ABSTRACT

An association of gallbladder carcinoma with cholelithiasis suggests that chronic inflammation may modulate tumorigenesis and/or progression of the carcinoma. An enhanced expression of cyclooxygenase-2 (COX-2) is observed frequently in advanced carcinomas of gastrointestinal tracts, which in turn suggests that potentiated arachidonate metabolism may play a crucial role in tumor biology. In the present study, the expression levels of COX-2 and prostaglandin E receptor subtypes were determined in 16 cases of pT1-pT4 gallbladder carcinomas, in which their mRNAs and protein were determined in the adjacent stroma and its biological effect via EP4 on the carcinoma cells may contribute to tumor growth and progression of gallbladder carcinoma.

INTRODUCTION

Gallbladder carcinoma has been associated with a dismal overall prognosis (1–5). The 5-year survival rate after surgery has been reported recently to be between 5 and 13% (1–5). Although the clinical course of gallbladder carcinoma has been thought to depend on the depth of tumor invasion (6–8), tumorigenesis of gallbladder carcinoma, as well as its growth and progression, is complex and not completely understood. An association of gallbladder carcinoma with cholelithiasis (9) or an anomalous arrangement of the pancreaticobiliary duct (10, 11) suggests that long-term inflammation may modulate tumorigenesis and/or progression of the carcinoma. Supporting this notion, in situ lesions of dysplasia are found frequently in the epithelia adjacent to gallbladder carcinoma associated with gallstones (12), which in turn suggests the possibility that the histogenesis of epithelial dysplasia may be attributable to a chronic inflammatory stimulus on the gallbladder by gallstones and that some of the precursor lesions may yield carcinoma. In addition, inflammatory changes are often observed in noncancerous epithelia adjacent to advanced carcinoma (12).

Because a number of studies has shown that arachidonate (13) and PGE2 levels (14, 15) are increased in human carcinoma, the expression of COX-2 and prostaglandin E-receptor in situ hybridization revealed the existence of COX-2 mRNA and protein in both the cancerous epithelia and adjacent stroma of pT1-pT4 carcinomas. Only in pT3 and pT4 carcinomas was intense expression of COX-2 observed in the adjacent stroma. The tissue concentration of PGE2 was significantly increased in pT3 and pT4 carcinomas. The mRNAs of PGE receptor subtypes EP2, EP3, and EP4 were amplified in pT1-pT4 gallbladder carcinomas, in which their mRNAs and EP4 protein were expressed mostly in the cancerous epithelia. Treatment with a specific EP agonist, as well as PGE2 but not EP2 and EP3 agonists, up-regulated the expression of c-fos, an induced growth response gene, and increased colony formation. In advanced gallbladder carcinoma, enhanced expression of COX-2 is observed in the adjacent stroma rather than in the cancerous epithelia, and the stroma is a potent source of PG synthesis. In epithelial-stromal interactions, the increased PGE2 synthesis in the adjacent stroma and its biological effect via EP4 on the carcinoma cells may contribute to tumor growth and progression of gallbladder carcinoma.
COX-2 and EPs in Gallbladder Carcinoma

...noma tissues, interest has been focused on the expression of inflammatory enzymes in the arachidonate cascade, COXs, and PLA2s in carcinoma of the gallbladder.

A second isof orm of COX, COX-2, is induced by mitogens (16), cytokines (16), and growth factors (17), and it produces PG-involved inflammation (18) and cell growth (19). Recent studies (20–23) have shown that COX-2 mRNA expression is markedly elevated in most tissues of human colorectal carcinomas and have suggested a putative role of COX-2 in the tumorigenesis, growth, and progression of carcinomas. Notifying a biological function of COX-2 in carcinoma tissues, overexpression of COX-2 in carcinoma cells is associated with biochemical changes, including activation of membrane metalloproteinase and PG synthesis (24), which in turn yield the phenotypic change of increased invasiveness of the carcinoma cells (24, 25). Besides COX-2, overexpression of sPLA2-IIA has been shown in gastric (26), breast (27), and hepatocellular carcinomas (28). The increased expression of sPLA2-IIA in carcinoma tissues has been shown to correlate with an increased malignant potential of the carcinoma cells (26, 27) and is likely to contribute to tumor development.

Diverse biological activity of PGE2 (29–31), i.e., physiological, inflammatory, and immunological functions, can be attributed to four specific G protein-coupled receptors, termed EPs. Recent studies have shown that EP mRNAs are distributed throughout the gastrointestinal tract (32, 33), and the biological effect of PGE2 in gastrointestinal tissues involves signaling via EP subtypes. The biological effect of COX-2-derived PGE2 via EPs on carcinoma cells may be involved in phenotypic changes of the carcinoma cells observed in the process of tumor progression, which may modulate the malignant behavior of carcinoma cells.

To elucidate the role of arachidonate metabolism in tumor growth and progression, the expression levels of COX-2 and sPLA2-IIA, as well as EP subtypes, were determined in gallbladder carcinoma tissues of different depths of invasion. Furthermore, in in vitro experiments, a growth promotion of gallbladder carcinoma cells in response to treatment with specific EP2, EP3, and EP4 agonists, as well as PGE2, was studied.

MATERIALS AND METHODS

Patients. Specimens from 16 patients (7 males and 9 females) with gallbladder carcinomas (3 with pT1, 2 with pT2, 4 with pT3, and 7 with pT4 carcinomas) were included in the present study. The mean age of the patients was 65 years (range, 52–78 years). The patients had been diagnosed as having gallbladder carcinoma and had undergone operations between April 1997 and December 1999 in the University of Tsukuba School of Medicine Hospital. Gallbladder carcinoma was diagnosed on the basis of histological findings and classified according to the tumor node metastasis classification of the American Joint Committee on Cancer (34). In addition, intact gallbladder specimens were obtained by surgery from 10 subjects who had undergone hepatectomy because of metastatic liver carcinoma.

Immunohistochemical Expression of COX-2 and EP in Gallbladder Carcinoma. Immunostaining of COX-2 was performed by the avidin-biotin complex technique using a Vectastain Elite avidin-biotin complex kit (Vector, Burlingame, CA), as described previously (23). Formalin-fixed, paraffin-embedded specimens were serially sectioned at a thickness of 4 μm, placed onto microscopic slides, and then deparaffinized. The slides were immersed for 30 min in 0.3% hydrogen peroxide/methanol to deplete endogenous peroxidase. Then, nonspecific binding sites were blocked with 0.3% normal goat serum for 20 min. The primary Ab raised against COX-2 or the Ab raised against human EP3 (Cayman Chemical Co., Ann Arbor, MI) was used at a dilution of 1:100, applied to tissue sections, and incubated at room temperature for 30 min. After washing with PBS, biotinylated goat antirabbit IgG (Vector) was applied onto the tissue sections and incubated at room temperature for 30 min. After washing with PBS, a streptavidin peroxidase reagent was applied and incubated at room temperature for 10 min. Finally, the reaction product was visualized using developing color by incubating the slides in a solution of 0.3% hydrogen peroxide, diaminobenzidine tetrahydrochloride, and PBS. A negative control was made using BSA instead of the Ab against COX-2. Counter staining was done by hematoxylin. Specificity was determined by preabsorption of the anti-COX-2 Ab with the COX-2 synthetic polypeptide, which was used as an immunogen (17 amino acids, position 251–267: TVKD TQAE-MI YY PPQ VPE) for generation of the Ab (37) before staining. Immunostaining with normal rabbit serum and anti-COX-2 Ab absorbed with the synthetic COX-2 polypeptide was completely negative.

Evaluation of the sections was performed by a single pathologist who was blinded to the clinical characteristics and pathological grade of response. The total number of cancerous epithelia and adjacent stroma cells in each section was evaluated. The immunohistochemical expression of COX-2 in gallbladder carcinoma was evaluated in terms of the intensity and positive rate of the immunostaining in the cancerous epithelia or adjacent stroma cells. The intensity was defined by comparing the intensity in smooth muscles or vascular endothelia as internal built-in controls. Fig. 1 shows pictures representing the intensities of immunostaining of COX-2 in gallbladder tissues. The intensity was graded on a scale of 0–2: (a) grade 0 (G0), an intensity in epithelia or adjacent stroma cells being less intense than that in internal controls or the staining being negative; (b) grade 1 (G1), an intensity being similar to that in internal controls (Fig. 1, A and B); and (c) grade 2 (G2), an intensity being more intense than that in internal controls (Fig. 1C). When >5% of the total number of cancerous epithelia or adjacent stroma cells in each section was scored as grade 1 or 2, the section was judged as being positive for COX-2 staining, and then the positive rate (expressed as a percentage) was calculated by counting the epithelia or stroma cells expressing COX-2.
Assay of Tissue Concentration of sPLA_2-IIA in Gallbladder Carcinoma. The protein masses of sPLA_2-IIA in the tissues of gallbladders and gallbladder carcinomas (ng/mg X protein) were immunoradiometrically assayed as described recently (38). Assay kits were kindly supplied from the Pharmaceuticals Research & Development Division, Shionogi & Co., Ltd. (Osaka, Japan). All assays were performed in triplicate. Protein concentration in the supernatant was measured by the method described by Lowry et al. (39).

Assay of Tissue Concentration of PGE_2. Aliquots of tissue homogenates were assayed by a highly specific RIA (Ref. 40; anti-PGE_2 Ab; Amersham, London, United Kingdom) for PGE_2 in duplicate and at two dilutions. The protein contents in the supernatant were measured by the method described by Lowry et al. (39). The final results are expressed as pg PGE_2/mg X protein.

RNA Isolation and cDNA Synthesis. Total RNA was isolated from gallbladder carcinoma specimens using Trizol reagent by the modified method described by Chomczynsky and Sacchi (41). First-strand cDNAs were synthesized from total RNA with Moloney murine leukemia virus reverse transcriptase by the random primer method.

RT-PCR. Semiquantitative RT-PCR was performed using a DNA Thermal Cycler (model PJ 2000; Applied Biosystems, Inc., Foster City, CA). PCR was subjected to each cycle (G3PDH, 20; COX-1, 30; COX-2, 30; sPLA_2-IIA, 35; EP_1, 35; EP_2, 35; EP_3, 35; and EP_4, 35) at 94°C for 1 min, at 55°C for 2 min, and at 72°C for 2 min. Aliquots of the reaction mixture were electrophoresed on a 2% agarose gel. PCR primers were designed from cDNA sequences for human COX-1 (42), COX-2 (43), sPLA_2-IIA (44), EP_1 (45), EP_2 (46), EP_3 (47), and EP_4 (48) and then synthesized using an Applied Biosystems DNA Synthesis system.
synthesizer (model 392; Applied Biosystems, Inc.) as follows: G3PDH, sense 5'-GAACGGGAAGCCTGCACTGGGCA-3', antisense 5'-TATTAGGCACCAACCCGGTC-3'; COX-1, sense 5'-CCCTGGGTACCTACGTTGGA-3', antisense 5'-AGGCGGCAAGATACGATTG-3'; COX-2, sense 5'-AAAGGCTTCTCCTAATGCTTCC-3', antisense 5'-TACGACATCGCATCATC-3'; sPLA2-IIA, sense 5'-ACCATTGAAGACCTCTACTAGTGA-3', antisense 5'-GAAGAGGGAGCTCACTGGCATGG-3'; EP2, sense 5'-GGCGGTGGCCCTCTGCTTC-3', antisense 5'-CCCAGGCGAGGATGAAACCAC-3'; and EP4, sense 5'-GCTTCTCATTGTCTCG-3', antisense 5'-GAGGACTGAACG-3'. Plasmid vectors into which each objective coding region of human COX-1 (42), COX-2 (43), EP2, EP3, and EP4 were prepared in the laboratory of Dr. T. Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan). All plasmids were linearized, and antisense RNA probes were transcribed with RNA polymerase in the presence of Cytidine 5'-32P]dCTP by random primer extension as described previously (49). RT-PCR was done in triplicate. In the semiquantitative assessment, the amounts of fluorescence intensity were measured using a FluorImager (Molecular Dynamics, Sunnyvale, CA). The data were expressed relative to the amount of G3PDH mRNA present in each specimen and then averaged.

**Synthesis of cRNA Probes of COX-2 and EP Subtypes.** Riboprobes were synthesized from plasmid vectors into which each objective coding region of COX-2, EP2, EP3, and EP4 had already been inserted. Briefly, pBluescript KS containing the coding region of human COX-2 was prepared in the laboratory of Dr. T. Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan). All plasmids containing each coding region of human EP2, EP3, and EP4 were prepared in the laboratory of Prof. A. Ichikawa (Kyoto University, Kyoto, Japan). The inserted coding regions were a 414-bp fragment of human COX-2 (from 7634 to 8047 of D28235, GenBank/EMBL Data Bank; Ref. 43), a 1089-bp fragment of human EP3 (from 157 to 1245 of U19487, GenBank/EMBL Data Bank; Ref. 46), a 1186-bp fragment of human EP2 (from 45 to 1230 of B36875, GenBank/EMBL Data Bank; Ref. 47), and a 1251-bp fragment of human EP4 (from 362 to 1612 of L28175, GenBank/EMBL Data Bank; Ref. 48). These plasmids were linearized, and antisense RNA probes were transcribed with RNA polymerase in the presence of Cytidine 5'-[α-32P]triphosphate [32P]S] to a specific activity of 1 × 109 cpm/μg.

**ISH of COX-2 and EP Subtypes in GALLBLADDER Carcinoma.** Frozen sections of 8 μm in thickness were cut on a cryostat and thaw mounted onto poly-L-lysine-coated slides. The slides were fixed with 4% formaldehyde in phosphate buffer saline, rinsed in PBS twice, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl at room temperature.

ISH was performed as described previously (49). Hybridization was carried out in a buffer containing 50% formamide, 2 × SSC, 10 mM Tris-Cl (pH 7.5), 1 × Denhardt’s solution, 10% dextran sulfate, 0.2% SDS, 100 mM DTT, 500 μg/ml sheared single-stranded salmon sperm DNA, and 250 μg/ml yeast tRNA. Riboprobes were added to the hybridization buffer at 7 × 104 cpm/ml. The hybridization solution was applied to the slides, which were then covered with a coverslip and sealed by rubber cement. After incubation at 60°C for 5 h, the slides were immersed in 2 × SSC to remove the coverslips and then washed for 1 h by warming in 2 × SSC and 10 mM β-mercaptoethanol. The sections were then treated with 20 μg/ml RNase A in 0.5 mM NaCl, 10 mM Tris-Cl (pH 7.5), and 1 mM EDTA, followed by an additional wash in 0.1 × SSC at 60°C for 1 h. After dehydration, the slides were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with distilled water. After exposure for 4 weeks at 4°C, the dipped slides were developed in COPINAL (FUJIFILM, Tokyo, Japan), diluted 1:2 in distilled water, fixed, and counterstained with H&E.

**Cell Line and Culture Conditions.** Mz-ChA-1 and Mz-ChA-2, gallbladder adenocarcinoma cell lines (36), were obtained from Dr. A. Knuth (Johannes-Gutenberg University, Mainz, Germany). The cells were maintained in DMEM that contained 10% heat-inactivated FCS (Hyclone Laboratories, Inc., Logan, UT) in a humidified atmosphere with 5% carbon dioxide at 37°C. The biological properties of these two cell lines, i.e., the in vitro colony formation, the in vitro growth kinetics, and the tumor formation into nude mice, have been described by Knuth et al. (36).

Immunoblot analysis of COX-2 in cell protein lysates was performed in the same way as described before. Northern blot analysis was performed using total cellular RNA extracted from the cells. RNA specimens (each 20 μg) were separated on formaldehyde-agarose gels and blotted onto nitrocellulose filters. The blot was hybridized with cDNA probes labeled with [α-32P]dCTP by random primer extension as described previously (50). RT-PCR and ISH of EP mRNAs in the cells were performed in the same ways as described before. PGE2 production in the cells was determined by measuring PGE2 concentration in the media from the cells with and without 10 μM arachidonate treatment. The PGE2 concentration 6 h after the addition of arachidonate was assayed in triplicate by an RIA in the same way as described before (40).

Mz-ChA-2 cells were placed in 24-well tissue culture plates, precultured for 24 h, and then treated with test reagents. In experiments for c-fos expression, Mz-ChA-2 cells were grown in complete DMEM with 10% fetal bovine serum for 24 h and then in a medium with 1% FCS for 48 h before being harvested for RNA extraction.

**Colonies Formation Assay.** The colony number of Mz-ChA-2 cells was counted according to the method (51) described previously with minor modifications. Briefly, Mz-ChA-2 cells were plated in a 10-cm cell culture dish at a density of 1000 cells/dish with DMEM containing 10% FCS. A selective EP2 agonist (ONO-AE1–259; Ref. 52; Ono Pharmaceutical Co., Ltd., Osaka, Japan), EP3 agonist (ONO-AE-248; Ref. 52), and EP4 agonist (ONO-AE1–329; Ref. 52) at a concentration of 0.01, 0.1, 1, or 10 μM or PGE2 (Cayman Chemical Co.) at a concentration of 1 μM was added daily to selected cells, and the medium was replaced every day. The cells were incubated for 14 days, and then the colonies were visualized by staining with 0.2% methylene blue and counted manually. In each experiment, the assay was done in triplicate.

**Assay of C-fos Expression in GALLBLADDER Carcinoma Cells.** To elucidate the mechanism involved in the PGE2 or EP2 agonist-induced growth of gallbladder carcinoma cells, the steady-state mRNA level of c-fos, one of the earliest induced growth response genes (53), was determined in Mz-ChA-2 cells.

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*by K.M. Ishikawa, T. Tanabe, Y. Shiiki, H. Nakazawa, K. Endo, M. Kimura, and T. Fujita*
with or without a stimulation by semiquantitative RT-PCR. The cells were grown in complete DMEM supplemented with 10% FCS for 24 h and then in serum-free media for 48 h before being harvested for RNA isolation. PGE2 at a concentration of 1 μM or ONO-AE1–329 at a concentration of 0.01, 0.1, 1, or 10 μM was added to the cells, and the cells were harvested after 30 or 60 min. Isolation of total RNA from the cells and RT-PCR were performed in the same way as described before. PCR was subjected to each cycle (G3PDH, 20; c-fos, 25) at 94°C for 1 min, at 55°C for 2 min, and at 72°C for 2 min. PCR primers of c-fos were designed from cDNA sequences for human c-fos (54) and then synthesized in the same way as described before as follows: c-fos, sense 5′-GAATAAGATGGCTGACGCAAATGGCCGCAA-3′, antisense 5′-CAGTCAGATCAAGGGAAGCCACAGACATCT-3′. In each experiment, RT-PCR was done in triplicate. The data were expressed relative to the amount of G3PDH mRNA present in each specimen and then averaged.

Statistics. Values are given as means ± SE. Means of two groups were compared with the Mann-Whitney rank sum U test (two-tailed test), and multiple comparisons were performed by ANOVA. A two-sided χ2 test was used for comparison of clinicopathological data between groups. A P <0.05 was defined as statistically significant.

RESULTS

Immunoblot Analysis of COX-2 in Gallbladder Carcinoma. A limited number of carcinoma tissue specimens (5 specimens from 1 patient with chronic cholecystitis associated with cholelithiasis, 1 patient with pT2, 2 patients with pT3, and 1 patient with pT4 carcinoma) was subjected to immunoblot analysis (Fig. 2). Parallel to the depth of invasion, the carcinoma tissues yielded a prominent band for COX-2; in densitometric analysis, the abundance of the band (expressed as COX-2/β-actin ratio) was significantly higher in 11 specimens of pT1 and pT2 carcinomas (0.88 ± 0.08, mean ± SE, P < 0.01) than in 5 specimens of pT1 and pT2 carcinomas (0.53 ± 0.02) and 10 specimens of normal gallbladders (0.24 ± 0.02).

Immunohistochemical Expression of COX-2 in Gallbladder Carcinoma. In tissue specimens of intact gallbladders, immunostaining of COX-2 was observed in the epithelia and smooth muscles (Fig. 1A). The expression levels were weak. However, in tissue specimens of gallbladder carcinomas, intense immunostaining of COX-2 was observed in cancerous epithelia in the specimens of pT1 and pT2 carcinomas (data not shown) and in both cancerous epithelia and stroma adjacent to the epithelia in the specimens of pT3 and pT4 carcinomas (Fig. 1C). The COX-2 stainings in the stroma included fibroblasts (arrowheads in Fig. 1D), vascular endothelial cells (arrowheads in Fig. 1E), and inflammatory mononuclear cells (arrowheads in Fig. 1F). Immunostaining of COX-2 was observed in >80% of cancerous epithelia of pT1-pT4 gallbladder carcinomas and normal epithelia of the gallbladders (Table 1). No significant difference in the expression rate of COX-2 was found among the cancerous and normal epithelia. In the epithelia (Table 1), grade 2 intensity of COX-2 expression was observed at a high frequency in pT1 and pT2 carcinomas (60%, P < 0.05) and pT1 and pT4 carcinomas (82%, P < 0.05), compared with that in normal epithelia (0%). Grade 1 intensity was observed at a high frequency in normal epithelia (80%). In the stroma adjacent to the cancerous epithelia (Table 1), grade 2 intensity was observed only in the stroma adjacent to pT3 and pT4 carcinomas (55%), and the rate of grade 2 intensity in these stroma was significantly higher than that in the stroma adjacent to the normal epithelia (0%, P < 0.05). Grade 1 intensity was observed in the stroma of pT1 and pT2 carcinomas (20%) and pT3 and pT4 carcinomas (27%).

Tissue mRNA Levels of COX-1 and -2, sPLA2, and EP Subtypes in Gallbladder Carcinoma. Fig. 3 shows the PCR-assisted amplifications of COX-1, COX-2, sPLA2-IIA, and EP2,4 subtypes in the tissues of normal gallbladders and gallbladder carcinomas of different depths of invasion. The mRNA level of COX-2 was significantly higher in 5 specimens of pT1 and pT2 carcinomas (91 ± 17% of G3PDH, mean ± SE, P < 0.01) and in 11 specimens of pT3 and pT4 gallbladder carcinomas (136 ± 11%, P < 0.01), compared with the level in 10 specimens of normal gallbladders (54 ± 5%) as shown in Table 2, whereas the level of COX-1 was not significantly different among the specimens. In terms of the depth of invasion, the mRNA level of COX-2 was significantly higher in the pT1 and pT4 carcinomas than in the pT1 and pT2 carcinomas (P < 0.01). Of the sPLA2 isoforms, the mRNA of sPLA2-IIA was expressed strongly in tissues of gallbladder carcinomas, whereas it was only expressed weakly in tissues of normal gallbladder. Similar to the COX-2 expression pattern, the mRNA level of sPLA2-IIA
was significantly higher in 5 specimens of pT1 and pT2 carcinomas (120 ± 20%, P < 0.01) and in 11 specimens of pT3 and pT4 gallbladder carcinomas (200 ± 16%, P < 0.01), compared with the level in 10 specimens of normal gallbladders (88 ± 10%). In terms of the depth of invasion, the mRNA level was significantly higher in the pT1 and pT2 carcinomas than in the pT3 and pT4 carcinomas (P < 0.01).

Of the EP subtypes, the mRNAs of EP2, EP3, and EP4 were amplified in both tissues of normal gallbladders and gallbladder carcinomas (Fig. 3). The mRNA levels of EP2-4 subtypes determined by the semiquantitative assessment were not significantly different among normal gallbladders and pT1-pT4 carcinomas. In contrast to COX-2 expression, the expression levels of EP2, EP3, and EP4 mRNAs did not differ significantly in terms of the depth of invasion.

**ISH of mRNAs of COX-2 and EP Subtypes in Gallbladder Carcinoma Tissues.** COX-2 mRNA was expressed widely in the epithelia and focally in the adjacent stroma in the specimens of pT4 gallbladder carcinoma tissues (Fig. 4, A and B). The extent and distribution of COX-2 mRNA resembled that of COX-2 protein in individual cases (data not shown). EP2, EP3, and EP4 mRNAs were predominantly expressed in the cancerous epithelia (Fig. 4, C, D, and F), and the extent and distribution of EP4 mRNA was consistent with that of EP4 protein (Fig. 4E).

**Tissue Concentration of sPLA2-IIA in Gallbladder Carcinoma.** The tissue concentration of sPLA2-IIA in gallbladder carcinoma was significantly increased in 5 specimens of pT3 and pT4 carcinomas (1.5 ± 0.3 ng/mg × protein, mean ± SE, P < 0.01) and in 11 specimens of pT1 and pT3 gallbladder carcinomas (3.9 ± 0.6, P < 0.01), compared with the concentration in 10 specimens of normal gallbladders (0.9 ± 0.1) as shown in Table 2. In terms of the depth of invasion, the concentration was significantly higher in the pT1 and pT2 carcinomas than in the pT3 and pT4 carcinomas (P < 0.01).

**Tissue Concentration of PGE2 in Gallbladder Carcinoma.** In association with the increased COX-2 and sPLA2-IIA expression levels, the tissue concentration of PGE2 was significantly increased in 5 specimens of pT1 and pT2 carcinomas (106.2 ± 16.1 pg/mg × protein, mean ± SE, P < 0.01) and

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**Table 1** Immunohistochemical expression of COX-2 in the epithelia and stroma of gallbladder carcinoma of different depths of invasion*

<table>
<thead>
<tr>
<th></th>
<th>Normal gallbladder (10)</th>
<th>pT1-pT2 carcinoma (5)</th>
<th>pT3-pT4 carcinoma (11)</th>
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</thead>
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<tr>
<td>Epithelia</td>
<td>Positive rate G0, G1, G2</td>
<td>Positive rate G0, G1, G2</td>
<td>Positive rate G0, G1, G2</td>
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<td>Stroma</td>
<td>10 2 0</td>
<td>4 1 0</td>
<td>2 3 6</td>
</tr>
</tbody>
</table>

* Immunostaining of COX-2 was evaluated in terms of the positive rate and intensity (see "Materials and Methods").
  * P < 0.01, significantly different from other groups.
  + P < 0.05.

**Table 2** Steady-state mRNA levels of sPLA2-IIA, COX-1, and COX-2 and tissue concentrations of sPLA2-IIA and PGE2 in gallbladder carcinoma of different depths of invasion

<table>
<thead>
<tr>
<th></th>
<th>Normal gallbladder (10)</th>
<th>pT1-pT2 carcinoma (5)</th>
<th>pT3-pT4 carcinoma (11)</th>
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<tbody>
<tr>
<td>Messenger RNA</td>
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<tr>
<td>sPLA2-IIA</td>
<td>88 ± 10</td>
<td>120 ± 20</td>
<td>200 ± 16**</td>
</tr>
<tr>
<td>COX-1</td>
<td>105 ± 5</td>
<td>107 ± 12</td>
<td>98 ± 12</td>
</tr>
<tr>
<td>COX-2</td>
<td>54 ± 5</td>
<td>91 ± 17</td>
<td>136 ± 11**</td>
</tr>
<tr>
<td>Tissue concentration</td>
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<tr>
<td>sPLA2-IIA</td>
<td>0.9 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>3.9 ± 0.6**</td>
</tr>
<tr>
<td>PGE2</td>
<td>59.2 ± 7.9</td>
<td>106.2 ± 16.1</td>
<td>257.9 ± 26.1**</td>
</tr>
</tbody>
</table>

* P < 0.01, significantly different from normal gallbladder.
+ P < 0.01, significantly different from pT1-pT2 carcinoma.
+ P < 0.05.
+ P < 0.05.

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**Fig. 3** PCR-assisted amplifications of mRNAs of sPLA2-IIA, COX-1, COX-2, EP2, EP3, and EP4 in gallbladder carcinoma tissues. Plasmid vectors into which each objective coding region of human COX-1 (42), COX-2 (43), sPLA2-IIA (44), EP2 (46), EP3 (47), and EP4 (48) had already been inserted were used as positive controls. Lane P, positive controls; Lane I, a case of normal gallbladder; Lane II, a case of pT1 carcinoma; Lane III, a case of pT2 carcinoma; Lane IV, a case of pT3 carcinoma; Lane V, a case of pT4 carcinoma. The abundance of G3PDH mRNA was determined as an internal standard. The PCR products were 449 bp in size for sPLA2-IIA, 309 bp for COX-1, 531 bp for COX-2, 392 bp for EP2, 416 bp for EP3, 212 bp for EP4, and 311 bp for G3PDH.
To elucidate whether the effect of PGE\textsubscript{2} via the PLA\textsubscript{2}/COX-2 pathway on tumorigenicity in the Mz-ChA-2 cells is mediated by EPs, we evaluated the effect of EP\textsubscript{2-4} agonists or PGE\textsubscript{2} treatment on colony formation in a monolayer culture (Fig. 5C). Because the number of tumor colonies can be affected by increased proliferation of carcinoma cells or a decreased rate of apoptosis, a colony formation assay was performed. The Mz-ChA-2 cells were used for the experiments because endogenous production of PGE\textsubscript{2} was observed to be very low in the cells, and, thus, the signaling pathway via the EP\textsubscript{2-4} may be less activated. A dose-dependent increase in the colony number after treatment with 0.01, 0.1, 1, and 10 \(\mu\text{M}\) EP\textsubscript{2} agonist (ONO-AE1-329) for 14 days (a 1.4-fold increase at a concentration of 1 \(\mu\text{M}\)), as well as a 1.3-fold increase after 1 \(\mu\text{M}\) PGE\textsubscript{2} treatment, was observed in the cells. However, the colony number of Mz-ChA-2 cells after treatment with EP\textsubscript{2} agonist (ONO-AE1-259; Ref. 52) and the number with EP\textsubscript{4} agonist (ONO-AE-248; Ref. 52) were 109 \(\pm\) 5\% (at a concentration of 1 \(\mu\text{M}\)) and 103 \(\pm\) 6\% (at a concentration of 0.1 \(\mu\text{M}\)) of the nontreated cells, respectively. Treatment with an EP\textsubscript{2} or EP\textsubscript{4} agonist did not cause significant changes in the colony number of the cells. PGE\textsubscript{2} production via the PLA\textsubscript{2}/COX-2 pathway and its related EP\textsubscript{4} activation could be important components in mediating colony growth of gallbladder carcinoma cells.

Furthermore, to investigate the signals mediating the biological functions of PGE\textsubscript{2} or the EP\textsubscript{4} agonist, i.e., cell growth and proliferation, the expression of c-fos, a growth-related proto-oncogene, in the Mz-ChA-2 cells with or without treatment was determined. After (30 min) the addition of PGE\textsubscript{2} or the EP\textsubscript{4} agonist, total RNA was isolated from the cells and was subjected to RT-PCR analysis to observe the expression level of c-fos mRNA in the cells. The expression level of the c-fos product after being normalized to G3PDH was increased in a dose-dependent manner in the cells in response to treatment with the EP\textsubscript{4} agonist. c-fos mRNA was \(\sim 3\)-fold higher in the cells treated with 1 \(\mu\text{M}\) the EP\textsubscript{4} agonist than in the nontreated cells. The magnitude of increase in c-fos mRNA in the cells treated with 1 \(\mu\text{M}\) the EP\textsubscript{4} agonist was comparable with that in the cells treated with 1 \(\mu\text{M}\) PGE\textsubscript{2}. The expression level of EP\textsubscript{4} mRNA did not change significantly in these cells. These data strongly suggest that PGE\textsubscript{2} up-regulates c-fos expression in gallbladder carcinoma cells, at least partly, through activation of the EP\textsubscript{4}.

**DISCUSSION**

Overexpression of COX-2 has been reported in various types of gastrointestinal carcinomas (21–24, 56). However, the tissue localization of COX-2 in carcinoma tissues is not well understood. Localization of COX-2 is observed in tumor-derived epithelial cells of colonic adenocarcinomas (23), whereas the localization is found in stroma cells in tissues of colonic adenoma (57) and colorectal carcinoma (22, 23). In addition, the localization of COX-2 is found in interstitial cells of colonic adenomatous polyps formed in Apc\textsuperscript{Min} knockout mice (58), Apc\textsuperscript{Min} mice (59), and interleukin-10-deficient mice (60). These discrepant findings should be sorted out to determine the role of COX-2 in not only carcinogenesis but also

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**Fig. 4** ISH of COX-2 and EP\textsubscript{2-4} in gallbladder carcinoma tissue. A specimen of gallbladder carcinoma tissues from a case of pT\textsubscript{4} carcinoma was used for analysis. In **A**, COX-2 mRNA is expressed diffusely in the corresponding cancerous epithelia (original magnification: \(\times 200\)). In **B**, COX-2 mRNA is expressed focally in the corresponding fibroblasts (original magnification: \(\times 200\)). **C**, **D**, and **F**, EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4} mRNAs were diffusely expressed in the corresponding cancerous epithelia (original magnification: \(\times 200\)). In **E**, EP\textsubscript{4} protein is expressed in the cancerous epithelia (original magnification: \(\times 200\)).

---

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE\textsubscript{2} Concentration ((\mu\text{M}))</th>
<th>Colony Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>100 (Basal)</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td>0.01</td>
<td>1.4-fold increase</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td>1</td>
<td>1.3-fold increase</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td>10</td>
<td>No significant change</td>
</tr>
</tbody>
</table>

---

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EP\textsubscript{4} Concentration ((\mu\text{M}))</th>
<th>c-fos mRNA Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>100 (Basal)</td>
</tr>
<tr>
<td>EP\textsubscript{4}</td>
<td>0.01</td>
<td>3-fold increase</td>
</tr>
<tr>
<td>EP\textsubscript{4}</td>
<td>1</td>
<td>Significant increase</td>
</tr>
<tr>
<td>EP\textsubscript{4}</td>
<td>10</td>
<td>No significant change</td>
</tr>
</tbody>
</table>

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**Note:** The data in Table 1 and 2 were analyzed using a statistical software package (SigmaStat, Systat Software Inc., San Jose, CA).

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**Fig. 5** Mz-ChA-2 cells were observed to produce only trace amounts (Fig. 5A). The Mz-ChA-1 cells were observed to produce significant amounts of PGE\textsubscript{2} in response to treatment with arachidonate, whereas the Mz-ChA-2 cells were observed to produce only trace amounts (Fig. 5A). PGE\textsubscript{2} production in the cells appeared to be dependent on the expression level of COX-2, as reported for colorectal carcinoma cells (24, 55). The mRNAs of EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4} mRNAs were amplified in the Mz-ChA-2 cells (Fig. 5B), whereas EP\textsubscript{1} mRNA was not detected. In the ISH, EP\textsubscript{2-4} mRNAs were diffusely and strongly expressed in the cells (Fig. 5B).

---

**Effect of EP2-4 Agonists on Colony Formation and C-fos Expression in Gallbladder Carcinoma Cells.** COX-2 protein and mRNA were expressed strongly in the Mz-ChA-1 cells but only slightly in the Mz-ChA-2 cells (Fig. 5A). The Mz-ChA-1 cells were observed to produce significant amounts of PGE\textsubscript{2} in response to treatment with arachidonate, whereas the Mz-ChA-2 cells were observed to produce only trace amounts (Fig. 5A). PGE\textsubscript{2} production in the cells appeared to be dependent on the expression level of COX-2, as reported for colorectal carcinoma cells (24, 55). The mRNAs of EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4} mRNAs were amplified in the Mz-ChA-2 cells (Fig. 5B), whereas EP\textsubscript{1} mRNA was not detected. In the ISH, EP\textsubscript{2-4} mRNAs were diffusely and strongly expressed in the cells (Fig. 5B).

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in 11 specimens of pT\textsubscript{1} and pT\textsubscript{4} gallbladder carcinomas (257.9 \(\pm\) 26.1 pg/mg \(\times\) protein, \(P < 0.01\)), compared with the concentrations in 10 specimens of normal gallbladders (59.2 \(\pm\) 7.9 pg/mg \(\times\) protein; Table 2). Similarly, in terms of the depth of invasion, the concentration was significantly higher in the pT\textsubscript{3} and pT\textsubscript{4} carcinomas than in the pT\textsubscript{1} and pT\textsubscript{2} carcinomas (\(P < 0.01\)).
tumor growth and progression of human carcinomas in terms of epithelial-stromal interactions.

The important finding in the present study was that the expression levels of COX-2 in gallbladder carcinoma was increased in parallel to the depth of invasion; in pT3 or pT4 carcinoma of the gallbladder, a substantial increase in COX-2 mRNA and protein levels was observed compared with the levels in pT1 or pT2 gallbladder carcinoma or normal gallbladder tissue. In addition, ISH and immunohistochemistry revealed increased expression of COX-2 mRNA and protein in stroma cells adjacent to the cancerous epithelia of advanced carcinoma. Therefore, the main sources of COX-2 in the tissues of pT3 or pT4 gallbladder carcinoma may be not only the cancerous epithelia but also the adjacent stroma, and both the epithelia and stroma probably produce PGE2, which regulates tumor biology in terms of epithelial-stromal interactions.

Besides COX-2, it is well known that sPLA2-IIA is involved in the inflammatory response and can provide arachidonate for prostanooid production. Previous studies have shown that, like COX-2, PLA2 activity (13) and arachidonate levels (61) are increased in human colorectal carcinoma. As overexpression of sPLA2-IIA has been found in other carcinomas (26–28), the expression level of sPLA2-IIA mRNA was significantly increased in pT3 and pT4 gallbladder carcinomas compared with the concentration in pT1 and pT2 carcinomas and normal gallbladder tissues. The high level of sPLA2-IIA mRNA expression in advanced gallbladder carcinoma, in conjunction with the elevated expression of COX-2, could provide a substrate for COX-2 and lead to increased PG production. In another regard, sPLA2 itself could be directly related to growth and differentiation in the human gastrointestinal tract, because the sPLA2 receptor-mediated biological responses include stimulation of cellular proliferation (DNA synthesis; Ref. 62) and prostanooid production (63).

Interest should be focused on the biological effects of either COX-2 itself or PLA2/COX-2-derived PGE2 on tumor growth and progression of gallbladder carcinoma, because the tissue concentration of PGE2 was increased significantly in pT3 and pT4 gallbladder carcinomas in the present study. As indicated in several reports (50, 64–66), PGE2 produced by COX-2-expressing carcinoma cells and stroma cells may play an important role in tumor growth and progression. This is because PGE2 may stimulate carcinoma cell proliferation (51), inhibit apoptosis in carcinoma cells (51), promote immunosuppression in carcinoma tissues by preventing activation of inflammatory cells (67, 68), and induce growth factors important for the
progression of carcinomas (66). Furthermore, COX-2-derived PGE$_2$ may play an important role in the formation and maintenance of the stroma and vessel structure in carcinoma tissues, because PGE$_2$ stimulates mitogenesis in fibroblasts (66) and induces angiogenesis (69, 70). A markedly increased production of hepatocyte growth factor in COX-2-expressing human fibroblasts via a PG-mediated pathway (71) is most interesting in epithelial-stromal interactions and may explain the crucial role of stromal cells adjacent to carcinoma cells in tumor growth and progression. Thus, “field-effect” alterations in stromal cell biology might contribute to the development of gallbladder carcinoma.

It is of particular interest to determine the effect of the PLA$_2$-COX-2-derived PGE$_2$ on the biology of gallbladder carcinoma. In an experiment to determine the effect of PGE$_2$ treatment on the formation of colonies by plating gallbladder carcinoma cells (Mz-ChA-2) in a monolayer culture, we observed an increase in the number of Mz-ChA-2 cells in response to PGE$_2$ treatment through an up-regulation of c-fos expression. Supporting this, PGE$_2$ has been shown to potentiate a replication of gallbladder carcinoma cells (72). As found in several studies (73–75), the biological effect of PGE$_2$ in gastrointestinal tissues involves signaling via EP subtypes. Importantly, treatment with an EP$_4$ agonist was found to increase the number of Mz-ChA-2 cells to a similar degree through an up-regulation of c-fos expression. A key step by which PGE$_2$ potentiates growth of gallbladder carcinoma cells may be the activation of the EP$_3$, as observed recently in colorectal carcinoma cells (76). The activation of EP$_3$ in turn would mediate signals inside the nucleus to induce c-fos gene transcription, and the increased expression of c-fos, a growth-related proto-oncogene, may, at least in part, account for the increased number of colonies of the carcinoma cells as observed previously (54, 77). In contrast, treatment with an EP$_3$ or EP$_4$ agonist did not cause significant changes in the colony formation.

In summary, the results of the present study suggest that in cases of advanced pT$_1$ and pT$_3$ carcinoma of the gallbladder, the enhanced expression of COX-2 mRNA and protein is observed in the adjacent stroma rather than in the cancerous epithelia and that the stroma in these advanced gallbladder carcinomas is a potent source of PG synthesis. In epithelial-stromal interactions, the increased production of PLA$_2$-COX-2-derived PGE$_2$ in the adjacent stroma and its biological effect via EP$_4$ on the carcinoma cells in a paracrine fashion may contribute to the development of gallbladder carcinoma.

REFERENCES


Expressions of Cyclooxygenase-2 and Prostaglandin E-Receptors in Carcinoma of the Gallbladder: Crucial Role of Arachidonate Metabolism in Tumor Growth and Progression

Toru Asano, Junichi Shoda, Tetsuya Ueda, et al.


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