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Prevention and Treatment of Pancreatic Cancer by Curcumin in Combination With Omega-3 Fatty Acids

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Pancreatic cancer BxPC-3 cells were exposed to curcumin, docosahexaenoic acid (DHA), or combinations of both and analyzed for proliferation and apoptosis. Pancreatic tumor xenografts were established by injecting BxPC-3 cells into each flank of nude mice. After the tumors reached a size of approximately 190–200 mm^3, animals were fed diets with or without 2,000 ppm curcumin in 18% corn oil or 15% fish oil + 3% corn oil for 6 more wk before assessing the tumor volume and expression of inducible nitric oxide synthase (iNOS), cyclooxygeanse-2 (COX-2), 5-lipoxinase (5-LOX), and p21. A synergistic effect was observed on induction of apoptosis (approximately sixfold) and inhibition of cell proliferation (approximately 70%) when cells were treated with curcumin (5 μM) together with the DHA (25 μM). Mice fed fish oil and curcumin showed a significantly reduced tumor volume, 25% ($P < 0.04$) and 43% ($P < 0.005$), respectively, and importantly, a combination of curcumin and fish oil diet showed >72% ($P < 0.0001$) tumor volume reduction. Expression and activity of iNOS, COX-2, and 5-LOX are downregulated, and p21 is upregulated in tumor xenograft fed curcumin combined with fish oil diet when compared to individual diets. The preceding results evidence for the first time that curcumin combined with omega-3 fatty acids provide synergistic pancreatic tumor inhibitory properties.

INTRODUCTION

Pancreatic adenocarcinoma is the fourth most common cause of cancer-related deaths in men and women in the United States (1). Despite significant progress made in treatment of several epithelial tumors, pancreatic cancer still remains a universally fatal disease, with mortality rates approaching the number of newly diagnosed cases. An estimated 40,000 pancreatic cancer cases will be diagnosed in the United States in 2008, and the majority of these patients will die within 6 mo (1). Gemcitabine has been considered the standard chemotherapeutic agent in the treatment of pancreatic cancer. Efforts to improve the efficacy of gemcitabine, either as monotherapy or as combination therapy with cytotoxic or molecular targeted agents, revealed only a marginal benefit of 1 to 2 mo at best overall survival (2). Novel, biologically efficacious agents for the prevention and treatment of pancreatic cancer are needed urgently.

Pancreatic ductal cancer results from a multistage carcinogenesis process that involves distinguishable but closely connected stages: normal cell → intraepithelial neoplasia-1, -2 and -3 → pancreatic ductal adenocarcinoma. Deregulated signal transduction pathways associated with inflammation act as key regulators in promotion of pancreatic carcinogenesis (3, 4). Substantial evidence for the role of inflammation in pancreatic cancer can be understood by the frequent upregulation of inflammation mediators such as nuclear factor kappa B (NF-κB), inducible nitric oxide synthase (iNOS), cyclooxygeanse-2 (COX-2), and 5-lipoxinase (5-LOX). Studies have shown that overexpression of iNOS, COX-2, and 5-LOX is associated with poor prognosis and reduced survival of pancreatic cancer patients (5–9). Thus, inhibition of tumor promoting inflammatory signal pathways are excellent targets for pancreatic cancer prevention and therapy.

Finding safe and efficacious anti-inflammatory agents is a challenge and most of the pharmaceutically designed steroidal and nonsteroidal anti-inflammatory drugs are associated with a constellation of side effects. Perhaps the best example is the cardiovascular system-related side effects recently identified with most coxibs. Thus, there is a great need for safer and efficacious anti-inflammatory agents. Numerous lines of evidence suggest that curcumin is a potent anti-inflammatory agent. Curcumin suppresses the activation of the transcription factor NF–κB, which regulates the expression of proinflammatory gene products such as expression of COX-2, an enzyme linked with most types of inflammations (10–13). Also, curcumin inhibits the expression of 5-LOX, another proinflammatory enzyme (13–15). Curcumin has been shown to bind to the active site of 5-LOX and COX-2 and inhibits the activities (15). In addition, curcumin has been shown to downregulate the expression of various cell surface adhesion molecules and expression of various inflammatory cytokines including tumor necrosis factor, interleukin

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(IL)-1, IL-6, IL-8, and chemokines (13, 14). Previously, we and others have shown that curcumin exhibits antitumorigenic effects in preclinical models of various organ sites (16–19). Recent clinical trials further support the potential usefulness of curcumin for prevention and treatment of various cancers (20).

Epidemiologic and biochemical evidence shows that n-6 polyunsaturated fatty acids (PUFAs) promote the pathogenesis of many diseases, including cancer, whereas n-3 PUFAs exert suppressive effects (21, 22). It has been estimated that the present Western diet is deficient in n-3 PUFAs with a ratio of approximately 15:1 n-6 to n-3 PUFAs and is a risk factor for many cancers (22). n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are generally found in fish oil. Arachidonic acid and EPA, which are incorporated into the *sn*2 position of major membrane glycerophospholipids, are immediate precursors for the synthesis of type-2-series and 3-series eicosanoids, respectively. The health benefits of n-3 PUFAs are thought to stem mainly from an increased production of anti-inflammatory type 3-series eicosanoids [e.g., prostaglandin E_3(PGE_3)] with suppressed generation of proinflammatory type 2-series eicosanoids (e.g., PGE_2) (23, 24). Preclinical studies using genetically modified animals and xenograft mouse models convincingly demonstrate that dietary intake of n-3 PUFAs (e.g., in the form of diets with a low n-6/ n-3 PUFA ratio) reduces the incidence and growth of various cancers (25–29). In chemically induced pancreatic carcinogenesis models, fish oils were capable of reducing the incidence of pancreatic cancers and hepatic metastases (30, 31). In addition, in vitro studies have demonstrated that n-3 PUFAs inhibit pancreatic cancer cell growth by induction of apoptosis (32,33). There is compelling evidence to support that combinational regimens, based on the rational mechanisms, should provide synergistic and/or additive tumor inhibitory effects (28, 34). Previously, we have shown that a low-dose combination of celecoxib and omega-3 fatty acids results in synergistic efficacy in the models of in vitro and in vivo (28). Although there is evidence to support the beneficial effects of curcumin or omega-3 fatty acids in suppression of pancreatic cancer cells, a comprehensive, comparative analysis of the effects of curcumin and n-3 PUFAs and, more important, their combined effects on pancreatic cancer growth at both in vitro as well as at in vivo have not been explored.

Our data provides evidence that a combination of curcumin and n-3 PUFA/DHA synergistically enhances the inhibitory properties of pancreatic tumor growth in in vitro and in vivo models.

MATERIALS AND METHODS

Materials

DHA, acridine orange, and protease inhibitor were purchased from Sigma (St. Louis, MO); ethidium bromide was purchased from Invitrogen (Carlsbad, CA); curcumin was procured from the National Cancer Institute Chemopreventive Drug Repository (Bethesda, MD). COX-2, 5-LOX, and iNOS antibodies and 15-(*R*)-hydroxy eicosatetraenoic acid (HETE) and 5-(*S*)-HETE were purchased from Cayman Chemicals (Ann Arbor, MI). α-Tubulin and antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Pancreatic Cancer Cell Lines

Human pancreatic cancer BxPC-3 (well to poorly differentiated, COX-2/5-LOX positive, and PANC-1 (poorly differentiated, COX-2/5-LOX negative) cell lines were kindly provided by Dr Rajesh Agarwal (University of Colorado Health Sciences Center, Denver, CO) and cultured as previously described (35). Before the experiments, subconfluent cells were cultured overnight in serum-deprived (0.5%) medium. Culture medium was then replaced with serum-free medium together with the various subtoxic concentrations of curcumin or DHA and/or combinations of both. DHA was precomplexed with bovine serum albumin (fatty acid free, 1 mg/ml, Sigma Chemical) for 30 min at 37°C before adding to the cells.

Cytotoxicity and Proliferation

Cell toxicity and viability was determined by using the 3-(4, 5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide assay and cell counting as described previously (36). Briefly, pancreatic cancer cells were incubated with the indicated reagents for 24 h in serum-free medium. Cell proliferation was measured by Hexosaminadase assay using a chromogenic substrate (p-nitropheny-N-acetyl-B-D-Glucosaminide; Sigma-Aldrich). The lysozyme enzyme (N-acetyl-B-D-hexosaminidase) released from the proliferating cells convert the substrate to p-nitrophenyl, which was measured at 405 nm in a microtitre plate reader, and cell viability was further confirmed by trypan blue staining method.

Apoptosis Assays

Human pancreatic cancer BxPC-3 and PANC-1 cells cultured for 24 h in the presence of various concentrations of DHA, curcumin, and/or combinations of both and washed with phosphate-buffered solution (PBS) and trypsinized. Of the cell suspension, 25 μl (5×10^6 per ml) were incubated with 1 μl of acridine orange/ethidium bromide (1 part each of 100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS) just before microscopy. A 10-μl aliquot of the gently mixed suspension was placed on microscope slides, covered with glass slips, and examined under an Olympus AX71 microscope (Tokyo, Japan) connected to a digital imaging system with SPOT RT software version 3.0. Acridine orange is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity.

Animals, Diets, and Curcumin

Athymic nude mice were obtained at 7 wk of age from the National Cancer Institute (Frederick, MD, Jackson Laboratories).

TABLE 1
Composition of experimental diets

	Percentage Composition (Modified AIN-76A Diet)[a]			
Ingredient	Control Diet	Fish Oil Diet	Curcumin Diet	Fish Oil + Curcumin Diet
Casein	23.5	23.5	23.5	23.5
Alphacel	5.9	5.9	5.9	5.9
Dextrose	9.02	9.02	9.02	9.02
DL-Methionine	0.35	0.35	0.35	0.35
Choline bitartrate	0.24	0.24	0.24	0.24
Corn starch	37.7	37.7	37.5	37.5
Corn oil	18.0	3.0	18.0	3.0
Fish oil	0	15.0	0	15.0
Mineral mix, AIN-76A	4.11	4.11	4.11	4.11
Vitamin mix, AIN revised	1.18	1.18	1.18	1.18
Curcumin	0	0	0.2	0.2

[a]Diet was formulated based on the American Institute of Nutrition Standard reference diet, with the modification of varying sources of carbohydrate (38).

Sterile (gamma-irradiated) ingredients for the semipurified diets were purchased from Bioserv, Inc. (Frenchtown, NJ) and stored at 4°C prior to diet preparation. Diets were based on the modified AIN-76A diet. Composition of experimental diets is shown in Table 1. Curcumin was premixed with a small quantity of casein and then blended into bulk diet using a Hobart Mixer (Troy, OH). Both control and experimental diets were prepared weekly and stored in a cold room. Menhaden fish oil (premium grade) was kindly provided by Omega Protein, Inc. (Houston, TX). Curcumin and fatty acid content in the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution.

Tumor Xenograft Assay

At 7 wk of age, mice were maintained on control diet, and pancreatic cancer BxPC-3 cells (2×10^6), suspended in 50 μl of culture medium, were injected subcutaneously into the each flank of nude mice. After the tumors reached a volume of approximately 200 mm^3, animals ($n = 8$ each) were randomly allocated to 4 experimental diets for 6 wk. Mice were individually tagged and had free access to diet and water. Food intake was monitored regularly, and body weight was determined twice weekly. Tumor volumes were measured every week until termination. After 6 wk on the experimental diets, all animals were killed, tumors were harvested, and the tumor volume was assessed using the formula $2/3\ \pi r^3$.

Western Blot Analysis of COX-2, 5-LOX, iNOS, and $p21^{WAF1/CIP}$

Tumor xenografts isolated from different experimental groups were homogenized in 1:3 vol of 100 mM Tris–hydrochloride (HCl) buffer (pH 7.2) with 2 mM calcium chloride ($CaCl_2$). After centrifugation at 100,000 *g* for 1 h at 4°C, the resulting separations were subjected to 8% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were electroplated onto polyvinylidine fluoride nitrocellulose membranes as described previously (26). These membranes were blocked for 1 h at room temperature with 5% skim milk powder and probed with primary antibodies for 1 h. The primary antibodies, COX-2, 5-LOX, and iNOS (Cayman Chemicals, Ann Arbor, MI) and p21 and caspase-3 (Santa Cruz Biotech., Santa Cruz, CA) were used at 1:500 dilutions. Blots were washed 3 times and incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotech) at 1:2500 dilutions for 1 h. The membranes were washed 3 times and incubated with Super-Signal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) for 5 min, exposed to Kodak XAR5 photographic film, and developed to detect proteins. Intensities of each band were scanned by a computing densitometer. α-Tubulin (Ab-1) mouse monoclonal antibody (Oncogene, San Diego, CA) was used at 1:1000 dilution as the internal standard for all Western blots.

5-LOX and COX-2 Synthetic Activity

5-LOX and COX-2 activities in tumor xenografts (6/group) were assayed using our previously published method (37, 38). In brief, the 5-LOX activity was assayed by measurement of ^{14}C-labeled 5(*S*)-HETE that will be formed from the [^{14}C]-AA. The reaction mixture (200 μl) containing 100 mM Tris-HCl (pH 7.2) and 2 mM $CaCl_2$ [^{14}C]AA (6 nmol, 480,000 dpm) and cytosol fraction (100 μg protein) will be incubated for 15 min at 37°C. To determine COX-2 activity, the reaction mixture was preincubated with 150 μM of ASA to block COX-1 activity and to modify COX-2 activity to 15-(*R*)-HETE. Each reaction mixture containing 150 μl [12 μM [^{14}C] AA (420,000 dpm), 1 mM

epinephrine, 1 mM glutathione in 50 mM phosphate buffer, and 50 μg of tumor microsomal protein] were incubated at 37°C for 15 min. After incubation, the reactions were terminated by adding 40 μl of 0.2 M HCl. The 5-LOX and COX-2 metabolites 5-(*S*)-HETE and 15-(*R*)-HETE were extracted 3 times with 0.5 ml of ethyl acetate. The combined extracts were evaporated to dryness under N_2, redissolved in chloroform, and subjected to TLC on Silica G plates. The TLC plates were developed in a solvent system containing a mixture of chloroform:methanol:acetic acid:water (100:15:1; 25:1, vol/vol/vol/vol) and were exposed in an iodide chamber for 5 min to visualize the standards. The metabolites of [^{14}C]-AA corresponding to [^{14}C]-5-(*S*)-HETE and [^{14}C]-15 (*R*)-HETE were detected by their comigration with authentic standards and radioactivity was counted with a BioScan Radiomatic detector. Results were expressed as *pmol* [^{14}C]-5-(*S*)-HETE formed/mg protein/min for 5-LOX activity and [^{14}C]-15 (*R*)-HETE for COX-2 activity.

iNOS Activity

To quantify iNOS (calcium-independent) activity, conversion of L-arginine to L-citrulline was measured as described previously (37). Briefly, the assay was carried out by adding 100 μg of sample protein to 150 μl of assay buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM dithiothreitol, 1 mM magnesium chloride, 5 mg/l pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 3 mg/l aprotinin, pH 7.4) containing 70 μM arginine, 250,000 dpm L-[^{3}H]-arginine, 2 mM NADPH, 5 μM tetrahydrobiopterin, 5 μM flavin adenine dinucleotide, 1 mM ethylene glycol-bis-aminoethylether-N,N,N′,N′-tetraacetic acid (without calcium) to determine Ca^{+2}-independent iNOS activity. After 30 min at 37°C, the reaction was stopped with 100 μl of 1 M trichloroacetic acid. The samples were adjusted to pH 4.6 by adding 500 μl of 20 mM HEPES and applied to Dowex AG 50W-X8 resin columns. L-[^{3}H]-citrulline was eluted and separated using TLC. Radioactivity was counted with a BioScan Radiomatic detector. Results were expressed as pmol L-[^{3}H]-citrulline/mg protein /min.

Statistical Analyses

All results are expressed as means ± standard error of the mean and were analyzed by Student's *t*-test. Differences were considered significant at the $P < 0.05$ level. All the statistics were done in GraphPad Prism 4.0 Software.

RESULTS

Cell Growth In Vitro

To evaluate the effects of DHA and curcumin (individually and in combination) on pancreatic cancer cell growth in vitro, 2 human pancreatic cancer cell lines (BxPC-3 and PANC-1) were exposed to incremental concentrations of DHA (1-100 μM) and curcumin (1–20 μM), and cell growth was determined after 24 h.

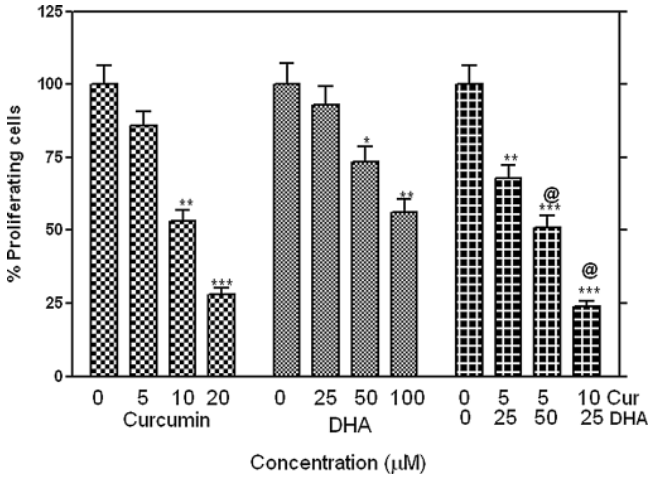

FIG. 1. Effect of curcumin and docosahexaenoic acid (DHA) treatment individually and in combination on the proliferation of BxPC-3 cells. Cells exposed to subtoxic concentrations of curcumin (0–20 μM), DHA (0–100 μM), or combinations of both for 24 h. Columns are mean of triplicate samples; bars are SE. $^*P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$, statistically significant from control group by Student's *t*-test. @, $P < 0.05$–0.001, statistically significant compared to same concentrations of individual treatments.

The results suggest that pretreatment with DHA and curcumin inhibits BxPC-3 cell growth at increasing concentrations in a dose-dependent manner. However, synergistic inhibitory effects on BxPC-3 cell growth was observed when cells were exposed to only doses of 5 μM of curcumin together with 25 μM of DHA (Fig. 1). Similar observations were made with the PANC-1 cell growth with treatment of DHA and curcumin, albeit to a much lesser extent (data not shown).

Induction of Apoptosis

Figure 2 summarize the effects of DHA and curcumin and/or combinations of these agents on BxPC-3 pancreatic cancer cell apoptosis. Pretreatment of BxPC-3 cells with DHA and curcumin induced apoptosis in a dose-dependent manner. However, stimulation of apoptosis occurred significantly at higher concentrations of DHA ($\leq$100 μM) and curcumin ($\geq$10 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 DHA (25 μM) along with curcumin (5 μM), a synergistic enhancement of apoptosis was observed in the BxPC-3 cells (Fig. 2). Combination of 25 μM DHA plus 10 μM curcumin induced fourfold to eightfold apoptosis in BxPC-3 cells when compared to individual treatments (Fig. 2).

Fish Oil-Enriched Diet With Curcumin Attenuates Pancreatic Cancer Growth In Vivo

Having demonstrated that the combination of DHA with curcumin suppresses pancreatic cancer cell growth and stimulates apoptosis in vitro, we sought to determine the possible synergistic efficacy of curcumin with n-3 PUFAs-enriched diet

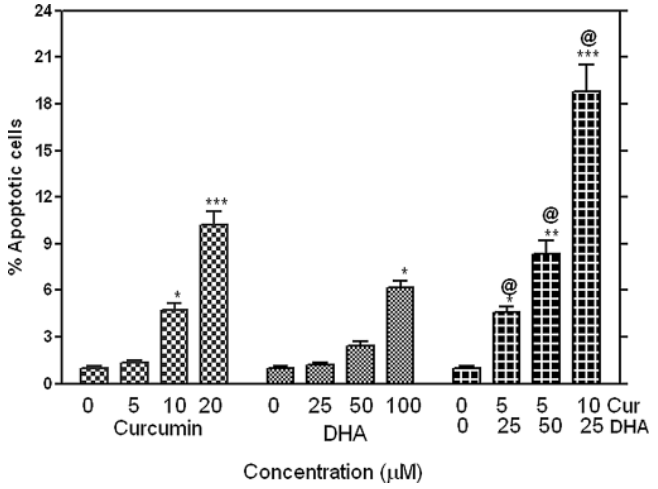

FIG. 2. Effect of curcumin and docosahexaenoic acid (DHA) treatment individually and in combination on the induction of apoptosis in BxPC-3 cells. Cells exposed to subtoxic concentrations of curcumin (0–20 μM), DHA (0–100 μM), or combinations of both for 24 h. Columns are mean of triplicate samples; bars are SE. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$, statistically significant from control group by t-test. @, $P < 0.01$–0.001, statistically significant compared to same concentrations of individual treatments.

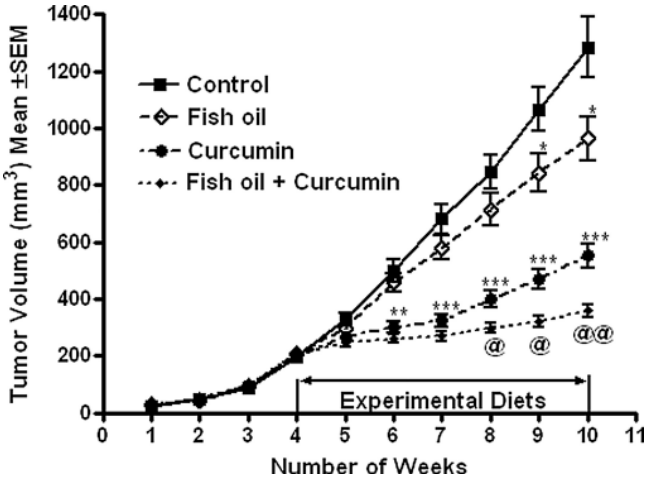

FIG. 3. Effect of corn oil (n-6 fatty acids) and fish oil (n-3 fatty acids) rich diets with or with out 2,000 ppm curcumin on pancreatic tumor latency in nude mice. Each dot represents weekly tumor volumes (mm^3) ± SE; *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$, statistically significant from control group by t-test.

on pancreatic cancer growth in a COX-2-positive BxPC-3 xenograft animal model in vivo. Figure 3 summarizes the effect of fish oil and curcumin individually and in combination on pancreatic cancer tumor latency. There were no significant differences in food intake (corn oil: 4.52 ± 0.24 g/mouse/day; fish oil: 4.60 ± 0.30 g/mouse/day; curcumin: 4.58 ± 0.26 g/mouse/day; fish oil + curcumin: 4.49 ± 0.27 g/mouse/day), and animal weight gain between both experimental groups was not statistically significant. Our results suggest that animals fed a n-PUFAs-rich diet showed retardation of tumor growth and a decrease in tumor latency when compared to corn oil diet; however, tumor growth was significantly ($P < 0.05$) reduced only after 5 wk of exposure to the n-3 PUFAs rich diet. Mice administered with curcumin in a n-6 PUFAs-rich diet showed significant ($P < 0.001$) delay in tumor latency within the 2 wk after exposure to the curcumin when compared to control diet. Importantly, mice fed with curcumin in a n-3 PUFAs-rich diet showed further delay in the tumor latency compared to either n-3 PUFAs rich diet or curcumin alone. Figure 4 summarizes the final tumor volumes of mice exposed to different experimental diets. As shown in Fig. 4, based on the final mean tumor volume, fish oil and curcumin diets showed 25% ($P < 0.01$) and 43% ($P < 0.0001$), respectively, suppression of pancreatic tumor xenografts compared to control diet. Importantly, mice fed with a combination of n-3 PUFAs with curcumin decreased the formation tumor xenograft by approximately 72% compared to tumors of the mice fed the control diet. Tumor data was further analyzed for possible synergistic effects in mice fed a curcumin and fish oil-rich diet; we observed that a combinational approach resulted in statistically significant suppression of pancreatic tumor xenograft growth ($P < 0.0004$–0.00001) compared to curcumin or fish oil diets alone.

Modulation of COX-2, 5-LOX, iNOS, and p21$^{waf1/cip}$ Expression

The effects of fish oil and curcumin (given individually and in combination) on COX-2, 5-LOX, iNOS, and p21$^{waf1/cip}$ expression levels in tumor xenografts are shown in Fig. 5. Tumors harvested from mice taking the fish oil diet showed a marginal reduction of COX-2, 5-LOX, and iNOS expression, but it was not statistically significant (P>0.05) compared to mice fed corn oil diet. Tumors from mice taking the curcumin diet showed

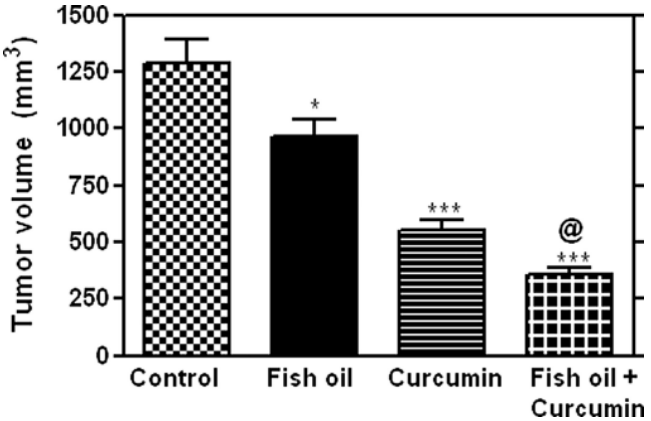

FIG. 4. Effect of corn oil (n-6 fatty acids), fish oil (n-3 fatty acids) rich diets with or with out 2,000 ppm curcumin on pancreatic tumor volume at the termination of the experiment. Tumor volume (mm^3) ± SE; *, $P < 0.05$; ***, $P < 0.0001$, statistically significant from control group by t-test; @, $P < 0.001$–0.0001, statistically significant when compared to either fish oil or curcumin diet groups.

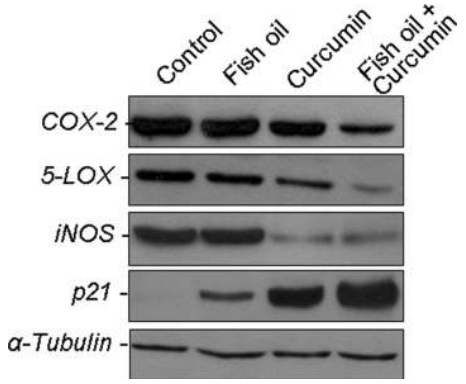

FIG. 5. Expression levels of cyclooxygeanse-2 (COX-2), 5-lipoxinase (5-LOX), nitric oxide synthase (iNOS) and $p21^{waf1/cip}$protein from BxPC-3 tumor xenografts of mice fed corn oil (n-6 fatty acids) and fish oil (n-3 fatty acids) rich diets with or with out 2,000 ppm curcumin. Tissue lysates were homogenized in lysis buffer and were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blotting as described in **Materials and Methods.** Membranes were probed for COX-2, 5-LOX, iNOS, and $p21^{waf1/cip}$specific primary antibodies and then peroxidase-conjugated appropriate secondary antibodies. Proteins were visualized with enhanced chemiluminescence detection system.

significantly reduced expression of COX-2 ($P < 0.02$), 5-LOX ($P < 0.005$), and iNOS ($P < 0.0001$) in comparison to tumor xenografts from control diet fed mice. Mice fed fish oil and curcumin together showed enhanced suppression of COX-2 and 5-LOX expression in tumors compared to the tumors of mice fed either fish oil diet or curcumin diet alone. Although the fish oil diet containing curcumin strongly suppressed iNOS expression in tumors compared to corn oil or fish oil diets alone, it was not statistically significant when compared to curcumin diet alone. We assessed the expression of $p21^{waf1/cip}$ in tumor xenografts as key marker of cell cycle progression. As shown in Fig. 5, the tumor xenografts of mice given the diet rich in corn oil showed no detectable expression of $p21^{waf1/cip}$, whereas mice fed fish oil or curcumin or a combination of both showed an increase of $p21^{waf1/cip}$ (increase as fish oil diet < curcumin diet < fish oil plus curcumin diet).

Modulation of COX-2, 5-LOX, and iNOS Activities

Because n-3 PUFAs and curcumin also affect the catalytic activity of COX-2, 5-LOX, and iNOS, we measured activities of these enzymes in tumor xenografts of mice fed various experimental diets. Results are summarized in Figs. 6A–6C. Tumors harvested from mice fed the fish oil diet showed a significant inhibitory effect on COX-2 ($P < 0.05$), 5-LOX ($P < 0.001$), and iNOS ($P < 0.05$) activities. Similarly, tumor xenografts from mice fed the curcumin diet also showed significantly reduced activity of COX-2 ($P < 0.001$), 5-LOX ($P < 0.0001$), and iNOS ($P < 0.0001$) in comparison to control-diet-fed mice tumor xenografts. Importantly, tumors of mice fed curcumin with fish oil together showed a greater inhibition of COX-2, 5-LOX, and iNOS activities ($P < 0.01$–0.001) when compared to the tumors of mice fed either fish oil diet or curcumin diet alone.

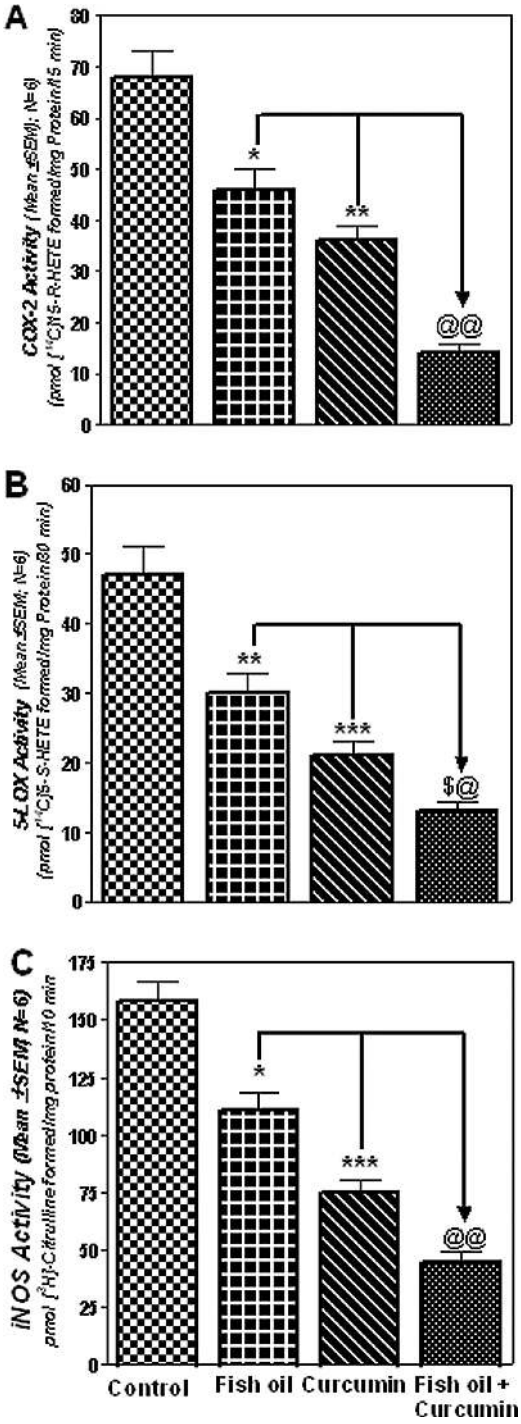

FIG. 6. of corn oil (n-6 fatty acids) and fish oil (n-3 fatty acids) rich diets with or with out 2,000 ppm curcumin on BxPC-2 tumor cyclooxygeanse-2 (COX-2), 5-lipoxinase (5-LOX), and nitric oxide synthase (iNOS) activity. A: COX-2 activity, as measured by AA-metabolism leading to 15-[*R*-]-hydroxy eicosatetraenoic acid in the presence of aspirin. B: 5-LOX activity, as measured by AA-metabolism leading to 15-[*R*-]- hydroxy eicosatetraenoic acid (HETE). C: iNOS activity, as measured by arginine conversion to citrulline, as described in **Materials and Methods** Values are mean ± SE; *, $P < 0.05$; **, $P < 0.001$, ***, $P < 0.0001$, statistically significant from control group by *t*-test; @@, $P < 0.01$–0.0001, statistically significant when compared to either fish oil or curcumin diet groups.

DISCUSSION

The specific objective of this study was to develop novel strategies for the prevention and treatment of pancreatic cancer in both preclinical and clinical settings. A key factor in testing the effectiveness of n-3 fatty acids and curcumin is to increase their efficacy while minimizing the side effects, if any, associated with long-term administration of curcumin. Here, we examined the effects of omega-3 fatty acids/DHA and curcumin in pancreatic cancer models of in vitro and in vivo. Taken together, our results clearly suggest that combining n-3 fatty acids with curcumin provides synergistic pancreatic cancer inhibitory properties. Previous studies have also suggested the potential usefulness of omega-3 fatty acids and curcumin for pancreatic cancer prevention and treatment (30–33,39–41). Although the combinational approaches to improve the preventive and therapeutic efficacy for cancer treatment is not new, selection of combinational strategy in this study is based on the clinical relevance defined by overexpression of COX-2, 5-LOX, and iNOS in pancreatic cancer patients associated with decreased survival rates (7–9).

Experimental and epidemiological evidence support that fish oils rich in n-3 fatty acids are thought to suppress the pathogenesis of several human diseases including cancer (21,22,42). However, systematic reviews of epidemiologic and cohort studies on fish-oil-associated cancer risk have provided controversial results (43,44). In contrast, highly controlled preclinical studies have convincingly demonstrated preventive and therapeutic efficacies of n-3 fatty acids on cancer development and growth (23,45). In patients with advanced pancreatic cancers, fish oil supplements rich in n-3 fatty acids are known to counteract the metabolic catabolism seen in these patients (46,47). Our in vitro studies suggest that DHA suppresses pancreatic cancer cell growth and stimulates apoptosis. This is in agreement with published reports that have used colon, breast, prostate, and pancreatic cells (36,48–50). Further, our in vitro data was corroborated by a xenograft mouse model that demonstrated antitumor efficacy of a diet enriched in n-3 fatty acids. Recently, Funahashi et al. (51) showed opposing effects of diets rich in omega-6 and n-3 fatty acids on pancreatic tumor xenografts in nude mice. Fish oil thereby served as the source of n-3 fatty acids, mostly EPA and DHA. The findings are in accordance to other mouse models that have illustrated therapeutic efficacy of omega-3 fatty acid-enriched diets (25–28). Thus, this observation on the pancreatic cancer inhibitory is supported by the preceding studies.

It is important to note that curcumin alone suppressed pancreatic cell growth and tumor xenografts in nude mice. Previous studies have confirmed the antipancreatic cancer effects of curcumin in both in vitro and in vivo models (39–41). However, this is the first report to show that curcumin enhances growth inhibitory and apoptotic effects of DHA in cultured pancreatic cancer cells. There are a number of reports that have suggested curcumin, either alone or in combination, synergies the pancreatic cancer cell growth by induction of apoptosis (40,52,53). In this study, DHA alone had a minimal effect on apoptosis in BxPC-3, but when combined with low-doses of curcumin, the apoptosis was increased several fold in BxPC-3 cells. Similarly, in pancreatic tumor xenograft assay, feeding of mice with omega-3 rich diet showed modest suppression of tumor xenografts (25%); however, when combined with curcumin, the inhibition of tumor growth was almost threefold (>72%), suggesting clear synergistic efficacy. In another report similar to our xenograft experiment, curcumin administered by gavage (1 g/Kg body weight/day) potentiates antitumor activity of gemcitabine in nude mice BxPC-3 tumor xenograft (40). Previously, we showed that combining omega-3 fatty acid-rich diets with COX-2 selective inhibitor, celecoxib, synergistically suppresses chemically induced colon cancer in rats (28). This information emphasizes the importance of developing combinational strategies for the prevention and treatment of pancreatic cancer.

Dietary intake of omega-3 fatty acids will modify the fatty acid composition of membrane phospholipids and increase the availability of omega-3 fatty acids as substrates for COX-2 and 5-LOX for the formation of biologically less potent eicosanoids. This notion is supported by a recent study of experimental prostate cancer that described a 7.8-fold higher omega-6/omega-3 fatty acid ratio in tumor membranes in animals fed an omega-6 fatty-acid-rich diet compared to an omega-3 fatty-acid-rich diet (27). In contrast to several other reports, our data demonstrated that an n-3 fatty-acid-enriched diet had no significant effect on intratumoral COX-2, 5-LOX, and iNOS protein levels (27,48,49). However, dietary intake of n-3 fatty acids in this study decreased COX-2 and 5-LOX activity in pancreatic tumor xenograft, suggesting that the antitumor effect of the n-3 fatty-acid enriched diet was indeed, to a certain extent, through a COX-2- and 5-LOX-dependent mechanism. In addition, the decrease in tumor growth with the n-3 fatty-acid-rich diet correlated with suppression of iNOS activity and an increase in $p21^{waf1/cip}$protein in the tumors, which has also been observed in other models (26,28,33,54).

Curcumin has been shown to modulate a wide range of molecular pathways associated with inflammation and cancer (55–57). Previously, we and others have shown that curcumin modulate the arachidonic acid metabolism by suppressing expression and activities of COX and LOX enzyme pathways (14–16). The results of this study show that curcumin indeed suppresses the COX-2 and 5-LOX expression and activity, leading to reduced levels of protumorigenic eicosanoids. Unlike omega-3 fatty acids, curcumin acts at both the expression and activity of tumor promoting enzymes, such as COX-2, 5-LOX, and iNOS, resulting in greater tumor inhibitory effects of curcumin when compared to n-3 fatty acids. Further, curcumin significantly enhanced n-3 fatty-acid-rich diet-induced inhibition of COX-2, 5-LOX, and iNOS activity in tumors, suggesting possible complementary inhibitory effects. It has been well established that $p21^{waf1/cip}$upregulation was associated with inhibition of cell growth and evidenced by little or no expression

$p21^{waf1/cip}$protein in aggressively growing tumors (58–60). In this study, combining curcumin with fish oil resulted in a significant upregulation of $p21^{waf1/cip}$in tumors when compared to either curcumin or fish oil alone and is well correlated with the inhibition of tumor growth. Mechanisms by which n-3 fatty acids and curcumin synergistically inhibit the pancreatic tumor growth are not fully known; it is likely due to the complementary modes of action of these agents when given in combination. At present, most of the existing evidence supports modulation of arachidonic acid metabolism as a primary mechanism by which fish oil and curcumin combination effectively suppress the tumor growth (28,40). Because curcumin has been shown to modulate numerous cellular targets, it is difficult to implicate the specific targets for the synergistic effects observed in this study. However, there is growing need for identification of particular cellular target(s) that would be modulated by the combinational approach but not by individual agents. Discovery of cellular target(s) that are modulated by dietary agent combinations is pivotal in understanding synergistic effects.

In summary, this study shows, for the first time, that dietary administration of a diet rich in omega-3 fatty acid with curcumin suppresses the pancreatic tumor xenograft with increased efficacy compared with either n-3 fatty acids or curcumin given alone. We have also shown a dose-dependent suppression of cell growth and stimulation of apoptosis in pancreatic cancer BxPC-3 cells treated with a combination of DHA and curcumin. Furthermore, our results suggest that synergistic pancreatic tumor inhibitory effects by n-3 fatty acids and curcumin may be responsible, in part, due to the inhibition of COX-2, 5-LOX, and iNOS activities, leading to the activation of $p21^{waf1/cip}$. Although our understanding of the tumor inhibitory mechanisms by combinational approaches are not yet fully appreciated, given limited treatment strategies for the pancreatic cancer development of effective combinations using dietary molecules will serve as a practical approach toward the design of effective clinical trials in humans.

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