Elevated cyclooxygenase-2 expression in patients with early gastric cancer in the gastric pylorus

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Background. Duodenogastric reflux after surgery increases the risk of gastric carcinoma. To determine whether bile reflux influences the development of gastric cancer in patients who have not had surgery, we compared cyclooxygenase-2 (COX-2) immunoreactivity in early gastric cancer originating from the gastric pylorus and that originating from other locations. We also examined the effects of bile acids on the expression and activity of COX-2 in gastric cells in vitro. Methods. Tumor sections from 79 patients who underwent endoscopic mucosal resection for early intestinal-type gastric carcinoma were stained using a COX-2-specific monoclonal antibody. Immunoblotting of COX-2 was used to assess the effects of bile acids on COX-2 expression and activity in human gastric cell lines. Results. Among the 79 early gastric cancer lesions studied, 13 (16%) arose in the gastric pylorus. In this group, COX-2 immunoreactivity was negative to weak in 38% (5 of 13 lesions) and moderate to strong in 62% (8 of 13 lesions). In the control group, COX-2 immunoreactivity was negative to weak in 70% (46 of 66 lesions) and moderate to strong in 30% (20 of 66 lesions). COX-2 expression was significantly elevated in early gastric cancer located in the gastric pylorus, compared with that in the other locations. In human gastric cell lines, bile acids induced COX-2 expression, mediated by the ERK 1/2 mitogen-activated protein kinase pathway. Conclusions. COX-2 expression is elevated in early gastric cancer of the gastric pylorus, a common site of gastric cancer. Bile acids induced COX-2 expression in human gastric cell lines, suggesting a role of bile reflux in gastric carcinogenesis.

Key words: COX-2, gastric pylorus

Introduction

Factors associated with an increased risk of gastric cancer include smoking, Helicobacter pylori infection, high salt intake, and low consumption of raw vegetables and fresh fruit.1–3 Duodenogastric reflux is also associated with gastrointestinal carcinogenesis. Increased duodenogastric reflux after surgery increases the risk of gastric cancer.4 In particular, the Billroth II procedure is reported to result in a higher incidence of subsequent cancer than the Billroth I procedure. This is likely because patients usually have a larger amount of duodenal contents containing bile in the gastric stump after Billroth II gastrectomy than do those after Billroth I. Furthermore, duodenal reflux induces gastric adenocarcinoma in rats with gastrojejunal stomata.5 Of note, healthy subjects may have as much bile reflux as gastric ulcer patients, which indicates that duodenal reflux occurs physiologically.6 In fact, duodenal reflux through the pylorus can often be found on gastric endoscopy. However, the relationship between gastric carcinoma and duodenogastric reflux in subjects who have not had surgery remains unclear.

Nonsteroidal anti-inflammatory drugs (NSAIDs) cause regression of colorectal adenomatous polyps in patients with familial adenomatous polyposis (FAP).7 Similarly, several epidemiologic studies have shown that the prolonged use of aspirin is associated with a reduced risk of colorectal cancer.8–10 The best known target of NSAIDs is cyclooxygenase (COX), the rate-limiting enzyme in the conversion of arachidonic acid to prostanooids.11,12 Several isoforms of COX enzymes have been reported. COX-1 is a housekeeping enzyme. In contrast, COX-2 is an inducible immediate-early gene, and its pathophysiological role has been connected to inflammation, the immune system, ovulation, and carcinogenesis.13–15 Many kinds of stimulus induce COX-2 expression. Recently, bile acids were reported to induce COX-2 expression in many cell-culture systems, includ-
ing cells from the gastrointestinal tract, which indicated a relationship with carcinogenesis.\textsuperscript{16–18} Furthermore, there may be other forms of COX enzyme; COX-3, an isoform of COX-1, was identified recently.\textsuperscript{19} COX-3 is selectively inhibited by analgesic drugs such as acetaminophen. However, whether COX-3 is involved in tumorigenesis is not known.

To examine the possible involvement of bile reflux in the development of gastric cancer in nonsurgical patients, we compared COX-2 immunoreactivity in early gastric cancer originating from the gastric pylorus with that originating from other gastric locations. We also examined the effects of bile acids on the expression and activity of COX-2 in gastric carcinoma cell lines. The hypothesis tested herein is that the reflux of bile acids might affect the pyloric mucosa directly and cause the subsequent development of cancer in this area. The results indicated that COX-2 expression was elevated in early gastric cancer of the gastric pylorus. Bile acids induced COX-2 expression in human gastric carcinoma cells in vitro.

**Patients and methods**

**Patients**

Between 1996 and 2003, 79 patients underwent endoscopic mucosal resection (EMR) for early intestinal-type gastric carcinoma at the Division of Gastroenterology, Showa University Fujigaoka Hospital, Yokohama, Japan. The indication criteria for endoscopic resection were intramucosal differentiated (papillary or tubular) adenocarcinoma, no ulcer fibrosis, and a tumor diameter of less than 2cm. In Japan, gastric carcinoma is diagnosed according to nuclear and structural criteria, even when invasion is absent.\textsuperscript{20} Paraffin-embedded archival tissues were studied retrospectively.

**Immunohistochemistry**

Sections (4\mu m) were cut and incubated overnight with anti-human COX-2 monoclonal antibody (no. 160112; Cayman Chemical, MI, USA) diluted at 1:200. We used Cayman’s anti-COX-2 antibody because it is well-characterized.\textsuperscript{21–24} A biotin-avidin-immunoperoxidase method (Vector, CA, USA) was used for localization, according to the manufacturer’s instructions. As positive and negative controls for Cayman’s anti-COX-2 antibody, we used a gastric hyperplastic polyp, which is reported to possess COX-2 immunoreactivity in stromal cells.\textsuperscript{23} We observed strong immunoreactivity in stromal cells and very little immunoreactivity in epithelial cells with Cayman’s anti-COX-2 antibody (Fig. 1A).

The specimens were scored based on the intensity and extent of COX-2 immunoreactivity. The scoring criteria we used for the tumor cells were those of Buskens et al.:\textsuperscript{23} 0, no staining; 1+, weak diffuse cytoplasmic staining; 2+, moderate to strong granular cytoplasmic staining in 10%–90% of the cancer cells; and 3+, more than 90% of the tumor cells stained with strong intensity. Scores of 2 and 3 were categorized as COX-2 high for the statistical analyses.

**Cell culture**

AGS, a well-differentiated human gastric adenocarcinoma cell line, provided by Dr. Hiroyuki Mutoh (Jichi Medical School, Tochigi, Japan), was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 20mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 10mM NaHCO\textsubscript{3} and antibiotics. MKN7, another well-differentiated human gastric carcinoma cell line, provided by Dr. Teiichi Motoyama (Yamagata University Yamagata, Japan), was maintained in RPMI 1640 medium supplemented with 10% FCS, 20mM HEPES, 10mM NaHCO\textsubscript{3}, and antibiotics. All the cultures were incubated at 37°C under 95% air and 5% CO\textsubscript{2}.

**Western blotting**

Cells were solubilized in RIPA buffer (1x phosphate-buffered saline [PBS]/1% Nonidet P-40[Nacalai Tesque, Japan]/0.5% sodium deoxycholate/0.1% sodium dodecylsulfate [SDS]), containing 1mM phenylmethylsulfonyl fluoride (PMSF) and 100 units/ml aprotinin. Lysates were sonicated for 20s on ice and centrifuged at 10000g for 10min. Proteins (25\mu g/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to nitrocellulose membranes. The filters were probed with an anti-human COX-2 antibody (IBL, Gunma, Japan) or an anti-COX-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Visualization was performed with an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, UK).

**PGE\textsubscript{2} production**

Cells (1 \times 10^5/well) were plated in 24-well dishes and grown to 60% confluence in DMEM containing 10% FCS. The medium was then replaced with DMEM containing vehicle or bile acid for 12h. At the end of the treatment period, the medium was replaced with fresh DMEM and 10mM sodium arachidonate. After 30min, the medium was collected for the analysis of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). The levels of PGE\textsubscript{2} released by an cells were measured by an enzyme immunoassay (Cayman