Expression of Cyclooxygenase-2 (COX-2) in Hepatocellular Carcinoma and Growth Inhibition of Hepatoma Cell Lines by a COX-2 Inhibitor, NS-398

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ABSTRACT

Cyclooxygenase-2 (COX-2) has been suggested to be associated with carcinogenesis. In hepatocellular carcinoma (HCC), the expression pattern of COX-2 protein has been well correlated with the differentiation grade, suggesting that abnormal COX-2 expression plays an important role in hepatocarcinogenesis. We investigated the expression pattern and clinical significance of COX-2 in HCC tissues. In addition, we evaluated the efficacy of a selective COX-2 inhibitor, NS-398, in three hepatoma cell lines. Thirty-six HCC tissues, 15 hepatoma cell lines, 1 colorectal cell line (HT-29), and 1 fibroblast cell line (SV80) were included in the study. We evaluated serological tests and histological and radiological evaluations of HCC tissues. Immunohistochemical staining for COX-2 was performed on 36 HCC tissues and 17 cancer cell lines. A cell viability assay for growth inhibition of NS-398 in five cell lines was performed. Immunohistochemically, all six well-differentiated HCCs were positive, whereas 83% (10 of 12) of the poorly differentiated HCCs were negative. There was no significant relationship between the intensity of COX-2 expression and the level of α-fetoprotein, tumor size, presence of portal vein thrombosis, tumor capsule and metastasis, Tumor-Node-Metastasis staging, and growth types (P > 0.05). According to the cell viability assay, NS-398 suppressed the growth of all cell lines, independent of the degree of COX-2 expression.

The inhibitory effect on each cell line was identified in 10 μM NS-398 and was significantly strong in 100 μM NS-398. All cell lines exhibited apoptosis, which was identified by 4′-6-diamidino-2-phenylindole staining. In conclusion, COX-2 may be a determinant of the differentiation grade of HCC, and the inhibition of COX-2 can induce growth suppression of hepatoma cell lines via induction of apoptosis.

INTRODUCTION

HCC is the leading cause of malignant cancer death in the world (1). However, the exact molecular mechanism of hepatocarcinogenesis is still unclear. COX-2, a rate-limiting enzyme in the pathway of PG synthesis, is one of the interesting cellular factors and has been suggested to be associated with carcinogenesis in colorectal cancer (2–5). In addition, expression of COX-2 has also been found to be high in other types of cancers including prostate (6), breast, gastric (7, 8), pancreatic (9), lung (10), and head and neck cancers (11). Empirically, inhibition of COX-2 with NSAIDs such as aspirin could induce the regression of colon polyps in patients with familial adenomatous polyposis (12). Experimentally, the size and frequency of intestinal polyps were markedly reduced in mice with a double knockout of adenomatous polyposis coli and COX-2 genes (13). Furthermore, a selective inhibition of COX-2 brought the nearly complete suppression of azoxymethane-induced colon cancer (14).

COX, known as PG synthase, catalyzes the metabolism of arachidonic acid to PGs and thromboxanes (15), and two isoforms, COX-1 and COX-2, have been identified. COX-1 is constitutively present in many cell types and is responsible for various cytoprotective prostanoids in a number of organs, such as the gastric mucosa and the kidneys, whereas COX-2 is usually absent under basal conditions but inducible in certain cells by mitogens, cytokines, and other factors (16, 17). Aspirin, which acetylates a serine on the substrate binding site of COX-1 and COX-2, completely blocks the substrate use of COX-1 and PGH2 formation by COX-2 (18, 19). Thus, the ability of NSAIDs like aspirin to inhibit COX-2 may well explain the therapeutic utility. In contrast, inhibition of COX-1 may explain the unwanted side effects of nonspecific NSAIDs, such as gastric toxicity and renal dysfunction (20). Recently, new NSAIDs, which are selective inhibitors of COX-2, were discovered, and these exert therapeutic efficacy without the toxic

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3 The abbreviations used are: HCC, hepatocellular carcinoma; COX-1, -2, cyclooxygenase-1, -2; NSAIDs, nonsteroidal anti-inflammatory drugs; HBV, hepatitis B virus; HCV, hepatitis C virus; PG, prostaglandin; AFP, α-fetoprotein; TNM, Tumor-Node-Metastasis staging system; DAPI, 4′-6-diamidino-2-phenylindole; T, tumor; NT, nontumor.
effects from the inhibition of COX-1. A recent study has demonstrated that a selective COX-2 inhibitor reduced the growth of colon cancer in carcinogen-treated rats and also inhibited the various cancer cell lines (21–23).

In HCC, the expression pattern of COX-2 protein was found to be well correlated with the differentiation grade, suggesting that abnormal COX-2 expression plays an important role in hepatocarcinogenesis (24, 25). In the present study, the expression pattern of COX-2 was investigated in HCC tissues for evaluation of its clinical significance. Furthermore, we demonstrated that a selective COX-2 inhibitor, NS-398, strongly inhibited the proliferation of hepatoma cell lines.

**MATERIALS AND METHODS**

**Materials**

**Human Tissues.** A total of 36 paired specimens of tumor and surrounding nontumor liver tissues were obtained from HCC patients, who were treated surgically in Kangnam St. Mary’s Hospital, The Catholic University of Korea, Seoul, Korea, between 1994 and 1999. Nontumor tissues, which were comprised of 5 chronic active hepatitis-based livers and 31 chronic active hepatitis tissues with cirrhosis, were examined. Before surgical resection of HCC, 15 patients were treated with transarterial chemotherapy. All specimens for this study were fresh tissues without necrosis from transarterial chemotherapy. The characteristic features of the patients are summarized in Table 1.

**Cancer Cell Lines.** A total of 15 hepatoma cell lines (HepG2, HepG2.2.15, HLE, HLF, HuH-7, Hep3B, PLC/PRF-5, SNU182, SNU354, SNU368, SNU387, SNU398, SNU423, SNU449, and SNU475), one colorectal cell line (HT-29), and one fibroblast cell line (SV80) were all obtained from the Korea Cell Bank (Seoul, Korea).

**Methods**

**Serological Tests.** HBV surface antigen and anti-HCV antibody were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kit (Organon Teknika, Durham, NC) according to the manufacturer’s instructions.
immunoassay kits (Abbott Laboratories, Abbott Park, IL). Serum AFP titer was measured by a RIA kit (Abbott Laboratories).

**Histological Interpretation (H&E Staining).** The differentiation grade was classified into three types according to the criteria proposed by the Liver Cancer Study Group of Japan: (a) well-differentiated; (b) moderately differentiated; and (c) poorly differentiated types (26).

**Radiological Evaluation of HCC.** Radiological features for the TNM staging of HCC before operation, such as size and growth types, were evaluated by computed tomography scan. The growth types were evaluated for gross histological interpretation according to the method suggested by the Liver Cancer Study Group of Japan: type I, single nodular type; type II, single nodular with perinodal type; type III, multinodular confluent type; type IV, multinodular discrete type; type V, massive type; and type VI, diffuse type (26).

**Statistical Analysis.** We evaluated the intensity of COX-2 expression, according to the level of AFP, tumor size, presence of portal vein thrombosis, tumor capsule and metastasis, differentiation grade by Liver Cancer Study Group of Japan, TNM staging, and growth types. For such analysis, COX-2 staining was classified into three different types by the intensity of staining: ++, strongly positive; +, positive; and −, negative (Fig. 1A–C). The statistical significance was determined by using the χ² test, and the significance level was set at P < 0.05.

**Culturing of Cell Lines.** Each cell line was grown on a tissue culture plastic dish in MEM (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum with

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**Fig. 1** Three patterns of the intensity of COX-2 staining in primary HCCs (×400). A, the strong intensity of COX-2 staining. The intensity of COX-2 in the tumor portion (T) is stronger than that in the non-tumor portion (NT) in well-differentiated HCC. B, the positive intensity of COX-2 staining. The intensity of COX-2 in the tumor portion (T) is the same as that in the non-tumor portion (NT) in moderately differentiated HCC. C, the negative intensity of COX-2 staining. COX-2 in the tumor portion (T) showed no staining in the poorly differentiated HCC, contrasted with that in non-tumor portion.
Table 2  Relationship between the intensity of COX-2 expression in the cytoplasm and differentiation grade

<table>
<thead>
<tr>
<th>COX-2 expression</th>
<th>Total (n = 38)</th>
<th>WD(^{a}) (n = 6)</th>
<th>MD(^{b}) (n = 20)</th>
<th>PD(^{c}) (n = 12)</th>
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<tr>
<td></td>
<td>NT/T(^{d})</td>
<td>No. (%)</td>
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<tr>
<td>+/+</td>
<td>7</td>
<td>18%</td>
<td>2</td>
<td>33%(^e)</td>
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<td>-/+</td>
<td>5</td>
<td>13%</td>
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<td>50%(^f)</td>
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\(^{a}\)WD, well-differentiated.
\(^{b}\)MD, moderately differentiated.
\(^{c}\)PD, poorly differentiated.
\(^{d}\)NT, nontumor; T, tumor.
\(^{e}\)Increased expression of COX-2 in WD HCCs; \(P < 0.03\).
\(^{f}\)Decreased expression of COX-2 in PD HCCs; \(P < 0.03\).

Fig. 2  The expression patterns of COX-2 at heterogeneity of the differentiation grade in a single tumor. A1, the borderline area between the well-differentiated (WD) HCC and poorly differentiated (PD) HCC components (×40). A2, WD HCC area (×400). A3, PD HCC area (×400); the borderline area showed a distinct expression for COX-2. B1, the mixed area included moderately differentiated (MD) HCC and poorly differentiated (PD) HCC (×400). B2, MD HCC area (×400). B3, PD HCC area (×400); the intensity of COX-2 staining was different according to the differentiation grade.
COX-2 Expression in Hepatocellular Carcinoma

In a CO₂ incubator at 37°C in a humidified atmosphere containing 5% CO₂ and 95% humidity, specimens, tissue sections (4 μm) mounted on silanized slides (DAKO Japan, Kyoto, Japan) were deparaffinized with xylene and dehydrated in a graded series of ethanol. After rehydration in absolute ethanol for 15 s, the slides were heated by microwave in 10 mmol/liter citrate buffer (pH 6.0; Zymed Lab Inc., San Francisco, CA) for 10 min. After washing in a PBS bath for 10 min at 4°C, the specimens were preblocked for 10 min in an autoblocker (Research Genetics, Huntsville, AL). Then, they were incubated overnight with mouse antihuman COX-2 monoclonal antibody (dilution, 1:100; Transduction Lab Inc., Franklin Lakes, NJ) at room temperature. After three washes in PBS, the sections were incubated with biotinylated antimouse IgG (Zymed Lab Inc., San Francisco, CA) for 8 min. After washing in PBS, 3-amino-9-ethylcarbazole (Lab Vision, Fremont, CA) solution was added. Counterstaining was done with hematoxylin (Research Genetics).

Immunohistochemical Staining for COX-2. For liver specimens, tissue sections (4 μm) mounted on silanized slides (DAKO Japan, Kyoto, Japan) were deparaffinized with xylene and dehydrated in a graded series of ethanol. After rehydration in absolute ethanol for 15 s, the slides were heated by microwave in 10 mmol/liter citrate buffer (pH 6.0; Zymed Lab Inc., San Francisco, CA) for 8 min. After washing in a PBS bath for 10 min at 4°C, the specimens were preblocked for 10 min in an autoblocker (Research Genetics, Huntsville, AL). Then, they were incubated overnight with mouse antihuman COX-2 monoclonal antibody (dilution, 1:100; Transduction Lab Inc., Franklin Lakes, NJ) at room temperature. After three washes in PBS, the sections were incubated with biotinylated antimouse IgG (Zymed Lab Inc., San Francisco, CA) for 10 min and then, incubated with a labeled-avidin-biotin method (Zymed Lab Inc., San Francisco, CA). After washing in PBS, 3-amino-9-ethylcarbazole (Lab Vision, Fremont, CA) solution was added. Counterstaining was done with hematoxylin (Research Genetics).

**Growth Inhibition of Hepatoma Cell Lines by NS-398.** Some cell lines were selected to evaluate the inhibitory growth effect of NS-398 (Cayman Chemicals, Ann Arbor, MI) according to the staining patterns of COX-2. The cells were plated at 5 × 10⁵ cells/well in six-well plastic dishes with 2 ml of 10% fetal bovine serum-supplemented medium for 24 h. The media were changed with serum-free (0.1% BSA) and phenol red-free ones containing various concentrations of NS-398, and cells were incubated for 5 days with refeeding at day 3.

For concentration-dependent experiments, media containing various concentrations of NS-398 were used, and the cells were harvested daily for 5 days. The cells were detached with 800 μl of trypsin-EDTA for 10 min at 37°C, washed, and resuspended in the medium. An aliquot was combined with an equal volume of trypan blue dye (Life Technologies, Inc.). The surviving cells were counted using a hemocytometer.

**DAPI Staining.** DAPI, a DNA-binding fluorescent dye, was used to determine whether the mechanism of growth inhibition of these cell lines by NS-398 was apoptosis. After treatment with 100 μM NS-398 for 5 days, the cells were washed three times with PBS, fixed in a solution of 3.7% formaldehyde for 10 min, fixed once in 1 ml of methanol, and then stained with 4 μg/ml DAPI (Oncor, Gaithersburg, MD) for 20 min. The results were determined with visual observation of the nuclear morphology by fluorescence microscopy.
RESULTS

Clinicopathological Features. Thirty-six subjects consisted of 29 males and 7 females; their mean age was 57 years of age (range, 38–77 years). Sixteen were HBV surface antigen-positive, four were anti-HCV antibody-positive, one had both HBV and HCV, six had alcohol-related liver diseases, and six had other non-B, non-C liver diseases. Three patients were undetermined (Table 1).

The mean tumor size was 5.1 cm; 23 patients had HCC ≤5.0 cm, and 13 patients had HCC ≥5.0 cm. As determined by morphological types on the computed tomography scan, there were 21 of type I, 2 of type II, 2 of type III, 8 of type IV, 1 of type V, and 2 of type VI (Table 1).

Among the 36 HCC specimens, two cases had heterogeneity in a single tumor, showing a mixture of two cell groups in which the differentiation grades differed: well and moderately differentiated in one; and moderately and poorly differentiated in the other. Therefore, as determined by the Liver Cancer Study Group of Japan, there were 6 well-differentiated, 20 moderately differentiated, and 12 poorly differentiated types (Table 1).

COX-2 Expression in Tumor and Nontumor Liver Tissues. In 36 nontumor specimens, COX-2 staining was positive in 26 (72%) and negative in 10 (28%). The nontumor specimens expressed mild intensity of COX-2 staining, because most cases had mild to moderate inflammatory activity. In 36 HCC specimens, COX-2 staining was positive to various degrees in 17 samples (47%) and negative in 19 (53%) samples.
Among the 17 COX-2-positive HCC specimens, 5 were strongly positive (Table 1).

Compared with that of the surrounding nontumor liver tissues, five different patterns of COX-2 staining could be identified in the tumor tissues: NT/T, +/+++; NT/T, −/++; NT/T, +/+; NT/T, +/−; NT/T, −/− (Table 2). When analyzed according to an individual differentiation grade, all of the 6 well-differentiated HCCs were positive, whereas 83% (10 of 12) of the poorly differentiated HCCs were negative. In the 20 moderately differentiated HCCs, 50% were negative in COX-2 expression and 50% were positive (Table 2 and Fig. 1).

Among the two HCC specimens that demonstrated heterogeneity in differentiation grade in a single tumor, one showed positive COX-2 expression in the area of well-differentiated HCC and negative in the area of moderately differentiated HCC. In the other, COX-2 expression was positive in the area of moderately differentiated HCC and negative in the area of poorly differentiated HCC (Fig. 2).

There was no significant relationship between the intensity of COX-2 expression and the level of AFP, tumor size, presence of portal vein thrombosis, tumor capsule and metastasis, TNM staging, and growth types (P > 0.05; Tables 3, 4, and 5).

**COX-2 Expression in Hepatoma Cell Lines.** In 12 hepatoma cell lines, except PLC/PRF-5, SNU398, and SNU423, COX-2 protein was expressed in the cytoplasm. Unexpectedly, SV80, a fibroblast cell line, did not show COX-2 protein in the cytoplasm. HT-29, a colon cancer cell line, which is used as a positive control, showed a strong COX-2 expression in the perinuclear area (Fig. 3).

**Growth Inhibitory Effect of NS-398 to Cell Lines.** An inhibitory growth effect of NS-398 in the cell lines with high COX-2 expression was evaluated by a cell viability assay (Fig. 4). Five cell lines were selected for this experiment: HLE, Huh-7, HepG2, SV80, and HT-29. NS-398 suppressed the growth of all cell lines independent of the degree of COX-2 expression.

The degree of inhibition was dose-dependent. The inhibitory effect on each cell line was identified in 10 μM NS-398, but the effect was most significantly strong in 50 μM and μ 100 μM NS-398.

**Apoptotic Cell Death as a Mechanism of Growth Inhibition by NS-398.** In the 3 days after treatment with 100 μM of NS-398, DAPI staining was performed to identify the characteristic features of cell death. All cell lines exhibited condensed and fragmented nuclei, which were indicative of apoptosis (Fig. 5).

**DISCUSSION**

As key enzymes in the conversion of arachidonate to PG H2, COX-1, and COX-2 are involved in various biological processes via the production of cell-specific PGs, including the regulation of immune function, kidney development, reproductive biology, and gastrointestinal integrity. Regarding oncogenesis, COX-2 contributes to tumor formation or growth, although the in vivo mechanism by which COX-2 affects tumor growth has not been determined. In addition, both tumor and stromally derived COX-2 could influence tumor angiogenesis and/or immune function (30).

According to the histological grade, HCCs are classified into three types: well-differentiated, moderately differentiated, and poorly differentiated. Well-differentiated HCCs are common among the HCCs of <2 cm in diameter, and they are rare in advanced tumors. Moderately or poorly differentiated HCC is commonly found among advanced HCCs (31). In the present study, it was found that COX-2 expression was well correlated with the differentiation grade of HCC. COX-2 was up-regulated in all of the six well-differentiated HCCs, whereas it was down-regulated in 83% of the poorly differentiated HCCs. Such a close relationship between COX-2 expression and the differentiation grade of HCC has been reported previously (24, 25, 32). These suggest that the modulation of COX-2 expression may be a determinant of cellular differentiation in HCC. Such a biological role of COX-2 can be supported by a recent observation that, when epithelial cells are transfected with the COX-2 gene, the adhesion to the extracellular matrix increases and apoptosis is inhibited (33).

In the nontumor liver tissues, which were all cirrhotic, COX-2 expression was identified in 72%, whereas 28% were completely negative. These results are similar to those of Kondo et al. (81% of cirrhotic liver; Ref. 32) and Koga et al. (nearly all of cirrhotic liver; Ref. 24). They suggest that the up-regulation of COX-2 of nontumor cirrhotic liver tissues may be closely related to the active inflammation.

In the hepatoma cell line study, the immunohistochemical staining for COX-2 protein showed a strong expression in the cytoplasm of 12 hepatoma cell lines, with the exception of
intestinal lesions in rats. The IC50 values of NS-398, which is a novel anti-inflammatory agent that produces much fewer gas-
can inhibit the COX-2 activity in inflammation, is 10 μM whereas COX-1 activity is completely unaffected by 100 μM
(27). The selective COX-2 inhibitor, NS-398, can inhibit PG endoperoxidase synthase activity and prevent malignant trans-
formation of colon polyps in animal models (14, 36). We also found that treatment with various concentrations of NS-398,
particularly 100 μM, could inhibit the growth of three hepatoma cell lines dose-dependently via induction of apoptosis, as evidenced by the DAPI staining of the dying cells. In addition, similar to the previous observations by Elder et al. (37) and Piazza et al. (38), such an inhibitory effect of NS-398 on cell growth was irrelevant to COX-2 expression. These findings suggest that the antitumor activity of COX-2 inhibitors may be associated with another unknown mechanism(s). For example, the negative regulation of angiogenesis was recently found to be a biological function of COX-2 (39).

The present study demonstrates that COX-2 may be a determinant of the differentiation grade of HCC and that the inhibition of COX-2 can induce growth suppression of hepatoma cell lines via induction of apoptosis, although the exact mechanism is unclear. Therefore, additional studies are warranted for determining the prognostic value of the COX-2 expression pattern and the chemopreventive and therapeutic efficacy of some COX-2 inhibitors in HCC.

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REFERENCES


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