Cyclooxygenase-2 Expression in *Helicobacter pylori*-Associated Premalignant and Malignant Gastric Lesions

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Expression of cyclooxygenase-2 (COX-2) in various stages of the *Helicobacter pylori*-associated gastric carcinogenesis pathway has not been elucidated. We investigated the distribution and intensity of COX-2 expression in premalignant and malignant gastric lesions, and monitored the changes after *H. pylori* eradication. Gastric biopsies from *H. pylori*-infected patients with chronic active gastritis, gastric atrophy, intestinal metaplasia (IM), gastric adenocarcinoma, and noninfected controls were studied. Expression of COX-2 was evaluated by immunohistochemistry and *in situ* hybridization. Endoscopic biopsies were repeated 1 year after successful eradication of *H. pylori* in a group of IM patients for comparing COX-2 expression and progression of IM. In all *H. pylori*-infected patients, COX-2 expression was predominantly found in the foveolar and glandular epithelium and, to a lesser extent, in the lamina propria. In the non-infected group, only 35% of cases demonstrated weak COX-2 expression. Intensity of COX-2 was not significantly different between the chronic active gastritis, gastric atrophy, IM, and gastric adenocarcinoma groups. In 17 patients with IM, COX-2 expressions in the epithelial cells and stromal cells were reduced 1 year after *H. pylori* eradication. However, the changes in COX-2 expression did not correlate with progression/regression of IM. Both premalignant and malignant gastric lesions demonstrate strong COX-2 expression. Successful eradication of *H. pylori* leads to down-regulation of COX-2 expression but failed to reverse IM at 1 year. (Am J Pathol 2000, 157:729–735)

There is a wealth of evidence to support that cyclooxygenase-2 (COX-2), an inducible isoform of cyclooxygenase enzyme, which converts arachidonic acid to prostanooids, is strongly expressed in gastrointestinal tumors. Expression of COX-2 had been reported in colorectal

cancers,^{1,2} pancreatic cancer,³ hepatocellular carcinoma,^{4,5} esophageal cancers,^{6,7} and gastric cancers.^{8,9} Up-regulation of this inducible COX isoform in malignancies has also been found to associate with increased invasiveness and metastatic potentials of the tumors.^{10,11} Recently, there are also studies demonstrating COX-2 expression in premalignant lesions. These include colonic adenoma,¹ Barrett's esophagus,¹² and atypical alveolar epithelium in asbestosis and idiopathic fibrosing alveolitis,¹³ implying that this enzyme may be involved in the early process of carcinogenesis. Although the exact mechanism of how COX-2 contributes to cancer development is still unclear, existing data suggests that inhibition of COX-2 induces apoptosis, suppresses cellular proliferation, down-regulates bcl-2 expression,^{14–18} enhances the growth of H-ras-transformed cells,¹⁹ and induces expression of epidermal growth factor.²⁰

H. pylori has been classified as class 1 carcinogen for gastric cancer by the International Agency for Research on Cancer in 1994.²¹ According to Correa's²² model, gastric cancer develops in a multistep process from chronic active gastritis (CAG), gastric atrophy (GA), intestinal metaplasia (IM), dysplasia, and finally, gastric cancer. Recent studies also showed that *H. pylori* induced COX-2 expression in human gastric mucosa.^{23–26} Furthermore, McCarthy et al²⁶ showed that COX-2 expression in antral mucosa was reduced but not eliminated in the epithelium after successful eradication of *H. pylori*. The role of *H. pylori*-induced COX-2 expression in gastric carcinogenesis is not clear. This study attempted to address the issue from two perspectives. First, we studied COX-2 expression at different stages of gastric carcinogenesis from inflammation, premalignant lesions, to gastric cancer. Second, we followed the changes of COX-2 expression after eradication of *H. pylori* infection and attempted to correlate these changes with improvement or worsening of gastric premalignant lesions.

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Patients and Methods

Gastric biopsy tissues from *H. pylori*-infected patients with diagnoses of GA ($n = 20$), IM ($n = 51$), and gastric adenocarcinoma ($n = 25$) were studied. None of these patients were taking aspirin or NSAID. Intensity of COX-2 expression was examined by immunohistochemistry and was graded as 0 (negative), 1 (<5% cells with positive staining), 2 (5 to 30%), 3 (30 to 60% and with strong staining), and 4 (>60% and with very strong staining). Scoring was done by two independent investigators and the mean score was taken in each case. The localization of COX-2 protein detected by immunohistochemistry was verified by *in situ* hybridization. The scorings of COX-2 expression in various histological types were compared to patients with CAG ($n = 40$) and noninfected controls ($n = 40$).

In a group of patients with IM, endoscopic biopsy and immunohistochemistry were repeated at 1 year after 1 week of anti-*Helicobacter* therapy (omeprazole, amoxicillin, and clarithromycin). Antral biopsies were taken from the greater and lesser curvature within 2 to 3 cm from the pylorus for assessment of COX-2 staining and grading of IM as stipulated by the Updated Sydney Grading System.²⁷ Eradication of *H. pylori* was confirmed by negative results on the rapid urease test and histology.

Immunohistochemistry

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer-thick sections were deparaffinized, and endogenous peroxidase activity was blocked with 3% H₂O₂ in Tris-buffered saline (TBS). Non-specific binding was blocked with 5% rabbit serum (DAKO, Glostrup, Denmark) in TBS, and the tissues were incubated with antibody to COX-2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) in TBS containing 2% rabbit serum and 1% bovine serum albumin for 2 hours. This was followed by incubation with biotinylated rabbit anti-goat immunoglobulins (1:400; DAKO) for 45 minutes and then with avidin-biotin-peroxidase complex (DAKO) for 45 minutes. The color was developed in diaminobenzidine solution (Sigma Chemical Co., St. Louis, MO). The sections were then counterstained with Mayer's hematoxylin. Tissues were incubated with TBS containing 2% rabbit serum and 1% bovine serum albumin without the primary antibody as control. To confirm the cellular localization of COX-2, the immunohistochemical staining was repeated with another primary antibody against COX-2 (Cayman, Ann Arbor, MI) in a subset of samples ($n = 25$).

Generation of COX-2 RNA Probes

Human COX-2 cDNA fragment was prepared by reverse transcription-polymerase chain reaction of gastric carcinoma. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA). Five micrograms of total RNA was reverse-transcribed with the SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies, Inc., Gaithersburg, MD) using random oligonu-

cleotide priming in a 20- μ l reaction. COX-2-specific primers (forward: TTCAAATGAGATTGTGGGAAAATTGCT; reverse: AGATCATCTCTGCCTGAGTATCTT) were used to amplify a fragment of 305 bp. Polymerase chain reaction was performed for 40 cycles at 94°C for 35 seconds, 55°C for 35 seconds, and 72°C for 30 seconds in a 25- μ l volume containing 0.2 mmol/L dNTPs, 1 $\times$ polymerase chain reaction buffer (20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl), 1.5 mmol/L MgCl₂, and 0.625 U of *Taq* DNA-polymerase (Life Technologies, Inc.). The polymerase chain reaction products were analyzed in 2% agarose gel. The desired fragments were cut and cloned in pMOSBlue vectors using the pMOSBlue Blunt-Ended Cloning Kit (Amersham, Buckinghamshire, UK). The DNA fragment was sequenced to confirm identity and orientation of the insert. The anti-sense RNA probe was then synthesized using the DIG (digoxigenin) RNA Labeling Kit (Boehringer Mannheim, Germany). The integrity of the probe was determined by electrophoresis through an ethidium bromide-stained 2% agarose gel. Labeling efficiency was checked by dot blots as described by the manufacturer. A positive control experiment was conducted using glyceraldehyde-3-phosphate dehydrogenase RNA probe. For the negative control experiment, COX-2 sense RNA probe was prepared.

In Situ Hybridization

The method was modified from Komhoff et al.²⁸ Cryostat sections (8- μ m thick) were mounted onto 3-aminopropyltriethoxysilane-coated slides (Marienfeld, Badmergenheim, Germany) and fixed for 10 minutes in 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.4. After treatment in 0.2 N HCl for 20 minutes, the slides were incubated with 5 μ g/ml of proteinase K (Sigma) at 37°C for 15 minutes. Sections were then acetylated in 0.1 mol/L of triethanolamine with 0.25% acetic anhydride for 5 minutes and then 0.5% acetic anhydride for an additional 5 minutes. Prehybridization was performed with hybridization solution containing 50% formamide, 1 $\times$ Denhardt's solution, 10% dextran sulfate, 0.2 mg/ml of salmon sperm DNA, 1 mg/ml of tRNA, 1 mmol/L of ethylenediaminetetraacetic acid, and 2 $\times$ standard sodium citrate (SSC) devoid of cDNA probes for 1 hour. Hybridization was performed with 1 ng/ μ l digoxigenin-labeled COX-2 anti-sense RNA probe, and incubated at 65°C for 18 hours in a moist chamber. The slides were washed in 2 $\times$ SSC at room temperature for 1 hour, 1 $\times$ SSC at room temperature for 45 minutes, 0.1 $\times$ SSC at 60°C for 30 minutes, and 0.1 $\times$ SSC at room temperature for 15 minutes. Immunological detection was conducted using the DIG Nuclear Acid Detection Kit (Boehringer Mannheim). After the color development, the slides were counterstained with nuclear fast red. For negative controls, sections were hybridized with COX-2 sense RNA probe or treated with RNase before hybridization.

Statistical Analysis

Scoring of COX-2 staining between the five study groups (CAG, GA, IM, gastric adenocarcinoma, and control)

Table 1. Scoring of COX-2 Expression in Noninfected Patients (Control) and *H. pylori*-Infected Patients with Chronic Active Gastritis (CAG), Gastric Atrophy (GA), Intestinal Metaplasia (IM), and Gastric Cancer (CA)

Score	Control (n = 40)	CAG (n = 40)	GA (n = 20)	IM (n = 51)	CA (n = 25)
0	26	0	0	0	0
1	14	6	4	21	11
2	0	32	11	24	4
3	0	2	4	6	9
4	0	0	1	0	1

Intensity of COX-2 expression was graded as 0 (negative), 1 (<5% positive), 2 (5 to 30%), 3 (30 to 60% and strong staining), and 4 (>60% and very strong staining).

Significant difference was found between the different groups by the Kruskal-Wallis nonparametric ANOVA test ($P < 0.0001$). Using Dunn's multiple comparison test, significant difference was detected between the control group and all *H. pylori*-infected group. Among the four groups with *H. pylori* infection, no difference was found.

were compared by Kruskal-Wallis nonparametric analysis of variance test, using Dunn's multiple comparison test for post hoc comparison. Expression of COX-2 and grading of IM before and at 1 year after eradication of *H. pylori* was compared by the Mann-Whitney *U* test. Statistical significance was taken at $P < 0.05$.

Results

COX-2 expression was found in all gastric biopsies of *H. pylori*-infected patients, including 40 cases of CAG, 20 cases of GA, 51 cases of IM, and 25 cases of CA, with intensity scoring ranged from 1 to 4 (Table 1). *H. pylori*-associated gastritis exhibited strong expression of COX-2 in foveolar and glandular epithelium but with a lower intensity in mononuclear inflammatory cells, myofibroblasts, and endothelial cells (Figure 1, A and B). Strong expression of COX-2 was also found on intestinal epithelium of GA (Figure 1C) and IM (Figure 1, D and E). Both diffuse and intestinal types of gastric cancer cells expressed COX-2 protein on immunohistochemistry and there was no significant difference in intensity of expression (median score, 2 versus 2; Figure 1, F and G). The localization of COX-2 was confirmed by the second polyclonal antibody (Cayman antibody; Table 2) as well as by *in situ* hybridization (Figure 2). On the contrary, only 14 (35%) of noninfected patients expressed COX-2 protein in the gastric biopsy. The intensity of COX-2 expression was significantly higher in the *H. pylori*-infected group when compared to the noninfected control ($P < 0.0001$). However, there was no difference in the COX-2 expression between the four groups of patients with *H. pylori* infection (Table 1).

Seventeen patients with IM and successful eradication of *H. pylori* were followed-up for 1 year with repeated endoscopy. Comparing the paired samples from the same patient before and after *H. pylori* eradication reviewed significant reduction of COX-2 expression in the epithelial cells ($P = 0.049$) and stromal cells ($P = 0.001$) (Table 3). Changes in COX-2 expression in individual cases are shown in Figure 3. Although there was an

overall reduction in COX-2 expression at 1 year, changes in the foveolar epithelium failed to reach statistical significance. Furthermore, in all 17 cases, COX-2 expression was still detected in the epithelium albeit reduced expression. Reduction in COX-2 expression was more prominent in the stromal cells (Figure 1, H and I). In seven cases, total disappearance of COX-2 in the lamina propria was documented at follow-up. Changes in IM before and after eradication of *H. pylori* were highly diversified in these 17 patients. Progression of IM at 1-year follow-up was found in six patients, regression of IM in five, and unchanged histology in six. Overall, there was no significant change in the severity of IM after *H. pylori* eradication. There was no correlation between changes in IM and COX-2 down-regulation in this group of patients.

Discussion

Although previous studies confirmed that COX-2 expression in gastric mucosa is found in the presence of *H. pylori*-induced gastritis,²³⁻²⁶ their localization in the gastric tissue varies between different studies. In our study, COX-2 staining was predominantly found in the foveolar and glandular epithelium which was in keeping with that reported by Sawaoka et al.²³ On the contrary, Fu et al²⁴ reported that in *H. pylori*-positive gastritis, COX-2 staining is primarily localized in the mononuclear cells and myofibroblast in the lamina propria with no detectable staining in the epithelium. The difference in localization of COX-2 staining could be the result of variations in antibodies used in different studies and patient heterogeneity.

However, we have confirmed the cellular localization of COX-2 with a second set of antibodies (Table 2) and also by *in situ* hybridization (Figure 2) which strongly supported the validity of our findings.

In this study, we have shown that COX-2 is expressed in the epithelial lining of the stomach in the whole spectrum of *H. pylori*-associated gastric carcinogenesis pathway from CAG, to GA and IM, and finally to gastric cancer. This finding implies that COX-2 might be involved in the early stages of gastric cancer development ie, GA and IM. Similar phenomena have been reported in colonic adenoma and colorectal cancer as well as Barrett's esophagus and esophageal cancers.^{1,12} In a study on colorectal cancers, Hau et al²⁹ reported enhanced COX-2 expression in colonic adenoma when compared with normal mucosa but there was an insignificant difference between adenoma and adenocarcinoma of the colon. This observation parallels the relationship between GA, IM, and gastric cancer. Although the mechanism of COX-2 expression in carcinogenesis is still unclear, studies suggest that overexpression of COX-2 is associated with cellular resistance to apoptosis whereas treatment with specific COX-2 inhibitor suppresses cellular proliferation and induces apoptosis.^{6,14-18} This is postulated to be the possible mechanism of chemoprevention of colorectal cancer by nonsteroidal anti-inflammatory drugs. Moreover, prostaglandin, the product of cyclooxygenase, may also be related to the carcinogenesis process as PGE2 levels are increased in colorectal tumors³⁰ and

Figure 1. Immunohistochemical staining of COX-2 in: CAG showing strong expression in foveolar and glandular epithelium (A) and mild expression in mononuclear inflammatory cells, myofibroblasts, and endothelial cells in the lamina propria (B); GA showing strong expression in the atrophic glands of the gastric body (C); IM showing strong expression in intestinal epithelium and goblet cells (D and E); gastric cancer showing strong expression in cancer cells of both intestinal type (F) and diffuse type (G); IM before (H) and 1 year after eradication of *H. pylori* (I). There was an overall reduction in COX-2 expression with more dramatic changes in the glandular epithelium and stromal cells in the lamina propria. Residual expression of COX-2 was still present in the foveolar epithelium.

Table 2. Cellular Localization of COX-2 by the Use of Two Different Primary Antibodies (Santa Cruz; Santa Cruz, CA, and Cayman; Ann Arbor, MI) in Gastric Epithelium

		Santa Cruz antibody									
		Foveolar epithelium		Glandular epithelium		Stroma		Intestinal metaplasia		Tumor	
		+	-	+	-	+	-	+	-	+	-
Cayman antibody	+	10	0	8	3	10	1	5	0	5	0
	-	4	1	0	4	0	4	0	0	0	0

There were good correlation between the use of two antibodies in tumor and intestinal metaplasia ($n = 5$). The expression of COX-2 in the stroma, foveolar, and glandular epithelium also showed a very similar pattern by the use of the two different antibodies ($n = 15$).

eicosanoids stimulate the proliferation of colonocytes.³¹ It would be helpful to study the concentration of prostaglandin³² in gastric samples. Because of the unavailability of fresh samples, we were not able to examine the prostaglandin levels in this study. Interestingly, the addition of prostaglandins do not seem to affect the actions of NSAID in colon and gastric cancer cells, which argue against a role for the eicosanoids in tumorigenesis.^{33,34}

On the other hand, COX-2- or prostaglandin-independent pathways are also likely to have a role to play in chemoprevention. It has been shown that salicylic acid and sulindac sulfone, which do not appreciably affect the

catalytic activity of COX, also induce apoptosis in colonic cell lines.³³ Recently, other potential mechanisms for NSAID-related cancer prevention have also been suggested, which include alterations in cell cycle-machinery such as p21.³⁵

Because COX-2 expression is induced by *H. pylori* infection, we intended to investigate whether eradication of the bacterium, by resolution of inflammation, would lead to down-regulation of COX-2 expression and plausibly reverse the carcinogenesis process. Previous studies have shown that COX-2 expression in the antral epithelium is reduced but not eliminated after successful

Figure 2. A: *In situ* hybridization demonstrated that mRNA of COX-2 was predominantly located in the epithelial cells of the foveolar and glandular epithelium. **B:** Negative control by using sense COX-2 probe was shown.

Table 3. COX-2 Expression in 17 Patients with Intestinal Metaplasia before (HP+) and at 1-Year after (HP-) Eradication of *H. pylori*

Score	Epithelial cells		Stromal cells	
	HP+	HP-	HP+	HP-
0	0	0	0	7
1	1	7	7	9
2	5	6	10	1
3	11	3	0	0
4	0	1	0	0

Intensity of COX-2 expression was graded as 0 (negative), 1 (<5% positive), 2 (5 to 30%), 3 (30 to 60% and strong staining), and 4 (>60% and very strong staining).

Significant reduction in COX-2 expression was found in both epithelial cells ($P = 0.049$) and stromal cells ($P = 0.001$).

eradication of *H. pylori*.²⁶ In that study, biopsies were taken from patients with chronic gastritis and comparison was made at 4 weeks after antibiotic therapy. The present study examines a wider spectrum of gastric histology and a much longer follow-up period of 1 year after successful eradication of *H. pylori*. We found that in patients with IM, despite reduction of COX-2 expression at 1 year after eradication of *H. pylori*, there was no evidence of regression of IM or hence, the carcinogenic process. Specifically, there was no correlation between down-regulation of COX-2 and regression of IM. The most convenient explanation would be that 1-year follow-up is not sufficiently long enough for the regression of IM in the gastric

tissue to be detected. In fact, most studies following patients after *H. pylori* eradication do not show significant improvement in premalignant gastric lesions, such as atrophy and IM.³⁶⁻³⁸ However, the follow-up period of these studies may not be long enough to show any significant change in histology. Down-regulation of COX-2 and regression of IM, if it ever occurs, is likely to be a slow process.

Results from this study also showed that despite eradication of *H. pylori* in the stomach, there was only a modest reduction of COX-2 in the gastric epithelium. On the contrary, COX-2 expression in the lamina propria was markedly reduced. With the resolution of mucosal inflammation, COX-2 expression in mononuclear cells, myofibroblasts, and endothelial cells were greatly diminished. This dissociation in COX-2 expression between the epithelium and the lamina propria implies that expression of COX-2 in the former is not solely caused by inflammation. Other factors in the gastric milieu might contribute to perpetuation of COX-2 overexpression after successful *H. pylori* eradication. Thus, treatment of *H. pylori* infection alone, with the associated improvement in gastric inflammation, may not be sufficient to reverse the gastric carcinogenesis process. It would be interesting to examine the role of NSAID, or selective COX-2 inhibitors, on changes of COX-2 expression in premalignant gastric lesions after *H. pylori* eradication. However, human studies are lacking in this aspect. In addition to its role in carcinogenesis, animal studies suggested inhibition of COX-2 may impair ulcer healing³⁹ and resolution of allergic edema.⁴⁰ We had recently demonstrated the expression of both COX-1 and COX-2 in *H. pylori*-associated gastric ulcer.⁴¹ Whether the use of a COX-2 inhibitor, which is generally thought to be safe for the stomach, will actually impair the healing of ulcer in human still await further evaluation.

In conclusion, COX-2 protein is expressed in the gastric epithelium throughout the multistep gastric carcinogenesis cascade. Curing of *H. pylori* infection leads to down-regulation of COX-2 in the lamina propria but less so in the gastric epithelium. Hence, eradication of *H. pylori* may not be sufficient for the prevention of gastric cancer. Specific COX-2 inhibition should be tested for its efficacy in halting the process of carcinogenesis.

Figure 3. **A:** Changes in COX-2 expression in foveolar epithelium, glandular epithelium, and stromal cells of the stomach before (HP+) and at 1 year after (HP-) eradication of *H. pylori* in 17 patients with IM. Thickness of each line represents number of patients in that category (with numbers indicated). COX-2 expression was significantly reduced in glandular epithelium ($P = 0.036$) and stromal cells ($P = 0.0012$). No significant reduction in foveolar epithelium ($P = 0.099$) could be demonstrated. **B:** The percentage of samples showing increased, unchanged, and reduced in COX-2 expression at 1-year follow-up.

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