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\* H pylori \*

Roles of Helicobacter pylori infection and cyclooxygenase-2 expression in gastric carcinogenesis

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INTRODUCTION

Gastric cancer remains the world’s second, and the Chinese first, commonest cause of cancer related deaths[1]. There is epidemiological evidence that Helicobacter pylori (H pylori) infection is causally linked to gastric cancer[2,3]. It has been classified as a class I biological carcinogen by the World Health Organization[4]. However, the exact mechanism responsible for the development of gastric cancer in H pylori-infected patients still remains obscure. According to Correa’s [5] model, gastric cancer develops in a multistep process from chronic active gastritis, gastric glandular atrophy (GA), intestinal metaplasia (IM), dysplasia, and finally to gastric cancer. Recent studies have shown that H pylori infection induces cyclooxygenase-2 (COX-2) expression in human gastric mucosa[6,8]. COX-2, an inducible isoform of cyclooxygenase enzyme, which converts arachidonic acid to prostanooids, is strongly expressed in colorectal cancer[9,10], pancreatic cancer[11], hepatocellular carcinoma[12,13], esophageal cancer[14,15], and gastric cancer[16,17]. Several studies have also shown that COX-2 expression is increased in premalignant lesions including colonic adenoma[9], Barrett’s esophagus[18,19], and gastric adenomas[20], indicating that this enzyme may be involved in the early process of carcinogenesis.

It is well known that H pylori infection causes inflammation, and COX-2 is involved in inflammatory responses and also related to carcinogenesis. However, COX-2 expression in various stages of H pylori-associated gastric carcinogenesis pathway has not been elucidated. To clarify the role of H pylori induced COX-2 expression during carcinogenesis in the stomach.

METHODS: Gastric biopsies from 138 subjects [30 cases of chronic superficial gastritis (CSG), 28 cases of gastric glandular atrophy (GA), 45 cases of gastric mucosal intestinal metaplasia (IM), 12 cases of moderate gastric epithelial dysplasia and 23 cases of gastric cancer] were enrolled. H pylori infection was assessed by a rapid urease test and histological examination (modified Giemsa staining). The expression of COX-1 and COX-2 in human gastric mucosa was detected by immunohistochemical staining.

RESULTS: H pylori infection rate was 64.3% in GA and 69.5% in gastric cancer, which was significantly higher than that (36.7%) in CSG (P<0.05). The positive expression rates of COX-2 were 10.0%, 35.7%, 37.8%, 41.7% and 69.5% in CSG, GA, IM, dysplasia and gastric cancer, respectively. From CSG to GA, IM, dysplasia and finally to gastric cancer, expression of COX-2 showed an ascending tendency, whereas COX-1 expression did not change significantly in the gastric mucosa. The level of COX-2 expression in IM and dysplasia was significantly higher in H pylori-positive than in H pylori-negative subjects (P<0.01).

CONCLUSION: COX-2 expression induced by H pylori infection is a relatively early event during carcinogenesis in the stomach.

Abstract

AIM: Cyclooxygenase (COX-2) is over expressed in gastrointestinal neoplasm. Helicobacter pylori (H pylori) infection is causally linked to gastric cancer. However, the expression of COX-2 in various stages of H pylori-associated gastric carcinogenesis pathway has not been elucidated. Therefore, the aim of this study was to clarify the role of H pylori induced COX-2 expression during carcinogenesis in the stomach.

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was performed by one pathologist according to the updated Sydney system. GA was defined as loss of glandular tissue and fibrous replacement of lamina propria. IM or exchange of crypts by intestinal epithelium was recognized by the presence of goblet cells and absorptive cells.

**Detection of H pylori infection**

*H. pylori* infection was identified by histological examination using modified Giemsa stain and RUT (CLO test, Delta West, Bentley, Australia). Patients were classified as *H. pylori* positive if any of the two examinations yielded a positive result. Subjects were considered to be *H. pylori* negative only when both assays were negative for the organism.

**Immunohistochemistry**

Immunohistochemical staining for COX-1 and COX-2 was performed by the avidin-biotin-peroxidase complex (ABC) method using a Vectastain kit (Vector Laboratories, Burlingame, CA). In brief, 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 3 mL/L H2O2 in methanol for 30 min to block the endogenous peroxidase activity. Nonspecific binding was blocked with 50 mL/L rabbit serum (Dako, Glostrup, Denmark) in phosphate-buffered saline (PBS), and the tissues were then incubated with goat polyclonal antibody against COX-1 or COX-2 (1:200, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) in PBS containing 20 mL/L rabbit serum and 1 mL/L Triton 100 overnight at 4°C in a humidity chamber. After being rinsed with PBS, the sections were subsequently incubated with biotinylated secondary rabbit anti-goat immunoglobulins (1:400) for 45 min and then with avidin-biotin-peroxidase complex for another 45 min. The color was developed in 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) solution containing 0.3 mL/L H2O2. Nuclei were counterstained with Mayer’s hematoxylin (Merck, Darmstadt, Germany). Tissues of part sections were incubated with PBS containing 20 mL/L rabbit serum and 1 mL/L Triton 100 without the primary antibody as a negative control.

**Evaluation of COX-1 and COX-2 immunostaining**

In each section, 5 high-power fields were selected, and a total of at least 1,000 cells were calculated. The percentage of positive staining cells was graded semiquantitatively, and each sample was assigned to one of the following categories: - (negative, 0% to 4%); + (weak, 5% to 29%); ++ (moderate, 30% to 59%); or + ++ (strong, more than 60%). All immunostained sections were evaluated independently by two investigators who were blind to the pathological and clinical data. Evaluations were similar among assessors, with less than 10% disagreement. A final consensus was achieved between the 2 assessors using a multilabeled microscope.

**Statistical analysis**

Expressions of COX-1 and COX-2 between the 5 study groups (CSG, GA, IM, dysplasia and gastric cancer) were compared by Kruskal-Wallis nonparametric analysis of variance test, using Dunn’s multiple comparison tests for post hoc comparison. The association between COX-2 expression and *H. pylori* infection was analyzed using Fisher’s exact test. Statistical significance was taken at *P*<0.05.

**RESULTS**

**Histopathologic characteristics and the prevalence of *H pylori* infection**

Of the 138 patients, 63 were *H pylori*-positive and 75 were *H pylori*-negative. Table 1 shows the histopathologic characteristics and *H pylori* status. Histopathologic diagnosis in this population included CSG (*n*= 30), GA (*n*= 28), IM (*n*= 45), moderate dysplasia (*n*= 12) and gastric cancer (*n*= 23). The rates of *H pylori* infection in GA and gastric cancer were significantly higher than that in CSG.

**Table 1** Rates of *H pylori* infection in various gastric mucosal lesions

| Pathological diagnosis | *n* | *H pylori* rate (%) |
|------------------------|-----|--------------------|
| CSG                    | 30  | 36.7               |
| GA                     | 28  | 64.3*              |
| IM                     | 45  | 31.1               |
| Dysplasia              | 12  | 33.3               |
| Gastric cancer         | 23  | 69.5*              |

*P*<0.05 vs CSG.

**Expression of COX-1 and COX-2 in human gastric mucosa with various lesions**

COX-1 was clearly detected in the gastric foveolar and glandular epithelium including parietal cells. Patchy cytoplasmic staining for COX-1 was also seen in the inflammatory mononuclear cells and macrophages, myofibroblasts, as well as endothelial cells in the lamina propria. Spotty cytoplasmic staining for COX-1 was seen in gastric cancer cells (Figure 1). Perinuclear and cytoplasmic staining for COX-2 was mainly seen in the foveolar and glandular epithelium, and mild staining in mononuclear inflammatory cells and macrophages in the lamina propria. Strong expression of COX-2 was also found on glandular epithelium of IM and dysplasia. Immunoreactivity of COX-2 protein showed diffuse staining in the cytoplasm of gastric cancer cells (Figure 2). Furthermore, scattered expression for COX-2 was detected in interstitial cells such as vascular endothelial cells and myofibroblasts. Table 2 shows the positive rates of COX-1 and COX-2 expression in the gastric mucosa with various lesions. From CSG to GA, IM, dysplasia and finally to gastric cancer, the expression of COX-2 showed an ascending tendency, whereas COX-1 expression did not change significantly in the gastric mucosa with various lesions.

**Table 2** Expression of COX-1 and COX-2 in gastric mucosa with various lesions

| Pathological diagnosis | *n* | COX-1 expression (%) | COX-2 expression (%) |
|------------------------|-----|----------------------|----------------------|
| CSG                    | 30  | 56.6                 | 10.0                 |
| GA                     | 28  | 53.8                 | 35.7*                |
| IM                     | 45  | 53.3                 | 37.8b                |
| Dysplasia              | 12  | 41.7                 | 41.7*                |
| Gastric cancer         | 23  | 43.4                 | 69.5c                |

*P*<0.05*;*P*<0.01 vs CSG; *P*<0.05 vs GA.

**Relationship between *H pylori* infection and COX-2 expression in the gastric mucosa**

COX-2 expression was found in 57% (36/63) *H pylori*-infected patients, including one case of CSG, eight cases of GA, twelve cases of IM, four cases of dysplasia, and eleven cases of gastric cancer, with intensity scoring ranged from + to +++ (Table 3). *H pylori*-associated gastritis exhibited strong expression of COX-2 in foveolar and glandular epithelium but with a lower intensity in mononuclear inflammatory cells. On the contrary, only 20% (15/75) of non-infected patients expressed COX-2 protein in the gastric biopsy. The intensity of COX-2 expression in IM and dysplasia was significantly higher in *H pylori*-
positive than in \textit{H pylori}-negative subjects (P<0.01). However, there was no significant difference in COX-2 expression in CSG, GA and gastric cancer patients with or without \textit{H pylori} infection (Table 3).

**DISCUSSION**
Epidemiological studies have shown that long-term use of NSAIDs reduces the risk of colon cancer development by 40\%\([22,23]\) and the risk of esophageal cancer development by up to 90\%\([24,25]\).
In addition, NSAIDs could induce regression of adenomatous polyps in patients with familial adenomatous polyposis[26-27], as well as in an Apc Min mouse model[20]. Although the exact mechanisms of NSAIDs on cancer prevention have not been clarified, one possible role of NSAIDs is via the inhibition of COX enzymes, leading to chemopreventive effect. COX exists in two isoforms, of which COX-1 is constitutively expressed in many tissues, including the stomach, and COX-2 showing 61% homology with COX-1 is expressed at low concentrations or is even undetectable in unstimulated cells or tissues, but is readily induced by various stimuli including mitogens, cytokines, growth factors, and tumor promoters in inflammatory and certain cell types, such as fibroblasts, macrophages and endothelial cells[25-30]. It is well known that COX-2 is strongly expressed in gastric cancer[17-20]. More than 90% of gastric cancers are adenocarcinomas, which are divided into intestinal and diffuse histological types. Pathogenesis of the intestinal-type gastric cancer has been connected to precursor changes such as GA, IM, and dysplasia. The present study clearly showed that COX-2 protein was expressed not only in gastric cancer cells but also in the glandular epithelium of IM and dysplasia as detected by immunohistochemistry. From CSG, GA to IM and dysplasia and finally to gastric cancer, the expression of COX-2 showed an ascending tendency, whereas COX-1 expression did not change significantly in premalignant and malignant gastric lesions. These results provided evidence that COX-2 might contribute to an early event in gastric carcinogenesis[31], and suggesting the possibility that the use of selective COX-2 inhibitors may provide a chemopreventive strategy against gastric carcinogenesis.

H pylori infection is an important risk factor for adenocarcinoma of the distal stomach in humans[22-23], but the mechanism whereby H pylori infection contributes to gastric carcinogenesis is still hypothetical. Recent studies have shown that H pylori infection induces COX-2 expression in human gastric mucosa[6-8]. Furthermore, McCarthy et al[32] showed that COX-2 expression in antral mucosa was reduced but not eliminated in the epithelium after successful eradication of H pylori. Kimura et al[33] reported that immunoreactivity of COX-2 was observed in all cases of IM even after the cure of H pylori infection. Thus, cure of H pylori infection may decrease the risk of gastric carcinogenesis due to COX-2-related compounds in gastric mucosa but not in those patients with IM. In this study, we demonstrated that COX-2 was expressed in epithelial lining of the stomach in the H pylori-associated gastric carcinogenesis pathway from CSG, to GA and IM and dysplasia, and finally to gastric cancer. The level of COX-2 expression in patients with IM and dysplasia was significantly higher in H pylori-negative than in H pylori-positive subjects. In contrast, COX-1 protein was constitutively expressed in different gastric mucosal lesions with or without H pylori infection. Thus, COX-1 is considered as a housekeeping gene, and prostanooids synthesized via the COX-1 pathway are thought to be responsible for cytoprotection of the stomach, for vasodilatation in the kidney, and for production of a proaggregative prostanooid, thromboxane, by the platelets. In contrast, COX-2 is an inducible immediate-early gene, and its role has been connected to inflammation, reproduction, and carcinogenesis. Although the exact mechanism of COX-2 expression in carcinogenesis is still unclear, some studies have suggested that overexpression of COX-2 is associated with cellular resistance to apoptosis whereas treatment with specific COX-2 inhibitors could suppress cell proliferation and induces apoptosis[14,34-36]. It has been hypothesized that the alteration of the balance between apoptosis and proliferation of gastric epithelial cells, induced by H pylori infection, contributes to either gastric injury or carcinogenesis of the stomach[37-39]. Although H pylori could decrease epithelial cell proliferation and induce apoptosis in vitro[40-43], H pylori infection in vivo was associated with both increased apoptosis and proliferation[44-46].

These differences between the in vivo and in vitro results suggest that in vivo factors including inflammatory cells, extracellular matrix proteins, cytokines, and adhesion molecules may also contribute to epithelial cell turnover. On the other hand, COX-2 expression has been reported to correlate with invasion of the lymphatic vessels, lymph node metastasis, and advanced tumor stage in gastric cancer[47-48]. In conclusion, COX-2 over-expression plays an important role in the initiation of gastric carcinogenesis. The use of selective COX-2 inhibitors may provide a chemopreventive strategy against gastric carcinogenesis.

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