Modulation of cyclooxygenase-2 activities by the combined action of celecoxib and decosahexaenoic acid: Novel strategies for colon cancer prevention and treatment


Chemoprevention and Nutritional Carcinogenesis Program, Institute for Cancer Prevention, American Health Foundation-Cancer Center, Valhalla, NY

Abstract

To develop efficient synergistic or additive combinations of chemopreventive and nutritional agents to reduce the risk of colon cancer, experiments were designed to test the application of a selective cyclooxygenase-2 (COX-2) inhibitor together with dietary ω-3 polyunsaturated fatty acids (PUFAs), such as decosahexaenoic acid (DHA). Thus, individual application of celecoxib, a COX-2 inhibitor, DHA, a ω-3 PUFA, and combinations of both were tested for their effectiveness using cell proliferation, apoptosis, and COX-2 expression as markers in the human colon cancer HCA-7 cell line. HCA-7 cells exposed to various subtoxic doses of celecoxib, DHA, or combinations of both were analyzed for inhibition of cell proliferation by trypan blue exclusion and proliferating cell nuclear antigen methods, induction of apoptosis by 4',6-diamidino-2-phenylindole method, and COX-2 by reverse transcription-PCR and Western blot analysis. In addition, we examined the inhibitory potential of celecoxib and DHA on 14C-arachidonic acid metabolism mediated by COX-2 in the HCA-7 cell line. We found that treatment with celecoxib (50–150 μM) or DHA (150–225 μM) individually induces apoptosis and inhibits cell proliferation only at high concentrations in HCA-7 cell lines. A synergistic effect was observed on induction of apoptosis and inhibition of proliferation when cells were exposed to low doses of celecoxib (50–100 μM) together with DHA (75 μM). At high concentrations, celecoxib and DHA blocked the increase in COX-2 protein and mRNA expression in HCA-7 cells. Importantly, the inhibition of COX-2 expression was more pronounced in cells treated with low-dose combinations than with individual agents at high concentrations. In addition, celecoxib and DHA at low-dose levels inhibited 14C-arachidonic acid metabolism (50–85%, P < 0.0001) leading to very low levels of type 2 series prostaglandin formation. These findings provide the basis for the development of combinations of low-dose regimens of a COX-2 inhibitor and ω-3 PUFAs such as DHA for the prevention and treatment of colon cancer. We are currently testing this concept in preclinical models. [Mol Cancer Ther. 2004;3(2):215–221]

Introduction

Colorectal cancer is one of the leading causes of cancer deaths in Western countries in both men and women. In the United States, about 151,500 new cases of colon cancer and 57,600 related deaths are estimated to occur in 2003 (1). Etiological studies indicate that the risk of developing colon cancer may be attributable to combined actions of environmental and endogenous factors (2–4). On the basis of comparative data and case control studies in Japan and the United States in the late 1960s, Wynder et al. (5) suggested that dietary factors in general and dietary fat in particular might play a role in the etiology of colon cancer. Laboratory animal model studies carried out in our laboratory and elsewhere have consistently provided evidence that the colon tumor-promoting effect of dietary fat depends on the type and amount of dietary fat (6–8). Diets rich in ω-3 fatty acids (marine oils) reduce the risk of chemically induced colon carcinogenesis compared with diets high in ω-6 fatty acids and/or saturated fatty acids. This suggests that the fatty acid composition of dietary fat is more critical to colon cancer risk than the total amount of fat consumed (8–10). Eating a diet high in polyunsaturated fatty acids (PUFAs) that are rich in ω-3 fatty acids may decrease the risk of colorectal cancer; this has been the hypothesis in relation to fish and fish oil (11). Caygill and Hill (12) reported an inverse correlation between fish and fish oil consumption and colorectal cancer. Several in vitro and in vivo studies support the hypothesis that ω-6 fatty acids promote colon tumorigenesis, whereas ω-3 fatty acids lack promoting activity (13–15). Several studies using decosahexaenoic acid (DHA) or eicosapentaenoic acid have shown inhibition of tumour cell proliferation and induction of apoptosis (16, 17).

Cellular and molecular mechanisms by which ω-3 fatty acids inhibit the growth of tumor cells remain to be understood. However, existing evidence clearly suggests that ω-3 fatty acids modulate colon tumor cell growth.
through the alteration of membrane phospholipid turnover, release of membrane arachidonic acid (AA) from phospholipids, and prostaglandin (PG) synthesis via cyclooxygenase-2 (COX) enzymes (18, 19). It is noteworthy that elevated levels of COX isoforms, particularly COX-2, have been observed in human colon tumors and chemically induced colon tumors in rodents indicating the significance of these enzymes in colon tumor growth. Importantly, studies carried out in our laboratory suggest that administration of diets rich in ω-3 fatty acids significantly reduces chemically induced colonic tumor COX-2 expression and activities (20). In support of this, human colon cancer cells exposed to DHA showed down-regulation of several proinflammatory genes, including inducible nitric oxide synthase and COX-2 (21).

An effective preventive strategy for colon cancer could be envisioned on the basis of recent epidemiologic, clinical, and laboratory investigations that combine to present an inverse relationship between the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and colorectal cancer development (22, 23). These studies have consistently reinforced the evidence that NSAIDs, particularly COX-2-selective agents, are indeed very effective chemopreventive agents against colon cancer development. Compelling experimental evidence for the role of COX-2 in colon carcinogenesis is well established through several investigations (24–26). For example, there are studies to indicate that colonic epithelial cells that overexpress the COX-2 gene develop altered adhesion properties and resist undergoing apoptosis (27). Studies also suggest that progressive targeted knockout of COX-2 by genetic manipulation significantly reduces the number of intestinal polyps in APC<sup>−/−</sup> mice (28). These observations clearly suggest that development of strategies to inhibit COX-2 expression and its activity should be receiving increasing attention for the prevention and treatment of colon cancer. Importantly, chemoprevention studies with the COX-2-selective inhibitor, celecoxib, in laboratory animals and, at the clinical level, with familial adenomatous polyposis patients clearly encourage such strategies (29, 30). The efficacy observed with celecoxib in familial adenomatous polyposis patients (~30% reduction in total polyp number) compels development of yet more efficacious strategies for the prevention of colon cancer (31).

We believe that intervention with chemopreventive agents alone may not be sufficient for secondary prevention of colorectal cancer in high-risk patients. Furthermore, administration of high-dose levels of COX-2 inhibitors over longer periods may not be devoid of side effects, as was observed in recent studies (32–35). However, combinations of chemopreventive agents at low doses with dietary supplements can either abolish or delay the development of neoplasms and would therefore be an ideal strategy for the prevention of colorectal cancer. This approach is extremely important to enhance the efficacy of a promising chemopreventive agent, such as celecoxib, and to eliminate unwanted side effects in preclinical and clinical studies.

Mindful of the significance of ω-3 fatty acids (DHA) and celecoxib in colon carcinogenesis, experiments were designed to assess the effectiveness of DHA and celecoxib individually and in combination on cell proliferation, induction of apoptosis, and modulation COX-2 in the HCA-7 human colon cell line.

**Materials and Methods**

**Cell Lines and Reagents**

The HCA-7 human colon cancer cell line was kindly provided by Dr. Raymond DuBois (Vanderbilt University, Nashville, TN). Cells were maintained in McCoy’s 5A medium with 10% fetal bovine serum (FBS) in an atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. Celecoxib was provided by Pharmacia (St. Louis, KA) and DHA was purchased from NutCheck (Boston, MA). All experiments were performed with cells at about 70–80% confluence. HCA-7 cells were grown and treated with various concentrations of celecoxib or DHA individually and in combination to study cell proliferation, induction of apoptosis, and COX-2 protein and mRNA expression studies.

**Toxicity and Cell Growth**

Toxicity and cell viability levels of each test agent were determined by the trypan blue exclusion method and further assessed by proliferating cell nuclear antigen (PCNA; Ref. 36). Briefly, HCA-7 cells (0.5 × 10<sup>6</sup>) were fixed in 100% methanol for 10 min at −20°C, pelleted (5000 rpm for 10 min at 4°C), resuspended, and incubated in PBS containing 1% FBS/0.5% NP40 on ice for 5 min. Cells were washed twice in PBS/1% FBS, pelleted, and resuspended in 50 μl of a 1:10 dilution of the anti-PCNA primary antibody (PC-10; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS/1% FBS for 60 min at room temperature. Nonspecific IgG1/IgG2 was used as an isotypic control. Cells were then washed and incubated with goat anti-mouse phycoerythrin antibody (1:50) for 60 min at room temperature in the dark before assessing the PCNA-positive cells.

**Apoptosis**

Induction of apoptosis was measured by the 4′, 6-diamidino-2-phenylindole (DAPI) staining method. Briefly, human colon HCA-7 cancer cells were cultured for 24 h in the presence of various subtoxic concentrations of celecoxib, DHA, or combinations of both. Prior to staining, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then washed with PBS. DAPI was added to the fixed cells for 1 h, after which cells were visualized under a fluorescence microscope, using a blue filter. For each sample, at least five fields were examined. The morphological criteria for apoptosis included cytoplasmic and nuclear shrinkage, chromatin condensation, and cytoplasmic blebbing with maintenance of the integrity of the cell membrane. Apoptotic cells were identified by condensation and fragmentation of nuclei.

**Western Blot Analysis of COX-2**

COX-2 purified protein (Cayman Chemical Co., Ann Arbor, MI) was used as electrophoresis standard. Briefly, total protein (30 μg/lane) extracted from cells was separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. These membranes were blocked with...
Tris-buffered saline with Tween 20 blocking solution [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.01% (v/v) Tween 20] and 5% dry milk for 1 h. These membranes were probed with purified polyclonal antibodies at 1:1000 dilution for 1 h after being washed with buffer three times at 10-min intervals; the membranes were then treated with anti-antibodies and conjugated with horseradish peroxidase at 1:5000 dilution for 1 h. The proteins were visualized with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA Extraction and Reverse Transcription-PCR

Cultured cells were extracted and purified using the total RNA isolation kit (Ambion, Inc., Austin, TX). Purified RNA was suspended in diethyl pyrocarbonate-treated water and used for further experiments. The amount of extracted RNA was calculated from absorbance measurements at 260 nm (1OD 260–40 μg/ml). Total RNA was used to generate first-strand complementary DNA by employing SuperScript Reverse Transcriptase (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer’s recommended procedures. Briefly, 1 μg of total RNA was added to reverse transcriptase (RT) buffer [67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl2, 16.6 mM NH4SO4, 6.8 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM deoxynucleotide triphosphate] and was incubated for 45 min at 42°C in the presence of 40 units of RNase Out and 200 units of SuperScript Reverse Transcriptase. Then, PCR was performed for COX-2 from the complementary DNA samples by using Eppendorf PCR thermal cycles. Oligonucleotide primers for COX-2 were 5′-AGACAGATCATTGCTGGCC-GGGT-3′ and 5′-TCCAGATGAGACTGAAATTGAGG-3′. The housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase served as internal control. PCR amplification was initiated with 0.5 μg of cDNA, 5′ and 3′ primers (each 1 μg of final concentration) were added, and samples were subjected to 35 cycles of denaturation (30 s at 94°C), annealing (1 min at 56°C), and extension (30 s at 72°C) in the presence of Taq polymerase (Master mix form). The PCR products were then visualized by UV illumination after electrophoresis through a 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. The gel photographs were scanned with a computerized densitometer.

14C-AA Metabolism

Modulation of COX-2 activity in human HCA-7 colon cancer cells by celecoxib and DHA was measured by using a slight modification of a previously published method (37). Briefly, 150 μl of the reaction mixture containing 12 μM 14C-AA (300,000 dpm), 1 mM epinephrine, and 1 mM glutathione in 50 mM of phosphate buffer (pH 7.4), and 50–70 μg of microsomal protein (extracted from HCA-7 cells exposed to test agents for 24 h) were incubated at 37°C for 20 min. The reaction was terminated by adding 40 μl of 0.2 M HCl. The COX-2-mediated metabolites of AA were extracted with ethyl acetate (three times, 0.5 ml). The combined extracts were evaporated to dryness under N2, redissolved in acetonitrile, and subjected to reverse-phase high-performance liquid chromatography. 14C-AA metabolites were separated by two serially connected Bondaclone 10-μm C18 columns with a mobile phase of water and acetonitrile in a gradient program defined by a flow rate of 1.8 ml/min; solvent A: 65:35 water/acetonitrile (pH 3.5) and solvent B: acetonitrile (100%); sequences: 0–25 min, 100% solvent A; 25–40 min, 50% solvent A and 50% solvent B; 40–50 min, 80% solvent A and 20% solvent B; 50–60 min, 100% solvent A. Elution of each 14C-AA metabolite was detected by a radiomatic detector equipped with β-detector (Radiomatic Instruments, Tampa, FL) and corresponding individual eicosanoids were detected by RT values established by UV absorption at 235A for PGs and thromboxane B2, 306A PGE1 and PGI2, and 195A hydroxyeptadecatrienoic acid and hydroxyeicosaatetraenoic acid (Waters 990 Photodiode Array Detector, Milford, MA) with authentic standards.

Statistical Analysis

All experiments were carried out in triplicate at least twice. Data are presented as means ± SE or percentage means. Data were analyzed by Student’s t test and differences were considered significant at P < 0.05.

Results

Cell Proliferation

We tested the effect of celecoxib and DHA individually and in combination to assess the rate of cell proliferation in HCA-7 human colon cancer cells (Fig. 1). HCA-7 cells were exposed to subtoxic concentrations of the COX-2 inhibitor celecoxib (0–150 μM), the ω-3 fatty acid DHA (0–225 μM), or combinations of both for 18 h. Cell growth was measured by the trypsin blue exclusion method followed by PCNA for the assessment of proliferating cells. The results suggest that pretreatment with celecoxib or DHA inhibits cell proliferation only at high concentrations. However, synergistic inhibitory effect on cell proliferation was observed when cells were exposed to only doses of 50 μM of celecoxib together with 50 μM of DHA. Similar observations were made with the PCNA method (data not shown).

Figure 1. Effect of celecoxib and DHA treatment individually and in combination on the proliferation of HCA-7 cells. Cells exposed to subtoxic concentrations of celecoxib (0–150 μM), DHA (0–225 μM), or combinations of both for 18 h. Columns, mean of triplicate samples; bars, SE. *, P < 0.05; **, P < 0.001; ***, P < 0.0001, statistically significant from control group by Student’s t test.
Induction of Apoptosis

Figure 2 and 3 summarize the effects of celecoxib or DHA and/or combinations of these agents on HCA-7 colon cancer cells. As shown in Fig. 2, apoptotic cells were visualized in terms of characteristic morphological changes by the DAPI method (such as blebbing of the membrane, chromatin aggregation, and nuclear and cytoplasmic condensation pertaining to apoptosis). Pretreatment of HCA-7 cells with celecoxib induced apoptosis in a dose-dependent manner. However, stimulation of apoptosis occurred only at high concentrations of celecoxib (100–150 μM) and DHA (150–225 μM). Thus, at high concentrations, both agents induced apoptosis with inductive capacities of low to moderate levels. Interestingly, when cells were treated with low concentrations of celecoxib (50 μM) along with DHA (75 μM), a synergistic enhancement of apoptosis was observed in the HCA-7 cells (Fig. 3).

COX-2 Expression and Activity

We studied the expression levels of COX-2 protein in HCA-7 cell lines treated with various concentrations of celecoxib and DHA individually and in combination for 24 h. The results are summarized in Fig. 4. Treatment of the HCA-7 cell lines in the range of 50–150 μM celecoxib or 75–225 μM DHA decreased the expression of COX-2 protein in a dose-dependent manner; however, low concentrations had only limited effect (Fig. 4a). When cells were exposed to a combination of 50 μM celecoxib and 75 μM DHA, the inhibition of COX-2 protein was ~99% (Fig. 4b). Further analysis of COX-2 mRNA expression by RT-PCR produced similar observations. As indicated in Fig. 5, the combined application of 50 μM celecoxib and 75 μM DHA completely abolished COX-2 mRNA expression in HCA-7 cells.

The effects of celecoxib and DHA alone or in combination on COX-2 activity with regard to 14C-PGE2 formation from 14C-AA are shown in Fig. 6. Celecoxib and DHA individually suppressed 14C-PGE2 formation in a dose-response manner. We observed that even celecoxib at 25 μM or DHA at 50 μM suppressed 14C-PGE2 formation moderately (~17–22%) yet significantly (P < 0.03); however, exposure to a combination of 25 μM celecoxib and 50 μM DHA significantly enhanced the inhibition of COX-2 activity (~42%, P < 0.001). Similarly, the combination of 50 μM celecoxib and 75 μM DHA synergistically suppressed COX-2 activity by >93%.

Discussion

The present study is part of a large-scale investigation toward development of novel strategies for the prevention of colorectal cancer. As discussed in “Introduction,” the
key factors in testing the effectiveness of combinations of celecoxib and DHA are to increase their efficacy and to minimize side effects associated with long-term administration of such agents. In the present study, treatment with subtoxic concentrations of celecoxib or DHA suppressed proliferation in the human colon cancer HCA-7 cell line and induced apoptosis in a dose-dependent manner. Because unregulated cell proliferation and resistance to apoptosis are critical contributors to colon tumor growth, simultaneously counteracting both processes provides a more effective approach toward inhibiting or eliminating colon tumor growth (27, 38, 39). Although it has been well established that administration of NSAIDs or other COX-2 inhibitors can halt colon tumor growth by curbing cell proliferation and stimulating apoptosis (27, 40, 41), the precise mechanisms for such action are not clear. In the present study, we observed that in comparison with DHA, celecoxib predominately affects proliferation of HCA-7 cells and has a very modest effect on apoptosis, suggesting that COX-2 inhibitors at (pre)clinically relevant dose levels cause tumor inhibition mainly by suppressing cell growth. Unlike celecoxib, DHA at moderate to high dose levels inhibited cell proliferation and stimulated apoptosis in HCA-7 cells. It is not yet clear how DHA causes simultaneous effects on cell proliferation and apoptosis; however, there are several studies supporting the notion that DHA/ω-3 fatty acids act through multiple mechanisms, including those studied here and those as discussed below. Importantly, the results of the present study demonstrate for the first time that a combination of low-dose levels of celecoxib and DHA synergistically suppresses the growth of HCA-7 cells and enhances apoptosis 4–6-fold compared with the actions of individual agents. These effects are likely due to the differential modulation of COX-2 and other key enzymes that occur when agents are administered together. Our present study further supports our previous observation that agents with different modes of action (e.g., lovastatin and celecoxib) synergistically stimulate apoptosis in colon cancer cell lines (42).

It has been well established that celecoxib is an inhibitor of COX-2 activity and DHA is a modifier of COX-mediated metabolism (20, 43). In the present study, we examined the effects of celecoxib and DHA on COX-2 protein expression and mRNA expression to understand how combinations of these agents modulate COX-2 activity.

Figure 3. Effect of celecoxib and DHA individually and in combination on induction of apoptosis in HCA-7 cells. Cells exposed to subtoxic concentrations of celecoxib (0 – 150 μM), DHA (0 – 225 μM), or combinations of both for 24 h. Columns, mean of triplicate samples; bars, SE. **, P < 0.001; ***, P < 0.0001, statistically significant from control group by Student’s t test.

Figure 4. Effect of celecoxib (Co-xxib) and DHA individually (A) and in combination (B) on COX-2 protein expression in HCA-7 cells.

Figure 5. Effect of celecoxib and DHA individually and in combination on COX-2 mRNA expression in HCA-7 cells by RT-PCR method.

Figure 6. Effect of celecoxib and DHA individually and in combination on COX-2 activity/PGE2 formation in HCA-7 cells by reverse-phase high-performance liquid chromatography. *, P < 0.05; **, P < 0.01; ***, P < 0.001–0.0001, statistically significant from control group by Student’s t test.
Colon tumor cells. Clearly, treatment with moderate to high dose levels of celecoxib and/or DHA suppresses COX-2 expression and activity (PGE2 levels) in human colon HCA-7 cancer cells. As anticipated, the concentration of celecoxib required to block expression of COX-2 is much higher than the dose needed for PGE2 inhibition. However, combinations of low-dose levels of celecoxib and DHA synergistically suppressed COX-2 expression and caused more pronounced COX-2 activity. For example, treatment with 25 µM celecoxib or 50 µM DHA inhibits the formation of PGE2 by 17% and 21%, respectively; however, combining 25 µM celecoxib with 50 µM DHA results in PGE2 inhibition up to 42%. Thus, a combination of low-dose levels of celecoxib and DHA enhances inhibition of both COX-2 expression and activities as compared with high doses of these agents tested individually. The concentrations of celecoxib and DHA in cell culture test systems are higher than those established as human therapeutic dose levels; however, higher concentrations of test agents or drugs are being applied in most of the in vitro studies that are exploratory in nature. Systematic evaluation of the in vitro mechanisms will be followed by appropriate preclinical assays in laboratory animals before any considerations of appropriate dosages of celecoxib and DHA for clinical trials in humans.

The precise mechanisms by which celecoxib and DHA inhibit proliferation and stimulate apoptosis of colon tumor cells are not yet fully understood. However, on the basis of the present study and earlier investigations as depicted in Fig. 7, we propose a possible mechanism by which a COX-2-selective inhibitor and ω-3 fatty acids synergistically suppress colon tumor cell growth. As shown in Fig. 7, it has been well established that replacement of ω-3 fatty acids with ω-6 fatty acids in membrane phospholipids minimizes AA levels and ω-3 fatty acids become competing substrates for COX-2. In fact, these ω-3 fatty acids, in part metabolized by COX-2 to yield type 3 PGs and thrombox-

Figure 7. Possible mechanisms that lead to synergistic modulation of colon tumor cell proliferation and apoptosis by celecoxib and DHA.

References
Modulation of cyclooxygenase-2 activities by the combined action of celecoxib and decosahexaenoic acid: Novel strategies for colon cancer prevention and treatment
