil. Our aim for the present study was also to determine whether celecoxib is effective in inhibiting colon carcinogenesis in animals fed the high-fat diets. The results of this investigation clearly show that consuming a diet rich in ω-3 PUFAs (HFFO) had a higher potential to inhibit colon tumorigenesis compared with a Western-style diet high in mixed lipids, including saturated fats of animal origin as well as ω-6 PUFAs (HFML).

In our previous study, the incidence of adenocarcinomas was inhibited by 31% in the rats fed the HFFO diet (15) and in the

Table 2. Effects of celecoxib administered in HFML and HFFO diets on total COX activity and COX-2 activity in colon tumors

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<thead>
<tr>
<th>Experimental group</th>
<th>Total COX activity</th>
<th>COX-2 activity</th>
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<tr>
<td>HFML diet</td>
<td>6.84 ± 0.49</td>
<td>3.60 ± 0.14</td>
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<tr>
<td>HFML diet + 250 ppm celecoxib</td>
<td>3.21 ± 0.28*</td>
<td>1.22 ± 0.16†</td>
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<tr>
<td>HFFO diet</td>
<td>4.22 ± 0.29↑</td>
<td>2.50 ± 0.16↑</td>
</tr>
<tr>
<td>HFFO diet + 250 ppm celecoxib</td>
<td>2.28 ± 0.11*</td>
<td>0.78 ± 0.18↑</td>
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NOTE: As detailed in Materials and Methods, microsomal fractions of colonic tumors from animals fed the HFML and HFFO diets containing celecoxib at 0.250 ppm were assayed for COX-2 and COX activities. The data represents mean ± SE (n = 3-6 tumors). Total COX activity is expressed as nanomoles of [14C]arachidonic acid metabolized per milligram of protein per 15 minutes. COX-2 activity is expressed as nanomoles of [14C]15-R-hydroxyeicosatetraenoic acid formed per milligram of protein per 15 minutes.

*Significantly decreased from their respective controls at P < 0.01 to P < 0.001 by Student’s t test.
†Significantly different from their respective controls at P < 0.003 to P < 0.001 by Student’s t test.
↑Significantly decreased from HFML diet at P < 0.01 by Student’s t test.
*Significantly decreased from HFML diet at P < 0.05 by Student’s t test.
current study the inhibition reached ~70% compared with those fed the HFML diet. The major differences in these two studies were the composition of HFFO diet and duration of study period. In the current study, HFFO diet contained 10% mixed lipids and 10% fish oil, and the animals were on the experimental diets for ~24 weeks; whereas, in our previous study, the HFFO diet was formulated to contain 17% fish oil and 3% corn oil, and all animals received this diet for 38 weeks (15). Earlier studies have shown that 1,500 ppm celecoxib administered in a low-fat AIN-76A diet 1 week before, during, and after azoxymethane treatment (initiation and post-initiation stage) and 500 and 1,500 ppm celecoxib in a low-fat diet during the promotion and progression stage significantly suppressed colon carcinogenesis in rats (26, 28). Differences among these studies compared with present study on the degree of inhibition of colon adenocarcinomas by different levels of celecoxib might be explained on the basis of when this agent was administered (i.e., during the initiation, postinitiation, and/or promotion and progression stage of colon carcinogenesis), what type of basal diet was used, and how many weeks the animals were on the experimental diets. These aspects might explain the difference in tumor inhibition between the two studies.

To our knowledge, this is the first study to show that administration of celecoxib at 250 ppm representing very low dose in HFML and HFFO diets significantly suppresses colon carcinogenesis. Furthermore, low and moderately high doses (250, 500, and 1,000 ppm) of celecoxib suppressed the colon tumor incidence and multiplicity by ~80% to 100% even in rats fed the HFML diet. This finding is of clinical significance. It should be recognized, however, that administration of daily dose of 400 or 800 mg of COX-2 inhibitors have been shown to induce cardiovascular side effects in humans as recently reported. Extrapolation of the results of the present study in rats consuming 88 kcal/d to humans consuming on the average 2,000 kcal/d indicates that celecoxib at 250, 500, and 1,000 ppm dose levels used in our study translates to approximately daily doses of 100, 200, and 400 mg, respectively, in humans. Although it is presently unknown that these moderately low doses (100 and 200 mg) of celecoxib inhibit colorectal cancer in humans, it is likely that in humans these dose levels will have a favorable safety profile compared with a daily dose of 800 mg that have been used recently in ongoing studies.

Our present study provided evidence that administration of a very low dose of celecoxib in ω-3 PUFA–rich diet would be an ideal strategy for prevention of colon cancer. This approach is extremely important to enhance the efficacy of a promising chemopreventive agent, such as celecoxib, and to eliminate untoward side effects, if any, of very high dose of this agent. The results of the low dose of celecoxib administered in HFML and HFFO diets are further evidence for the potential of this compound as a chemopreventive agent against colon carcinogenesis. Another important observation of the present study is that the colon tumor multiplicity is significantly lower in animals given 250 ppm celecoxib in HFFO diet compared with those administered 250 ppm celecoxib in HFML diet. To our knowledge, this is the first demonstration of a moderate but significant chemopreventive effect of low dose of celecoxib administered in ω-3 PUFA–rich diet (healthy lifestyle) compared with that of mixed-lipid diet.

The present study shows that feeding of HFFO diet decreased the expression and formation of eicosanoids from the arachidonic acid (COX activity) in colon tumors. Arachidonic acid is metabolized to eicosanoids via COX enzyme. The results of the present study support the findings of our earlier study that colon tumor inhibition by ω-3 PUFA–rich diet is mediated through the suppression of COX activity and COX-2 expression (15). As discussed above, several reports indicate that eicosanoids have a role in the pathogenesis of colon cancer (16, 33). Also, some studies indicate that dietary ω-3 PUFAs may protect against carcinogenesis by either decreasing DNA adduct formation and/or enhancing DNA repair as well as increasing apoptosis (13–15). It has been shown that the inhibition of colon carcinogenesis by ω-3 PUFAs is mediated, in part, through the activation of retinoid X receptors (RXR), an obligatory component of a large number of nuclear receptors (37–39), suggesting that ω-3 PUFAs mediate growth inhibitory effects in the colon RXR subunit of nuclear receptor heterodimers. Members of the nuclear receptor superfamily are transcription factors that selectively regulate cell proliferation (40). With regard to the mode of action of celecoxib against colon cancer, administration of celecoxib suppresses both activity and expression of COX-2. Whereas it is well established that celecoxib predominately modulates colon tumor growth by acting on COX-2, there are studies to indicate that higher doses of COX-2 inhibitor modulates several COX-2–independent pathways, including cell cycle arrest and enhanced apoptosis, strengthening the concept of several investigators that more than one molecular mechanism is involved in tumor inhibition by these chemopreventive agents (38–42).

**Figure 4.** Effects of 250 ppm of celecoxib administered in HFML and HFFO diets on Western analysis of COX-2 expression in colon mucosa (M) and tumors (T) of rats. 1 to 4, colon tumors of rats fed 20% HFML (Western style) diet, 20% HFML diet + 250 ppm celecoxib, 10% fish oil + 10% HFML (HFFO) diet, and HFFO diet + 250 ppm celecoxib, respectively. Note that COX-2 expression was not detected in colonic mucosa. Colon tumors of rats fed HFFO diet + 250 ppm celecoxib (4T) showed decreased COX-2 expression compared with tumors of rats fed HFML diet + 250 ppm celecoxib (2T).
In summary, the present study shows that a high-fat diet containing fish oil and mixed lipids (saturated and unsaturated fats) induced fewer colon adenocarcinomas than did a high-fat diet containing mixed lipids. We also show for the first time that varying doses of celecoxib administered in Western-style HFML and HFFO diets suppressed colon carcinogenesis. Interestingly, low-dose level of celecoxib administered in HFFO diet caused a significant inhibition of COX-2 activity and expression and tumor incidence compared with low dose of celecoxib in HFML diet. Any human clinical trials using COX-2 inhibitors to prevent colorectal cancer in high-risk individuals should consider all relevant data that reflect on the efficacy and safety of these agents at very high dose levels. The ability of celecoxib at moderately high, low, and very low dose levels tested in the present study may have important preventive implications in human clinical trials. The use of low dose of celecoxib in combination with healthy lifestyles seems to be a promising approach that may evolve into a better chemopreventive strategy for future human clinical trials.

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References