

Cyclooxygenase-2 Promotes Human Cholangiocarcinoma Growth: Evidence for Cyclooxygenase-2-Independent Mechanism in Celecoxib-Mediated Induction of p21^{waf1/cip1} and p27^{kip1} and Cell Cycle Arrest

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ABSTRACT

The expression of cyclooxygenase-2 (COX-2) is increased in human cholangiocarcinoma. However, the biologic function and molecular mechanisms of COX-2 in the control of cholangiocarcinoma cell growth have not been well established. This study was designed to examine the direct effect of COX-2 and its inhibitor celecoxib on the growth of human intrahepatic cholangiocarcinoma cells. Overexpression of COX-2 or treatment with prostaglandin E₂ (PGE₂) enhanced human cholangiocarcinoma cell growth, whereas antisense depletion of COX-2 in these cells decreased PGE₂ production and inhibited growth. These findings demonstrate a direct role of COX-2-mediated PGE₂ in the growth regulation of human cholangiocarcinoma cells. Furthermore, the COX-2 inhibitor celecoxib induced a dose-dependent inhibition of cell growth, cell cycle arrest at the G₁-S checkpoint, and induction of cyclin-dependent kinase inhibitors p21^{waf1/cip1} and p27^{kip1}. However, the high concentration of celecoxib (50 μM) required for inhibition of growth, the incomplete protection of celecoxib-induced inhibition of cell growth by PGE₂ or COX-2 overexpression, and the fact that overexpression or antisense depletion of COX-2 failed to alter the level of p21^{waf1/cip1} and p27^{kip1} indicate the existence of a COX-2-independent mechanism in celecoxib-induced inhibition of cholangiocarcinoma cell growth.

INTRODUCTION

Cholangiocarcinoma is a malignant neoplasm originating from epithelium of the biliary tree with high mortality (1). It often occurs in patients with background conditions that cause long-standing inflammation, injury, and reparative biliary epithelial cell proliferation, such as primary sclerosing cholangitis, clonorchiasis, hepatolithiasis, or complicated fibropolycystic diseases. This association strongly indicates that chronic inflammatory processes involving bile duct epithelium predispose the development of cholangiocarcinoma. However, the exact molecular mechanisms by which inflammatory cascades promote malignant transformation of biliary epithelium are not well understood. Recent evidence reveals that cyclooxygenase-2 (COX-2), an inducible enzyme controlling the synthesis of lipid inflammatory mediator prostaglandins (PGs), is increased in cholangiocarcinoma cells (2–5), which suggests a potential role of COX-2-mediated PG pathway in biliary carcinogenesis.

The synthesis of PGs is controlled by coordinated activation of eicosanoid-forming enzymes, including COXs (6). There are two isoforms of COXs, COX-1 and COX-2, which catalyze the same enzymatic reaction but are regulated differently. Whereas COX-1 is expressed constitutively in most tissues, COX-2 is an immediate early gene that is induced by a variety of stimuli, such as cytokines,

hormones, mitogens, and growth factors (6–20). Recent evidence shows that the expression of COX-2 is increased in human cancers and that the COX-2-generated PGs promote tumor cell proliferation, survival, and angiogenesis (6–12, 21–25). Targeted expression of COX-2 in mammary gland (26) and skin (27) induces tumorigenesis in mice; knockout of the COX-2 gene suppresses intestinal tumorigenesis (28). These findings highlight the important role of COX-2-controlled PG metabolism in cancer development and progression.

The contribution of PG pathway in cancer growth is also supported by the substantial epidemiologic, experimental, and clinical studies using the COX inhibitor nonsteroidal anti-inflammatory drugs (NSAIDs). Large epidemiologic studies reveal that aspirin and other NSAIDs reduce the risk of colorectal cancer (7, 9, 13, 14, 16, 19) and several other cancers (15, 17, 18, 29–31). Randomized clinical studies show that NSAIDs decrease colon polyps in patients with familial adenomatous polyposis (32–35). Experimental studies using various *in vitro* and *in vivo* models demonstrate that NSAIDs decrease the growth of human cancer cells, including cholangiocarcinoma (3, 5, 25, 36). The proposed mechanisms by which NSAIDs prevent cancer growth include inhibition of cell proliferation, potentiation of immune response, inhibition of angiogenesis, and induction of apoptosis (7, 9, 19, 20, 37, 38). However, interpretation of the NSAID-mediated antitumor effect is complicated by the presence of COX-independent effect (7, 19, 21, 39–43). Celecoxib belongs to the new generation of NSAIDs that selectively inhibits COX-2 activity without inhibition of COX-1 and thus lacks the side effects associated with the traditional NSAIDs. Recent studies have shown that celecoxib inhibits the growth of several tumor cell types (35, 44–52), although its effect on cholangiocarcinoma growth remains to be further defined.

Despite increased COX-2 expression in human cholangiocarcinoma, the direct biologic role of COX-2-mediated prostanoids in the control of cholangiocarcinoma cancer growth has not been established with molecular approach. In this study, we performed *in vitro* studies to address the direct role of COX-2 in cholangiocarcinoma cell growth by overexpression and antisense depletion of COX-2 and to evaluate the efficacy and mechanisms of celecoxib in the growth of human intrahepatic cholangiocarcinoma cells.

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. (Rockville, MD). PGE₂ was purchased from Calbiochem (San Diego, CA). The cell proliferation assay reagent WST-1 was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Pharmacia (Chicago, IL) provided the COX-2 inhibitor celecoxib (SC58635). The antibody for human COX-2 was purchased from Cayman Chemical Company (Ann Arbor, MI). The antibodies against human p27, p21, cyclin E, and cyclin-dependent kinase 2 (cdk2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antihuman β-actin monoclonal antibody was purchased from Sigma (St. Louis, MO). The horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The PGE₂ enzyme immunoassay system also was from Amersham Pharmacia Biotech, Inc. The Bio-Rad protein assay system was obtained from Bio-Rad Laboratories (Hercules, CA). The Tris-glycine gels

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were obtained from Invitrogen Life Technologies, Inc. (Carlsbad, CA). The Tet-On antisense COX-2 construct (containing a 1.93-kb human COX-2 cDNA in antisense orientation that is controlled by the tetracycline response element) was provided by Dr. J. Morrow at Vanderbilt University (53). Dr. T. Hla at the University of Connecticut Health Center 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. Three human intrahepatic cholangiocarcinoma cell lines—HuCCT1 (Ref. 54; obtained from Japanese Cancer Research Resources Bank), SG231 (55), and CCLP1 (56)—were used in this study. HuCCT1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 50 $\mu\text{g}/\text{ml}$ gentamicin. SG231 cells were cultured in α -MEM with 2 mM L-glutamine, 50 $\mu\text{g}/\text{ml}$ gentamicin, 10 mM HEPES, and 10% FBS. CCLP1 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 50 $\mu\text{g}/\text{ml}$ gentamicin. The cells were cultured at 37°C in a humidified CO₂ incubator. The experiments were performed when cells reached ~80% confluence and conducted in serum-free medium with serum deprivation for 24 h before experiments.

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 0.5×10^4 cells) were plated in each well of 96-well plates. After 24-h culture to allow reattachment, the cells then were treated with specific reagents such as PGE₂ or celecoxib for indicated time points. At the end of each experiment, the cell proliferation reagent WST-1 (10 μl) was added to each well, and the cells were incubated at 37°C for 0.5–5 h. A_{450 nm} was measured using an automatic ELISA plate reader.

Cell Cycle Analysis. The cell cycle of human cholangiocarcinoma cells was analyzed by flow cytometry. Cells were fixed in ice-cold 70% ethanol, washed with PBS, treated with RNase (Sigma; 0.2 mg/ml) at room temperature for 30 min, and stained with propidium iodide (Sigma; 0.05 mg/ml). Cell cycle analysis was performed using EPICS XL flow cytometry (Beckman Coulter, Fullerton, CA). Fifteen thousand cells were analyzed for each point, and quantitation of cell cycle distribution was performed using the EXP032 software program provided by the manufacturer.

Immunoprecipitations. Equal amount of cellular protein from the treated cells was incubated with 10 μl of mouse antihuman cdk2 monoclonal antibody at 4°C for 1 h, followed by addition of 20 μl Protein A/G PLUS agarose (Santa Cruz Biotechnology). The mixture was incubated overnight and then washed three times with the cell lysis buffer [50 mM HEPES (pH 7.55), 1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail tablets from Roche Diagnostics (Basel, Switzerland)]. The final pellets were dissolved in 20 μl 1 \times protein loading buffer, and the samples were subjected to SDS-PAGE and Western blot analysis using 1:1000 dilution rabbit antihuman p27 or p21 polyclonal antibodies and enhanced chemiluminescence (Amersham Pharmacia Biotech, Inc.) Western blot detection system.

cdk2 Kinase Activity Assays. The cdk2 kinase was immunoprecipitated from 100 μg of cellular extracts with polyclonal antibodies against cdk2 (Upstate Biotechnology, Inc., Lake Placid, NY) for 2 h at 4°C. The obtained immunoprecipitates then were used to measure cdk2 kinase activity with histone H1 as the substrate using the kinase assay kit (Upstate Biotechnology, Inc.). The reaction mixture consisted of 20 mM 4-morpholinepropanesulfonic acid (MOPS; pH 7.2), 15 mM MgCl₂, 50 mM ATP, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, and 10 μCi of [γ -³²P] ATP in a 50- μl volume in the presence of 4 μM protein kinase C inhibitor peptide, 0.5 μM protein kinase A inhibitor peptide, and 0.5 μM compound R24517. The reaction was started by addition of the enzyme source from immunoprecipitates and was allowed to proceed for 20 min at 30°C and stopped by taking 25- μl aliquots onto p81 phosphocellulose squares and then washing three times with 0.75% phosphoric acid. After acetone rinse, the radioactivity was measured by scintillation counting.

Transfection of COX-2 Expression Plasmid in Human Cholangiocarcinoma Cells. CCLP1 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) or pCDNA3 control vector for 4 h. Following removal of the transfection mixtures, fresh medium with 10% FBS was added. On day 2, the medium was replaced with fresh medium containing 10% FBS and 800 $\mu\text{g}/\text{ml}$ G418 sulfate (Calbiochem). Colonies of resistant cells appeared after ~14 days and were subcultured at ~18 days. Subsequent cultures of

selected cells were routinely grown in the presence of the same selective pressure. Western blot analysis for COX-2 then was performed in the selected cells transfected permanently with the COX-2 expression plasmid or control plasmid. The selected cells with successful overexpression of COX-2 protein were used for subsequent experiments.

Antisense Inhibition of COX-2 in HuCCT1 Cells. The HuCCT1 cells (containing high basal level of COX-2 protein) were used for antisense inhibition of COX-2. The cells were transfected with the Tet-On antisense COX-2 plasmid (containing a 1.93-kb human COX-2 cDNA in antisense orientation that is controlled by the tetracycline response element) according to the previously described procedure with modification (53). Briefly, the cells were exposed to the mixture of Lipofectamine plus reagents and the antisense COX-2 construct in serum-free medium for 4 h. Following removal of the transfection mixtures, the cells were cultured in RPMI 1640 medium containing 10% FBS. On day 2, fresh medium containing 10% FBS and 800 $\mu\text{g}/\text{ml}$ G418 sulfate was added. Colonies of resistant cells appeared after ~14 days and were subcultured at ~18 days. Subsequent cultures of selected HuCCT1 cells were routinely grown in the presence of the same selective pressure. The level of COX-2 expression was determined 96–120 h after cells were exposed to doxycycline (2 $\mu\text{g}/\text{ml}$) by Western blot analysis. The selected cells with optimal depletion of COX-2 were used for subsequent experiments.

RESULTS

Antisense Depletion of COX-2 Inhibits Cholangiocarcinoma Cell Growth. The direct effect of COX-2 on cholangiocarcinoma cell growth was evaluated using antisense inhibition of COX-2. The HuCCT1 cells were selected for this approach because these cells contain high basal level of COX-2 protein. The HuCCT1 cells were transfected with the COX-2 antisense plasmid containing a 1.93-kb human COX-2 cDNA in antisense orientation that is controlled by the tetracycline response element (53). Fig. 1 shows that ~80% reduction of COX-2 protein was achieved in cells with antisense inhibition of COX-2. The cells with antisense depletion of COX-2 exhibited significant reduction of PGE₂ and decrease of cell growth. Furthermore, addition of PGE₂ partially reverses the growth inhibition caused by antisense depletion of COX-2 (Fig. 1). These findings demonstrate a direct effect of COX-2-controlled PGE₂ in human cholangiocarcinoma cell growth.

Overexpression of COX-2 Promotes Cholangiocarcinoma Cell Growth. The direct contribution of COX-2 to cholangiocarcinoma cell growth was examined further by stable transfection of a COX-2 expression plasmid. The CCLP1 cells were selected for this approach because these cells contain low basal level of COX-2 protein. The cells were transfected with the human COX-2 constitutive expression plasmid pCDNA3-COX-2 and the control vector pCDNA3. After transfection, the cells were cultured in media containing 800 ng/ml G418 to select the cells with stably transfected cells. The selected cells transfected with COX-2 expression plasmid then were analyzed for their growth *in vitro* under spontaneous culture conditions and showed significantly increased cell growth over the control vector cells (Fig. 2). The efficiency of COX-2 expression was confirmed by Western blot analysis showing that the COX-2 overexpressed cells exhibit ~12-fold increase of COX-2 protein when compared with the vector control cells as evaluated by densitometry (see below; Fig. 7). These observations further demonstrate an important role of COX-2 in human cholangiocarcinoma cell growth.

Celecoxib Inhibits Growth of Human Cholangiocarcinoma Cells. We then examined whether the selective COX-2 inhibitor celecoxib would prevent cholangiocarcinoma cell growth. Fig. 3A shows that celecoxib treatment induced a dose-dependent inhibition of cell growth in SG231, HuCCT1, and CCLP1 cells. Despite the higher basal level of COX-2 expression in HuCCT1 cells, a comparable degree of inhibition of growth was observed in all of the three human cholangiocarcinoma cell lines.

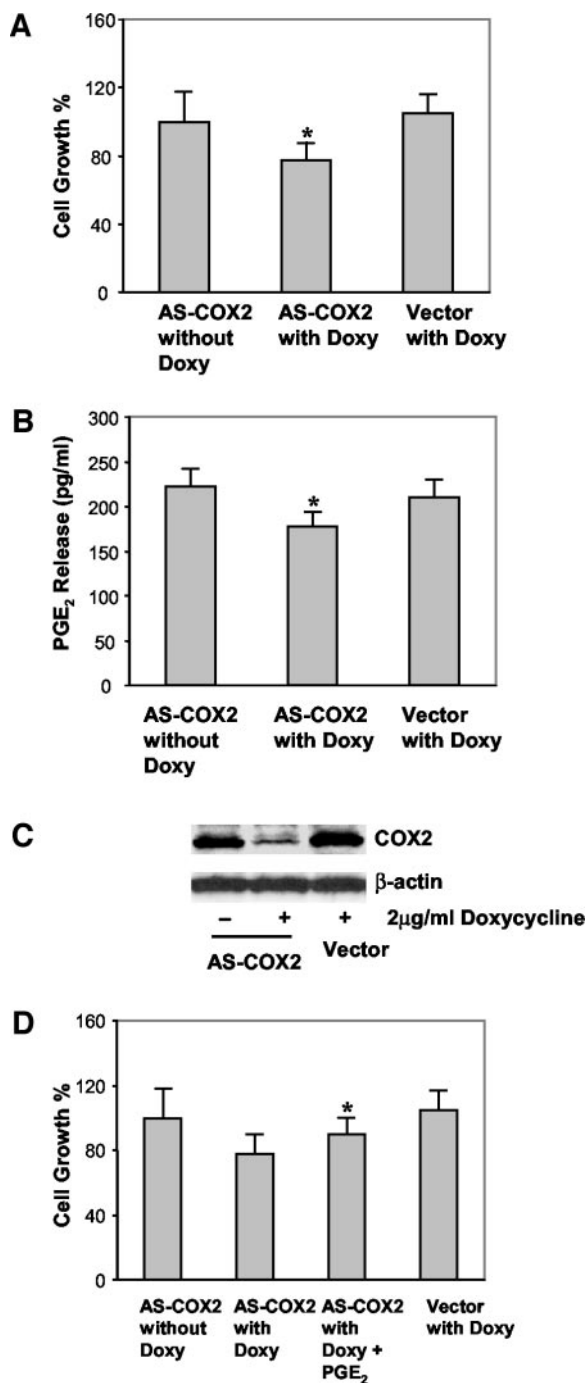


Fig. 1. Antisense depletion of cyclooxygenase-2 (COX-2) inhibits cholangiocarcinoma cell growth. The HuCCT1 cells were transfected with the COX-2 antisense plasmid (pUHD.2 neo) containing a 1.9-kb human COX-2 cDNA in antisense orientation that is controlled by the tetracycline response element (Ref. 53; provided by J. Morrow at Vanderbilt University). The HuCCT1 cells stably transfected with the COX-2 antisense plasmid were selected using G418, and the expression of antisense COX-2 RNA was induced by treatment with doxycycline (2 μg/ml for 4–5 days). The cells transfected with the empty control vector were selected as control. A, decreased growth of HuCCT1 cells with antisense inhibition of COX-2. Equal numbers of cells (1×10^5 /ml) were seeded onto the 96-well plates and cultured in serum-free medium for 48 h either with induction by 2 μg/ml doxycycline or without induction. The cell growth was determined by using WST-1 assay, and the data are presented as percentage of control \pm SD from six experiments ($*P < 0.01$ compared with antisense COX-2 cells without doxycycline treatment or control vector cells with doxycycline treatment). B, prostaglandin (PG) E₂ production in HuCCT1 cells with or without antisense inhibition of COX-2. Equal numbers of cells (3×10^5 /ml) were cultured on 12-well plates, and the 8-h spent media were collected. One hundred μl of centrifuged supernatant from each sample were analyzed for PGE₂ production using the PGE₂ enzyme immunoassay system. The production of PGE₂ in cells with antisense inhibition of COX-2 is decreased significantly when compared with controls (antisense COX-2 cells without doxycycline treatment or control vector cells with doxycycline treatment; $*P < 0.05$). The results were obtained

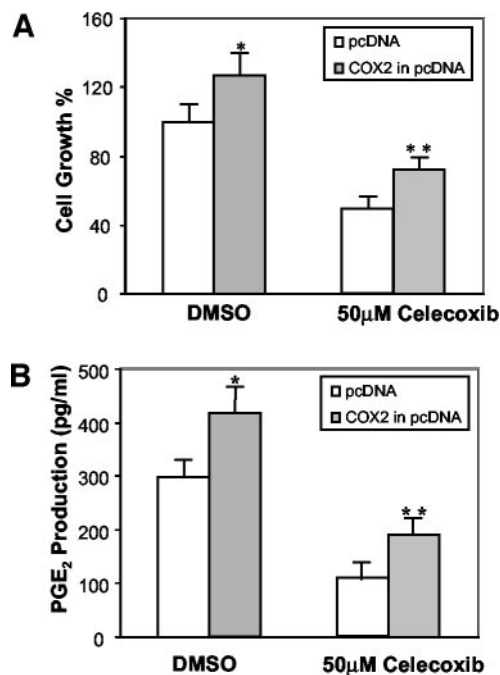


Fig. 2. Overexpression of cyclooxygenase-2 (COX-2) promotes human cholangiocarcinoma cell growth. CCLP1 cells were transfected with the COX-2 expression vector or control vector (pcDNA3), and the stably transfected cells were selected using G418 as described in "Materials and Methods." A, effect of COX-2 overexpression on cell growth. Equal numbers of cells were seeded onto the 96-well plates, and the cell growth was determined using WST-1 assay. The data are presented as percentage of control \pm SD from six experiments. Cells transfected with COX-2 expression plasmid show ~26% increase in cell growth when compared with cells transfected with pcDNA3 vector ($*P < 0.05$). In addition, the cells stably transfected with the COX-2 expression plasmid or pcDNA3 control vector were also treated with the COX-2 inhibitor celecoxib (50 μM) in serum-free medium for 24 h to determine cell growth. COX-2 overexpression partially prevents the celecoxib-induced inhibition of cell growth ($**P < 0.01$ compared with pcDNA3 cells treated with celecoxib). B, prostaglandin (PG) E₂ production in cells with or without COX-2 overexpression. Equal numbers of cells (3×10^5 /ml) were cultured on 12-well plates; the 8-h supernatants were collected for PGE₂ measurement; and the result is expressed as mean \pm SD from four experiments. The production of PGE₂ in cells with overexpression of COX-2 is significantly higher than in cells transfected with control vector under spontaneous culture conditions ($*P < 0.05$; $n = 4$). Increased PGE₂ production is also observed in COX-2 overexpressed cells in the presence of celecoxib (50 μM; $**P < 0.01$ compared with pcDNA3 cells treated with celecoxib; $n = 4$).

Celecoxib-Induced Inhibition of Cholangiocarcinoma Cell Growth Is Partially Reversed by COX-2 and PGE₂. Additional experiments were performed to examine whether COX-2 could protect cholangiocarcinoma cells from celecoxib-induced inhibition of growth. As shown in Fig. 2, overexpression of COX-2 in CCLP1 cells partially prevented the celecoxib-mediated inhibition of growth; increased PGE₂ production was observed in COX-2 overexpressed cells in the presence of celecoxib. In addition, cells treated with celecoxib showed decreased PGE₂ production (Fig. 2), and exogenous PGE₂ partially prevented the celecoxib-induced inhibition of growth (Fig. 3B). Consistent with these findings, celecoxib induced more prominent inhibition of growth in HuCCT1 cells with antisense depletion of COX-2 (see below; Fig. 6). These observations suggest that COX-2

from four individual experiments and are presented as mean \pm SD. C, Western blot analysis showing successful reduction of COX-2 protein level in cells with antisense inhibition of COX-2. Equal amount of cellular protein (20 μg) was subjected to SDS-PAGE, followed by Western blot analysis for COX-2. The same blot then was stripped and reprobed for β-actin as loading control. D, addition of PGE₂ partially reconstitutes the growth inhibition caused by antisense depletion of COX-2. HuCCT1 cells expressing antisense COX-2 were incubated with or without 10 μM PGE₂ in serum-free medium for 48 h, and the cell growth was determined using WST-1 assay. The data are presented as percentage of control \pm SD from six experiments ($*P < 0.05$ when compared with doxycycline-treated antisense COX-2 cells).

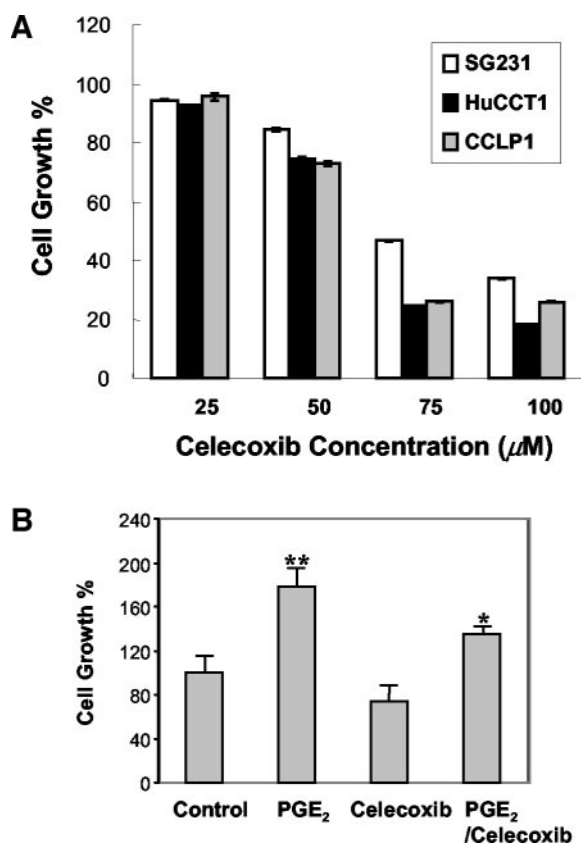


Fig. 3. The cyclooxygenase-2 (COX-2) inhibitor celecoxib inhibits human cholangiocarcinoma cell growth, which is partially prevented by prostaglandin (PG) E₂. **A**, SG231, HuCCT1, and CCLP1 cells were treated with increasing concentrations of celecoxib (25–100 μM) or vehicle DMSO (0.1%) for 24 h, and the cell growth was measured by WST-1 assay. The cells treated with celecoxib showed a dose-dependent inhibition of cell growth ($P < 0.01$). The results were obtained from at least six individual experiments for each time point and are expressed as percentage of control \pm SD. **B**, addition of PGE₂ partially prevents the celecoxib-mediated inhibition of growth. HuCCT1 cells were treated with 50 μM celecoxib for 24 h in the presence or absence of 10 μM PGE₂. The data are presented as percentage of control \pm SD from six experiments (** $P < 0.01$ compared with control; * $P < 0.05$ compared with celecoxib-treated cells).

and PGE₂ can partially prevent the celecoxib-mediated inhibition of cholangiocarcinoma cell growth.

Celecoxib-Induced Inhibition of Cell Growth Is Mediated in Part through G₁-S Arrest. To document further the mechanisms by which celecoxib inhibits cholangiocarcinoma cell growth, flow cytometry was performed to determine the cell cycle progression in cells with or without celecoxib treatment. As shown in Table 1, celecoxib induced a significant inhibition of cell cycle progression at the G₁-S checkpoint, with no effect on G₂-M transit. This result suggests that the celecoxib-induced inhibition of cholangiocarcinoma cell growth is mediated, at least in part, through cell cycle inhibition at the G₁-S checkpoint.

Celecoxib Increases Expression of cdk Inhibitors p21^{waf1/cip1} and p27^{kip1}. Because p21 and p27 play an important role in human cholangiocarcinoma growth (57–60), additional experiments were performed to examine whether celecoxib treatment might alter their expression. Fig. 4 shows that celecoxib treatment induced a significant increase of p21 and p27 in human cholangiocarcinoma cells. Basal level of p27 is detected in all of the three human cholangiocarcinoma cell lines (HuCCT1, SG231, and CCLP1); its expression is elevated significantly after treatment with 50 μM celecoxib. In HuCCT1 and CCLP1 cells, the induction of p27 appeared at 6 h (with ~2-fold increase) and progressed with longer time periods (~4.5-fold increase is observed at 48 h). In SG231 cells, the induction of p27 occurred at 12 h and peaked at 48 h. Whereas HuCCT1 cells express little or no p21 at baseline culture

conditions, ~2.5-fold increase of p21 was observed 6 h after celecoxib treatment, and this effect peaked at 12 h. Celecoxib treatment also increased the expression of p21 in SG231 and CCLP1 cells. In contrast, the levels of p18^{INK4c} (another G₁ cdk inhibitor) and GADD45 (cdc2 inhibitor) were not altered after celecoxib treatment (data not shown). These results suggest the participation of p21^{waf1/cip1} and p27^{kip1} in celecoxib-mediated G₁-S arrest in human cholangiocarcinoma cells.

We then measured the G₁-S cdk2 activity in cells with or without celecoxib treatment. Fig. 5A shows that celecoxib significantly inhibited the cdk2 activity in all of the three human cholangiocarcinoma cell lines ($P < 0.05$). The cells treated with 50 μM celecoxib for 24 h exhibited ~40–60% reduction of cdk2 activity. Western blot analysis showed that celecoxib did not alter the protein levels of cdk2 and cyclin E, indicating that the celecoxib-induced reduction of cdk2 activity is not mediated through cyclin E degradation or alteration of cdk2 protein level (Fig. 5B). Additional experiments were performed to evaluate whether celecoxib would alter the *in vitro* binding between cdk2 and p27 or p21. Human cholangiocarcinoma cells were treated with 50 μM celecoxib or DMSO vehicle for 24 h, and the cell lysates were obtained for immunoprecipitation using anti-cdk2 antibody, followed by Western blot for p27 and p21. Fig. 5C shows that treatment with 50 μM celecoxib increases the binding of p27 to cdk2 kinase complex in all three of the human cholangiocarcinoma cell lines. Celecoxib treatment also increased the association between cdk2 complex and p21 in HuCCT1 and SG231 cells, although this effect is weak in CCLP1 cells. These results further demonstrate that celecoxib induces G₁-S cell cycle arrest through induction of p27 and p21 in human cholangiocarcinoma cells.

Effect of Altered COX-2 Expression on Celecoxib-Induced Cell Growth, PGE₂ Production, and Expression of p21^{waf1/cip1} and p27^{kip1}. The direct effect of COX-2 on the expression of p21 and p27 in human cholangiocarcinoma cells was examined further by antisense inhibition and overexpression of COX-2. For antisense depletion of COX-2, HuCCT1 cells stably transfected with COX-2 antisense plasmid or control vector were cultured with or without doxycycline, and the cells were treated with celecoxib. Fig. 6A shows that celecoxib induced more prominent inhibition of growth in HuCCT1 cells with antisense depletion of COX-2 when compared with the control cells (cells transfected with the COX-2 antisense plasmid but without doxycycline induction or cells transfected with the empty vector plus doxycycline treatment). However, the COX-2 antisense cells and control cells show a comparable degree of PGE₂ reduction after celecoxib treatment (Fig. 6B). Furthermore, the celecoxib-mediated induction of p21 and p27 was not altered by antisense depletion of COX-2. As shown in Fig. 6C, although ~80% reduction of COX-2 protein was achieved in cells expressing antisense COX-2, the level of p27 and p21 was not significantly altered either under spontaneous culture conditions or after celecoxib treatment. These

Table 1 Cell cycle analysis of celecoxib-treated cholangiocarcinoma cells

Data are presented as mean (%) \pm SD ($n = 5$). Cholangiocarcinoma cells were subjected to celecoxib treatment (50 μM) for 48 h. Cells then were harvested, and the cell cycle profile was analyzed using flow cytometry.

Cell line	Phase of the cell cycle	Vehicle	Celecoxib
CCLP1	G ₁	62.1 \pm 0.1	74.4 \pm 0.8 ^a
	S	11.8 \pm 0.1	6.6 \pm 0.3
	G ₂ /M	21.7 \pm 0.2	16.6 \pm 0.8
HuCCT1	G ₁	61.8 \pm 2.5	77.6 \pm 0.7 ^a
	S	10.2 \pm 0.3	3.1 \pm 0.1
	G ₂ /M	23.5 \pm 2.2	17.3 \pm 0.6
SG231	G ₁	58.8 \pm 2.3	74.0 \pm 0.6 ^a
	S	9.2 \pm 0.3	6.5 \pm 0.2
	G ₂ /M	26.6 \pm 2.0	15.3 \pm 0.5

^a $P < 0.01$ compared with the vehicle treatment.

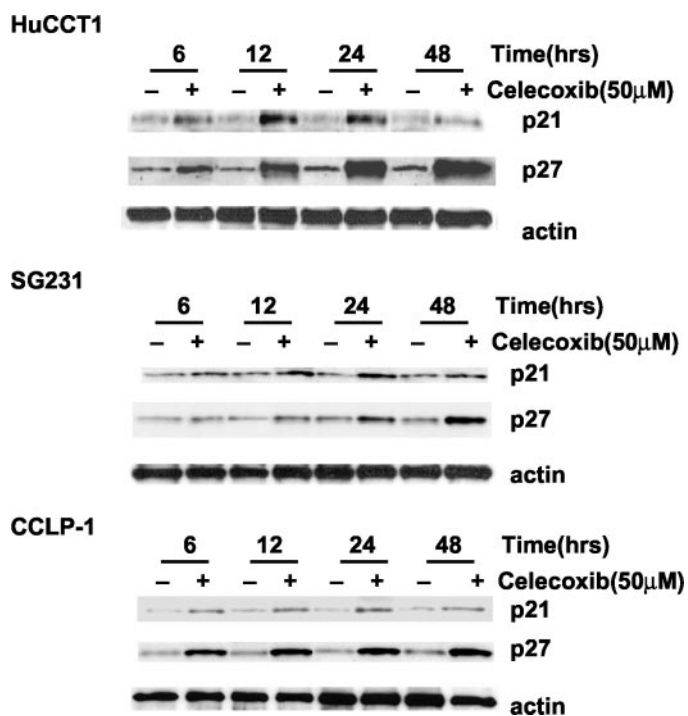


Fig. 4. Celecoxib induces the expression of p27 and p21 in human cholangiocarcinoma cells. HuCCT1, SG231, and CCLP1 cells were treated with 50 μM celecoxib or vehicle DMSO (0.1%) for 6–48 h, and the cell lysates were obtained for Western blot analysis. Equal amount of cellular protein (20 μg) was subjected to SDS-PAGE, followed by Western blot analysis for p27 and p21. Western blot for β -actin was used as loading control.

findings suggest that celecoxib-induced growth inhibition is mediated, at least in part, through mechanisms independent of COX-2 inhibition.

We then performed experiments to examine whether overexpression of COX-2 would alter celecoxib-induced p21 and p27 expression. CCLP1 cells were transfected with the human COX-2 expression plasmid or control vector, and the cell lysates were obtained to determine the expression of p27 and p21. Fig. 7 shows that although an \sim 12-fold increase of COX-2 protein was achieved after transfection with the COX-2 expression plasmid, the level of p27 and p21 in these cells was not significantly altered when compared with the vector control cells either under basal culture conditions or after celecoxib treatment. Taken together, these findings suggest that the celecoxib-mediated induction of p21^{waf1/cip1} and p27^{kip1} in cholangiocarcinoma cells is mediated predominantly through mechanism(s) independent of COX-2 inhibition.

DISCUSSION

The results in this study establish a direct role of COX-2 in the growth control of intrahepatic human cholangiocarcinoma cells. This conclusion is based on observations that overexpression of COX-2 increases PGE₂ production in cholangiocarcinoma cells and enhances cell viability, that antisense depletion of COX-2 inhibits PGE₂ production in these cells and decreases cell viability, and that PGE₂ increases cholangiocarcinoma cell growth. Moreover, our findings demonstrate that the COX-2 inhibitor celecoxib potentially inhibits cholangiocarcinoma cell growth, and this effect is mediated, in part, through inhibition of cell cycle progression. Because this study uses only intrahepatic cholangiocarcinoma cells, the potential role of COX-2 and celecoxib in the growth of extrahepatic cholangiocarcinoma cells remains to be further defined.

In eukaryotes, the cell cycle is regulated tightly at G₁-S and G₂-M

checkpoints by several protein kinases composed of a cdk subunit(s) and corresponding regulatory cyclin subunit(s), and cdk inhibitors (61, 62). The G₁-S progression is mediated by the cdks (cdk2, cdk4, and cdk6) in complex with the G₁ cyclins (cyclin D1 and cyclin E), which phosphorylate Rb, resulting in cell cycle transit. The G₁-S checkpoint is controlled by the CIP/KIP and INK4 families of cdk inhibitors, including p21^{waf1/cip1}, p27^{kip1}, and p18^{ink4c}, which bind to

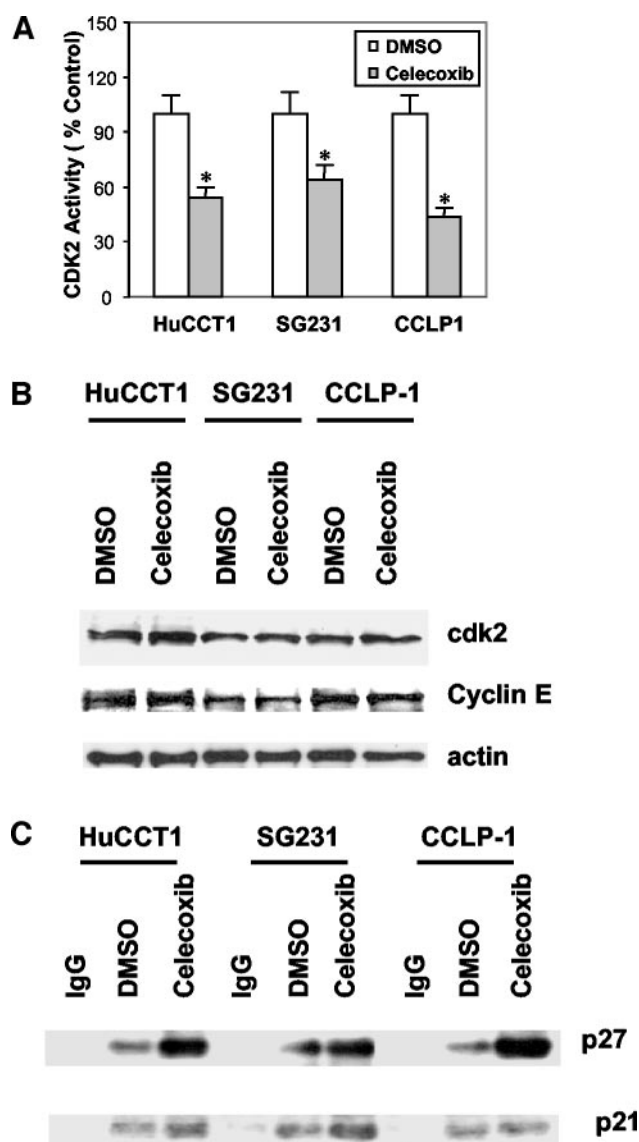


Fig. 5. Effect of celecoxib on cyclin-dependent kinase 2 (cdk2) kinase activity and cdk2/cyclin E protein level in cholangiocarcinoma cells. **A**, celecoxib inhibited cdk2 kinase activity. Human cholangiocarcinoma cells (HuCCT1, SG231, and CCLP) were treated with 50 μM celecoxib or 0.1% DMSO as control in serum-free medium for 24 h. The cell lysates were then obtained, and the cdk2 kinase was immunoprecipitated from 100 μg of cellular protein with antibody against cdk2 for 2 h at 4°C. The kinase assay was performed in 50 μl reaction buffer containing 20 μg histone H1 substrate and 10 μCi of [γ -³²P] ATP as described in "Materials and Methods." Celecoxib significantly inhibited the cdk2 kinase activity in all of the three cholangiocarcinoma cell lines ($*P < 0.01$ compared with DMSO control). The data are presented as percentage of control \pm SD from four different experiments. **B**, celecoxib did not alter the protein levels of cdk2 and cyclin E. The cells were treated with 50 μM celecoxib or 0.1% DMSO in serum-free medium for 24 h. Equal amount of cell lysate was obtained for Western blot analysis using antibodies against cdk2 and cyclin E with β -actin as loading control. **C**, celecoxib increased binding of p27 and p21 to cdk2 kinase complex. Cholangiocarcinoma cells were treated with 50 μM celecoxib for 24 h, and the cell lysates were obtained for immunoprecipitation using monoclonal anti-cdk2 antibody, followed by Western blot analysis for p27 and p21. Cell lysate incubated with mouse IgG was used as the negative control for immunoprecipitation. The cells treated with celecoxib showed increased association between cdk2 kinase complex and p27 or p21.

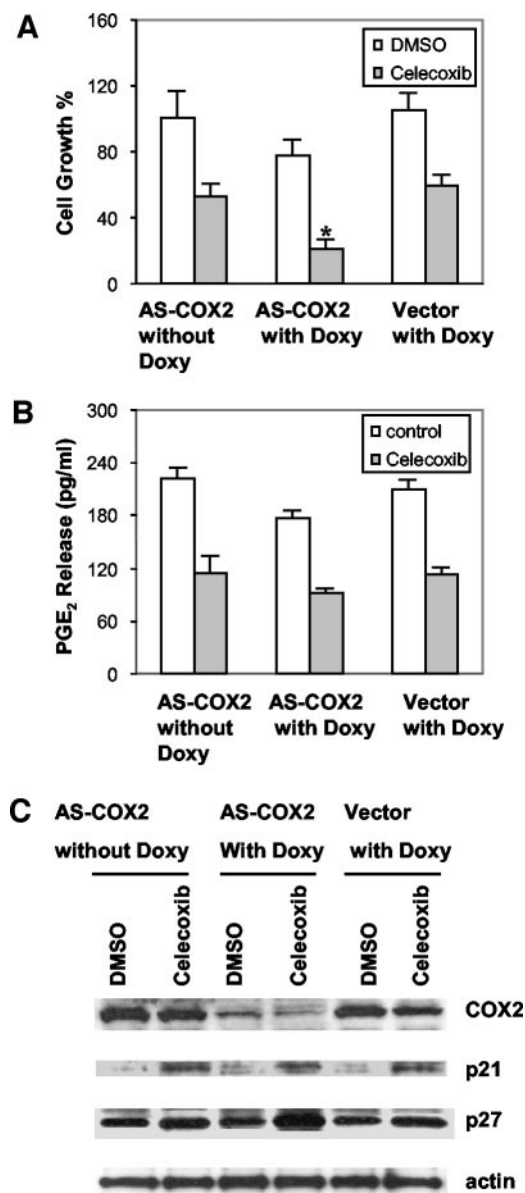


Fig. 6. Effect of celecoxib on prostaglandin (PG) E₂ production, p21/p27 expression, and cell growth in cholangiocarcinoma cells with or without antisense depletion of cyclooxygenase-2 (COX-2). **A**, effect of celecoxib on cell growth. HuCCT1 cells stably transfected with COX-2 antisense plasmid or control vector were cultured with or without doxycycline (2 μ g/ml; 5 days). Equal numbers of cells with or without COX-2 antisense depletion (1×10^5 /ml) were seeded onto the 96-well plates and treated with celecoxib or DMSO vehicle for 48 h. The cell growth was determined using WST-1 assay. Although celecoxib inhibits growth in cells without COX-2 depletion, this inhibitory effect is more prominent in cells with COX-2 depletion ($*P < 0.05$ compared with cells transfected with COX-2 antisense vector without doxycycline induction or cells transfected with control vector plus doxycycline treatment). The data represent percentage of control \pm SD from six individual experiments. **B**, effect of celecoxib on PGE₂ production. Equal numbers of cells (3×10^5 /ml) were cultured on 12-well plates and treated with celecoxib or DMSO vehicle for 8 h. The supernatants were collected for PGE₂ measurement. Celecoxib treatment resulted in a similar reduction of PGE₂ release in cells with or without antisense depletion of COX-2. The data are presented as mean \pm SD from four experiments. **C**, representative Western blot analysis showing the levels of COX-2, p21, and p27 in cells with antisense COX-2 depletion and celecoxib treatment. HuCCT1 cells transfected with COX-2 antisense plasmid or control vector were cultured in the presence or absence of doxycycline and treated with or without 50 μ M celecoxib for 24 h. Equal amount of cellular protein (20 μ g) was subjected to SDS-PAGE, followed by Western blot analysis for COX-2, p21, and p27. The same blot then was stripped and reprobed for β -actin as the loading control.

G₁ cyclin/cdk complexes and inhibit their catalytic activity, thus preventing the transition of cells from G₁ to S phase (63, 64). The transition from G₂ to M is regulated mainly by the G₂-specific kinases consisting of cdc2 and cyclin B1 (65, 66). The G₂-M checkpoint is

controlled by regulatory molecules, including GADD45 (65, 66), which interact with the cdc2/cyclin B1 complex and inhibit the cdc2 activity, thus preventing the transition of cells from G₂ to M phase. The expression of p27 is increased in lung cancer cells after treatment with the COX-2 inhibitor NS-398, suggesting that cdk inhibitors may be potential targets for COX-2 inhibitor-mediated inhibition of tumor growth (67). The cdk inhibitors p21^{waf1/cip1}, p27^{kip1}, and GADD45 have been shown recently to play a critical role in the regulation of human intrahepatic cholangiocarcinoma growth (57–60, 68). For example, decreased expression of p21^{waf1/cip1} and p27^{kip1} in human cholangiocarcinoma tissue is associated with poor patient survival (57–60); induction of GADD45 is an important mechanism for agonist-induced G₂-M phase arrest in human cholangiocarcinoma cells (68). In the present study, we found that celecoxib treatment significantly increased the p21^{waf1/cip1} and p27^{kip1} protein level in a dose- and time-dependent fashion in human cholangiocarcinoma cells, whereas the protein levels of p18^{ink4c} and GADD45 were not altered. The cells treated with celecoxib show increased binding of p21 and p27 to cdk2 kinase complex and decreased cdk2 kinase activity but no change in cdk2 and cyclin E protein levels. Consistent with these findings, flow cytometric analysis showed that celecoxib induced G₁-S arrest with no significant effect on G₂-M transition. These results provide a novel link between p21/p27 and celecoxib-mediated inhibition of intrahepatic cholangiocarcinoma cell growth.

Our findings provide evidence for the involvement of COX-2-independent mechanism in celecoxib-mediated inhibition of human intrahepatic cholangiocarcinoma cell growth. The observations that celecoxib inhibits the production of PGE₂ and that overexpression of COX-2 or addition of exogenous PGE₂ partially protects the cells from celecoxib-induced inhibition of growth suggest the involvement of COX-2 inhibition. However, the high concentration of celecoxib (50 μ M) required for inhibition of growth, the incomplete protection of celecoxib-induced inhibition of cell growth by PGE₂ or COX-2 overexpression, and the fact that overexpression or antisense depletion of COX-2 failed to alter the level of p21^{waf1/cip1} and p27^{kip1} indicate the existence of COX-2-independent effect. Thus, although celecoxib potentially inhibits human cholangiocarcinoma cell growth, its antitumor effect is mediated, at least in part, through mechanisms independent of COX-2 inhibition.

The exact mechanism by which COX-2 promotes cholangiocarcinoma cell growth remains to be defined further. Because the level of p21 and p27 was not altered significantly by COX-2 overexpression and antisense depletion or by PGE₂ treatment, it is likely that COX-2 and PGs may promote cholangiocarcinoma cell growth through other

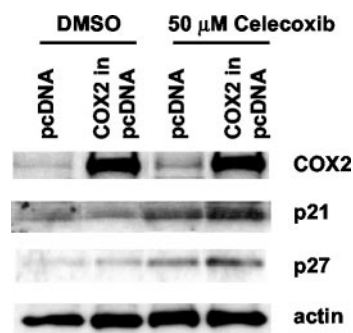


Fig. 7. The level of p27 and p21 in human cholangiocarcinoma cells with overexpression of cyclooxygenase-2 (COX-2). CCLP1 cells transfected with COX-2 expression plasmid or control vector pcDNA3 were treated with 50 μ M celecoxib or DMSO vehicle for 24 h. Equal amount of cellular protein (20 μ g) was subjected to SDS-PAGE, followed by Western blot analysis for COX-2, p27, and p21. Western blot analysis for β -actin was used as the loading control. Overexpression of COX-2 did not alter the basal level or celecoxib-induced expression of p21 and p27.

intracellular targets. In light of the limited survival benefit from current chemotherapy and the lack of effective chemoprevention for cholangiocarcinomas (69), additional studies delineating the molecular mechanisms by which COX-2 and its inhibitors modulate cholangiocarcinogenesis will likely provide important future therapeutic implications.

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