Expression of Cyclooxygenase-2 in Dysplasia of the Stomach and in Intestinal-type Gastric Adenocarcinoma

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ABSTRACT

**Purpose:** Cyclooxygenase (Cox) is the key enzyme in conversion of arachidonic acid to prostanoids. Two Cox genes have been cloned, and expression of Cox-2 mRNA and protein has been shown to be elevated in several human malignancies and in animal models of carcinogenesis. The purpose of this study was to investigate Cox-2 protein expression in human gastric dysplasias and adenocarcinomas.

**Experimental Design:** Performance of several Cox-2 antibodies was evaluated, after which Cox-2 protein expression was studied in 67 gastric cancer specimens and in eight definitive dysplasias by using immunohistochemistry.

**Results:** Cox-2 positivity was detected in 58% (25/43) of the intestinal-type (well-differentiated) tumors and 6% (1/18) of diffuse-type (poorly differentiated) tumors. Consistent with these data, we detected higher expression of Cox-2 mRNA, protein, and enzymatic activity in well-differentiated gastric cancer cell lines (MKN-28 and MKN-74) when compared with poorly differentiated cell lines (HSC-39 and KATO III). Cox-2 immunoreactivity was localized to the carcinoma cells, but the stroma of the tumors was negative. However, strong Cox-2 positivity was consistently detected in stromal cells at sites of erosions and ulcerations. Furthermore, four of nine (44%) definitive dysplasias of the stomach that showed no evidence of invasion were positive for Cox-2.

**Conclusions:** Cox-2 is expressed by the neoplastic cells in the intestinal-type gastric adenocarcinoma and by precancerogenic (dysplastic) lesions leading to this disease.

INTRODUCTION

Cancer of the stomach is one of the most frequent and lethal malignancies in the world (1). Etiopathogenesis of gastric cancer is complex and incompletely understood, but diet, infections, and genetic factors are involved. More than 90% of gastric cancers are adenocarcinomas, which are divided into two histological types (intestinal and diffuse) by the Laurén classification (2). Pathogenesis of the intestinal-type cancer has been connected to precursor changes such as chronic atrophic gastritis, intestinal metaplasia, and dysplasia, whereas the diffuse type lacks well-recognized precursor lesions (1, 3). Furthermore, these two types of gastric cancer seem to express distinct genetic backgrounds (1, 4). Thus, the Laurén classification based on histology divides gastric adenocarcinoma into two different disease entities. In contrast with adenocarcinoma of the stomach, incidence of adenocarcinoma of the gastric cardia has increased (1, 5), which may reflect a closer relationship in regard to risk factors with the adenocarcinoma of the esophagus rather than with adenocarcinoma of the stomach.

Record linkage studies have found a lower incidence of gastrointestinal cancers among patients with rheumatoid arthritis (6–8). Because these patients use extensive amounts of NSAIDs, it was suggested that the use of NSAIDs could be responsible for the reduction in the cancer incidence. This antineoplastic effect of NSAIDs is supported by both observational and controlled epidemiological studies that have shown that prolonged use of aspirin and other NSAIDs is associated with a 40–50% reduction in the risk of colorectal cancer (9–11). Furthermore, sulindac, another NSAID, causes regression of colorectal adenomatous polyps in patients with FAP, and several NSAIDs inhibit chemically induced carcinogenesis in rodents (10, 11). Interestingly, the effect of NSAIDs does not seem to be restricted to the colorectal carcinoma, because the use of aspirin has been connected to reduced incidence of both cancer of the esophagus and the stomach (12–17).

The best known target of NSAIDs is Cox, the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids (18). Two Cox genes have been cloned (Cox-1 and Cox-2) that share over 60% identity at amino acid level and have similar enzymatic activities (19, 20). The most striking difference be-
The tumors were divided to intestinal type (n = 43), diffuse type (n = 18), and unclassified (n = 6) as evaluated by the same pathologist (P. S.). Each histological group contained samples from Finland and the Netherlands. Nine gastric biopsy specimens with definitive dysplasia but without evidence of invasive cancer were also examined (as evaluated by P. S.).

Table 1  Characteristics of gastric adenocarcinoma cases and results of Cox-2 immunohistochemistry*

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Site of cancer (n)</th>
<th>Sex (F/M)</th>
<th>Age (mean ± SE)</th>
<th>Cox-2 positivity (n)</th>
<th>Positive for Cox-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal type</td>
<td>Cardia (21)</td>
<td>4/17</td>
<td>65 ± 2</td>
<td>10/8/3</td>
<td>52%</td>
</tr>
<tr>
<td></td>
<td>Noncardia (22)</td>
<td>5/17</td>
<td>70 ± 3</td>
<td>8/5/9</td>
<td>64%</td>
</tr>
<tr>
<td>Diffuse type</td>
<td>Cardia (4)</td>
<td>1/3</td>
<td>53 ± 7</td>
<td>4/0/0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>Noncardia (14)</td>
<td>8/6</td>
<td>40 ± 6</td>
<td>13/1/0</td>
<td>7%</td>
</tr>
<tr>
<td></td>
<td>All (18)</td>
<td>9/9</td>
<td>43 ± 5</td>
<td>17/1/0</td>
<td>6%</td>
</tr>
<tr>
<td>Unclassified</td>
<td>Cardia (4)</td>
<td>2/2</td>
<td>67 ± 5</td>
<td>3/1/0</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Noncardia (2)</td>
<td>1/1</td>
<td>64 ± 21</td>
<td>1/1/0</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>All (6)</td>
<td>3/3</td>
<td>66 ± 6</td>
<td>4/2/0</td>
<td>33%</td>
</tr>
</tbody>
</table>

* Cox-2 positivity was almost exclusively restricted to the intestinal type adenocarcinomas (25/43) when compared with those of the diffuse type (1/18) (P < 0.0001).

Recent studies suggest that Cox-2 is one of the targets of NSAIDs in prevention of intestinal carcinogenesis (19, 21). Elevated levels of Cox-2 mRNA and protein, but not those of Cox-1, are found in human colon carcinoma (22–24). Additionally, selective Cox-2 inhibitors suppress neoplasia formation in rodent models of intestinal carcinogenesis (25–29), and genetic disruption of Cox-2 suppresses the polyp formation in Apcmin mutant and in Min mice, which are models for FAP (25, 30). Finally, a Cox-2 selective inhibitor reduces the number and size of colorectal polyps in patients with FAP (31). In addition to colon cancer, elevated Cox-2 expression has been detected in several human malignancies (32). We reported recently that human gastric adenocarcinoma tissues contain elevated levels of Cox-2 mRNA but not that of Cox-1 (33). Several groups have reported an identical pattern of Cox protein expression in stomach tumors as detected by immunoblotting (34–37). Because very little is known about the cellular localization of Cox-2 in gastric neoplasias and about its distribution in different histological types of gastric cancer, we investigated the expression of Cox-2 in dysplasia of the stomach and in gastric adenocarcinoma by using immunohistochemistry and several Cox-2 antibodies.

MATERIALS AND METHODS

Patient Samples. Primary gastric adenocarcinomas of cardia (n = 29) and those distal to the cardia (n = 38) were obtained from surgically removed tissues (Table 1), which were fixed in 10% neutral-buffered formalin and then embedded in paraffin. The tumors were divided to intestinal type (n = 43), diffuse type (n = 18), and unclassified (n = 6) as evaluated by the same pathologist (P. S.). Each histological group contained samples from Finland and the Netherlands. Nine gastric biopsy specimens with definitive dysplasia but without evidence of invasive cancer were also examined (as evaluated by P. S.).
However, this antibody stained mononuclear inflammatory cells in the stroma of the tumors and in the lamina propria of the nonneoplastic epithelium, which could not be blocked by using recombinant human Cox-2 protein (100 μg/ml). Indeed, it was reported recently that this mAb may cross-react with Cox-1 (38). Because of this unspecific staining, this antibody was not additionally analyzed.

Specificity of the antibodies was determined by preadsorption of the antimouse Cox-2 antibodies with a mouse Cox-2 control peptide (20 μg/ml; Cayman Chemical) and antihuman Cox-2 antibodies with a human Cox-2 control peptide (20 μg/ml; Cayman Chemical) for 1 h in room temperature before the staining procedure. An α-smooth muscle cell actin peptide (50 μg/ml; DAKO, Glostrup, Denmark) was used as a non-Cox-2 peptide. Nonimmune rabbit serum (1:300; PG 27c; Oxford Biomedical Research) and IgG (1:10; sc-2027; Santa Cruz Biotechnology) were used as additional controls. The intensity of the staining was estimated on a scale from 0 to 3 (0: absent, 1: weak, 2: moderate, and 3: strong) and the area of positivity by values 1 (focal or <10%), 2 (10–49%), and 3 (>50%) in consensus of three investigators (P. S., A. R., and B. v. R.).

**Cell Culture.** The gastric carcinoma cell lines that originated either from well-differentiated intestinal tumors (MKN-28 and MKN-74) or from poorly differentiated ones (HSC-39 and KATO III). The cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, and antibiotics (Life Technologies, Inc., Grand Island, NY).

**RNA Isolation and Northern Blot Analyses.** Total RNA was extracted by using the Trizol Reagent (Life Technologies, Inc.). For Northern blot analysis, 20 μg of RNA was denatured in 1 M glyoxal, 50% DMSO, and 10 mM phosphate buffer at 50°C for 60 min and then electrophoresed through 1.2% agarose gel. The RNA was transferred to nylon membranes (Micron Separation Inc., Westbrook, MA). Then the blots were baked for 1 h at 80°C. cDNAs from human open reading frame of Cox-2 and Cox-1 (33) and a 304-bp fragment of human β-actin were labeled using [α-32P]dCTP (DuPont- New England Nuclear, Boston, MA) and Prime-a-Gene kit (Promega, Madison, WI). Probes were purified with nick columns (Pharmacia, Uppsala, Sweden) and used at 1 × 10^6 cpm/ml. Hybridizations were performed at 60°C for 16 h in ExpressHyb Hybridization solution (Clontech Laboratories, Palo Alto, CA). Membranes were rinsed several times and washed three times at 55°C for 15 min each time and twice for 20 min in 0.1 × SSC and 0.1% SDS and visualized by autoradiography.

**Western Blot.** The cells were lysed in radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 50 mM Tris (pH 8.0)] supplemented with Complete mini protease inhibitor mixture tablets (Boehringer Mannheim, Mannheim, Germany) and centrifuged at 14,000 g for 15 min. Protein concentration was measured with BSA protein assay (Pierce, Rockford, IL). Proteins (100 μg) were resuspended in sample loading buffer [74 mM Tris-HCl (pH 6.8), 2% SDS, 12% glycerol, 5% β-mercaptoethanol, and 0.015% bromphenol blue] and separated by SDSPAGE (12%). The proteins were transferred electrophoretically to Hybond-C extra nitrocellulose membranes (Amersham, Buckinghamshire, United Kingdom). Nonspecific binding was blocked by TBS-NP40, 5% low-fat dry milk solution, over night at 4°C. For immunodetection, the membrane was incubated with the monoclonal Cox-2 antibody (1:1000 dilution; Cayman Chemical) or the monoclonal Cox-1 antibody (dilution 1:500; 160110; Cayman Chemical) for 1 h at room temperature. The membrane was washed three times in TBS-NP40 and incubated with sheep antinmouse antibodies conjugated to horseradish peroxidase (1:2000 dilution; ECL Western blotting analysis system; Amersham) for 1 h at room temperature. After four washes with TBS-NP40, Cox-2 proteins were visualized by enhanced chemiluminescence (Amersham). Loading was controlled by goat antihuman β-actin antibody (1:500 dilution; Santa Cruz Biotechnology) with donkey antigoat antibodies conjugated to horseradish peroxidase (1:2000 dilution; Santa Cruz Biotechnology) as the secondary antibody.

**Measurement of PGE2.** The gastric cancer cell lines were first incubated with 10 ml of the complete growth media in 10-cm tissue culture dishes for 24 h after which the cells were washed once with PBS and additionally incubated with arachidonic acid (10 μM; Sigma Chemical Co.) for 15 min. PGE2 was analyzed with EIA (Cayman Chemical).

**Statistical Analysis.** The Spearman correlation was used for calculating the significance of the correlation (Fig. 3A). Statistical significance was calculated with the Wilcoxon signed-rank test (Fig. 3B) or Fisher’s exact test (Cox-2 positivity between intestinal type versus diffuse type and cardia cancer versus noncardia cancer). All of the results are shown as mean ± SE, and P < 0.05 was selected as the statistically significant value.

**RESULTS**

We first evaluated Cox-2 immunoreactivity in eight intestinal type gastric adenocarcinoma specimens, which had been shown previously to express elevated levels of Cox-2 mRNA (33). One polyclonal antibody preparation (160116) stained 8/8 tumors, another polyclonal product (160106) 5/8 tumors, the monoclonal Cox-2 antibody (1:1000 dilution; Cayman Chemical) or the monoclonal Cox-1 antibody (dilution 1:500) blocked by TBS-NP40, 5% low-fat dry milk solution, over night at 4°C. For immunodetection, the membrane was incubated with the monoclonal Cox-2 antibody (1:1000 dilution; Cayman Chemical) or the monoclonal Cox-1 antibody (dilution 1:500; 160110; Cayman Chemical) for 1 h at room temperature. The membrane was washed three times in TBS-NP40 and incubated with sheep antinmouse antibodies conjugated to horseradish peroxidase (1:2000 dilution; ECL Western blotting analysis system; Amersham) for 1 h at room temperature. After four washes with TBS-NP40, Cox-2 proteins were visualized by enhanced chemiluminescence (Amersham). Loading was controlled by goat antihuman β-actin antibody (1:500 dilution; Santa Cruz Biotechnology) with donkey antigoat antibodies conjugated to horseradish peroxidase (1:2000 dilution; Santa Cruz Biotechnology) as the secondary antibody.

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Further-more, neither the mAb nor the affinity-purified polyclonal antibodies stained the unspecific areas observed with the nonpurified polyclonal antibodies (Fig. 2). Immunoreactivity obtained by the mAb and by the affinity-purified polyclonal antibody preparation colocalized in each of the positively stained tumor specimens. There also existed a positive correlation between the staining intensity obtained by these two antibodies (Fig. 3A). Thus, the Cox-2 immunoreactivity obtained by both the monoclonal and the affinity-purified antibodies seemed to be specific, but all of the nonaffinity-purified polyclonal antibody preparations suffered from either poor sensitivity or specificity. However, because the signal intensity of the mAb was stronger than that obtained by the affinity-purified antibodies (Fig. 3B), additional evaluation of Cox-2 expression in cancer specimens was performed by using the mAb.

Cox-2 immunoreactivity was investigated by staining 67 gastric adenocarcinoma specimens. Cox-2 immunopositivity

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Fig. 1 Immunostaining specific for Cox-2 in an intestinal-type adenocarcinoma of the gastric cardia. Antibodies that were used included A, polyclonal antiserum (160116, dilution 1:1000) and B, its preadsorption control; C, mAb (160112, dilution of 1:50) and D, its preadsorption control; E, affinity-purified polyclonal antibody preparation (160126, dilution of 1:200) and F, its preadsorption control (see Material and Methods for details). Cytoplasmic Cox-2 immunoreactivity was evident in carcinoma cells but not in the stroma of the tumor. ×400.

Fig. 2 Nonspecific immunoreactivity in two intestinal-type adenocarcinoma specimens of the stomach. Antibodies used for Sample 1 included A, polyclonal antiserum (160116, dilution of 1:1000) and B, its preadsorption control; C, mAb (160112, dilution of 1:50); D, affinity-purified polyclonal antibody preparation (160126, dilution of 1:200). Antibodies used for Sample 2 included E, polyclonal IgG (160106, dilution of 1:1000) and F, its preadsorption control; G, the mAb and H, the affinity-purified polyclonal antibody preparation (see above). The unspecific tumor cell staining that could not be preadsorbed with the antigenic peptide was only recognized by the nonpurified polyclonal antibodies. ×600.
Our first goal was to characterize several Cox-2 antibodies by using immunohistochemistry. This was achieved by performing preadsorption control experiments with the antigenic peptides or with the recombinant Cox-2 protein, which was necessary because a simple omission of the primary antibody or its replacement with a control serum or IgG was not sufficient. Furthermore, it became apparent that several cancer specimens needed to be examined, because some nonpurified polyclonal antibodies gave both specific and unspecific signals in the cancer cells. In fact, only the affinity-purified polyclonal antibody preparation and the mAb stained the tumor cells in a specific manner, which is supported by the following observations: (a) all of the tumor-cell staining obtained by the monoclonal or the affinity-purified antibodies was blocked by the preadsorption protocol; (b) the cellular distribution of immunostaining obtained by these two antibodies was similar; and (c) the intensity of immunoreactivity obtained by them correlated.

Our data suggest that Cox-2 is expressed predominantly by the intestinal type but not by the diffuse-type gastric adenocarcinoma as detected by immunohistochemistry. Furthermore, unclassified tumors expressed Cox-2 only at sites of better differentiation. Interestingly, colorectal tumors that show a histological pattern of signet ring cells, a typical feature of diffuse-type gastric cancer (1, 2), contain low levels of Cox-2 immunoreactivity (39). Furthermore, recent reports indicate that genotypic features (e.g., defective mismatch repair) have an effect on expression of Cox-2 in both gastric and colorectal cancers (37, 39). Because a different set of genetic alterations may take place in the sequence leading to the two histologically distinct types of gastric carcinoma (1, 4), it is possible that a cellular event(s) typical for intestinal-type carcinogenesis is responsible for induction of Cox-2 expression.

Only a limited number of observations have been made about expression of Cox-2 in gastric cancer by using immunohistochemistry. These data show quite variable frequency (43–100%) and cellular distribution of Cox-2 protein (33, 35, 40, 41). In our specimens, the frequency of Cox-2 positive intestinal-type gastric adenocarcinomas was 58%, and the Cox-2 immunoreactivity localized to the carcinoma cells. Although carcinomas have consistently been shown to express the highest levels of Cox-2 in esophageal, gastric, and colorectal tumors, the extent of stromal cell positivity remains unclear (24, 35, 40–45). Variable results may depend on specificity and sensitivity issues related to different Cox-2 antibodies and staining protocols or alternatively on tissue collection and handling procedures.

In addition to immunohistochemistry, elevated levels of Cox-2 mRNA and protein have been detected in gastric cancer specimens by Northern and Western blot analyses (33, 34), and elevated Cox-2 protein levels have been detected in 67–83% of gastric adenocarcinomas (35–37, 46). However, elevation of Cox-2 expression was found in both intestinal- and diffuse-type gastric adenocarcinomas (35, 39). Because a different set of genetic alterations may take place in the sequence leading to the two histologically distinct types of gastric carcinoma (1, 4), it is possible that a cellular event(s) typical for intestinal-type carcinogenesis is responsible for induction of Cox-2 expression.

DISCUSSION

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In addition to immunohistochemistry, elevated levels of Cox-2 mRNA and protein have been detected in gastric cancer specimens by Northern and Western blot analyses (33, 34), and elevated Cox-2 protein levels have been detected in 67–83% of gastric adenocarcinomas (35–37, 46). However, elevation of Cox-2 expression was found in both intestinal- and diffuse-type tumors (33, 35, 46). This apparent discrepancy between our present immunohistochemical data and that obtained by analyzing whole tissues may, at least in part, depend on our observation that gastric tumor specimens have a high frequency of Cox-2 positive erosions and ulcerations. This injury-associated Cox-2 expression localized to the stromal cells that were positioned close to the lumen of the stomach. However, when no mucosal injury was observed or when intestinal-type carcinomas below the mucosa were observed, no Cox-2 immunoreactivity was detected in the stromal cells of the tumors. Thus, the frequency of Cox-2 positive tumors may be overestimated when whole tissue preparations are analyzed, because a proportion of the signal may originate from nonneoplastic cells at the site of a mucosal injury. This is especially important in tumors that
express low or nondetectable levels of Cox-2, such as diffuse-type gastric adenocarcinomas.

Our results suggest, for the first time, that Cox-2 is expressed in definitive dysplasias of the stomach that do not show evidence for invasion. In this respect they represent true pre-neoplastic lesions of the intestinal-type gastric adenocarcinoma (3). Cox-2 immunoreactivity localized to the dysplastic epithelial cells, which is consistent with the data published on Barrett’s dysplasia (42, 47). In addition to neoplastic epithelial cells, Cox-2 is also expressed in the stromal compartment of intestinal adenomatous polyps (25, 45, 48). These stromal cells have the morphological and immunohistochemical characteristics of macrophages, myofibroblasts, and sometimes also vascular endothelial cells. Although the exact nature of these cells remains a topic of discussion (48), a recent report suggests that stromal expression of Cox-2 may contribute to carcinogenesis (49).

*Helicobacter pylori* have been reported to induce expression of Cox-2 in a gastric carcinoma cell line (50), expression of Cox-2 was detected in connective tissue cells and inflammatory mononuclear cells rather than in the epithelial cells in *H. pylori* gastritis (51–55). However, one report indicates that the highest *H. pylori*-associated staining is in the epithelial cell compartment including metaplastic epithelium (56). It is unclear to what extent the infection itself is responsible for Cox-2 expression in cancer cells *in vivo*, but we would like to propose that the elevated Cox-2 expression in transformed cells is more likely to depend on intrinsic events within the neoplastic cell such as the activation of oncogenes or the inactivation of tumor suppressor genes. This is supported by our observations that well-differentiated gastric cancer cell lines express the constitutively biologically active Cox-2 enzyme and because invasive gastric cancer cells, which are not in contact with the infection, express Cox-2 with comparable or even higher levels than the superficial cancer cells. It is, however, possible that chronic inflammation-
induced expression of Cox-2 in stromal cells contributes to the sequence leading to neoplastic transformation, for example, by inducing immunosuppression (57).

Cox-2 expression has been reported to correlate with invasion of the lymphatic vessels, lymph node metastasis, and advanced tumor stage in gastric cancer (36, 37). Interestingly, experimental data suggest that expression of Cox-2 may lead to increased invasive potential (58). Furthermore, a Cox-2 selective inhibitor reduced the growth of a Cox-2-expressing human gastric carcinoma cell line but not that of nonexpressing cell lines (59, 60), and inhibition of Cox-2 also reduces growth of the Cox-2-expressing gastric cancer cell line in nude mice mainly by inducing apoptosis (61). It was recently reported that Cox-2 expression correlates with density of CD34-positive microvascular endothelial cells, which may implicate that Cox-2 overexpression is associated with angiogenesis in gastric cancer (46). This is supported by experimental animal studies in which a Cox-2-selective inhibitor suppressed angiogenesis and tumor growth of a Cox-2-expressing gastric cancer cell line in nude mice (62). Interestingly, a nonselective Cox inhibitor reduced angiogenesis in xenografts of a non-Cox-2-expressing cell line (62) indicating that inhibition of vascular endothelial Cox-1 may also be an antiangiogenic target (63–65).

Our data suggest that expression of Cox-2 is elevated in intestinal-type gastric adenocarcinoma and that this expression is not restricted to the invasive cancer, because Cox-2 was also detected in definitive dysplasias of the stomach. Whether Cox-2 inhibitors can be used in treatment of patients who have pre-neoplastic gastric lesions or suffer from gastric cancer remains to be studied.

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