Suppression of Tumor Cell Growth Both in Nude Mice and in Culture by n-3 Polyunsaturated Fatty Acids: Mediation through Cyclooxygenase-independent Pathways

Mary D. Boudreau, Kyung Hee Sohn, Sang Hoon Rhee, Sam W. Lee, Jay D. Hunt, and Daniel H. Hwang

Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana 70808; [M. D. B., K. H. S., S. H. R., D. H. H.]; Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112; [J. D. H.]; and Department of Medicine, Beth Israel Hospital, Harvard Medical School and Harvard Institute of Medicine, Boston, Massachusetts 02215 [S. W. L.]

ABSTRACT

Dietary n-3 polyunsaturated fatty acids (PUFAs), as compared with n-6 PUFAs, suppress cellular production of prostaglandins and tumor cell growth both in vitro and in vivo. However, the mechanism by which n-3 PUFAs suppress tumor growth is not understood. We investigated whether the suppression of tumor cell growth by dietary n-3 PUFAs is mediated through inhibition of cyclooxygenase (COX). A colon tumor cell line, HCT-116, that does not express COX was stably transfected with the constitutively expressed COX-1 or the inducible COX-2 cDNA using a retroviral transfection and infection system. Athymic nude mice transplanted with the cells expressing enzymatically active COX were fed isocaloric diets containing either safflower oil or fish oil for 2 weeks before the start of the experiment and for an additional 21 days after transplantation. Both tumor volume and tumor burden (tumor volume/body weight) were significantly reduced in mice fed the fish oil diet as compared with safflower oil-fed mice. This reduction occurred even in control mice that received injections with cells infected with the retroviral vector alone in the nude mice and in soft agar. N-3 PUFAs, as compared with n-6 PUFAs, also inhibited the growth of these cells in culture. This suppression of tumor cell growth by dietary n-3 PUFAs is mediated through inhibition of COX-2.

INTRODUCTION

The rates of cancer incidence are generally low in Eskimos of Alaska and Greenland as compared with North Americans and other Western population groups (1–6). Results from epidemiological studies (7–10) demonstrated an inverse association of fish consumption with colon cancer. Results from clinical studies (11–12) also demonstrated a decrease in the intestinal hyperproliferation in subjects at risk of colon cancer by consuming n-3 PUFAs derived from FO. In many animal tumorigenesis studies (13–18), it has been shown that diets containing FO or n-3 PUFAs have a protective effect on chemically induced colon carcinogenesis. N-3 PUFAs, as compared with n-6 PUFAs, also suppress tumor cell growth both in vitro and in vivo (19–25). The mechanism by which n-3 PUFAs suppress tumor cell growth is not understood.

Marine oils are rich in n-3 PUFAs, such as eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3). It is well documented that metabolic competition exists between n-3 and n-6 PUFAs (26–29). Increased intake of n-3 PUFAs leads to reduced levels of arachidonic acid (20:4n-6) in tissue lipids and, subsequently, to suppressed production of prostanooids derived from arachidonic acid. Numerous studies (30–34) have demonstrated that the levels of PGs in various tumors or the biosynthetic capacity of the tumor for PGs is greater when compared with normal tissues. COX, PG endoperoxide synthase, catalyzes the conversion of arachidonic acid to PG endoperoxides. This is the rate-limiting step in PG and thromboxane biosynthesis. Two isoforms of COX have been cloned from various animal cells: constitutively expressed COX-1 (35–39) and mitogen-inducible COX-2 (40–45). It has been shown that the inducible form of COX is overexpressed in colon and other tumor tissues (46–49). Many epidemiological studies (50–58) have demonstrated that aspirin and other NSAIDs can reduce the incidence of colon cancers. The well-documented pharmacological action of aspirin and other NSAIDs is inhibiting COX. Thus, it has been a prevailing hypothesis that the suppression of tumor cell growth by dietary n-3 PUFAs, as compared with n-6 PUFAs, is mediated through the inhibition of COX. However, experimental evidence to support this hypothesis has not been demonstrated.

To determine whether or not suppression of tumor cell growth by n-3 PUFAs is mediated through inhibition of COX, we selected a colon tumor cell line, HCT-116, which does not express COX, and transfected these cells with COX cDNAs to overexpress COX. If the suppression of tumor cell growth by n-3 PUFAs both in vitro and in vivo is primarily mediated through inhibition of COX, n-3 PUFAs should not affect the growth of cells that do not express COX. Results from these studies should provide a new insight into understanding the mechanism by which n-3 PUFAs mediate the suppressive action on tumor cell growth and other cellular effects that cannot be explained solely based on the inhibition of PG production.

MATERIALS AND METHODS

Cell Line

The human colon cancer cell line (HCT-116) was obtained from the American Type Culture Collection and cultured in McCoy’s 5A medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Intergen Co., Purchase, NY), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Preparation of COX-1/2-Flag Expression Constructs in Tetracycline-regulated Retroviral Vector

Flag (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) epitope tag sequence was added at COOH-terminal ends of human COX-1 and COX-2 cDNAs using overlapping extension PCR. For the COX-1-Flag construct, PCR was performed with the primer P1-F1 and P1-R1 first using human COX-1 cDNA as a template. Then, the PCR fragment was used as the template for the second PCR with P1-F2 and P1-R2. The final PCR product was subcloned into BamHI and NotI sites in pcDNA3.1(zeo−−) (Invitrogen, Carlsbad, CA). For the COX-2-Flag expression construct, the primers P2-F and P2-R1 were used for the first PCR. The PCR product was then used as the template for the second PCR with the

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2 To whom requests for reprints should be addressed, at Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70808. Phone: (225) 763-2518; Fax: (225) 763-3030; E-mail: hwangdh@pbrc.edu.

3 The abbreviations used are: PUFAs, polyunsaturated fatty acids; COX, cyclooxygenase; FO, fish oil; NSAIDs, nonsedient anti-inflammatory drugs; PG, prostaglandin; PPARα, peroxisome proliferator-activated receptors; SO, safflower oil.
primers P2-F and P2-R. The COX-2-Flag PCR fragment was inserted into BamHI site in pcDNA3.1/zeo(−). The Pme 1 restriction fragments of these constructs were subcloned into Hpa I site of the tetracycline-regulated retroviral vector, pLinx (59). The integrity of these expression constructs was verified by sequencing the complete coding regions. The primers used for PCR are as follows: P1-F1, 5′-AACCGGCGGATCCGCGGAATCTC-3′; P1-R1, 5′-TTATATCNGGATCCATCTATTGATCGG-3′; P1-F2, 5′-CTACTGATACCGCGGATCCATCTATTGATCGG-3′; P1-R2, 5′-CTACTGATACCGCGGATCCATCTATTGATCGG-3′. Preparation of Stable Clones (HCT-116) Overexpressing Enzymatically Active COX-1 or COX-2 in a Tetracycline-regulated Manner

Transfection of the amphotropic packaging cell line (d8N; Dr. Garry Nolan, Stanford University, California) was performed as described by Pear et al. (60). Briefly, the packaging cells (d8N cells) in a 60-mm dish were transfected with 5 µg of the retroviral plasmid DNA using SuperFect (Qiagen, Valencia, CA) reagent. Plasmid DNA was prepared using endofree plasmid Maxi kit (Qiagen). The medium containing the virus was removed 72 h after transfection and centrifuged at 1500 rpm for 5 min to pellet cell debris. Target cells (HCT-116) at a density of 5 × 10^6 cells/60-mm dish were infected in the presence of polybrene (5 µg/ml; Sigma Chemical Co.) with 4 ml of centrifuged medium containing the virus. Transfection and infection were carried out in the presence of tetracycline (8 µg/ml). Infected cells were selected in the presence of genetin (800 µg/ml). Individual positive clones were propagated, and the expression of enzymatically active COX-1 or COX-2 was determined. To detect COX protein derived from the plasmids, cell lysates were immunoprecipitated with polyclonal anti-Flag antibodies and immunoblotted with COX antibodies. COX enzyme activity was determined by RIA for PGE2 produced in these cells as described in our previous studies (61).

Preparation of Cell Lysates, Immunoprecipitation, Immunoblot, and Assay for COX

These were carried out as described in our previous studies (61, 62). Polyclonal anti-Flag antibodies were prepared in rabbits with the Flag peptide (Cys-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) conjugated to keyhole lymp hemocyanin at the Core Laboratory, Louisiana State University Health Sciences Center, New Orleans, LA.

Nude Mice Studies

Animals. Female athymic nude mice (NCI-nu/nu) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana) at 3 to 4 weeks of age and maintained in microisolator cages within a pathogen-free isolation facility. There were 51 mice in each of the two dietary groups. Mice were housed four per cage. All of the protocols were approved by the Institutional Animal Care and Use Committees of the Pennington Biomedical Research Center and the Louisiana State University Health Sciences Center.

Diets. The experimental diets were prepared by BioServ (Frenchtown, NJ) and were based on the purified American Institute of Nutrition (AIN-93G) rodent diet. Two experimental diets were used: the SO diet or the FO diet, supplemented with SO (20%; w/w) or a mixture of SO (2%; w/w) and FO (18%; w/w), respectively. The SO was provided by BioServ, whereas the menhaden oil was provided gratis by Omega Protein (Reedsdale, VA). The diets were isocaloric with 4.467 kcal/g for the AIN-93G-modified SO diet and 4.48 kcal/g for the AIN-93G-modified menhaden FO diet. The diets were sterilized by γ-irradiation (2–4 mRAD) and stored at −20°C in heat-sealed mylar bags that were filled with nitrogen and packaged approximately 200 g/bag. Feeding the experimental diets was commenced 14 days before injection of the tumor cells so that the desired lipid environment was present during the initial stage of tumor proliferation and angiogenesis. Fresh food was provided three times weekly.

Experimental Procedures. Mice were fed the experimental diets for 2 weeks before tumor cell implantation to allow for dietary fatty acids to be incorporated into tissue lipids. A randomization method was used to assign one of two diets and one of three cell types to each mouse. Cell suspensions in HBSS were drawn into a sterile 1-ml disposable syringe using an 18GA needle, and the syringe was depleted of air. Mice received injections with 2 × 10^6 tumor cells (200 µl of cell suspension) s.c. into the subscapular region using a 25GA needle. Selection of 2 × 10^6 cells for injection was based on pilot studies that showed 100% tumor development and a reasonable latency period for this cell line (data not shown) until completion of the study. The experiment was terminated 21 days after injection of tumor cells.

Fatty Acid Analyses. Extraction of tumor tissue lipids and analysis of fatty acid composition were carried out as described previously (63).

Statistical Analyses. A two-way repeated measure analysis with an unstructured covariance structure incorporated into the model was used as the primary analysis design to test the main effects of diet, cell line, and their interaction. Comparisons were adjusted using the Bonferroni method. The measures of tumor burden and fatty acid profiles incorporated a two-way ANOVA. For all of the statistical analyses of this study, SAS System 6.12 software was used.

Cell Viability Assay. HCT-116 cells (0.75 × 10^6 cells/well) were plated to a 96-well plate. The cells were serum-starved for 24 h by replacing the culture media with McCoy’s 5 A medium containing 0.25% fetal bovine serum. Then, the media were removed, and fresh media containing fatty acids were added. The fatty acids were combined with fatty acid-free albumin at a molar ratio of 10:1 (fatty acid:albumin) in serum-poor medium. After culturing for 48 h, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) solution (5 µg/ml) were added to each well and incubated for another 4 h. Insoluble formazan precipitates formed in media were solubilized with 100 µl of 10% SDS-0.1 N HCl solution. Absorbance at 595 nm was measured using a Bio-Rad plate reader.

Soft-agar Clonogenic Assay. HCT-116 cells overexpressing COX-2 or cells transfected with the vector without COX-2 cDNA were plated to a 100-mm plate. The cells were serum-starved for 24 h by replacing the culture media with McCoy’s 5 A media containing 0.25% fetal bovine serum. Then, the media were removed, and fresh media containing fatty acids were added. The fatty acids were combined with fatty acid-free albumin at a molar ratio of 10:1 (fatty acid:albumin) in serum-poor medium. After culturing for 48 h, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) solution (5 µg/ml) were added to each well and incubated for another 4 h. Insoluble formazan precipitates formed in media were solubilized with 100 µl of 10% SDS-0.1 N HCl solution. Absorbance at 595 nm was measured using a Bio-Rad plate reader.

RESULTS

Expression of Enzymatically Active COX Proteins in HCT-116 Cells Transfected with COX cDNAs. HCT-116 cells were infected with retroviral particles harboring COX-1 or COX-2 cDNA. We selected the clones expressing enzymatically active COX-1 (Fig. 1, A and B) or COX-2 protein in a tetracycline regulated manner (Fig. 1, C and D). Overexpression of COX can lead to significantly increased concentrations of PGs in the culture media. Cells may undergo adaptive change under high concentrations of PGs in the media. Thus, cells were cultured in the presence of tetracycline to suppress the expression of COX during the subcultivation, but tetracycline was removed from the culture media before in vitro and in vivo studies.

Growth Suppression of Colon Tumor Cells (HCT-116) in Athymic Nude Mice by FO Diet as Compared with SO Diet. Of the mice that received injections s.c. with 2 × 10^6 tumor cells, 100% developed detectable tumors, and there were no differences among the dietary groups when the percentage of tumor incidence was compared. Analysis of fatty acids indicated significant incorporation of n-3 PUFAs from diets into tumor tissue lipids (Table 1). All of the tumors derived from HCT-116 cells transfected with Flag-epitope-tagged COX-2 or COX-2 cDNA showed expression of respective COX protein (Fig. 2). The growth of tumor cells xenografted into athymic nude mice was suppressed by the FO diet as compared with SO diet regardless of whether COX is expressed or not in the cells (Fig. 3, A–C). Tumor growth was suppressed by the FO diet even in the animals xenografted with the cells that do not express COX (Fig. 3A). This suppression was not because of the difference in body weight,
because the pattern of suppression by the FO diet was the same as the tumor volume if the data were expressed in terms of tumor burden (tumor weight/final body weight; Fig. 3D). The body weight gains among the various dietary treatment groups were not significantly different. Additionally, body weight gain was not affected by the cell line injected into the mice or by the interaction of diet and cell line.

Overexpression of COX-1 or COX-2 in HCT-116 Cells Does Not Affect Tumor Growth in Nude Mice. The growth of the tumor cells overexpressing COX-1 or COX-2 was not different as compared with the control cells transfected with the control vector (Fig. 3, A–C).

The growth of the tumor cells overexpressing COX-2 in the mice fed the FO diet (○ in Fig. 3C) or the cells overexpressing COX-1 in the mice fed the SO diet (● in Fig. 3B) appeared to be slower as compared with the respective control cells transfected with the vector (Fig. 3A). However, this difference was not statistically significant.

Inhibition of Cell Proliferation by n-3 PUFAs as Compared with Linoleic Acid. Cell proliferation was inhibited by n-3 PUFAs, whereas it was not inhibited by linoleic acid (Fig. 4). This inhibition was more pronounced by docosahexaenoic acid as compared with eicosapentaenoic acid. In addition, the inhibition of cell proliferation by docosahexaenoic acid was more pronounced in the cells transfected with the vector as compared with the cells overexpressing COX-1 or COX-2 (Fig. 4). These in vitro results corroborate the in vivo results shown in the nude mice studies (Fig. 3, A–D).

Overexpression of COX-2 in HCT-116 Cells Does Not Affect Anchorage-independent Cell Growth. We next evaluated whether the inhibition of COX-2 expression had an effect on the in vitro transformed phenotype using soft-agar colony-forming assay. As shown in Fig. 5, colony sizes of COX-2-transfected cells grown in the absence of tetracycline were similar to those grown in the presence of tetracycline. In addition, control vector-transfected cells also formed similar size colonies in soft agarose.

DISCUSSION

The inhibitory effects of dietary n-3 PUFAs on the growth of tumor cells, both in culture and as solid tumors in nude mice, were demonstrated in many tumor cell lines and transformed cells (14–22). It was suggested that these inhibitory effects are attributed to the inhibition of prostanoid synthesis from arachidonic acid. However, experimental evidence supporting the proposition has not been demonstrated.

In our studies, the diet containing n-3 PUFAs suppressed the growth of the colon tumor cell line (HCT-116), which does not express COX, both in culture and in nude mice as solid tumors (Fig. 3 and Fig. 4). Overexpression of COX in this cell line did not affect the cell growth in culture or in nude mice. In addition, overexpression of COX-2 in this cell line did not affect anchorage-independent growth in soft agar (Fig. 5). Together, these results suggest that the inhibitory effects of n-3 PUFAs on tumor cell growth are not mediated through inhibition of COX activity.

Sheng et al. (64) showed that administration of a COX-2 specific inhibitor to the nude mice resulted in growth suppression of the tumors derived from HCA-7 cells that express endogenous COX but not the tumors derived from HCT-116 cells that do not express COX. These results were interpreted as evidence that COX-2 expression is linked to tumor cell growth. However, the two colon cancer cell lines may differ from each other not only in regards to the status of endogenous COX expression but also in mutations of different genes.
Thus, the difference in the rate of tumor growth in response to the COX-2 inhibitor in nude mice for these cell lines may not be entirely because of the status of COX expression. If the expression of COX is linked to tumor growth, overexpression of COX in HCT-116 should enhance growth of tumor cells. However, our results showed that the growth of HCT-116 cells both in culture or in nude mice was not affected by overexpression of COX-1 or COX-2. Anchorage-independent growth of HCT-116 cells in soft agar was also not affected by overexpression of COX-2. These results suggest that COX expression is not linked to tumor cell growth in this cell line.

It was demonstrated that crossbreeding of APC\(^{Δ716}\) knockout mice with COX-2 knockout mice or administration of COX-2 inhibitor to APC\(^{Δ716}\) knockouts resulted in a reduction of the number and size of the intestinal polyps (66). In addition, overexpression of COX-2 was shown to enhance tumorigenic phenotypes, metastatic potential, and angiogenesis (67–69). On the other hand, it was shown that overexpression of COX-2 induces cell cycle arrest in many cell types (70) and that the anti-inflammatory drug sulindac causes rapid regression of intestinal tumors in Min\(^+/−\) mice independent of PG biosynthesis (71).

Fig. 3. Growth suppression of colon tumor cells (HCT-116) in athymic nude mice by FO diet as compared SO diet. The nude mice received injections with the control cells that do not express COX (A), cells expressing COX-1 (B), or cells expressing COX-2 (C). Tumor volumes were determined as described in “Materials and Methods.” Values represent tumor volumes (mean ± SE; n = 15). D, tumor burden was expressed by tumor weight/final body weight. *, significantly reduced as compared with the group fed SO diet (P < 0.05).

Fig. 4. Suppression of cell proliferation by n-3 PUFAs in HCT-116 cells that express no COX (A; transfected with the empty pLinx vector), COX-1 (B), or COX-2 (C). Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in “Materials and Methods.” LA, linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values are mean ± SE (n = 6).

Fig. 5. Effects of COX-2 overexpression on anchorage-independent growth of HCT-116 cells. Colony formation in soft agarose was determined in the presence or absence of tetracycline (tet) as described in “Materials and Methods.”

HCT116/pLinx HCT116/Cox2

(+ tet) (- tet)
evidence for involvement of COX in tumorigenesis in colon cancer. However, recent evidence indicates that NSAIDs have diverse biological actions in addition to their inhibitory effect on COX enzyme activity.

Many NSAIDs bind and activate PPARs, which regulate the expression of a broad array of gene products (72, 73). Some NSAIDs and other PPAR activators inhibit cell proliferation and induce apoptosis in many cell lines, including HCT-116 (74–79). Results from our previous study (80) indicated that some NSAIDs suppress the expression of mitogen-inducible COX-2 and other inflammatory marker genes. Therefore, it is likely that the beneficial effect of NSAIDs in reducing the risk of colon cancer is mediated through not only inhibition of COX enzyme activity but also COX-independent pathways.

It has been well documented that fatty acids and their metabolites also bind and activate PPARs (81–86). Xu et al. (81) reported the crystal structure demonstrating that eicosapentaenoic acid binds to the ligand-binding domain of the PPARs. Many ligands for PPARs, including NSAIDs, are known to inhibit tumor cell growth both in vitro and in vivo (74–79). Therefore, whether suppression of tumor cell growth by n-3 PUFAs is mediated through differential activation of PPARs would be an appealing hypothesis to be tested.

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