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Expression of Cyclooxygenase-2 in Human Gastric Carcinoma[1]

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ABSTRACT

Epidemiological studies suggest that the use of aspirin decreases the incidence of and mortality from gastrointestinal cancers. The best known target of aspirin and other nonsteroidal anti-inflammatory drugs is cyclooxygenase (Cox), the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids. Two Cox genes have been cloned, of which *Cox-2* is an inducible immediate-early gene. It is still unknown how nonsteroidal anti-inflammatory drugs act as chemopreventive agents, but they may target Cox-2. Cox-2 mRNA and protein were recently found to be expressed in human colon carcinoma. We have now studied the expression of Cox-2 in human gastric adenocarcinoma tissues which contained significantly higher levels of Cox-2 mRNA when compared with paired gastric mucosal specimens devoid of cancer cells. In contrast, Cox-1 mRNA levels were not elevated in the carcinoma. However, Cox-2 mRNA was not expressed in mucinous ovarian carcinoma samples as detected by Northern blot hybridization. Immunohistological detection of Cox-2 protein showed cytoplasmic staining in the gastric carcinoma cells but not in the surrounding stroma. Some hyperplastic glands showed intense staining, whereas glands of normal morphology were negative. Our data thus suggest that Cox-2 is expressed by human gastric adenocarcinoma.

INTRODUCTION

Gastric cancer is one of the most frequent and lethal malignancies in the world (1). It is the fourth most common malignancy in Finnish males and the fifth in females, and accounts for 5% of all malignancies in Finland (2). Early detection of stomach cancer is difficult, and in most western countries the 5-year survival rate is less than 20% (3). More than 90% of stomach cancers are adenocarcinomas, which are divided into intestinal and diffuse types by the Laurén classification (4). Pathogenesis of gastric cancer is complex and not completely understood, but in the case of the intestinal type certain precursor changes, such as chronic atrophic gastritis, intestinal metaplasia, and epithelial dysplasia, have been associated with the disease (5). In contrast, the diffuse type lacks well-recognized precursor lesions. Since a different combination of genetic changes have been found in these two histologically distinct types of gastric cancer, they may possess different genetic backgrounds (6, 7).

NSAIDs[3], such as aspirin, indomethacin, and sulindac, inhibit chemically induced carcinoma of the colon in animal models (8, 9). Record linkage studies in Finland and Sweden, which were partially motivated by the hypothesis that chronic use of NSAIDs might increase the cancer risk, found a lower incidence of cancer in the gastrointestinal tract among patients with rheumatoid arthritis than in the general population (10–12). Since these patients use aspirin and other NSAIDs in high doses for prolonged periods of time, it is possible that these drugs are responsible for the reduction in the cancer incidence. Indeed, both observational and controlled human studies have shown that NSAIDs, especially sulindac, cause regression of colorectal adenomatous polyps in patients with FAP, which is an inherited condition leading to colorectal cancer (13). Similarly, several epidemiological studies have shown that prolonged use of aspirin is associated with reduced risk of colorectal cancer by 40–50% (see Refs. 9 and 14 and references therein; Ref. 15). In addition, in a large prospective mortality study, the use of aspirin was associated with reduced risk of esophagus, gastric, and colorectal cancers, but not in the case of cancers outside the gastrointestinal tract (16).

The best known target of NSAIDs is Cox, the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids (17, 18). Two Cox genes have been cloned (*Cox-1* and *Cox-2*) that share over 60% identity at the amino acid level and have similar enzymatic activities (19–21). *Cox-1* is considered as a housekeeping gene, and prostanoids synthesized via the *Cox-1* pathway are thought to be responsible for cytoprotection of the stomach, vasodilation in the kidney, and production of a proaggregatory prostanoid, thromboxane, by the platelets. In contrast, *Cox-2* is an inducible immediate-early gene, and its pathophysiological role has been connected to inflammation, ovulation, and carcinogenesis. Recent studies suggest that Cox-2 is connected to colon carcinogenesis and may thus be the target for the chemopreventive effect of NSAIDs: (*a*) genetic disruption of the *Cox-2* gene or treatment with a Cox-2-specific drug suppress the polyp formation in a mouse model for FAP (22); (*b*) overexpression of *Cox-2* in rat intestinal epithelial cells alters their rate of programmed cell death and their adhesion to the extracellular matrix (23); and (*c*) two different Cox-2-selective inhibitors suppress chemically induced aberrant crypt foci in the rat colon (24, 25). Furthermore, elevated levels of Cox-2 mRNA and protein, but not those of *Cox-1*, are found in chemically induced rat colon carcinoma tissues (26) and in human colon carcinoma when compared with normal mucosa (27–31). Because it is not known whether *Cox-2* is present in gastric carcinomas, we studied its expression in adenocarcinomas of the stomach.

MATERIALS AND METHODS

Patient Samples. Twelve gastric adenocarcinoma (Table 1) and 12 ovarian carcinoma specimens of mucinous histology were obtained from surgically removed tissues that were frozen in liquid nitrogen and stored at −70°C until analyzed. One case of gastric carcinoma, because it showed strong autolysis in histological examination, was excluded from analysis. In the case of gastric carcinoma, paired samples of gastric mucosa, which contained no macroscopic tumor tissue or histologically detectable cancer cells, were obtained from the antrum (n = 10) and corpus (n = 10). All stomach cancers were primary adenocarcinomas, of which eight were intestinal and three of diffuse type (4) as evaluated by the same pathologist (P. S.).

RNA Isolation and Northern Blot Analysis. Total RNA was isolated according to the method of Chomczynski and Sacchi (32) with RNAzol B reagent (Tel-Test, Friendswood, TX) and quantitated by absorbance at 260 nm. RNA samples (15 μg) were denatured in 1 M glyoxal, 50% DMSO, and 10 mM phosphate buffer at 50°C for 60 min, electrophoresed through a 1.2% agarose gel, and transferred to Hybond-N nylon membranes (Amersham International, Aulesbury, United Kingdom), which were then UV irradiated for 6 min with a Reprostar II UV illuminator (Camag, Muttenz, Switzerland). Purified cDNA

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[3] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatous polyposis; Cox, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.

Table 1 *Characterization of 11 gastric carcinoma cases*

Case	Age (yr)	Sex	Cancer site	Cancer type	Cancer (%)[a]	Antrum sample[b]	Corpus sample[b]
1	88	Male	Angulus	Intestinal	50	Mild	Mild
2	82	Male	Corpus	Intestinal	100	No sample	Moderate[c]
3	69	Male	Antrum	Intestinal	20	Severe	Mild
4	67	Male	Whole stomach	Diffuse	30	Mild	No sample
5	66	Male	Antrum, pylorus	Intestinal	50	Mild	Not present
6	70	Female	Angulus	Intestinal	40	Moderate	Not present
7	85	Male	Pylorus	Intestinal	80	Mild	Mild
8	75	Male	Antrum	Diffuse	90	Not present	Mild
9	62	Male	Corpus	Intestinal	60	Not present	Severe
10	82	Male	Angulus	Intestinal	60	Mild	Mild
11	73	Male	Antrum, prepylorus	Diffuse	30	Not present	Not present

[a] Percentage of tumor cells in the gastric carcinoma samples.
[b] Severity of atrophic gastritis and intestinal metaplasia in specimens containing no cancer cells.
[c] Yield of RNA was insufficient for Northern blot analysis.

fragments of human Cox-1 open reading frame (1.8 kb), Cox-2 open reading frame (1.8 kb), and GAPDH (0.8 kb) were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; DuPont New England Nuclear, Boston, MA) and the Prime-a-Gene kit (Promega, Madison, WI). Probes were purified with nick columns (Pharmacia, Uppsala, Sweden) and used at 1×10^6 cpm/ml in hybridization solution containing 50% formamide, 6× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 100 μg/ml herring sperm DNA, 100 μg/ml yeast RNA, and 0.5% SDS at 42°C for 16 h. Filters were washed three times with 0.1–1× SSC and 0.1% SDS at 50°C. Northern blots were quantitated with Fujifilm IP-Reader Bio-Imaging Analyzer BAS 1500 (Fuji Photo Co., Tokyo, Japan) and the MacBas software supplied by the manufacturer and visualized using autoradiography.

RT-PCR. Total RNA (1 μg) was converted to cDNA with Superscript II (Life Technologies, Inc., Gaithersburg, MD) with both oligo(dT) (Pharmacia) and random hexamers (Life Technologies, Inc.). To obtain semiquantitative results, three parameters were optimized: number of cycles, concentration of primers, and annealing temperature. The cDNA (4 μl) was PCR amplified in 100 μl of reaction mixture that contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.2 mM deoxynucleotide triphosphates, 1.5 mM MgCl, 0.2 μg (Cox-1), or 2 μg (Cox-2) of sense and antisense primers (33) and 2.5 units of Dynazyme II DNA polymerase (Finnzymes, Espoo, Finland). Samples were amplified for 30 (Cox-1) or 32 (Cox-2) cycles of denaturation at 96°C for 1 min, annealed at 60°C (Cox-1) or 46°C (Cox-2) for 1 min, and extended at 72°C for 1 min. Amplified cDNAs were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. The amplified products were quantitated with a high-performance CCD camera (Cohu 4910 series with on chip integration; Cohu, Inc., San Diego, CA) and with Scion Image 1.57 software (Scion Corp., Frederick, MD) on a Macintosh personal computer.

Immunohistochemistry. Tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned (4–5 μm), and deparaffinized. The slides were first immersed in 0.3% hydrogen peroxide for 30 min and then in normal goat serum (1.5%) for 20 min to block endogenous peroxidase activity and unspecific binding sites, respectively. Immunostaining was performed with a rabbit polyclonal IgG specific for human Cox-2 (Cayman Chemical Co., Ann Arbor, MI) in a dilution of 1:40 at room temperature for 30 min. The sections were thereafter treated with biotinylated secondary antibodies in a dilution of 1:200 (Vector Laboratories, Burlingame, CA) and antibody-binding sites were finally visualized by avidin-biotin peroxidase complex solution (ABComplex, Vectastain; Vector Laboratories) and 3,3′- diaminobenzidine.

Statistical Analysis. Statistical significance was calculated with the Wilcoxon signed rank test, and $P < 0.05$ was selected as the statistically significant value. All results are shown as means ± SE.

RESULTS

Gastric carcinoma tissues expressed significantly higher levels of Cox-2 mRNA than did antrum or corpus samples, which were devoid of cancer cells, as detected by Northern blot hybridization (Fig. 1). The Cox-2 transcripts were expressed both by intestinal and diffuse adenocarcinomas. Levels of Cox-2 mRNA did not correlate with the proportion of carcinoma tissue in the specimens. As shown in Fig. 1*C*, levels of Cox-1 transcripts were not elevated in the carcinoma tissues when compared with the levels in their respective controls. Ovarian carcinoma samples did not contain Cox-2 mRNA as detected by Northern blot assay (data not shown).

Three gastric carcinoma samples (specimens 5, 9, and 10) expressed low levels of Cox-2 mRNA as detected by the Northern blot assay (Fig. 1*A*). To further evaluate the level of Cox-1 and Cox-2 expression in these samples, we performed a semiquantitative RT-PCR, with sample 1 as a positive control. As shown in Fig. 2, the ratio of Cox-2 mRNA:Cox-1 mRNA was higher in carcinoma samples than in paired antrum or corpus samples that contained no cancer cells.

Immunohistological staining with Cox-2-specific polyclonal antibodies showed cytoplasmic staining in the cancer cells, but not in the surrounding stroma (Fig. 3*A*). Some hyperplastic glands showed intense staining, whereas glands of normal morphology were negative (Fig. 3*B*). Inflammatory cells in the gastric mucosa did not stain for Cox-2 protein (data not shown). When the primary antibody was omitted, tissue sections exhibited no staining.

DISCUSSION

We found elevated levels of Cox-2 mRNA, but not those of Cox-1, in human gastric adenocarcinoma tissues. A similar pattern of Cox expression has previously been found in human colon carcinoma (27–31). Overexpression of Cox-2 in malignancies does not, however, seem to be a general phenomenon, since Cox-2 protein was not found in human breast carcinoma (28) and Cox-2 mRNA was not expressed in mucinous ovarian carcinoma (present work). In gastric carcinoma, Cox-2 protein was primarily localized in the cancer cells. This was also the case for Cox-2 mRNA (31) and protein (29) in human colon carcinoma. Similarly, Cox-2 protein was localized in the dysplastic epithelium in the mouse model for FAP (34). In contrast, a Cox-2 promoter-driven β-galactosidase expression system was active in the interstitial, rather than in epithelial, compartment in a similar mouse model (22). Explanation for these apparently conflicting results is presently unknown, but the latter observation suggests that in the early phase of polyp formation expression of Cox-2 may not be limited to the epithelial cells.

Nondetectable or low levels of Cox-2 mRNA were present in nonmalignant gastric tissues using 15 μg of total RNA in the Northern blot hybridization assay. O'Neill and Ford-Hutchinson (35) reported previously that Cox-1 and Cox-2 mRNAs are expressed in human stomach using 3 μg of poly(A)$^+$ in a similar assay. This is consistent with our data using RT-PCR, which showed that all nonmalignant tissues contain some Cox-2 mRNA. However, the specific source of this basally expressed Cox-2 mRNA signal is unknown, since we could not detect clear Cox-2 immunoreactivity in tissues other than carcinoma cells and in some hyperplastic glands. Furthermore, the

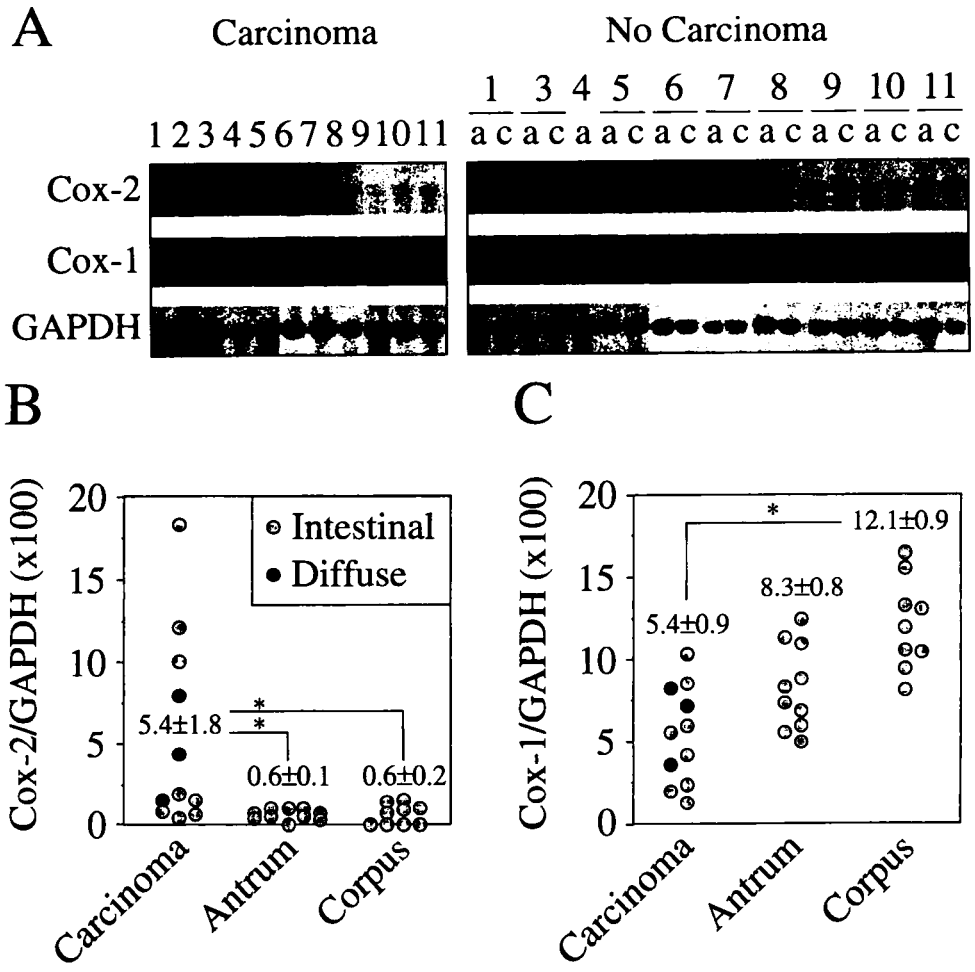

Fig. 1. Northern blot hybridization analysis of total RNA extracted from gastric carcinoma specimens 1–11 and from their paired control samples that contained no cancer cells (*a*, antrum; *c*, corpus). *A*, hybridization was performed with probes for human Cox-1 and Cox-2 and with GAPDH as the loading control. *B*, ratio of Cox-2 mRNA:GAPDH mRNA (×100) is shown. *C*, ratio of Cox-1 mRNA:GAPDH mRNA (×100) is shown. Values (means ± SE) represent the ratio of Cox mRNA:GAPDH mRNA (×100) calculated from the arbitrary densitometric units. *Asterisks*, significant ($P < 0.05$) differences between carcinoma samples and their paired controls.

significance of this finding is not clear, since Kargman *et al.* (36) reported recently that they could not detect Cox-2 protein using immunohistochemistry or Western blotting or a Cox-2 enzyme activity assay in normal gastrointestinal tract tissues of several species, including human stomach.

Elevated expression of Cox-2 was not limited to the intestinal type, since each of the three diffuse carcinomas analyzed contained higher levels of Cox-2 mRNA than their respective controls. Thus, overexpression of Cox-2 is one of the properties shared by these two histologically and genetically distinct diseases, which may suggest that it is involved with the early stage of carcinogenesis (7). Indeed, we found that some nonmalignant hyperplastic gastric glands that may represent premalignant lesions stained for the Cox-2 protein. Similarly, expression of Cox-2 was previously found in mouse epidermis during hyperplastic transformation (37). Since normal colonic epithelium expresses only low levels of Cox-2 mRNA and elevated levels are found in more than 40% of premalignant colonic adenomas and in almost 90% of colon carcinomas (27), it is possible that such gradient of gene expression is also found in the stomach. Whether Cox-2 is expressed in premalignant lesions of gastric carcinoma needs further investigation.

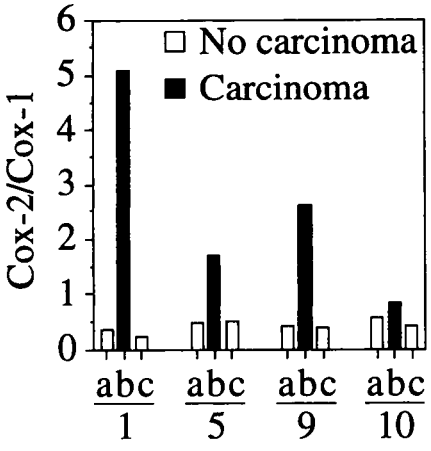

Fig. 2. Cox-1 and -2 mRNA levels were detected by RT-PCR in gastric carcinoma specimens (*b*) of cases 1, 5, 9, and 10 and from their respective controls that were devoid of cancer cells (*a*, antrum; *c*, corpus). Total RNA was first reverse transcribed. Then the cDNA samples were amplified with PCR using isoenzyme-specific primers for human Cox-1 and Cox-2. Finally, the PCR products were analyzed and quantitated (see "Materials and Methods"). Ratio of Cox-2 mRNA:Cox-1 mRNA is shown.

NSAIDs are very active chemopreventive agents against colon carcinoma in animal models (8, 9). They also inhibit experimental tumor formation in the bladder, skin, esophagus, small intestine, pancreas, breast, and uterine cervix (8). However, in a rat model of gastric carcinoma, the NSAID flurbiprofen enhanced tumor growth (38), which may suggest that prostanoids produced by the gastric mucosa protect against the action of carcinogens in rodents. It is unclear whether this experimental model is relevant to human carcinogenesis. Indeed, epidemiological studies suggest that NSAIDs may reduce the risk of human gastric cancer (10–12, 16). Interestingly, Tsuji *et al.* (39) reported recently that a Cox-2-specific inhibitor suppressed growth of a gastric and a colon carcinoma cell line that expressed high steady-state levels of Cox-2 mRNA. This was not the case in cell lines with low levels of Cox-2 mRNA. All this supports

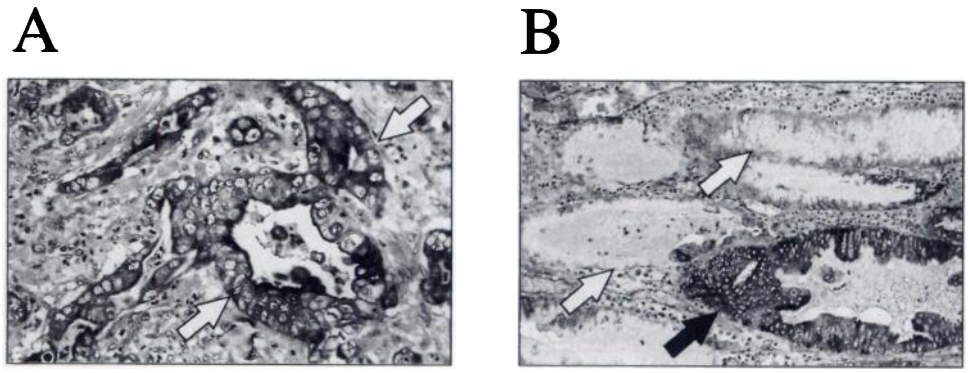

Fig. 3. Immunostaining for Cox-2 in gastric carcinoma tissues. *A*, immunoreactive Cox-2 was expressed in glandular structures composed of cancer cells (*arrows*). *B*, immunoreactivity for Cox-2 was found also in nonmalignant hyperplastic glands (*black arrow*) but not in gastric glands of normal morphology (*white arrows*).

the idea that the chemopreventive effect of NSAIDs is targeted against Cox-2, although it is important to emphasize that Cox enzymes are not the exclusive targets of NSAIDs (40, 41). However, the mechanism of elevated Cox-2 expression in gastrointestinal tumors remains unknown. It was recently reported that a Cox-2 promoter construct, which is silent in nonmalignant cells without an exogenous stimulation, is constitutively active in a colon cancer cell line (31). This may suggest that transcription of the *Cox-2* gene is activated in some malignant cells, which may be due to activation of oncogenes or inactivation of antioncogenes (42). Because Cox-2 mRNA is very unstable and its stability can be regulated (33, 43, 44), it is possible that dysregulation of post-transcriptional processing of Cox-2 transcripts is also associated with carcinogenesis.

Prostanoids produced by Cox-2 may facilitate tumor progression by several mechanisms: they may act as growth and differentiation factors, as immunosuppressors, and as angiogenic agents (45, 46). Indeed, in a mouse skin cancer model, the antitumor effect of indomethacin was reversed by prostaglandin $F_{2\alpha}$ (47). However, the mechanism behind the chemopreventive effect of NSAIDs may be independent of prostanoids, since Cox enzymes themselves can participate in activation and formation of carcinogens (45). Furthermore, NSAIDs induce apoptosis and inhibit proliferation of v-*src*-transformed chicken embryo fibroblasts (48) and in colon (49) and gastric cancer cell lines (50), which were not reversed by prostaglandins. Interestingly, Cox enzymes have been found to physically interact with an apoptosis-associated protein (51), but the biological significance of this finding is unknown.

We have shown that Cox-2 is expressed in human gastric adenocarcinoma. Expression of Cox-2 in human carcinomas seems, at least thus far, to be restricted to the gastrointestinal tract. Whether Cox-2 promotes malignant transformation in humans and whether the potentially beneficial effect of NSAIDs in reducing the risk of human gastrointestinal cancers is Cox-2 dependent needs further investigation.

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