Cyclooxygenase-2-derived Prostaglandin E₂ Promotes Human Cholangiocarcinoma Cell Growth and Invasion through EP₁ Receptor-mediated Activation of the Epidermal Growth Factor Receptor and Akt*}

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Cyclooxygenase-2 (COX-2)-mediated prostaglandin synthesis has recently been implicated in human cholangiocarcinogenesis. This study was designed to examine the mechanisms by which COX-2-derived prostaglandin E₂ (PGE₂) regulates cholangiocarcinoma cell growth and invasion. Immunohistochemical analysis revealed elevated expression of COX-2 and the epidermal growth factor (EGF) receptor (EGFR) in human cholangiocarcinoma tissues. Overexpression of COX-2 in a human cholangiocarcinoma cell line (CCLP1) increased tumor cell growth and invasion in vitro and in severe combined immunodeficient mice. Overexpression of COX-2 or treatment with PGE₂ or the EP₁ receptor agonist ONO-DI-004 induced phosphorylation of EGFR in enhanced tumor cell proliferation and invasion, which were inhibited by the EP₁ receptor small interfering RNA or antagonist ONO-8711. Treatment with PGE₂ or ONO-DI-004 enhanced binding of EGFR to the EP₁ receptor and c-Src. Furthermore, PGE₂ or ONO-DI-004 treatment also increased Akt, which was blocked by the EGFR tyrosine kinase inhibitor AG 1478 and an EGFR antagonist. Inhibition of the EP₁ receptor and c-Src. Furthermore, PGE₂ or ONO-DI-004 treatment also increased Akt, which was blocked by the EGFR tyrosine kinase inhibitor AG 1478 and an EGFR antagonist. This study reveals a novel cross-talk between the EP₁ receptor and Akt that synergistically promotes cancer cell growth and invasion.

Cholangiocarcinoma is a highly malignant epithelial neoplasm of the biliary tree with a high rate of mortality (1–3). Although it composes ~10–15% of hepatobiliary neoplasms, its incidence is increasing (1–4). The tumor often arises from background conditions that cause long-standing inflammation, injury, and reparative biliary epithelial cell proliferation, such as primary sclerosing cholangitis, liver fluke infestation (Opisthorchis viverrini and Clonorchis sinensis), hepatitis, and complicated fibropolycystic diseases. Early diagnosis of cholangiocarcinoma is difficult, and there is presently no effective treatment for patients with the advanced disease or any effective therapy for its chemoprevention. Although it is well known that the chronic inflammatory conditions involving the bile ducts predispose patients to the development of cholangiocarcinoma, the molecular mechanisms linking chronic inflammation to malignant transformation remain to be further defined.

Recent evidence suggests that cyclooxygenase-2 (COX-2) and the epidermal growth factor (EGF) receptor family, in gallbladder and biliary tree epithelia results in the development of gallbladder adenocarcinoma in mice (10). Bile acids (including obstruction and the tumor promoter in cholangiocarcinogenesis, COX-2 expression through transactivation of cultured non-neoplastic and neoplastic cholangiocytes further promotes tumor growth (12). Consistent with these findings, COX-2 inhibits Fas ligand-mediated apoptosis in cholangiocarcinoma cells (13), and treatment with exogenous PGE₂ increases cholangiocarcinoma cell growth and prevents apoptosis (12–17). Moreover, prostaglandin signaling also mediates cholangiocarcinoma cell growth induced by hepatocyte growth factor and interleukin-6 (15) and subverts mito-inhibition induced by transforming growth factor-β (18). Conversely, inhibitors of COX-2 prevent cholangiocarcinoma cell growth and induce apoptosis, even though their actions may involve both COX-2-dependent and COX-2-independent mechanisms (12, 14, 16, 17).

Whereas evidence for COX-2 and prostaglandin signaling in cholangiocarcinogenesis is compelling, the mechanism for their actions remains largely unknown. Prostanoids exert their biological actions primarily via their respective G-protein-coupled receptor (GPCR) superfamily of seven-transmembrane spanning G-proteins on the cell-surface membrane (19, 20). The most abundant prostaglandin in cholangiocarcinoma cells is PGE₂ (15). There are four EP receptor subtypes that can bind to PGE₂: EP₁, EP₂, EP₃, and EP₄. The EP₁ receptor is coupled with the Gₛ protein and thus signals through phospholipase C activation of the molecular pathways that mediate prostanoid actions.
and intracellular Ca\(^{2+}\). The EP\(_2\) and EP\(_4\) receptors are coupled with the G\(\alpha\) protein, signaling through elevation of intracellular cAMP levels and activation of protein kinase A. The EP\(_{1}\) receptor is coupled with the G\(\alpha\) protein and signals through reduction of intracellular cAMP levels. The interaction between prostaglandins and the specific GPCRs depends on the differential expression of individual receptor subtypes in tissues and cells, their binding affinity for prostaglandins, and the differential activation of each receptor (19–23). To date, there is no information on EP receptor subtypes or their specific functions in cholangiocarcinoma cells.

In light of recent evidence showing activation of EGFR by GPCRs (24–26) and enhanced EGFR activation in cholangiocarcinoma cells (27, 28), this study was designed to evaluate our hypothesis that the G-protein-coupled EP receptor may transactivate EGFR and that this mechanism may be important in cholangiocarcinogenesis. Our data reveal that COX-2-derived PGE\(_2\) transactivates EGFR through the EP\(_1\) receptor in human cholangiocarcinoma cells and that this process involves the c-Src protein. Transactivation of EGFR subsequently induces Akt phosphorylation and enhances tumor cell proliferation and invasion. Furthermore, we show that activation of EGFR by EGF increases COX-2 expression, whereas blocking PGE\(_2\) synthesis or the EP\(_1\) receptor attenuates EGF-induced EGFR phosphorylation, suggesting that the COX-2/PGE\(_2/\)EP\(_1\) receptor pathway also modulates activation of EGFR induced by its cognate ligand. Our findings reveal a novel interaction between COX-2-derived PGE\(_2\) and EGFR signaling that synergistically promotes cancer cell growth and invasion.

**EXPERIMENTAL PROCEDURE**

**Materials—** A Minimal essential medium (MEM) plus antibiotics, LipofectaminTM Plus reagent was purchased from Life Technologies; pCMV-EGFP was purchased from BD Biosciences. The COX-2 inhibitor NS-398, the Src family tyrosine kinase inhibitor PP2 (4-aminophenol), the EGFR tyrosine kinase inhibitors AG 1478 and PD 153035, PGE\(_2\), the EP receptor antagonists EP1 receptor antagonist ONO-8711, and the EP3 receptor agonist ONO-AE-248 were provided by the Ono Pharmaceutical Co., Ltd. (Osaka, Japan), EGF antagonist AG1024, and the EP2 receptor antagonist PGE\(_2\) alcohol were purchased from Cayman Chemical Co., Inc. (Ann Arbor, MI). \(^{3}H\)Thymidine was purchased from PerkinElmer Life Sciences. Antibodies against the human EGFR and phosphotyrosine (PY99) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Actin antibody and anti-phospho-Akt (Thr\(^{308}\)) antibody were purchased from Cell Signaling (Beverly, MA). Antibodies against the EP\(_1\), EP\(_2\), EP\(_3\), and EP\(_4\) receptors and COX-2 antibody were purchased from Cayman Chemical Co., Inc. Anti-\(\beta\)-actin antibody was purchased from Sigma. Horseradish peroxidase-linked streptavidin and chemiluminescence detection reagents were purchased from Amersham Biosciences. Severely combined immunodeficient (SCID) mice (4–5 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME).

**Cell Culture and Transfections—** Human cholangiocarcinoma cell lines, including CCLP1, SK21, and HuCCT1, were cultured according to our previously described methods (12, 15, 29). For transient transfection assays, the cultured cells were seeded at a concentration achieving 80\% confluent in 6-well plates 18 h before transfection. The cells were transfected with the COX-2 expression plasmid (cloned into pcDNA) or the pcDNA control vector (1 \(\mu\)g of plasmid for each transfection using LipofectaminTM Plus reagent). The cells with optimal overexpression of COX-2 were confirmed by immunoblotting and subsequently used for further experiments.

**Cell Invasion Assay—** The cell invasion assay was performed in Matrigel-coated Transwell chambers (BD Biosciences). Cells (4 \(\times\) 10\(^4\)) in 500 \(\mu\)l of serum-free medium were seeded in the upper chamber in the presence or absence of different inhibitors or EP\(_1\) receptor antagonist. Serum-free medium-containing vehicle, PGE\(_2\), or different EP receptor agonists were added to the lower chamber as chemoattractants. To determine the invasiveness of CCLP1 cells with antisense inhibition of EP receptors, the cells transfected with the antisense oligonucleotides for individual EP receptors or control cells were seeded in the upper chamber in serum-free medium, with the lower chamber containing vehicle or PGE\(_2\) in serum-free medium. After 24 h of incubation at 37 °C, the cells on the upper surface of the filter were mechanically removed with a cotton swab. The filter was fixed and stained using a Diff-Quik staining kit (Dade Behring Inc., Newark, DE) according to the manufacturer’s instructions. The invading cells on the lower surface were counted under a microscope (magnification \(\times 50\)). Five fields were counted per filter, and 4 wells were used for each treatment.

**Phosphorylation of EGFR—** CCLP1 cells were transfected with the COX-2 expression plasmid or EP\(_1\) receptor small interfering RNA (siRNA) or treated with PGE\(_2\), EP\(_1\) receptor agonist/antagonist, or EGFR inhibitors, and cell lysates were obtained. Equal amounts of the cell lysates were preincubated with 5 \(\mu\)g/ml rabbit anti-human EGFR polyclonal antibody at 4 °C, followed by the addition of 20 \(\mu\)l of protein A/G-agarose (Santa Cruz Biotechnology, Inc.). The mixtures were incubated overnight at 4 °C. After three washes with the same hypotonic buffer, the pellet was used for immunoblotting with anti-phosphotyrosine monoclonal antibody PY99.

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**Binding of EGFR to the EP\(_1\) Receptor and c-Src—** The binding complexes of the EGFR and the EP\(_1\) receptor and c-Src in CCLP1 cells were determined by immunoprecipitation and Western blotting. Confluent CCLP1 cells were serum-starved for 24 h and then treated with ONGDI-004 or PGE\(_2\) for 15 min. Cell lysates were subsequently prepared for immunoprecipitation with a antibody against the EP\(_1\) receptor or c-Src, and the immunoprecipitated proteins were subjected to SDS-PAGE and Western blotting. The specific sequences of EP receptor antisense oligonucleotides—Human EP\(_1\), EP\(_2\), EP\(_3\), and EP\(_4\) receptors and control phosphorothioate-modified DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Cor- nelius, OR). The specific sequences for the EP receptor antisense oligonucleotide were designed as previously described (30, 31) with modification and were as follows: EP\(_1\), 5'-GCAAAGCTCTAGCGACG-3' (nucleotides 113–130); EP\(_2\), 5'-ACTGGAATCTGAGG-3' (nucleotides 14–28); EP\(_3\), 5'-AGGTGGTACGTGG-3' (nucleotides 236–252); and EP\(_4\), 5'-AGGTGGTACGTGG-3' (nucleotides 208–222). The CCLP1 cell cultures at 50\% confluence were transfected with 5 \(\mu\)g EP receptor antisense or control phosphorothioate-modified oligonucleotide with LipofectaminTM 2000. After 24 h of incubation, the cells were harvested to analyze cell invasion in Matrigel-coated Transwell chambers as described above, and cell lysates were obtained for Western blotting to determine the protein levels of the corresponding EP receptors.

**RNA Isolation and Reverse Transcription-PCR—** Total RNA from the cultured cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The primers specific for each EP receptor were designed as described previously (31, 32) with modification and were synthesized by Integrated DNA Technologies, Inc. For analysis of COX-2 expression, the sequences of the primers were as follows: EP\(_1\), (32 bp), 5'-CTTGGTTCGATCAGTGGTGGT-3'; forward, nucleotides 1031–1035); EP\(_1\), (32 bp), 5'-GACACAGCTCAGCTGTCGAG-3'; (reverse, nucleotides 1312–1335); EP\(_4\), (654 bp), 5'-GTTCTCTCAACAGCTCTCAGC-3' (forward, nucleotides 364–387) and 5'-CTTGGTTCGATCAGTGGTGGT-3'; (reverse, nucleotides 970–1018); EP\(_3\), (582 bp), 5'-GACACAGCTCAGCTGTCGAG-3' (forward, nucleotides 364–387) and 5'-CTTGGTTCGATCAGTGGTGGT-3'; (reverse, nucleotides 970–1018); EP\(_1\), (343 bp), 5'-TGGTATTAGTGTCGAGC-3' (forward, nucleotides 782–837) and 5'-GACACAGCTCAGCTCAGC-3' (reverse, nucleotides 1178–1195). Amplification of each EP receptor with \(\beta\)-actin as an internal control in each reaction was carried out with a TitaniumTM two-step reverse transcription-PCR kit (BD Biosciences) according to the standard protocol.

**Gelatin Zymography—** The matrix metalloprotease (MMP) proteolytic activity in the supernatants of the treated cells was analyzed for the level of MMP-2 by zymography. Briefly, an equal amount of serum-free medium from cells with different treatments was loaded onto 10\% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Invitrogen). After electrophoresis, SDS was removed from the gel by incubation in 2.5\%
Triton X-100 at room temperature for 30 min with gentle shaking. The gel was washed well with distilled water and incubated at 37 °C for 18–36 h in a developing buffer containing 50 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, 5 mM CaCl₂, and 0.02% NP-40. The gel was then stained with a solution of 30% methanol, 10% glacial acetic acid, and 0.5% Coomassie Blue G-250 and then destained in the same solution without dye. Proteinase activity was detected as unstained bands on a blue background representing areas of gelatin digestion.

**Immunohistochemical Analysis for COX-2 and EGFR**—Eleven archival formalin-fixed, paraffin-embedded specimens of human cholangiocarcinoma and surrounding non-tumor liver tissue were obtained from the University of Pittsburgh Medical Center. The tissue specimens were utilized for immunohistochemical analysis for COX-2 and EGFR following the protocol recommended by the University of Pittsburgh. None of the cases used in this study had patient identifiers, and strict confidentiality was maintained. For immunohistochemical staining of COX-2 and EGFR using human cholangiocarcinoma tissue, 5-μm-thick tissue sections of formalin-fixed and paraffin-embedded sections were deparaffinized and rehydrated, followed by microwave retrieval of antigen according to standard procedures. The slides were incubated overnight at 4 °C with 1:100 diluted anti-human COX-2 monoclonal antibody (obtained from Cayman Chemical Co., Inc.) and anti-EGFR antibody (obtained from Dako Corp.). Following repeated washings, the slides were incubated with biotin-conjugated secondary antibody (1:100) in room temperature. After probing, the avidin-peroxidase complex was added, and finally, 3,3'-diaminobenzidine substrate was utilized for color development. The slides were then counterstained with hematoxylin. The intensity of staining for COX-2 and EGFR was scored in each specimen on a scale of 0–3, in which 0 = negative staining, 1 = weakly positive staining, 2 = moderately positive staining, and 3 = strongly positive staining. For each sample, 10 random high power fields were scored. The immunoreactivity for COX-2 and EGFR in each sequential section was documented and compared.

**Inoculation of CCLP1 Cells into SCID Mice**—Cultured CCLP1 cells were transfected with the COX-2 expression plasmid or pcDNA3 control vector, and the stably transfected cells were selected (5 × 10⁵) suspended in phosphate-buffered saline (PBS) and injected into the livers of SCID mice under anesthesia (33). After tumor cell implantation, the mice were divided into five groups: free conditions, fed standard chow, fed water, fed ethanol, and fed water. The mice were closely observed for up to 5 weeks after injection to determine tumor volume. Tumor volume was calculated using the formula (L × W × D) / 2, where L is length, W is width, and D is depth. The survival protocol used followed the recommendations of the University of Pittsburgh Institutional Animal Care and Use Committee.

**RESULTS**

**Expression of COX-2 and EGFR is Increased in Human Cholangiocarcinoma Tissues**—Immunohistochemical stains were utilized to determine expression of COX-2 and EGFR in sequential sections of human cholangiocarcinoma and non-neoplastic bile ducts. Eleven paired cholangiocarcinomas and their matched non-tumor liver tissues were analyzed. The average COX-2 staining intensity in cholangiocarcinoma (2.10 ± 0.54) was significantly higher than that in the non-neoplastic bile duct epithelium (0.64 ± 0.10; p < 0.01). The average EGFR staining intensity in cholangiocarcinoma (1.45 ± 0.92) was also significantly higher than that in the non-neoplastic bile duct epithelium (0.14 ± 0.10; p < 0.01). Sequential sections from individual tissue specimens revealed cytoplasmic staining of COX-2 and membrane staining of EGFR in the same tumor cells (Fig. 1).

**COX-2-derived PGE₂ Induces EGFR Phosphorylation in Cholangiocarcinoma Cells**—Increased expression of COX-2 and EGFR in human cholangiocarcinoma cells suggests a possible interconnection between these two signaling pathways during cholangiocarcinogenesis. Given that COX-2-derived prostaglandins mediate effects primarily through specific plasma membrane receptors that are coupled with G-proteins and that certain GPCRs are known to activate EGFR (24–26), we postulated that COX-2 may promote tumor growth through activation of EGFR. To evaluate this hypothesis, we first examined the potential effect of COX-2 on phosphorylation of EGFR in cultured human cholangiocarcinoma cells. As shown in Fig. 2A, overexpression of COX-2 in CCLP1 cells enhanced EGFR phosphorylation. The COX-2-overexpressing cells exhibited increased PGE₂ production compared with the control vector cells (430 versus 285 pg/ml). Consistent with this, treatment of CCLP1 cells with PGE₂ induced rapid phosphorylation of EGFR (Fig. 2B). These findings indicate that COX-2-derived PGE₂ enhances EGFR phosphorylation in human cholangiocarcinoma cells.

**The EP₁ Receptor Plays a Key Role in PGE₂-induced EGFR Phosphorylation**—Selective EP receptor subtype analogs were next utilized to determine their effects on PGE₂-induced EGFR phosphorylation. As shown in Fig. 2C, treatment with the EP₁ receptor agonist ONO-DI-004 caused a significant increase in EGFR phosphorylation, an effect similar to that induced by PGE₂. In addition, PGE₂-induced EGFR phosphorylation was inhibited by the selective EP₁ receptor antagonist ONO-8711 as well as by the EGFR tyrosine kinase inhibitors AG 1478 and PD 153035. These findings provide pharmacological evidence for the involvement of the EP₁ receptor in PGE₂-induced EGFR phosphorylation. The observation that PP2 partially prevented PGE₂-induced EGFR phosphorylation suggests the involvement of the Src protein in this process.

The role of the EP₁ receptor in PGE₂-induced EGFR phosphorylation was further examined by siRNA suppression of the EP₁ receptor. In this approach, CCLP1 cells transfected with EP₁ receptor siRNA or control RNA were treated with either
FIG. 2. PGE₂ induces EGFR phosphorylation via the EP₁ receptor in CCLP1 cells. A, effect of COX-2 overexpression on EGFR phosphorylation. CCLP1 cells were transfected with the COX-2 expression plasmid in serum-free medium for 24 h. EGFR phosphorylation was determined by immunoprecipitation with anti-EGFR antibody and immunoblotting with anti-phosphotyrosine antibody (first panel). Total EGFR in the immunoprecipitate was determined by reprobing the same blot with anti-EGFR antibody (second panel). An equal amount of the same cell lysate was used for Western blotting to detect COX-2 expression (third panel), which was reprobed with actin (fourth panel). B, effect of PGE₂ on EGFR phosphorylation. CCLP1 cells cultured in serum-free medium for 24 h were treated with 10 μM PGE₂ for the indicated times, and cell lysates were obtained. EGFR phosphorylation was determined by immunoprecipitation with anti-EGFR antibody, followed by immunoblotting with anti-phosphotyrosine antibody (upper panel). Total EGFR in the immunoprecipitates was determined by reprobing the same blot with anti-EGFR antibody (middle panel). Quantitative analysis of EGFR phosphorylation was performed by determining the ratio between the EGFR protein and phosphorylation levels from three different experiments (lower panel). *, p < 0.01 compared with the control. C, the EP₁ receptor antagonist ONO-8711 blocks PGE₂-induced EGFR phosphorylation. CCLP1 cells were serum-starved for 24 h before being treated with ONO-8711, AG 1478, PD 153035, or PP2 for 30 min. The cells were subsequently treated with 10 μM PGE₂ for 30 min, and cell lysates were obtained. EGFR phosphorylation was determined by immunoprecipitation with anti-EGFR antibody, followed by immunoblotting with anti-phosphotyrosine antibody (upper panel). Total EGFR in the immunoprecipitates was determined by reprobing the same blot with anti-EGFR antibody (middle panel). Quantitative analysis of EGFR phosphorylation was performed by determining the ratio between the EGFR protein and phosphorylation levels from three different experiments (lower panel). Increased EGFR phosphorylation was observed after treatment with ONO-DI-004 or PGE₂ at 10 μM (*, p < 0.01 compared with the control). PGE₂-induced EGFR phosphorylation was significantly blocked by pretreatment with the EP₁ receptor antagonist ONO-8711, the EGFR kinase inhibitor AG 1478 or PD 153035, or the c-Src inhibitor PP2 (**, p < 0.05 compared with PGE₂ treatment). D, RNAi suppression of the EP₁ receptor inhibits EGFR phosphorylation induced by PGE₂ and the EP₁ receptor agonist ONO-DI-004. CCLP1 cells were transfected overnight with EP₁ receptor siRNA or control RNA in serum-free medium and then treated with PGE₂ or ONO-DI-004 at 10 μM for 30 min. EGFR phosphorylation was determined by immunoprecipitation with anti-EGFR antibody and immunoblotting with anti-phosphotyrosine antibody (upper panel). Total EGFR in the immunoprecipitates was determined by reprobing the same blot with anti-EGFR antibody (middle panel). Quantitative analysis of EGFR phosphorylation was carried out by calculating the ratio between the EGFR protein and phosphorylation levels from three different experiments (lower panel). RNAi suppression of EP₁ receptor expression significantly inhibited EGFR phosphorylation induced by either ONO-DI-004 (*) or PGE₂ (**, p < 0.01). E, representative Western blot showing the level of EP₁ protein in CCLP1 cells transfected with EP₁ receptor siRNA or control RNA and non-transfected cells.
**PGE₂ Transactivates EGFR through the EP₁ Receptor**

PGE₂ or ONO-DI-004 at 10 μM to determine EGFR phosphorylation. RNAi suppression of the EP₁ receptor significantly inhibited phosphorylation of EGFR induced by PGE₂ or the EP₁ receptor agonist ONO-DI-004 (Fig. 2D). The efficacy of EP₁ receptor depletion in this system was verified by Western blot analysis, showing successful reduction of EP₁ protein in cells transfected with EP₁ receptor siRNA (Fig. 2E).

**Detection of the EP₁ Receptor-EGFR Complex in Human Cholangiocarcinoma Cells**—To further examine the role of the EP₁ receptor in PGE₁-induced EGFR activation, immunoprecipitation and Western blot experiments were performed to determine whether the EP₁ receptor associates with EGFR in cells. In these experiments, CCLP1 cells were treated with the EP₁ receptor agonist ONO-DI-004 or PGE₂ at 10 μM for 30 min, and cell lysates were then prepared and subjected to immunoprecipitation with anti-EP₁ receptor antibody, followed by immunoblotting with anti-EGFR antibody. Although a low level of the EP₁ receptor-EGFR complex was detected in the control cells, treatment with ONO-DI-004 or PGE₂ at 10 μM to determine EGFR phosphorylation in CCLP1 cells. Increased Akt phosphorylation was observed in cells treated with either PGE₂ or the EP₁ receptor agonist ONO-DI-004 (Fig. 4). PGE₂-induced Akt phosphorylation was blocked by the EP₁ receptor antagonist ONO-8711, the EGFR tyrosine kinase inhibitors AG 1478 and PD 153035, and the Src inhibitor PP2. These findings indicate that PGE₂-induced Akt phosphorylation is mediated, at least in part, through activation of EGFR by the EP₁ receptor and that this process may involve the Src protein family.

**Involvement of the EP₁ Receptor, EGFR, and Akt in COX-2- and PGE₂-induced Cholangiocarcinoma Cell Growth and Invasion**—To determine the direct effect of COX-2 on cholangiocarcinoma cell growth, cultured CCLP1 cells were transfected with the COX-2 expression plasmid or pcDNA3 control vector, and the stably transfected cells were selected using G418. The selected cells were then analyzed for tumor cell growth in vitro and in SCID mice. An ~26% increase in in vitro cell proliferation was observed in the COX-2-transfected cells compared with the control vector cells (\( p < 0.05 \)). When these cells were inoculated into the livers of SCID mice for 4 weeks, the COX-2-transfected cells developed larger tumor volumes compared with the control vector cells (1170.3 ± 261.8 mm³ for the COX-2 group versus 272.1 ± 40.0 mm³ for the control vector group (\( n = 4 \); \( p < 0.01 \)). The COX-2-transfected tumors also exhibited enhanced tumor invasiveness (invasion to the adjacent intestine, diaphragm, and abdominal wall) compared with the vector-transfected tumors (Fig. 5A). Consistent with these find-
FIG. 5. COX-2 and PGE\textsubscript{2} promote cholangiocarcinoma cell growth and invasion: effect of inhibiting EP\textsubscript{1} receptor-mediated EGFR activation. A, overexpression of COX-2 in CCLP\textsubscript{1} cells enhances tumor growth and invasion in SCID mice. CCLP\textsubscript{1} cells were transfected with the COX-2 expression plasmid or pcDNA3 control vector. The stably transfected cells selected using G418 were inoculated into the livers of SCID mice to monitor tumor growth. The gross photos depict representative tumors 4 weeks after hepatic inoculation. Tumor size was measured using a micrometer caliper. The COX-2-transfected tumor exhibited increased size and invasiveness (invasion to the adjacent intestine, diaphragm, and abdominal wall) compared with the vector-transfected tumor. B, PGE\textsubscript{2}-induced tumor cell invasion is inhibited by the EP\textsubscript{1} receptor antagonist ONO-8711, the Src family inhibitor PP2, the phosphatidylinositol 3-kinase inhibitor LY 294002, and the EGFR tyrosine kinase inhibitors AG 1478 and PD 153035. CCLP\textsubscript{1} cells (4 x 10\textsuperscript{4}) were seeded in the upper chamber in the presence or absence of ONO-8711, PP2, LY 294002, AG 1478, or PD 153035. Medium containing ONO-DI-004, PGE\textsubscript{2}, or vehicle was added to the lower chamber. After 24 h, the cells on the upper surface of the filter were removed, and the filter was fixed and stained. The cells on the lower surface were counted under a microscope (magnification x50). Five fields were counted per filter, and 4 wells were used for each treatment. The experiments were repeated three times. PGE\textsubscript{2} or ONO-DI-004 significantly increased cell invasiveness (*, \textit{p} < 0.05 compared with the control), and this effect was inhibited by ONO-8711, PP2, LY 294002, AG 1478, or PD 153035 (**, \textit{p} < 0.05 compared with PGE\textsubscript{2} treatment alone; ***, \textit{p} < 0.01 compared with ONO-DI-004 treatment alone). C, MMP-2 activity in CCLP\textsubscript{1} cells overexpressing COX-2 or treated with PGE\textsubscript{2}. CCLP\textsubscript{1} cells were transfected with the COX-2 expression plasmid or treated with PGE\textsubscript{2}, in the presence or absence of inhibitors. The supernatants were collected for zymographic analysis as described under "Experimental Procedures." Panel a, overexpression of COX-2 increases MMP-2 activity; panel b, PGE\textsubscript{2}-induced MMP-2 activity is blocked by ONO-8711, AG 1478, PP2, and LY 294002. D, PGE\textsubscript{2}-induced cell proliferation is blocked by ONO-8711, AG 1478, and PD 153035. Serum-starved CCLP\textsubscript{1} cells were cultured in the presence or absence of ONO-8711, AG 1478, or PD 153035 for 30 min prior to the addition of ONO-DI-004, PGE\textsubscript{2}, or vehicle. \textsuperscript{[3H]}Thymidine incorporation was measured during the final 24 h of culture. The data were obtained from four individual experiments. *, \textit{p} < 0.05 compared with the control; **, \textit{p} < 0.01 compared with PGE\textsubscript{2} treatment.
and proliferation were attenuated by the EP1 receptor antagonist ONO-8711. RNAi suppression of the EP1 receptor also inhibited PGE2-induced cholangiocarcinoma cell invasion and proliferation (Fig. 6). Moreover, PGE2-induced cell invasion and proliferation are transduced by the EGFR tyrosine kinase inhibitors AG 1478, or PD 153035, by the Src inhibitor PP2, and by inhibition of Akt with LY 294002 (Fig. 5, B–D). Similarly, the EP1 receptor agonist butaprost, the EP3 receptor agonist ONO-AE-248, and the EP4 receptor agonist PGE1 alcohol exhibited no effect on tumor cell invasion, whereas both PGE2 and the EP1 receptor agonist ONO-DI-004 increased tumor cell invasiveness under the same experimental conditions (*, p < 0.01 compared with the control).

For experiments with antisense inhibition of EP receptor subtypes, CCLP1 cells were transfected with the oligonucleotides specific for the EP1, EP2, EP3, and EP4 receptors, and the transfected cells were then analyzed for PGE2-induced cell invasion. As shown in Fig. 8B, PGE2-induced cell invasion was blocked by antisense inhibition of the EP1 receptor, but not by antisense inhibition of the EP2, EP3, or EP4 receptor. Taken together, the data from the above pharmacological approaches with specific agonists and antagonists as well as molecular approaches with antisense and siRNA all support the involvement of the EP1 (but not EP2, EP3, or EP4) receptor in PGE2-induced cholangiocarcinoma cell invasion.

**Activation of EGFR by EGF Increases COX-2 Expression and PGE2 Production**—The data presented above indicate that COX-2-derived PGE2 activates EGFR/Akt through the G-protein-coupled EP1 receptor and that this process is involved in cholangiocarcinoma cell growth and invasion. In light of the elevated expression of EGFR and COX-2 in human cholangiocarcinoma tissues (as shown in Fig. 1) and the known effect of receptor tyrosine kinase inhibition and COX-2 expression (9), we postulated that EGFR activation could influence COX-2 expression and regulate cholangiocarcinoma cell growth. To examine this hypothesis, cultured CCLP1 cells were treated with EGF for 24 h, and Western blotting was performed for Western blotting to determine the level of COX-2. As shown in Fig. 9A, EGF treatment significantly increased COX-2 expression; this effect appeared dose-dependent. Consistent with this hypothesis, cultured CCLP1 cells transfected with the EGFR tyrosine kinase inhibitors AG 1478 or NS-398, a similar effect was also observed with NS-398, a cytosolic phospholipase A2 inhibitor that reduces the availability of arachidonic acid substrate for COX-2. Moreover, EGF-induced phosphorylation of EGFR was partially inhibited by pretreatment with AACOCF3, NS-398, ONO-8711, AG 1478, or PD 153035 (Fig. 9C). These findings indicate that EGFR activation further induces COX-2 expression and PGE2 production and that this signaling is involved in activation of EGFR by its cognate ligand.

**DISCUSSION**

Several noteworthy findings are presented in this study. First, overexpression of COX-2 in human cholangiocarcinoma cells promoted tumor growth and invasion both in vitro and in a xenograft model in SCID mice. Second, the expression and localization of COX-2 and EGFR in human cholangiocarcinoma cells was documented using immunohistochemical stains on sequential sections of human cholangiocarcinoma tissues. Third, this study provides the first evidence for transactivation of EGFR by COX-2 and PGE2 in human cholangiocarcinoma cells and the key role of the EP1 receptor in this process. Finally, and most important, this study reveals a novel cross-talk between the COX-2-derived PGE2 pathway and EGFR signaling for cancer cell growth and invasion, as illustrated in Fig. 10.
provide the first evidence for the expression profiles of EP receptors in human cholangiocarcinoma cells and depict a pivotal role of the EP1 receptor in EGFR transactivation in human cancer cell proliferation and invasion. The latter conclusion is based on the following observations: 1) enhanced EGFR phosphorylation by COX-2 overexpression or PGE2 treatment; 2) induction of EGFR phosphorylation by the selective EP1 receptor agonist ONO-DI-004; 3) inhibition of PGE2-induced EGFR phosphorylation by the selective EP1 receptor antagonist ONO-8711; 4) attenuation of PGE2-induced EGFR phosphorylation by EP1 receptor siRNA; 5) increased binding of the EP1 receptor to EGFR in response to PGE2 or ONO-DI-004; 6) induction of Akt phosphorylation and cell invasion by ONO-DI-004, but not by the agonists for EP2, EP3 and EP4; 7) inhibition of PGE2-induced Akt phosphorylation, cell proliferation and invasion, and MMP-2 activity by ONO-8711 and the EGFR tyr-
PGE<sub>2</sub> Transactivates EGFR through the EP<sub>1</sub> Receptor

Cross-talk between different members of receptor families has become a well established concept in signal transduction. GPCRs as well as receptor tyrosine kinases constitute prominent families of cell-surface proteins regulating the responsiveness of cells to environmental signals (35, 36). Different classes of G-proteins have been shown to be involved in the transactivation of tyrosine kinase receptors, including the G<sub>i</sub>, G<sub>q</sub>, and G<sub>13</sub> proteins, although, to date, there have been no data available implicating G<sub>i</sub>-coupled receptors in EGFR signal transactivation (24, 26). Consistent with these observations, in this study, we demonstrated a predominant role of the G<sub>i</sub>-coupled EP<sub>1</sub> receptor (but not the G<sub>q</sub>-coupled EP<sub>2</sub> and EP<sub>4</sub> receptors) in EGFR transactivation and cell proliferation/invasion. Given that the G<sub>i</sub>-coupled EP<sub>2</sub> and EP<sub>4</sub> receptors mediate their effect via increasing intracellular cAMP levels and that activation of cAMP/protein kinase A signaling is known to enhance the proliferation and motility of cholangiocytes and cholangiocarcinoma cells, the lack of EP<sub>2</sub> and EP<sub>4</sub> receptor effect revealed in this study further underscores the importance of EP<sub>1</sub> receptor-mediated EGFR transactivation in cholangiocarcinoma progression. It is of further interest that the experiments with the EP<sub>1</sub> receptor agonist ONO-AE-248 and EP<sub>2</sub> receptor siRNA also failed to show involvement of the Gi-coupled EP<sub>1</sub> receptor, despite the fact that it is known to exert its effect through reduction in intracellular cAMP levels. Thus, our findings indicate that EP<sub>2</sub> receptors cannot directly stimulate EGFR transactivation. EGFR can be transactivated by extracellular release of reactive oxidative species, through intracellular molecules, or by receptor tyrosine kinases, and the inhibitory role of EP<sub>2</sub> receptor siRNA on EGFR-specific phosphorylation of EGFR in cholangiocarcinoma cells occurs through activation of downstream molecules, including the phosphoinositide 3-kinase/Akt pathway (34). Aberrant EGFR signaling due to overexpression, mutation, or autocrine signaling loops has been implicated in several other human cancers (for review, see Refs. 39–43). In this study, we showed that EGFR expression was increased in human cholangiocarcinoma tissue as determined by immunohistochemical analysis. Moreover, our data suggest the involvement of Akt in transducing the effects of EP<sub>1</sub> receptor-induced EGFR activation in human cholangiocarcinoma cells. The latter assertion is based on the findings that Akt was activated within 30 min after PGE<sub>2</sub> treatment of the CCLP1 cells and that inhibition of Src by PP2 prevented PGE<sub>2</sub>-induced Akt phosphorylation, tumor cell invasion, and MMP-2 activity. These findings are consistent with recent studies showing the involvement of Src in PGE<sub>2</sub>-induced transactivation of EGFR in colon cancer cells (37, 38).

The EGFR family consists of four receptor tyrosine kinases, EGFR (ErbB1), ErbB2, ErbB3, and ErbB4. EGFR controls a wide variety of biological responses, such as proliferation, migration, and modulation of apoptosis, and the effects are mediated through activation of downstream molecules, including the phosphoinositide 3-kinase/Akt pathway (34). Aberrant EGFR signaling due to overexpression, mutation, or autocrine signaling loops has been implicated in several other human cancers (for review, see Refs. 39–43). In this study, we showed that EGFR expression was increased in human cholangiocarcinoma tissue as determined by immunohistochemical analysis. Moreover, our data suggest the involvement of Akt in transducing the effects of EP<sub>1</sub> receptor-induced EGFR activation in human cholangiocarcinoma cells. The latter assertion is based on the findings that Akt was activated within 30 min after PGE<sub>2</sub> treatment of the CCLP1 cells and that inhibition of Src by PP2 prevented PGE<sub>2</sub>-induced Akt phosphorylation, tumor cell invasion, and MMP-2 activity. These observations are further corroborated by additional findings that the EGFR kinase inhibitors prevented PGE<sub>2</sub>-induced cholangiocarcinoma cell proliferation and invasion and MMP-2 activity.

Akt plays a key role in tumorigenesis and cancer progression by stimulating cell proliferation and invasion or by inhibiting apoptosis (44–46). Akt is composed of an N-terminal PH domain and a C-terminal kinase catalytic domain and is activated by a dual regulatory mechanism, including translocation to the...
PGE₂ Transactivates EGFR through the EP₁ Receptor

A

FIG. 9. EGF treatment increased COX-2 expression and PGE₂ production in CCLP1 cells. Blocking PGE₂ signaling partially inhibited the phosphorylation of EGFR induced by EGF. A. EGF induces COX-2 expression. Cultured CCLP1 cells were serum-starved for 24 h and then treated with 20 ng/ml EGF for the indicated times. Cell lysates were obtained for Western blotting to determine the level of COX-2. EGFR treatment increased COX-2 expression. This effect was observed at 6 h and peaked at 24–48 h. EGF increases COX-2 expression. The effects of the cytosolic phospholipase A₂ inhibitor AACOCF₃, the c-Src family inhibitor PP2, and the EGFR tyrosine kinase inhibitors AG 1478 and PD 153035 were determined. Serum-starved CCLP1 cells were pretreated with AACOCF₃, or NS-388, or with PP2, or with AG1478, or with PD 153035 for 30 min. The cells were then continuously treated with 20 ng/ml EGF for 16 h, and the media were subsequently collected to measure PGE₂ production (*, p < 0.01 compared with the control; **, p < 0.05 compared with EGF treatment alone). The results are presented as the mean ± S.D. of four experiments. EGF treatment significantly increased PGE₂ production (*, p < 0.01 compared with the control; **, p < 0.05 compared with EGF treatment alone). The results are presented as the mean ± S.D. of four experiments. EGF treatment significantly increased PGE₂ production (*, p < 0.01 compared with the control; **, p < 0.05 compared with EGF treatment alone). The results are presented as the mean ± S.D. of four experiments.

B

C

FIG. 10. Proposed mechanisms for COX-2- and PGE₂-mediated cholangiocarcinoma cell invasion and proliferation. Prostaglandin synthesis is controlled by coupled activation of cytosolic phospholipase A₂ (cPLA₂) and COX-2 along the cell membrane. The produced prostaglandins are released to the extracellular space by the prostaglandin transporter. The major prostaglandin in biliary epithelial and cancer cells is PGE₂. Once PGE₂ is released into the extracellular space, it binds to the membrane G protein-coupled EP receptors on the same cell or neighboring cell (paracrine). Although there are four receptors (EP₁, EP₂, EP₃, and EP₄), our data indicate that EP₁ plays a key role in COX-2- and PGE₂-mediated cholangiocarcinoma cell invasion and proliferation and EGFR phosphorylation by activation of EGFR/Akt. COX-2- and PGE₂-dependent Akt phosphorylation partially inhibits EGFR signaling through the EGFR/PI3K/Akt/PP2 pathway in human cholangiocarcinoma cells. Thus, at least in part, through the EP₁ receptor subtype, this is the first study detailing the role of EP receptor subtype mediating cellular effects, such as stimulation of cell cycle progress and invasiveness. Increased expression of phosphorylated Akt has recently been documented in human cholangiocarcinoma cells by immunohistochemical staining of human cholangiocarcinoma tissues (14). The latter observation and the findings that the COX-2 inhibitor celecoxib inhibits Akt phosphorylation in cultured cholangiocarcinoma cell lines (14, 16, 17) suggest a possible involvement of Akt activation in COX-2-mediated cholangiocarcinoma cell growth, although a direct effect of COX-2-derived prostaglandin on Akt phosphorylation was not demonstrated prior to this study in light of the presence of COX-2-independent effects associated with celecoxib. Our present data establish a direct effect of PGE₂ on Akt phosphorylation in human cholangiocarcinoma cells. To our knowledge, this is the first study detailing the role of EP receptor subtype in Akt phosphorylation and EGFR transactivation in human cancer cells.

In addition to EGFR transactivation by COX-2-derived PGE₂, our data also show that treatment of human cholangiocarcinoma cells with EGF increased COX-2 expression and PGE₂ synthesis, suggesting the involvement of EGFR in COX-2

phosphorylation. The generation of phosphatidylinositol 3,4,5-triphosphate on the inner layer of plasma membrane following phosphatidylinositol 3-kinase activation recruits Akt by direct interaction with its PH domain. At the membrane, Akt is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 and -2. Phosphorylated Akt dissociates from the membrane and enters the cytoplasm and nucleus, where it phosphorylates several key proteins mediating cellular effects, such as stimulation of cell cycle progress and invasiveness. Increased expression of phosphorylated Akt has recently been documented in human cholangiocarcinoma cells by immunohistochemical staining of human cholangiocarcinoma tissues (14). The latter observation and the findings that the COX-2 inhibitor celecoxib inhibits Akt phosphorylation in cultured cholangiocarcinoma cell lines (14, 16, 17) suggest a possible involvement of Akt activation in COX-2-mediated cholangiocarcinoma cell growth, although a direct effect of COX-2-derived prostaglandin on Akt phosphorylation was not demonstrated prior to this study in light of the presence of COX-2-independent effects associated with celecoxib. Our present data establish a direct effect of PGE₂ on Akt phosphorylation in human cholangiocar...
expression. In this context, our result is consistent with that of Yoon et al. (11), who showed the involvement of COX-2 in bile salt-induced COX-2 expression in cholangiocytes and cholangiocarcinoma cells. Collectively, these findings suggest that COX-2-derived PGE₂ signaling may mediate the actions of EGFR in cholangiocarcinogenesis. This assertion is further supported by the observations that EGFR phosphorylation induced by its cognate ligand (EGF) was partially inhibited by blocking PGE₂ synthesis with the cytosolic phospholipase A₂ inhibitor AACOCF₃ and the COX-2 inhibitor NS-398 or with the selective EP₁ receptor antagonist ONO-8711.

In summary, this study has established a novel EP₁ receptor-mediated transactivation of EGFR by COX-2-derived PGE₂, which is crucial for COX-2- and PGE₂-induced cholangiocarcinoma cell growth and invasion. Moreover, we have shown that activation of EGFR further up-regulates COX-2 expression and thus enhances EGFR signaling via activation of the EP₁ receptor. The cross-talk between these two key signaling systems likely plays an important role in COX-2- and receptor tyrosine kinase-induced cholangiocarcinogenesis. Given the recently reported side effect associated with the currently available COX-2 inhibitors in patients (47, 48), our findings suggest that combinational utilization of agents targeting the EP₁ receptor and EGFR may represent a promising cancer therapeutic strategy that deserves further investigation.

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