Increased Expression of Cyclooxygenase-2 Protein in Human Gastric Carcinoma

Ho Yeong Lim,[1] Hee Jae Joo, Jin Hyuk Choi, Jong Wook Yi, Mal Sook Yang, Do Yeun Cho, Hyun Soo Kim, Dong Ki Nam, Kyi Beom Lee, and Hugh Chul Kim

Departments of Hematology and Oncology [H. Y. L., J. H. C., J. W. Y., M. S. Y., D. Y. C., H. S. K., D. K. N., H. C. K.] and Pathology [H. J. J., K. B. L.], Ajou University School of Medicine, Suwon 442-721, Korea

ABSTRACT

Gastric adenocarcinoma is one of the most common malignancies in the world, and yet little is known about its molecular process of development and progression. Recent studies have suggested that ingestion of nonsteroid anti-inflammatory drugs reduces the risk of colon cancer, presumably by inhibiting the cyclooxygenase (COX) enzyme. COX-2, one isoform of the COX enzyme, is the rate-limiting enzyme in prostaglandin synthesis, and the function of this enzyme is thought to relate to inflammatory processes and carcinogenesis. To understand the role of COX enzyme in gastric cancer, we measured COX-2 expression in 104 human gastric carcinoma tissues by immunohistochemical analysis. We obtained tissue specimens from 104 surgically resected gastric adenocarcinoma patients. We performed immunohistochemical stain for human COX-2 with polyclonal antibody in gastric carcinoma. After curative resection and extensive lymph node dissection, all patients received adjuvant chemotherapy containing 5-fluorouracil. Expression of COX-2 showed cytoplasmic staining, not only in cancer cells but also in precancerous lesions such as metaplastic and adenomatous cells. We confirmed up-regulation of COX-2 in gastric cancer tissues compared with normal paired mucosa using Western blot analysis. There was no correlation between clinicopathological characteristics of gastric cancer patients and intensity of COX-2 protein expression. This study indicates that COX-2 protein overexpression may contribute to an early event of gastric cancer development, and it further suggests that selective inhibition of COX-2 may provide a chemopreventive effect against gastric carcinogenesis.

INTRODUCTION

Gastric adenocarcinoma is one of the most common malignancies in the world, especially in Eastern Asia including Korea and Japan (1). Although the incidence of gastric carcinoma has been declining in Western countries, gastric carcinoma is still the leading cause of cancer death worldwide (2). Nevertheless, recent statistics indicate that the overall survival in patients with gastric carcinoma has improved in part because of the high detection rate of early cancer and wider implementation of radical surgery (3, 4). Nonetheless, the treatment outcome of this common malignancy is still not satisfactory. The primary treatment modality for gastric cancer is curative resection, but the 5-year survival rate in patients after curative surgical resection hovers around 20–40%, and various chemotherapeutic attempts in an adjuvant setting have failed to improve the survival rate in gastric cancer. Therefore, prevention and early detection of the tumor are essential to reduce cancer death resulting from gastric cancer.

Little is known about the molecular events leading to its development and progression. Recent studies suggest that COX-2[2] is important in carcinogenesis of gastrointestinal cancers (5–8). The COX (2) enzyme has a function to catalyze the conversion of arachidonic acid to prostaglandin (9). Two isoforms of COX share over 60% identity at the amino acid level (10). COX-1 is constitutively expressed in most tissues and has been proposed as a housekeeping gene for cytoprotection of the stomach mucosa, vasodilation in the kidney, and control of platelet aggregation (11). In contrast, *COX-2* is an immediate-early gene and is induced by various stimuli including mitogens, cytokines, growth factors, and tumor promoters. Increased expression of COX-2 has been linked to inflammatory processes and carcinogenesis (5, 7, 11–13). COX-2 expression is especially prominent in gastrointestinal cancers, suggesting its important role in the development of gastrointestinal cancers (5–8). COX-2 mRNA in colon cancers and adenomatous polyps were found to be to 86 and 43% higher, respectively, than normal mucosa (5). In addition, recent studies indicate that COX-2 is involved in carcinogenesis in a mouse model of familial adenomatous polyposis, and inhibition of COX enzyme induces regression of colonic carcinogenesis (14–16). However, the expression of COX-2 has not been extensively studied in gastric carcinoma (17–19). In the present study, we measured the COX-2 protein immunohistochemically in gastric carcinoma and examined its role in the development of gastric cancer.

MATERIALS AND METHODS

Patient Samples. Tissue samples were obtained from surgically removed specimens of 104 patients with primary gastric adenocarcinoma who underwent curative radical gastrec-

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[1] To whom requests for reprints should be addressed, at Department of Hematology and Oncology, Ajou University School of Medicine, Wonchon-dong san 5, Paldal-ku, Suwon 442-721, Korea. Phone: 82-331-219-5990; Fax: 82-331-219-5983; E-mail; Hoyeong@unitel.co.kr.

[2] The abbreviations used are: COX, cyclooxygenase; NSAID, nonsteroid anti-inflammatory drug; TNM, Tumor-Node-Metastasis.

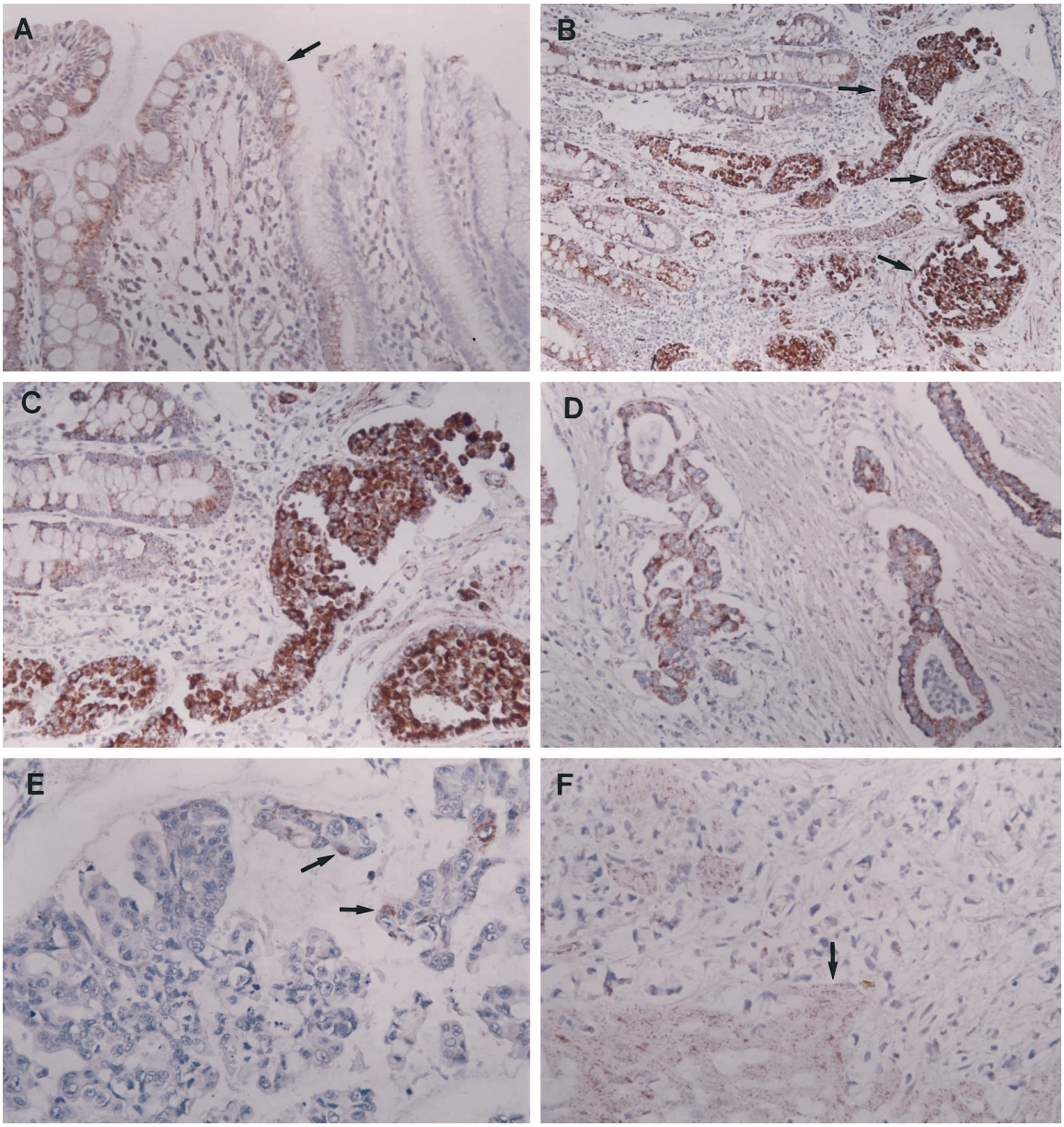

Fig. 1 Immunoreactivity of COX-2 in gastric mucosa. *A*, cytoplasmic immunoreactivity in intestinal metaplastic cells (*arrow*) in contrast to unreactive normal epithelium of the gastric mucosa (X200). *B*, more strongly reactive tumor cells (*arrows*) compared with metaplastic cells (×100). *C*, high-power view of *B* (X200). *D*, diffuse and strong immunoreactivity in tubular adenocarcinoma (×200). *E*, focal but strong immunoreactivity in mucinous adenocarcinoma (*arrows*; ×400). *F*, focal and weak immunoreactivity in signet ring cell carcinoma and internal positive control for COX-2 staining in smooth muscle cells (*arrow*; ×200).

tomy from June 1994 to December 1996 at Ajou University Hospital in Suwon, Korea. The surgical specimens were fixed in 4–10% buffered formaldehyde, embedded in paraffin, sectioned, and stained with H&E. These specimens were subjected to detailed pathological examination, which identified depth of invasion, nodal status, marginal involvement, and histological type of the tumors. The pathological tumor staging was determined according to the American Joint Committee on Cancer TNM classification (20). After curative resection, all patients received adjuvant chemotherapy containing 5-fluorouracil.

Immunohistochemical Staining. Paraffin-embedded blocks were sectioned at about 4-μm thickness, deparaffinized, and rehydrated. After microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval, slides were immersed in 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. After washing, slides were incubated overnight at 4°C with the polyclonal antibody against COX-2 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) in a dilution of 1:50. After a second incubation with a biotinylated antigoat antibody, slides were incubated with peroxidase-conjugated streptavidin

(DAKO LSAB+ kit; Dako Corp., Carpinteria, CA). Reaction products were visualized by immersing slides in diaminobenzidine tetrachloride and finally counterstained with Mayer's hematoxylin. We performed control immunostaining using preabsorption of anti-COX-2 antibody with human synthetic COX-2 peptide (Santa Cruz Biotechnology) to determine the specificity of primary antibody.

The immunohistochemical expression of COX-2 was examined independently by two pathologists using light microscopes without information of patients. Positive staining of smooth muscle cells within the gastric muscle coat provided an internal positive control for COX-2 staining. The percentage of positive tumor cells was graded semiquantitatively, and each sample was assigned to one of the following categories: 0 (0–4%); 1 (5–29%); 2 (30–59%); or 3 (60–100%). The intensity of immunostaining was determined as 0 (negative), 1 (weak), 2 (moderate, same intensity of smooth muscle cells), and 3 (strong). The immunoreactive score was calculated by multiplication of the grade determined by the percentage of positive cells and the staining intensity.

Western Blot Analysis. Frozen tissues were homogenized in ice-cold radioimmunoprecipitation buffer [150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, and 50 mM Tris (pH 8.0) supplemented with protease inhibitors leupeptin (1 μg/ml), aprotinin (1 μg/ml), and pepstatin (1 μg/ml)], and sonicated. The samples were then centrifuged (13,000 rpm for 10 min at 4°C), and supernatants were collected. Protein concentration was measured with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins (30 μg) were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was immersed in 0.5% skim milk for blocking. It was next incubated with a rabbit polyclonal IgG specific for human COX-2 (Santa Cruz Biotechnology) for 1 h at room temperature and then with peroxidase-labeled goat antirabbit IgG for 1 h at room temperature. Reaction bands were visualized by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

Statistical Analysis. Statistical analysis of the correlation between COX-2 expression in the tumors and clinicopathological parameters was calculated with the Student's *t* test and χ^2 test, and $P < 0.05$ was selected as the statistically significant value. Overall survival and disease-free survival were examined with the Kaplan-Meier method. Disease-free survival was defined as the time from the day of operation to a documented recurrence, or second primary cancer, or death from any other cause. Overall length of survival was measured from the day of operation. Overall survival and disease-free survival between two COX-2 expression groups were compared using the log-rank test.

RESULTS

We performed retrospective analysis for 104 patients who underwent curative resection for locally advanced gastric cancer. Reviewed hospital records were operative records, pathology reports, and clinical follow-up records of the patients. Curative resection was defined by the General Rules for Gastric Cancer Study in Surgery and Pathology of the Japanese Research Society for Gastric Cancer as: (*a*) no involvement of surgical stumps; (*b*) sufficient lymphatic dissection (R-number ≥ N-number); (*c*) no distant metastasis; (*d*) removal of involved adjacent organs and structures by combined en bloc resection; and (*e*) no gross residual disease (21). Postoperative adjuvant chemotherapy was started within 4 weeks after surgery for all patients. The chemotherapy regimens were not uniform, but all regimens consisted of 5-fluorouracil.

Table 1 Immunohistochemical expression of COX-2 protein in 104 gastric cancer patients

Grade[a]	1			2			3		
Intensity[b]	1	2	3	1	2	3	1	2	3
Immunoreactive score[c]	1	2	3	2	4	6	3	6	9
No. of patients	1	1	0	5	4	7	4	9	73

[a] Percentage of positive cells: 1, 5–29%; 2, 30–59%; 3, 60–100%.
[b] Staining intensity: 1, weak; 2, moderate; 3, strong.
[c] Immunoreactive score: grade multiplied by staining intensity ($a \times b$).

We investigated the expression and location of the COX-2 protein immunohistochemically in 104 gastric carcinoma tissues. All gastric cancer tissues showed positive staining with anti-COX-2 antibody (Fig. 1), and moderate to high immunoreactive scores were noted in the majority of the cases (Table 1), although normal gastric mucosa did not stain for COX-2. Immunoreactivity of COX-2 protein showed diffuse staining in the cytoplasm of tumor cells. Expression of COX-2 was also observed in smooth muscle cells and the fibroblasts and inflammatory mononuclear cells of the desmoplastic stroma. Additionally, epithelial cells showing intestinal metaplasia and adenoma were also strongly immunoreactive to COX-2 protein. Negative immunostaining with the synthetic COX-2 peptide confirmed the specificity of the primary anti-COX-2 antibody (data not shown). To confirm the results of the immunohistochemical investigations, we evaluated the expression of COX-2 at the protein level by Western blot analysis in gastric carcinoma tissues and normal paired gastric mucosa of same patients. We confirmed up-regulation of COX-2 in carcinoma tissues compared with normal paired mucosa. Cancer tissues showed intense immunoreactive bands of COX-2 protein, located at M_r 70,000, whereas normal gastric mucosa showed COX-2 protein expression at undetectable levels (Fig. 2).

Clinical and pathological characteristics of 104 gastric cancer patients are listed in Table 2. We evaluated the relationship between overexpression of COX-2 protein and various clinicopathological parameters of gastric cancer patients. We defined the group with high expression of COX-2 as tumors showing grade 3 and staining intensity 3; thus, their immunoreactive score would be 9. The majority of cases (73 of 104; 70.2%) exhibited high expression of COX-2 protein. However, there were no significant correlations between the levels of COX-2 expression and variable clinicopathological characteristics, such as histology, lymphatic invasions, and disease stages. The median follow-up duration of the survivors was 46 months (range, 31–58 months). There were no differences in recurrence and death between low and high expression of COX-2. Four-year disease-free survival rates of low and high expression of COX-2 were 60.7 and 57.8% ($P = 0.972$), respectively. Four-year

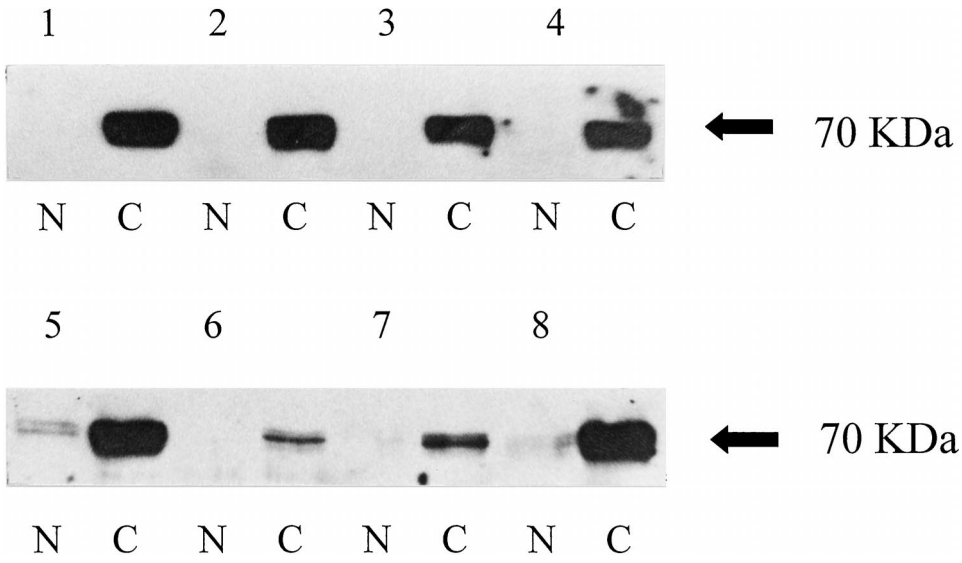

Fig. 2 Western blot analysis of COX-2 protein expression in gastric carcinoma tissues and normal paired mucosa. Total extracts (30 μg/lane) from both normal and gastric cancer tissues were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane and blotted with anti-COX-2 antibody. Bands were visualized with peroxidase-labeled goat antirabbit IgG antibody using chemiluminescence. *N*, normal gastric mucosa. *C*, cancer tissue. Numbers indicate each patient; *arrows*, molecular weight.

overall survival rate showed either no significant difference between low and high expression of COX-2 (59.6% *versus* 55.7%; $P = 0.950$; Table 2 and Fig. 3).

DISCUSSION

Recent epidemiological studies show that long-term use of NSAIDs reduces the risk of colon cancer development by 40% (22–24) and the risk of esophageal cancer development by up to 90% (25, 26). In addition, NSAIDs can induce regression of adenomatous polyps in patients with familial adenomatous polyposis (15, 27), as well as in Apc Min mouse model (28). Although the exact mechanisms of NSAIDs on cancer prevention have not been clarified, one possible role of NSAIDs is via the inhibition of COX enzyme, leading to chemopreventive effect.

In gastric cancer, several studies have shown enhanced expression of COX-2 in tumor tissues as compared with normal tissues, thus suggesting that COX-2 may play an important role in gastric carcinogenesis (17–19). Furthermore, our study demonstrated that overexpression of COX-2 is consistently observed in precancerous lesions such as metaplastic and adenomatous cells as well as in cancer cells of the stomach. Overexpression of COX-2 observed in metaplastic and adenomatous cells and not in normal mucosa in our study suggests that COX-2 may contribute to an early event in the gastric tumor formation. Similar results have been found in colon carcinoma and esophageal carcinoma. Although normal colonic epithelium expresses low levels of COX-2 mRNA, enhanced levels are expressed in 40% of colonic adenomas and in 90% of colon carcinoma (13). In addition, COX-2 was consistently up-regulated in Barrett's metaplastic tissues, a highly premalignant condition of the esophagus (29). The above results suggest that overexpression of COX-2 constitutes an early event in the gastrointestinal neoplastic transformation process. Ristimäki *et al.* (18) demonstrated that overexpression of COX-2 is one of the properties shared by gastric carcinoma of both intestinal and diffuse types, thus suggesting that COX-2 is connected to the early stages of carcinogenesis. Our results also suggest that COX-2 overexpression plays an important role in the initiation of gastric carcinogenesis. These findings suggest the possibility that the use of selective COX-2 inhibitors may provide a chemopreventive strategy against gastric carcinogenesis.

In the current study, COX-2 protein overexpression by immunohistochemical staining was found throughout all cancer tissues irrespective of clinicopathological characteristics of patients. This finding shows higher expression of COX-2 protein in comparison with previous studies for COX-2 expression in gastric cancer (17–19). One possibility for this result is high incidence of *Helicobacter pylori* infection in Korea. *H. pylori* has been known to contribute to initiating mucosal injury in the stomach and subsequent development of chronic atrophic gastritis (30, 31). Furthermore, *H. pylori* infection seems to play an important role in the development of gastric adenocarcinoma, particularly in the distal stomach (32). In patients who have gastric cancer with intestinal type, *H. pylori* infection has been identified in almost 90% of patients (33). A recent study shows that *H. pylori* up-regulates COX-2 mRNA expression and stimulates the release of prostaglandin E_2 in a gastric cancer cell line (34). Gastric tumors usually form more prostaglandins than their corresponding normal tissues (17). In Korea, recent studies revealed that the majority of adults have *H. pylori* infection (35), and this high infection rate of *H. pylori* may contribute to the higher expression of COX-2 protein in our study.

Several reports studied the relationship between COX-2 levels and clinicopathological characteristics of the tumors (36, 37). Fujita *et al.* (36) demonstrated that COX-2 levels significantly increased in colonic tumors with larger sizes and deeper invasion. In lung adenocarcinomas, markedly higher and more homogeneous COX-2 expression was observed in lymph node metastases than in the primary tumors (37). In contrast, COX-2 overexpression in gastric tumors did not correlate with clinicopathological characteristics, such as TNM staging, tumor histology, and lymphatic invasion in our study. This finding raises the possibility that COX-2 overexpression may be more inti-

Table 2 Relationship between COX-2 expression and clinicopathological characteristics in gastric cancer patients

Clinicopathological characteristics	Total	COX-2		*P*
		Low	High[a]	
No. of patients	104	31	73	
Sex				
Male	72	26	46	
Female	32	5	27	0.061
Age				
Median	53	54	53	0.776
Range	28–72	33–68	28–72	
Tumor size				
≤5 cm	53	13	40	
>5 cm	51	18	33	0.324
Histological differentiation				
Well	14	4	10	
Moderate	31	10	21	
Poor	37	8	29	
Signet ring cell	18	6	12	
Mucinous	4	3	1	0.258
Lauren classification				
Intestinal	58	17	41	
Diffuse	12	5	7	
Mixed	34	9	25	0.608
Lymphatic invasion				
No	25	8	17	
Yes	79	23	56	0.981
T Stage				
T_2	43	15	28	
T_3	58	15	43	
T_4	3	1	2	0.613
N stage				
N_0	18	6	12	
N_1	42	10	32	
N_2	28	8	20	
N_3	16	7	9	0.506
Disease stage				
IB	9	4	5	
II	29	8	21	
IIIA	31	7	24	
IIIB	17	4	13	
IV	18	8	10	0.416
Disease-free survival[b]	58.7%	60.7%	57.8%	0.972
Overall survival[b]	56.6%	59.6%	55.7%	0.950

[a] The group with high expression of COX-2 defined as tumors showing grade 3 and staining intensity 3, thus an immunoreactive score of 9.

[b] Disease-free survival and overall survival represent 4-year survival.

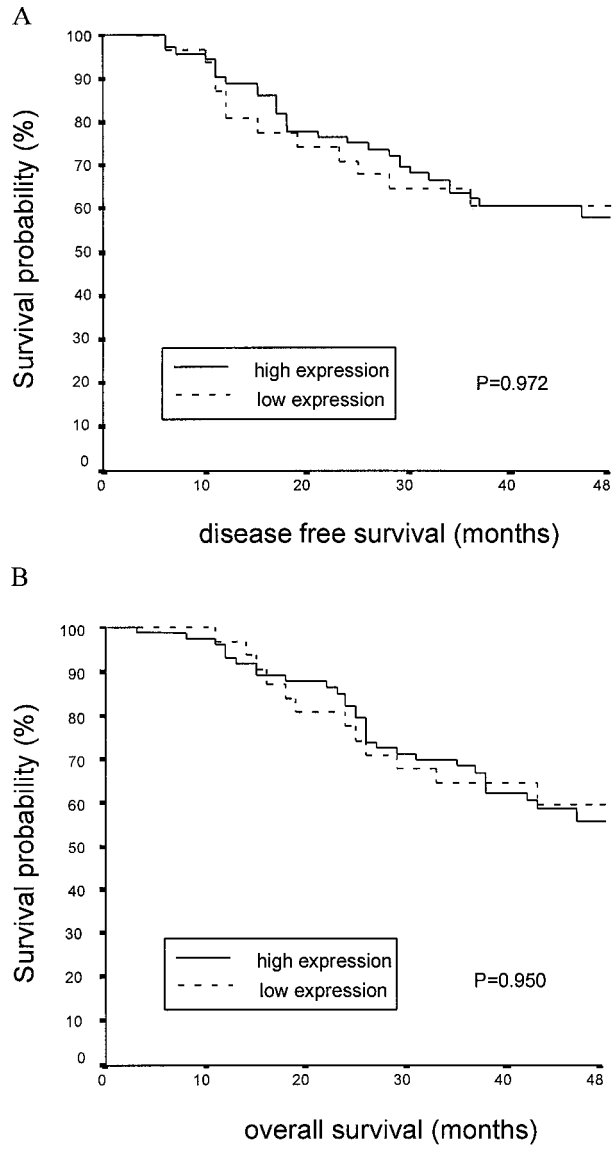

Fig. 3 Survival curves between low and high expression of COX-2 protein in gastric cancer patients. *A*, disease-free survival. *B*, overall survival.

mately involved in the initial development, not in the progression, of gastric cancer. However, further evidence that overexpression of COX-2 is involved in tumor growth and metastasis has come from experimental studies. Tsujii and DuBois (38) demonstrated that overexpression of COX-2 in intestinal epithelial cells developed alteration in adhesion to extracellular matrix proteins and inhibition of apoptosis after butyrate treatment. High Bcl-2 levels in these cells may relate to their resistance to undergo apoptosis, and in addition, down-regulation of E-cadherin and transforming growth factor β_2 receptors were found in cells transfected with COX-2. E-cadherin is related to cell-cell adhesion, and transforming growth factor β_2 receptors transduce signals important in modulating apoptosis.

COX-2-transfected cells acquired phenotypes showing increased invasiveness and metastatic potential. Biochemical changes associated with this phenotypic change included activation of membrane metalloproteinase-2 and increased RNA levels for the membrane type metalloproteinase-1 (39). These phenotypic changes were reversed by treatment with a COX-2 inhibitor, sulindac sulfide. In addition, they demonstrated that COX-2 leads to the release of proangiogenic prostaglandins. Prostaglandins stimulate angiogenic process by endothelial cell migration and tube formation (40). Therefore, inhibition of COX-2 overexpression provides a chemopreventive strategy against cancer development and progression. Because earlier used NSAIDs have properties for inhibiting both COX-1 and COX-2 activity, these drugs have induced many unwanted side effects, such as gastrointestinal ulceration or bleeding. Thus,

specific COX-2 inhibitors can reduce toxic side effects and enhance chemopreventive potency against carcinogenesis. A recent study by Sawaoka *et al.* (41) revealed that specific COX-2 inhibitors suppressed growth of tumor xenografts and cell replication and induced apoptosis in gastric cancer animal models. Our data show that COX-2 overexpression is an important and common event in initiating gastric carcinogenesis, and COX-2 inhibitors may be useful in the prevention of gastric cancer.

In conclusion, our study demonstrates that COX-2 protein is overexpressed in most metaplastic and adenomatous cells as well as cancer cells in gastric adenocarcinoma and suggests that COX-2 may be a strong potential target of chemoprevention in gastric carcinogenesis.

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