Omega-3 polyunsaturated fatty acids inhibit hepatocellular carcinoma cell growth through blocking β-catenin and cyclooxygenase-2

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Abstract
Hepatocellular carcinoma (HCC) is a common human cancer with high mortality, and currently, there is no effective chemoprevention or systematic treatment. Recent evidence suggests that cyclooxygenase-2 (COX-2)–derived PGE2 and Wnt/β-catenin signaling pathways are implicated in hepatocarcinogenesis. Here, we report that ω-3 polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) inhibit HCC growth through simultaneously inhibition of COX-2 and β-catenin. DHA and EPA treatment resulted in a dose-dependent reduction of cell viability with cleavage of poly ADP ribose polymerase, caspase-3, and caspase-9 in three human HCC cell lines (Hep3B, Huh-7, HepG2). In contrast, AA, a ω-6 PUFA, exhibited no significant effect. DHA and EPA treatment caused dephosphorylation and thus activation of GSK-3β, leading to β-catenin degradation in Hep3B cells. The GSK-3β inhibitor, LiCl, partially prevented DHA-induced β-catenin protein degradation and apoptosis. Additionally, DHA induced the formation of β-catenin/Axin/GSK-3β binding complex, which serves as a parallel mechanism for β-catenin degradation. Furthermore, DHA inhibited PGE2 signaling through downregulation of COX-2 and upregulation of the COX-2 antagonist, 15-hydroxyprostaglandin dehydrogenase. Finally, the growth of HCC in vivo was significantly reduced when mouse HCCs (Hepa1-6) were inoculated into the Fat-1 transgenic mice, which express a Caenorhabditis elegans desaturase converting ω-6 to ω-3 PUFAs endogenously. These findings provide important preclinical evidence and molecular insight for utilization of ω-3 PUFA for the chemoprevention and treatment of human HCC. [Mol Cancer Ther 2009;8(11):3046–55]

Introduction
Hepatocellular carcinoma (HCC) is the fifth most common human cancer with high mortality, and its incidence is increasing worldwide. The overall survival of patients with HCC is dismal, and currently, no efficient secondary prevention or systemic treatments are available. HCC usually develops in the presence of continuous inflammation and hepatocyte regeneration in the setting of chronic hepatitis and cirrhosis (1). Increased cellular turnover and regeneration within the context of a noxious chronically inflamed environment cause accumulation of chromosomal damages, which eventually affect the structure and expression of oncogenes and tumor suppressor genes leading to carcinogenesis. Recent studies have shown that mediators of inflammation, such as prostaglandins (PG), play an important role in hepatocarcinogenesis (2). For example, increased cyclooxygenase-2 (COX-2) expression has been found in human and animal HCCs and in dysplastic hepatocytes (3–9). Elevated levels of PGs, most notably PGE2, have also been detected in HCC (10). Overexpression of COX-2 or treatment with exogenous PGE2 increases human HCC cell growth and invasiveness (8, 11). The COX inhibitors, nonsteroidal anti-inflammatory drugs, inhibit the proliferation and induce apoptosis in cultured HCC cells and in animal models of hepatocarcinogenesis (2), although these inhibitors are known to mediate effects through both COX-dependent and COX-independent mechanisms.

In addition to upregulation of COX-2, Wnt/β-catenin activation has also been implicated in various stages of hepatic tumorigenesis, including the dysplastic foci, hepatic adenoma, hepatoblastoma, and HCC (12–16). Activation of the Wnt/β-catenin pathway occurs in approximately 30% to 40% of HCCs (17). Multiple mechanisms of β-catenin activation or stabilization have been reported in hepatic tumorigenesis, including mutations in the β-catenin gene (Ctnnb1), or components of its degradation machinery such as Axin and GSKβ inactivation (12–16). In mice, hepatic deletion of APC, another degradation component of β-catenin,
leads to HCC (18). Recently, upregulation of a member of Wnt receptors, Frizzled-7, has been shown as another possible mechanism of β-catenin activation in HCC (19). In addition, Wnt/β-catenin also plays an important role in regulation of hepatocyte proliferation, survival, liver regeneration, and in the maintenance and self-renewal of pluripotent stem cells and progenitor cells (12); hence, they may play a role in the maintenance of the cancer stem cell compartment. Indeed, β-catenin activation has been identified in oval cells (liver stem cells), which might be precursors of a subset of HCC (13). Thus, there seems to be multiple mechanisms of β-catenin activation leading to liver neoplasia. Although PGE2 has recently been shown to activate β-catenin in colon cancer cells (20, 21), it remains unknown whether the COX-2/PG and Wnt/β-catenin signaling pathways converge during hepatocarcinogenesis.

In contrast to the documented carcinogenic effect of the PGs (PGE2 in particular) derived from arachidonic acid [AA; an ω-6 polyunsaturated fatty acid (PUFA)], there is abundant experimental evidence that the ω-3 PUFAs rich in fish oil, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), prevent carcinogenesis (22, 23). However, the molecular mechanisms for the anticancer actions of ω-3 PUFAs remain incompletely understood. This study was designed to investigate the effect and mechanism of ω-3 PUFAs in HCC cells. Our results show that DHA and EPA inhibited the growth of three human HCC cells (Hep3B, Huh-7, HepG2), in vitro. The growth of HCC in vivo was also significantly reduced when mouse HCC cells (Hepa1-6) were inoculated into the syngeneic Fat-1 transgenic mice that carry a Caenorhabditis elegans desaturase converting ω-6 to ω-3 PUFAs. Moreover, our data reveal that COX-2–derived PGE2 activates β-catenin signaling pathways in human HCC cells and that ω-3 PUFAs inhibit HCC growth by simultaneously blocking β-catenin and COX-2 signaling pathways. These findings provide important preclinical evidence and molecular framework for utilization of ω-3 PUFAs in the chemoprevention and treatment of HCC.

Materials and Methods

Materials

α-MEM, DMEM, RPMI 1640, fetal bovine serum (FBS), glutamine, antibiotics, and Lipofectamine plus reagent were purchased from Life Technologies, Inc. PGE2 was purchased from Cayman Chemical Company. The antibodies against human Axin, β-catenin, poly ADP ribose polymerase (PARP), caspase-3, caspase-9, and c-Met were purchased from Santa Cruz Biotechnology. The anti-human β-actin monoclonal antibody was purchased from Sigma. The horseradish peroxidase–linked streptavidin and chemiluminescence detection reagents were from Amersham Pharmacia Biotech, Inc. The rabbit antibodies for phospho-Akt (Thr308), Akt, phospho-GSK-3β (Ser9), and GSK-3β were purchased from Cell Signaling Technology. Mouse monoclonal anti–GSK-3β was purchased from Transduction Laboratories, and cytochrome c was purchased from BD Bioscience. The Bio-Rad protein assay system was obtained from Bio-Rad Laboratories. The Tris-glycine gels were obtained from Invitrogen Life Technologies, Inc. Dr. T. Hla at the University of Connecticut Health Center, Farmington, CT, provided the COX-2 expression plasmid (containing full length of human COX-2 cDNA in sense orientation cloned in mammalian expression vector PCDNA3).

Cell Culture

The human HCC cell lines (Hep3B, HepG2, and Huh7) were obtained from American Type Culture Collection and cultured according to our previous described methods (8, 11, 24). Briefly, the cells were cultured in EMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, and penicillin/streptomycin. The cells were incubated at 37°C in a humidified CO2 incubator. The experiments were done when cells reached ~80% confluence and conducted in serum-free medium (with serum deprivation for 24 h before each experiment).

Cell Growth Assay

Cell growth was determined using the cell proliferation reagent WST-1, a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in viable cells. Briefly, 100 μL of cell suspension (containing 0.5–2 × 10^6 cells) were plated in each well of 96-well plates. After 24 h of culture to allow reattachment, the cells were then treated with specific reagents such as DHA, EPA, or Wnt3a-conditioned medium for indicated time points. At the end of each treatment, the cell proliferation reagent WST-1 (10 μL) was added to each well, and the cells were incubated at 37°C for 0.5 to 5 h. Absorbance at 450 nm was measured using an automatic ELISA plate reader.

Immunoprecipitations

Equal amount of cellular protein from the treated cells was incubated with 10 μL of rabbit anti-human Axin polyclonal antibody at 4°C for overnight, followed by addition of 20 μL Protein A/G PLUS agarose (Santa Cruz Biotechnology). The mixture was incubated for 2 h and then washed thrice with the cell lysis buffer [50 mmol/L HEPES (pH 7.55), 1 mmol/L EDTA, 1 mmol/L DTT, and protease inhibitor cocktail tablets from Roche Diagnostics]. The final pellets were dissolved in 20 μL 2× protein loading buffer, and the samples were subjected to SDS-PAGE and Western blot analysis using 1:1,000 dilution mouse anti-human GSK3-β or β-catenin monoclonal antibodies and enhanced chemiluminescence Western blot detection system (Amersham Pharmacia Biotech, Inc.).

Transient Transfection of COX-2 Expression Plasmid

Hep3B cells were exposed to the mixture of Lipofectamine plus reagents and COX-2 expression plasmid (full-length human COX-2 cDNA cloned in pcDNA3 vector), pcDNA3 control vector, or T-cell factor (TCF)/lymphoid enhancer factor (LEF)–Luc reporter plasmid for 4 h. Following removal of the transfection mixtures, the cells were cultured in fresh serum-free medium with or without...
specific treatment as indicated in the text. The expression of COX-2 was verified by immunoblotting.

**Luciferase Reporter Activity Assay**

The cultured cells were seeded at a concentration achieving 80% confluence in 12-well plates for 18 h before transfection. The cells were transiently transfected with 0.2 μg per well translucent TCF/LEF-Luc reporter vector, which was designed to measure the β-catenin transcriptional activity of TCF/LEF responsive genes. After transfection, the cells were treated with DHA, EPA, or AA at increasing concentrations (1–100 μmol/L) for indicated time periods (12–72 h). A, the effect of DHA, EPA, or AA on the viability of Hep3B cells (30 μmol/L, 24 h). The cell viability was determined using WST-1 assay. Columns, mean of six independent experiments; bars, SD.

**Immunoblotting**

At the end of each indicated treatment, the cells were scraped off the plates and centrifuged, washed twice with cold PBS containing 0.5 mmol/L phenylmethylsulfonyl fluoride and 10 μg/mL leupeptin, and resuspended in 5-fold volume of hypotonic buffer consisting of 50 mmol/L HEPES (pH 7.55), 1 mmol/L EDTA, 1 mmol/L DTT, and protease inhibitor cocktail tablets (Roche Diagnostics GmbH). After sonication, the whole-cell lysate was collected for Western blot analysis using antibodies against PARP, caspase-3, and caspase-9. A, DHA induces the cleavage of caspase-3, caspase-9, and PARP. PARP and release of cytochrome c in Hep3B cells. a, DHA induces the cleavage of caspase-3, caspase-9, and PARP. Hep3B cells were treated with DHA (30 μmol/L) for 24 h and the cell lysates were obtained for Western blotting analysis using antibodies against PARP, caspase-3, and caspase-9. b, DHA induces the release of cytochrome c in Hep3B cells. The levels of cytochrome c in the cytosolic and mitochondrial fractions were determined by Western blotting analysis. EPA was not used in these experiments.
Methods. DHA and EPA decreased for Western blot analysis using antibodies against c-Met expression, whereas AA had no effect. The cell lysates were obtained for luciferase activity assay following the formula: tumor volume = \( \pi \times W^2 \times 0.5 \). The animals were injected s.c. into the area overlying the right flank with 1.5 \( \times 10^6 \) mouse HCC cells (Hepa1-6) suspended in 100 \( \mu \)L serum-free medium. After inoculation, the animals were closely monitored for the development of s.c. tumor. The tumor size was measured with a caliper every 2 d. Upon sacrifice, the tumor volume was calculated according to the following formula: tumor volume = \( L \times W^2 \times 0.5 \). The animal experiments were carried out according to the protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee (0201740B).

Results

Immunohistochemical Stains for COX-2 and β-catenin in Human HCC Tissues

Twenty paired human HCCs and their matched nonneoplastic/nondysplastic liver tissues were analyzed by immunohistochemistry for the expression of COX-2 and β-catenin. Increased cytoplasmic staining for COX-2 and nuclear staining for β-catenin was observed in HCC cells when compared with the nontumor liver tissue (Supplementary Fig. S1). The average staining intensity for COX-2 in HCC is 2.10 ± 0.78, which is significantly higher than that in nontumor liver tissue (0.20 ± 0.09; \( P < 0.01 \), Student’s \( t \)-test). Whereas COX-2 is expressed exclusively in the cytoplasm of both HCC cells and to a less degree in hepatocytes, the expression pattern of β-catenin between HCC cells and nonneoplastic hepatocytes are distinctly different. In nontumorous hepatocytes, β-catenin is expressed exclusively in the plasma membrane with no significant cytoplasmatic staining and absence of nuclear staining in all 20 patients. In contrast, in HCC cells, nuclear staining for β-catenin was observed in 5 of 20 patients (25%), with focal cytoplasmatic staining and decreased membrane staining, indicating β-catenin nuclear translocation and activation. Thus, COX-2 and β-catenin signaling pathways are active in a significant percentage of human HCCs.

ω-3 PUFAs Induce HCC Cell Apoptosis

Human HCC cell lines were examined for their response to DHA, EPA, and AA treatment. As shown in Fig. 1A, treatment of Hep3B cells with two ω-3 PUFAs (30 μmol/L), DHA and EPA, induced a time-dependent reduction of cell viability; in contrast AA, a ω-6 PUFAs, had no significant effect. Treatment with 30 μmol/L EPA for 12, 24, 48, and 72 hours induced approximately 45%, 60%, 70%, and 75% reduction of viable cells, respectively. DHA seems to have more effect, with ~75% reduction of viable cells at 12 hours and >90% reduction at 24, 48 and 72 hours. The cells treated with DHA and EPA show morphologic features of cell death, characterized by shrinkage, roundness, and detachment. In contrast, AA treatment did not significantly alter the cell morphology. The effect of DHA and EPA is dose dependent in all three human HCC cell lines (Hep3B, Huh7, and HepG2; Fig. 1B). The observations that DHA induced the cleavage of PARP, caspase-3, and caspase-9, with concomitant release of cytochrome c from mitochondria to
cytosol confirm the induction of apoptosis (Fig. 1C). Taken together, these results document induction of apoptosis by ω-3 PUFAs in HCC cells. We have also tested the effect of DHA and EPA in primary cultures of liver parenchymal cells and these compounds were found to have no cytotoxic effect in primary cells.  

**DHA Decreases the Level of β-Catenin and Inhibits TCF/LEF Transcription Activity in HCC Cells**

Further experiments were done to assess the mechanisms by which ω-3 PUFAs induce HCC apoptosis. Because β-catenin activity is importantly involved in hepatocarcinogenesis, the potential effect of ω-3 PUFAs on β-catenin protein level and activity was examined. As shown in Fig. 2A and B, treatment with DHA or EPA reduced the level of β-catenin protein; this effect was time-dependent (observed 1–6 hours after treatment). As c-Met is a β-catenin-controlled downstream gene, the potential effect of DHA and EPA on c-Met protein expression was also examined. Indeed, DHA and EPA treatment also reduced the expression of c-Met. In contrast, treatment with AA did not alter β-catenin or c-Met level (Fig. 2C).

Because β-catenin regulates gene expression via binding as a transcription factor in complex with the TCF/LEF transcription factor family to the promoter region of target genes, we further examined the effect of DHA on TCF/LEF reporter activity. The TCF/LEF transcription activity was assayed after transient transfection of a luciferase reporter construct under the control of TCF/LEF response element. As shown in Fig. 2D, DHA treatment significantly inhibited the TCF/LEF reporter activity. This result further confirms suppression of β-catenin activity by DHA.

**ω-3 PUFAs Induce β-Catenin Degradation through Inhibition of GSK-3β Phosphorylation**

The level of β-catenin in cells is tightly controlled by its degradation complex composed of Axin, APC, GSK-3β, and β-catenin, in which GSK-3β phosphorylates β-catenin and thus triggers its ubiquitination and subsequent proteosomal degradation. The activity of GSK-3β is regulated by its phosphorylation status, with GSK-3β phosphorylation at Ser-9 being functionally inactive. To determine whether ω-3 PUFAs might induce β-catenin degradation through inhibition of GSK-3β phosphorylation, we examined the phospho-Ser-9-GSK-3β and total GSK-3β protein levels in Hep3B cells treated with PUFAs. As shown in Fig. 3A, DHA treatment reduced GSK-3β phosphorylation, whereas it had no effect on the protein level of total GSK-3β. Similarly,
EPA treatment also decreased the level of phosphor-GSK-3β, whereas AA had no effect. Because the phosphorylation of GSK-3β is controlled by Akt, we also examined the potential effect of DHA on Akt phosphorylation. Our data showed that DHA had no effect on Akt phosphorylation (Fig. 3A). Thus, DHA most likely inhibited GSK-3β phosphorylation through mechanism independent of Akt. Taken together, the above findings provide evidence for GSK-3β through mechanism independent of Akt. Against GSK-3β at different time points (1, 2, 3, 4, and 5 h). The cell lysates were immunoprecipitated with anti-Axin antibody followed by immunoblotting with antibodies against GSK-3β.

The GSK-3β Inhibitor, LiCl, Prevents DHA-Induced β-Catenin Degradation and Cell Death

To further determine the role of GSK-3β in DHA-induced β-catenin degradation, Hep3B cells were pretreated for 1 hour with LiCl before DHA treatment to determine the level of β-catenin protein, TCF/LEF reporter activity, and cell growth. As shown in Fig. 3B, inhibition of GSK-3β by LiCl prevented DHA-induced reduction of β-catenin protein and TCF/LEF reporter activity. Accordingly, LiCl pretreatment also prevented DHA-induced PARP cleavage and restored DHA-induced cell death (Fig. 3C). These findings further support the role of GSK-3β activation (dephosphorylation) in DHA-induced β-catenin degradation in HCC cells.

DHA Induces the Association of Axin with GSK-3β and β-Catenin in HCC Cells

The degradation of β-catenin strictly depends upon β-catenin phosphorylation, which occurs in a multiprotein complex containing Axin and GSK-3β and β-catenin. It is believed that in this complex assembled by Axin, GSK-3β phosphorylates the β-catenin primarily when it is bound to Axin. To determine whether DHA alters the assembly of the Axin/GSK-3β/β-catenin complex, immunoprecipitation and Western blot experiments were done to detect the Axin/GSK-3β/β-catenin binding complex. As shown in Fig. 4A to C, treatment of Hep3B cells with DHA induced the association of Axin with GSK-3β as well as β-catenin. This effect was observed within 1 hour and persisted at 5 hours. In contrast, AA did not affect the association between Axin and GSK-3β (Fig. 4D). These findings indicate that DHA induces the association of Axin with GSK-3β and β-catenin, thereby facilitating the formation of β-catenin destruction complex. Taken together, our data suggest that G3-PUFAs induce β-catenin degradation through dephosphorylation of GSK-3β and formation of β-catenin destruction complex in HCC cells.

Activation of β-Catenin by Wnt3a Partially Protects DHA-Induced HCC Cell Death

Because Wnt3a is known to activate β-catenin signaling in cells, further experiments were carried out to determine whether Wnt3a might protect HCC cells from DHA-induced apoptosis. Indeed, treatment of Hep3B cells with Wnt3a conditioned medium partially prevented DHA-induced cell death (Supplementary Fig. S2A). The effect of Wnt3a on β-catenin activation was confirmed by the observation that Wnt3a conditioned medium prevented DHA-induced reduction of TCF/LEF transcription activity (Supplementary Fig. S2B). These results further show that DHA inhibits HCC growth at least in part through downregulation of Wnt/β-catenin signaling pathway.

DHA Inhibits COX-2 Expression in HCC Cells

We next examined whether DHA might also affect the expression of COX-2 in HCC cells. As shown in Supplementary Fig. S3, DHA significantly inhibited the COX-2 promoter activity and COX-2 protein expression in HCC cells. These findings suggest that DHA inhibits the expression of COX-2 through suppression of gene transcription.

DHA Induces 15-PGDH Expression in HCC Cells

15-PGDH catalyzes the rate-limiting step of PG catabolism and thus represents a physiologic antagonist of COX-2 (26, 27). Recent emerging evidence suggests that elevated PGE2 in cancers may be the result of enhanced COX-2-mediated PGE2 synthesis as well as reduced 15-PGDH-mediated...
degradation of PGE2. Therefore, we sought to further determine whether DHA might affect 15-PGDH expression in HCC cells. As shown in Supplementary Fig. S4, DHA treatment enhanced the expression of 15-PGDH in a dose-dependent manner in HCC cells (Hepa1-6, HepG2, and HuH7). These data are consistent with the observation that DHA and EPA inhibit PGE2 production in Hepa1-6 cells (Supplementary Fig. S5).

**DHA Prevents PGE2 Induced β-Catenin Activation**

Because DHA reduces PGE2 level through concomitant inhibition of COX-2 and induction of 15-PGDH, we postulate that DHA might also inhibit β-catenin through inhibition of PGE2. To evaluate this hypothesis, further experiments were done to examine the direct effect of PGE2 on β-catenin activation and to determine whether DHA might prevent PGE2 effect. As shown in Fig. 5, PGE2 treatment resulted in dissociation of Axin from GSK-3β and enhanced TCF/LEF reporter activity in Hepa1-6 cells; these effects were significantly blocked by cotreatment with DHA. These findings suggest that suppression of PGE2 by DHA represents another mechanism for β-catenin degradation.

**ω-3 PUFAs Prevent HCC Growth In vivo**

After the in vitro effect of ω-3 PUFAs on HCC cell growth was documented, further experiments were carried out to evaluate the effect of ω-3 PUFAs on HCC growth in vivo. We implanted murine HCC cells (Hepa1-6) into the syngeneic Fat-1 transgenic and control mice (with C57BL/6 genetic background) and examined the growth of the inoculated tumor cells in these animals. The Fat-1 transgenic mice carry a *Caenorhabditis elegans* desaturase gene that adds a double bond into a saturated fatty acid hydrocarbon chain and converts ω-6 to ω-3 PUFAs, resulting in a significant increase in ω-3 PUFAs and reduction in ω-6 PUFAs in all the organs and tissues (25). The Hepa1-6 cell line was chosen because it was derived from HCC of C57BL/6 strain and can be grown to form tumors in mice with C57BL/6 genetic background. As shown in Fig. 6, there is a marked difference in the tumor size and tumor volume between Fat-1 transgenic (*n* = 10) and wild-type mice (*n* = 12). Over an observation period of 14 days, all wild-type mice developed a palpable tumor by day 4, whereas only 5 of 10 Fat-1 transgenic mice developed a minor tumor palpable by day 4 and the mass of all palpable tumor almost disappeared at day 12. Mice with homozygous mutation for the PG receptor EP1 (in C57BL/6 background) was used as an additional control, which showed a similar degree of tumor growth as the wild-type mice. These findings show that ω-3 PUFAs inhibit HCC growth, in vivo.

**The Effect of ω-3 PUFAs on Hepa1-6 Cell Growth In vitro**

Given the marked reduction of Hepa1-6 cell growth in the Fat-1 transgenic mice, we conducted subsequent experiments to evaluate ω-3 PUFA actions in Hepa1-6 cell growth, in vitro. Both DHA and EPA significantly reduced the viability of cultured Hepa1-6 cells (Supplementary Fig. S6). Treatment of Hepa1-6 with DHA led to reduction of β-catenin protein as well as TCF/LEF reporter activity (Supplementary Fig. S7). In parallel, DHA also inhibited the expression of COX-2 and induced the expression of 15-PGDH in Hepa1-6 because it was derived from HCC of C57BL/6 strain and can be grown to form tumors in mice with C57BL/6 genetic background. As shown in Fig. 6, there is a marked difference in the tumor size and tumor volume between Fat-1 transgenic (*n* = 10) and wild-type mice (*n* = 12). Over an observation period of 14 days, all wild-type mice developed a palpable tumor by day 4, whereas only 5 of 10 Fat-1 transgenic mice developed a minor tumor palpable by day 4 and the mass of all palpable tumor almost disappeared at day 12. Mice with homozygous mutation for the PG receptor EP1 (in C57BL/6 background) was used as an additional control, which showed a similar degree of tumor growth as the wild-type mice. These findings show that ω-3 PUFAs inhibit HCC growth, in vivo.

**Figure 5.** DHA prevents PGE2-induced dissociation of Axin/GSK-3β complex in HCC cells. **A,** PGE2 induces dissociation of Axin from GSK-3β in Hepa1-6 cells. The cells were treated with different concentrations of PGE2 in the serum-free medium for 1 h. The cell lysates were obtained for immunoprecipitation (IP) with Axin antibody followed by immunoblotting (IB) with antibodies against GSK-3β and Axin. **B,** the time-dependent effect of PGE2 on Axin/GSK-3β dissociation. Hepa1-6 cells were treated with PGE2 (10 μmol/L) for different time points in the serum-free medium. The cell lysates were obtained for immunoprecipitation with anti-Axin antibody followed by immunoblotting with antibodies against GSK-3β and Axin. **C,** DHA prevents PGE2-induced Axin/GSK-3β dissociation. Hepa1-6 cells were treated with PGE2 (10 μmol/L) or vehicle in the absence or presence of DHA (60 μmol/L) in serum-free medium for 1 h. The cell lysates were obtained for immunoprecipitation with anti-Axin antibody followed by immunoblotting with antibodies against GSK-3β and Axin. **D,** DHA inhibits PGE2-induced TCF/LEF reporter activity. Hep3B cells were transiently transfected with TCF/LEF-Luc reporter vector. After transfection, the cells were treated with PGE2 with or without DHA in the serum-free medium for 24 h. The cell lysates were obtained to determine the luciferase activity. Columns, mean of six independent experiments; bars, SD. DHA treatment significantly decreased TCF/LEF reporter activity (⁎, *P* < 0.01 compared with control; **, *P* < 0.01 compared with PGE2 treatment).
Hepa1-6 cells (Supplementary Fig. S8A). Consistent with the latter observations, DHA treatment also inhibited the production of PGE2 in Hepa1-6 cells (Supplementary Fig. S8B). Therefore, the effects of ω-3 PUFAs in Hepa1-6 cells are similar to those in human HCC cells. These findings show that ω-3 PUFAs inhibit β-catenin and COX-2 signaling in both murine and human HCC cells.

**Discussion**

Both the COX-2/PGE2 and Wnt/β-catenin signaling pathways are active in human HCCs. There is constitutively high expression and activation of COX-2 in human liver cancers and precancerous inflammatory liver diseases; COX-2 activation enhance the production of PGs from AA that subsequently promote hepatic inflammation and neoplasia (2). In parallel, the Wnt/β-catenin pathway is also activated in various stages of hepatic tumorigenesis (13–16, 28–31). Therefore, we postulate that therapies aimed at simultaneous disruption of the COX-2/PGE2 and Wnt/β-catenin pathways may produce effective chemopreventive and antitumorigenic effects. This study provides important experimental evidence and mechanisms for inhibition of both Wnt/β-catenin and COX-2/PGE2 signaling pathways by ω-3 PUFAs in HCC (Supplementary Fig. S9).

Compelling epidemiologic and experimental studies have indicated a relationship between PUFAs and the risk of cancer. For example, a high dietary intake of ω-6 PUFAs, such as linoleic acid (18:2ω-6), is associated with a high risk for colon cancer, whereas high intake of ω-3 PUFAs from fish oils, such as DHA (22:6ω-3) and EPA (20:5ω-3), decreases it (22, 23). Experimental data have shown that the ω-6 fatty acids stimulate carcinogenesis, tumor growth, and metastasis, whereas the ω-3 fatty acids exert suppressive effects. In this study, we used both in vitro and in vivo models to evaluate the effect of ω-3 PUFAs on HCC growth. Treatment with DHA and EPA induced a dose- and time-dependent growth inhibition and apoptosis in three human HCC cell lines. The induction of apoptosis is confirmed by cleavages of PARP, caspase-3, and caspase-9 and release of cytochrome c. To evaluate the antitumor effect of ω-3 PUFAs on HCC growth in vivo, we implanted murine HCC cells (Hepa1-6) into the syngeneic Fat-1 transgenic and control mice. The Fat-1 transgenic mice ubiquitously express a Caenorhabditis elegans desaturase, leading to significant increase in ω-3 PUFAs and reduction in ω-6 PUFAs in all the organs and tissues (25, 32). This model was selected because it provides a balanced ratio of ω-6 to ω-3 fatty acids in mouse tissues and eliminates the potential dietary variation associated with

**Figure 6.** Tumorigenicity of Hepa1-6 mouse HCC cells in Fat-1 transgenic and wild-type mice. A and B, tumor size and tumor volume in the Fat-1 transgenic and wild-type control mice. Hepa1-6 murine HCC cells were obtained from the American Type Culture Collection and cultured in DMEM containing 4 mmol/L L-glutamine and 10% FBS. Cells (1.5 × 10⁶) were injected s.c. into the right flank of each of Fat-1 transgenic and wild-type mice (8-wk-old male). Mice with homozygous mutation for the PG receptor EP1 (in C57BL/6 background) were used as an additional control. Tumor size was monitored at the indicated time with a caliper. Tumor volume was calculated on the basis of the following formula: Tumor volume = (length × width × width)/2. Points, mean of tumor size or volume of wild-type group (n = 12), EP1 knockout (n = 10), or Fat-1 transgenic (n = 10) mice; bars, SD. C, representative photographs showing tumor formation at three different time points after cell implantation.
Inhibition of HCC by ω-3 PUFAs

long-term feeding of PUFAs. A significant reduction of HCC tumor size and tumor volume was observed in the Fat-1 transgenic mice. These findings provide important in vivo evidence for inhibition of HCC by ω-3 PUFAs. The effect of dietary DHA and EPA on hepatocellular cancer growth remains to be further evaluated.

A prominent mechanism for the chemopreventive action of ω-3 PUFAs is their suppressive effect on the production of AA-derived prostanoids, particularly PGE2 (23, 33). This is important because PGE2 is implicated in multistages of tumorigenesis, including modulation of inflammation, cancer cell proliferation, differentiation, apoptosis, angiogenesis, metastasis, and host immune response to cancer cells (2). Our data in this study show that ω-3 PUFAs inhibit COX-2 expression in HCC cells, which is consistent with recently reported downregulation of COX-2 by ω-3 PUFAs in colon cancer cells (34). Moreover, our findings provide novel evidence for induction of 15-PGDH, a rate-limiting key enzyme in PG catabolism, by ω-3 PUFAs in human cancer cells. The latter observation is noteworthy, because 15-PGDH is a PG-degrading enzyme that physiologically antagonizes COX-2 and suppresses tumor growth.

In addition to modulation of COX-2 and 15-PGDH by ω-3 PUFAs, our results reveal that degradation of β-catenin is a novel parallel mechanism for ω-3 PUFA-mediated antitumor effect. β-Catenin is a key molecule in the canonical Wnt pathway that regulates multiple biological functions, including embryogenesis and tumorigenesis (35–38). In the absence of Wnt ligands, cytoplasmic β-catenin associates in a complex with GSK-3β, Axin, and APC, where it is phosphorylated and targeted for proteosomal degradation. Activation of Wnt signaling causes dissociation of the β-catenin degradation complex, leading to β-catenin accumulation in the cytoplasm and translocation into the cell nucleus. In the nucleus, β-catenin binds the transcription factor TCF/LEF that induce transcription of important downstream target genes implicated in cell proliferation, differentiation, and apoptosis (35–38). Recent evidence has shown that PGE2 induces the cytoplasmic and nuclear accumulation of β-catenin in human colon cancer cells. Castellone and colleagues (20) reported that PGE2 activates its G protein–coupled receptor, EP2, resulting in direct association of the G protein α subunit with the regulator of G protein signaling domain of axin; this causes release of GSK-3β from its complex with axin, thus leading to β-catenin accumulation. A separate study by Shao et al. (21) showed the involvement of cyclic AMP/protein kinase A pathway in PGE2–induced β-catenin accumulation in colon cancer cells. The current study provides evidence that PGE2 induces dissociation of GSK-3β from Axin, thus preventing β-catenin reduction in HCC cells.

Our data suggest that ω-3 PUFAs induce β-catenin degradation through three interrelated mechanisms. First, we show that DHA and EPA induce a rapid dephosphorylation of GSK-3β in HCC cells, suggesting that GSK-3β activation is involved in ω-3 PUFA–induced β-catenin degradation. This assertion is further supported by the observations that the GSK-3β inhibitor, LiCl, prevents DHA-induced reduction of β-catenin protein and transcription activity and restored DHA-induced cell death. Second, DHA treatment induces the association of Axin with GSK-3β forming β-catenin destruction complex. Third, ω-3 PUFAs suppress PGE2 signaling through concomitant inhibition of COX-2 and induction of 15-PGDH, thus preventing PGE2–induced β-catenin accumulation. The involvement of β-catenin degradation in ω-3 PUFA–induced inhibition of tumor growth is further supported by the observation that Wnt3a conditioned medium partially protects HCC cells from DHA-induced apoptosis.

In summary, this study provides encouraging preclinical evidence and important mechanism for utilization of ω-3 PUFAs in the chemoprevention and treatment of HCC, although the data should be interpreted with caution because the concentration of EPA and DHA used in the cultured cells is relatively high and most likely will not be achieved in vivo. Our findings have significant clinical implications, given that HCC is a common and highly malignant human cancer. It is conceivable that ω-3 PUFAs, either applied alone or in conjunction with current modalities, may represent an effective, nontoxic, and safe chemopreventive and therapeutic agent for patients with HCC or at high risks for development of this devastating tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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