
Chang Han,1 Jing Leng,1,2 A. Jake Demetris,1 and Tong Wu1

1Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, and 2Department of Pathology, Nanjing Medical University, Nanjing, China

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 p21waf1/cip1 and p27kip1. 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 p21waf1/cip1 and p27kip1 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, cholelithiasis, 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 prostanooids 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 p21, p27, cdc2, cyclin, and cyclin-dependent kinase 2 (cdk2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The vectors against human p27, p21, cyclin E, and cyclin-dependent kinase 2 (cdk2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antisense 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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Requests for reprints: Tong Wu, Department of Pathology, University of Pittsburgh School of Medicine, Presbyterian University Hospital C902, 200 Lothrop Street, Pittsburgh, PA 15213. Phone: 412-647-9504; Fax: 412-647-5237; E-mail: wut@msx.upmc.edu.

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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 μg/ml gentamicin. SG231 cells were cultured in α-MEM with 2 mM L-glutamine, 50 μg/ml gentamicin, 10 mM HEPES, and 10% FBS. CCLP1 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 50 μg/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 0.5–2 × 10^5 cells) were plated in each well of 96-well plates. After 24 h culture to allow reattachment, the cells were then 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 quantification of cell cycle distribution was performed using the EXP302 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 7.5), 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 X 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 were then 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-morpholinopropanesulfonic 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 μg/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 analyzed for the presence of the same selective pressure. Western blot analysis for COX-2 was then 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 μg/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 μg/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.
COX-2 PROMOTES HUMAN CHOLANGIOCARCINOMA CELL GROWTH

Fig. 1. Antisense depletion of cyclooxygenase-2 (COX-2) inhibits cholangiocarcinoma cell growth. The HuCCT1 cells were transfected with the COX-2 antisense plasmid (pHBD-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 ± SD. B, Western blot analysis showing successful reduction of COX-2 protein level in cells with antisense inhibition of COX-2. One hundred μl of centrifuged supernatant from each sample were collected. One hundred μl of supernatant were collected. One hundred μl of supernatant were collected. C, Western blot analysis showing successful reduction of COX-2 protein level in cells with antisense inhibition of COX-2. One hundred μl of supernatant from each sample were analyzed for PGE_2 production using the PGE_2 enzyme immunoassay system. The production of PGE_2 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 from four independent experiments and are presented as mean ± SD. C, Western blot analysis showing successful reduction of COX-2 protein level in cells with antisense inhibition of COX-2. Equal amount of total cell 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 PGE_2 partially reconstitutes the growth inhibition caused by antisense depletion of COX-2. HuCCT1 cells expressing antisense COX-2 were incubated with or without 10 μg PGE_2 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 ± SD from six experiments (*P < 0.01 when compared with pcDNA3 cells treated with celecoxib).
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 p18Ink4c (another G1 cdk inhibitor) and GADD45 (cdk2 inhibitor) were not altered after celecoxib treatment (data not shown). These results suggest the participation of p21<sup>wt/cip1</sup> and p27<sup>kip1</sup> in celecoxib-mediated G<sub>1</sub>-S arrest in human cholangiocarcinoma cells.

We then measured the G<sub>1</sub>-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 G<sub>1</sub>-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, PGE<sub>2</sub> Production, and Expression of p21<sup>wat/cip1</sup> and p27<sup>kip1</sup>.** 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 PGE<sub>2</sub> 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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Phase of the cell cycle</th>
<th>Vehicle</th>
<th>Celecoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCLP1</td>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>61.8 ± 1.6</td>
<td>66.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>11.8 ± 0.1</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>G&lt;sub&gt;2&lt;/sub&gt;/M</td>
<td>21.7 ± 0.2</td>
<td>16.6 ± 0.8</td>
</tr>
<tr>
<td>HuCCT1</td>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>61.8 ± 2.5</td>
<td>77.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>10.2 ± 0.3</td>
<td>31.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>G&lt;sub&gt;2&lt;/sub&gt;/M</td>
<td>23.5 ± 2.8</td>
<td>17.3 ± 0.6</td>
</tr>
<tr>
<td>SG231</td>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>58.8 ± 2.3</td>
<td>74.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>9.2 ± 0.3</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>G&lt;sub&gt;2&lt;/sub&gt;/M</td>
<td>26.6 ± 2.0</td>
<td>15.3 ± 0.5</td>
</tr>
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*P < 0.01 compared with the vehicle treatment.

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**Fig. 3.** The cyclooxygenase-2 (COX-2) inhibitor celecoxib inhibits human cholangiocarcinoma cell growth, which is partially prevented by prostaglandin (PG) E<sub>2</sub>. 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 ± SD. B, addition of PGE<sub>2</sub> 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 PGE<sub>2</sub>. The data are presented as percentage of control ± SD from six experiments (***P < 0.01 compared with control; *P < 0.05 compared with celecoxib-treated cells).**
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 ~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<sup>waf1/cip1</sup> and p27<sup>kip1</sup> 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 PGE<sub>2</sub> production in cholangiocarcinoma cells and enhances cell viability, that antisense depletion of COX-2 inhibits PGE<sub>2</sub> production in these cells and decreases cell viability, and that PGE<sub>2</sub> increases cholangiocarcinoma cell growth. Moreover, our findings demonstrate that the COX-2 inhibitor celecoxib potently 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 G<sub>1</sub>-S and G<sub>2</sub>-M checkpoints by several protein kinases composed of a cdk subunit(s) and corresponding regulatory cyclin subunit(s), and cdk inhibitors (61, 62). The G<sub>1</sub>-S progression is mediated by the cdks (cdk2, cdk4, and cdk6) in complex with the G<sub>1</sub> cyclins (cyclin D1 and cyclin E), which phosphorylate Rb, resulting in cell cycle transit. The G<sub>1</sub>-S checkpoint is controlled by the CIP/KIP and INK4 families of cdk inhibitors, including p21<sup>waf1/cip1</sup>, p27<sup>kip1</sup>, and p18<sup>ink4c</sup>, which bind to

![Fig. 4. Celecoxib induces the expression of p27 and p21 in human cholangiocarcinoma cells.](image)

![Fig. 5. Effect of celecoxib on cyclin-dependent kinase 2 (cdk2) kinase activity and cdk2/cyclin E protein level in cholangiocarcinoma cells.](image)
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^{ink4a} and GADD45 were not altered. The cells treated with celecoxib showed 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-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 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 COX-2-independent effect. Thus, although celecoxib potently 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 PGE2 may promote cholangiocarcinoma cell growth through other
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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