Promoter Methylation Regulates *Helicobacter pylori*-stimulated Cyclooxygenase-2 Expression in Gastric Epithelial Cells


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Abstract

Cyclooxygenase (COX)-2, the inducible form of the rate-limiting enzyme for prostaglandin synthesis, is up-regulated in gastrointestinal cancers and is a key mediator of epithelial cell growth. *Helicobacter pylori* is causally linked to gastric cancer. In *H. pylori* gastritis, COX-2 expression localizes to the subepithelial region, with variable levels in the epithelium. In contrast, in gastric cancer, COX-2 strongly predominates in the epithelium, suggesting that the transition to consistent epithelial COX-2 overexpression may be a critical molecular event in gastric carcinogenesis. Because aberrant promoter methylation inhibits expression of a variety of genes in gastrointestinal cancers, we sought to determine whether methylation of the COX-2 promoter could regulate the response to *H. pylori* in gastric epithelial cells. We assessed COX-2 expression and promoter methylation status in six gastric epithelial cell lines. In all four of the cell lines that exhibited basal expression of COX-2 and a significant increase in expression in response to *H. pylori*, the COX-2 promoter was unmethylated, whereas in the two cell lines that did not express COX-2, the COX-2 promoter was methylated. Treatment of COX-2-methylated cells with the demethylating agent 5-azacytidine had a modest effect on COX-2 expression, but when 5-azacytidine-treated cells were subsequently stimulated with *H. pylori*, there was a significant, 5-10-fold enhancement of both COX-2 mRNA and protein expression and release of the COX-2 product, prostaglandin E₂. In contrast, in COX-2-expressing cell lines that were unmethylated at the COX-2 promoter, 5-azacytidine had no effect on *H. pylori*-stimulated COX-2 expression. These findings suggest that loss of COX-2 methylation may facilitate COX-2 expression and promote gastric carcinogenesis associated with *H. pylori* infection.

Introduction

*Helicobacter pylori* is strongly linked to gastric cancer, with epidemiological evidence resulting in the classification of *H. pylori* as a class I carcinogen (1). From a molecular standpoint, this cancer risk has been attributed to DNA alterations associated with chronic inflammation, imbalance of epithelial proliferation and apoptosis, and growth of bacteria producing carcinogenic nitrogen metabolites in the setting of *H. pylori*-induced achlorhydria (2). COX-2 is the rate-limiting enzyme for the production of prostanooids (prostaglandins and thromboxanes) from arachidonic acid. In recent years, strong evidence has emerged that COX-2 is causally involved in colorectal carcinogenesis. COX-2 expression is increased in colorectal adenocarcinomas and adenomatous polyps (3, 4). There are substantial data that in humans and mice with mutations of the *adenomatous polyposis coli* gene, there is prevention of polyp formation and regression of polyps by both selective and nonselective COX-2 inhibitors (5, 6) or targeted deletion of the COX-2 gene (4). The mechanisms underlying the prooncologic effect of COX-2 include inhibition of apoptosis (7), stimulation of cellular proliferation (8, 9), and mutagenic activity (10).

COX-2 has also been implicated in upper gastrointestinal tract carcinomas. We and others have shown that COX-2 is overexpressed in premalignant metaplastic Barrett’s esophagus and associated adenocarcinomas (11, 12) and in *H. pylori* gastritis (13–15) and gastric cancer (14, 16, 17). As in the progression of Barrett’s esophagus to cancer, in *H. pylori* gastritis, COX-2 protein localizes to the lamina propria (13–15, 18) with variable levels in the epithelium (14, 18), but in gastric cancer, COX-2 is most strongly expressed in the epithelium of malignant and dysplastic glands (14, 16, 17). Similarly, in vitro, *H. pylori* induces large increases in COX-2 expression and activity in monocytes and macrophages (19), but a more variable effect appears to occur in gastric epithelial cells (20, 21).

Methylation of gene promoter DNA at areas of CpG islands has now been strongly linked to silencing of gene expression. In gastric and colon cancers, this effect has been demonstrated for DNA mismatch repair genes, such as hMLH1 (22–25), and tumor suppressor genes, such as *adenomatous polyposis coli* (26), E-cadherin (24, 27), and p16 (24, 25). Because a recent novel report demonstrated aberrant methylation of COX-2 in colorectal cancers and cell lines (28) and we have observed altered COX-2 methylation in gastric carcinomas, we sought to determine whether COX-2 promoter methylation regulates COX-2 expression and functional activity in gastric epithelial cells exposed to *H. pylori*.

Materials and Methods

Cell Lines and Treatments. MKN28, MKN45, AGS, and KATOII cells are established gastric adenocarcinoma cell lines maintained under appropriate conditions in our laboratory. HFE cell lines HFE145 and HFE145T5 are two lines immortalized with SV40 large T antigen and telomerase (provided by H. A. and D. T.S.). *H. pylori* strain UMAB41, a cagA-positive strain, was grown under microaerophilic conditions, and bacterial concentrations were determined by absorbance and validated by serial dilution and quantitative culture, all as described previously (29). Lysates of *H. pylori* were prepared in a French-pressure cell (29). Bacterial stimulation studies with both intact and lysed bacteria were conducted with antibiotics (penicillin, streptomycin, and gentamicin) and fetal bovine serum (5–20%, dependent on the cell line) present in the cell culture medium. For the demethylation experiments, cells were plated at low density (30% confluency) in six-well plates on day 1 and treated with 5-azacytidine (Sigma Chemical Co., St. Louis, MO) in concentrations from 1 to 10 μM on days 2 and 4. This was followed by stimulation with *H. pylori*.

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3 The abbreviations used are: COX, cyclooxygenase; HFE, human fundal epithelial; PGE₂, prostaglandin E₂.

4 M. Akhtar et al. Hypomethylation of the cyclooxygenase-2 promoter in gastric adenocarcinomas with microsatellite instability, manuscript in preparation.

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H. pylori preparations or vehicle on day 5. In parallel, cells were collected for RNA and protein 6 and 24 h after stimulation with H. pylori, respectively, and media supernatants were collected for PGE2 analysis by enzyme immunoassay (Assay Designs, Ann Arbor, MI) 24 h after stimulation. In some experiments, cells were treated with the COX-2 inhibitors NS-398 (Cayman Chemical Co., Ann Arbor, MI) and DFU (provided by Dr. C. C. Chan, Merck-Frosst, Pointe-Claire-Dorval, Quebec, Canada).

**Methylation-specific PCR.** DNA methylation patterns in the COX-2 promoter were determined by methylation-specific PCR, as described previously (22, 23, 26). This method distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated but not methylated, cytosines to uracil, and subsequent PCR using primers specific to either methylated or unmethylated DNA. One μg of genomic DNA was bisulfite modified (CpGenome DNA modification kit; Intergen Co., Purchase, NY). PCR was performed by using primer pairs described below with the following conditions. In 50 μl, the PCR mix contained 1 x PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM MgCl2, and 10 mM 2-mercaptoethanol), deoxynucleotide triphosphates (each at 1.25 mM), primers (20 pmol each of sense and antisense primers), bisulfite-modified DNA (50 ng), and 1.5 units of platinum Taq DNA polymerase (Life Technologies, Inc., Rockville, MD). Amplification was carried out for 35 cycles (30 s at 95°C, 30 s at 61°C, and then 30 s at 72°C, followed by a final 4-min extension at 72°C). Control PCRs lacking genomic DNA were performed for each set of reactions. PCR reaction products were loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV light, photographed, and analyzed with a digital gel fluorescence (Amersham) using exposure to Kodak BioMax MR film.

Reverse Transcription-PCR Analysis for COX-2. Detection of COX-2 mRNA was performed using our previously described methods and primer sequences (11, 13). Total RNA was isolated using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD), and 2 μg of RNA from each sample were reverse transcribed using SuperScript II RT (Life Technologies, Inc.) in a total reaction volume of 20 μl. One μl of reverse-transcription product (cDNA) was PCR amplified with AmpliTaq DNA polymerase (Perkin-Elmer/Applied Biosystems, Foster City, CA) and 15 pmol each of COX-2 sense and antisense primers and 3 pmol each of β-actin primers were included in the same reaction mix containing protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). After determining protein concentration of 14,000 μg/ml, cells were lysed in RIPA buffer containing protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Western blot analysis was performed using our previously described methods and primer sequences were included in the same reaction mix containing protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN).

**Western Blot Analysis for COX-2.** Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). After determining protein concentration of 14,000 μg/ml soluble supernatants (Detergent Compatible protein assay kit; Bio-Rad Laboratories, Hercules, CA), 100 μg/lane were separated by SDS-PAGE under reducing conditions and transferred onto Hybond-polyvinylidene difluoride membranes (Amerham, Inc., Arlington Heights, IL) by electroblotting. Equal loading and transfer of proteins were determined by reversible staining with Ponceau S prior to incubation with primary antibody. Membranes were blocked using 5% nonfat dry milk. COX-2 protein was detected by incubation of blots with a monoclonal antibody to a synthetic peptide from the human COX-2 sequence from Cayman Chemical Co. (Ann Arbor, Michigan) at a dilution of 1:500 overnight at 4°C, followed by a sheep antinouse secondary antibody conjugated to horseradish peroxidase and determination of enhanced chemiluminescence (Amerham) using exposure to Kodak BioMax MR film.

Results

**Increased Basal and H. pylori-stimulated COX-2 Expression Correlates with Absence of COX-2 Promoter Methylation.** As shown in Fig. 1, the cell lines MKN28, MKN45, HFE145, and HFE145T5 exhibited substantial basal production of PGE2, a predominant metabolite of COX-2 in the gastric mucosa (31) and in gastric cancer specifically (32). This basal production of PGE2 was readily decreased to undetectable levels by addition of the COX-2 inhibitors NS-398 and DFU (data not shown). In contrast, the cell lines AGS and KATOIII both had basal levels of PGE2 that were at the lower limits of detection of this sensitive immunoassay. As shown in Fig. 1, each of the cell lines with basal PGE2 production exhibited H. pylori-stimulated PGE2 release. This occurred in a concentration-dependent manner between 106 and 107 H. pylori/ml, a multiplicity of infection range of 2–2000 bacteria/cell. Peak PGE2 production was observed at ~106 bacteria/ml for each of the four cell lines MKN28, MKN45, HFE145, and HFE145T5. Response to the H. pylori lysates was comparable with that of the whole bacteria; data for the lysates are shown in Fig. 1. Treatment of cells with the COX-2 inhibitors NS-398 and DFU returned PGE2 release to basal levels when used at 1 μM or to below the lower limit of detection of PGE2 when added at 10 μM (data not shown). The COX-2 activity data were mirrored by similar increased basal and H. pylori-stimulated COX-2 mRNA and protein expression in the cell lines MKN28, MKN45, HFE145, and HFE145T5 but not the cell lines AGS and KATOIII: representative data are shown in Fig. 3.

Because we observed a marked stratification of COX-2 expression in the gastric cell lines, we assessed the COX-2 promoter methylation status. We identified a CpG dense region at nucleotides 262–829 of the COX-2 gene promoter just before the transcription start site at nucleotide 832 and before the 5′ untranslated region [nucleotides 832–965 (30); GenBank accession #U04636]. Therefore, primers for methylation-specific PCR were designed that identify unhypermethylated or methylated DNA sequences within this region of the promoter. By BLAST search analysis, the promoter sequence in GenBank U04636 is also present in other COX-2 nucleotide sequences submitted to GenBank (AF044206, HSU44805, D28235, AF276953, and HSU20548).

**Fig. 1.** PGE2 production in gastric epithelial cell lines and response to H. pylori. Representative data are shown for 108 lysed H. pylori/ml (media volume, 2 ml) in six-well plates to which 106 cells/well were added, equivalent to 200 bacteria/cell. Media supernatants from the six cell lines shown were collected 24 h after stimulation for PGE2 analysis. *, P < 0.05; **, P < 0.01 versus control by Student’s t test. Bars, SE.
Procedure used in versus control; Student-Newman-Keuls multiple comparisons performed on cell lysates harvested 24 h after treatments listed in A. Equal amounts of protein (100 μg) were loaded in each lane. D, PGE 2 levels measured by enzyme immunoassay in culture supernatants after 24 h of exposure to the stimuli shown and described in A. PGE 2 data are standardized to cellular protein concentration to correct for any cytotoxic effects of 5-azacytidine. n = 6. A–D, data for the 2 μM 5-azacytidine are shown; similar findings were observed with concentrations of 1, 4, and 5 μM. Statistical analysis in B and D, AGS cells: *P < 0.01 for AzaC + HP versus control; HP, or AzaC alone; MKN28 cells: **P < 0.01 for HP versus control; Student-Newman-Keuls multiple comparisons procedure used in B and D for both cell lines. Bars, SE.

Discussion

These results suggest, for the first time, that COX-2 promoter methylation regulates gastric epithelial cell COX-2 expression in response to H. pylori. Promoter methylation correlated with absence of basal COX-2 expression and activity and lack of response to H. pylori stimulation. In contrast, cells without COX-2 promoter methylation exhibited substantial basal COX-2 expression and activity and significant inducible COX-2 expression in response to H. pylori. Treatment of COX-2-methylated cells with the demethylating agent 5-azacytidine clearly facilitated COX-2 expression in response to H. pylori but had no significant effect on cells that were unmethylated at the COX-2 promoter.

Our findings of methylation of the COX-2 promoter in two of six
gastric epithelial cell lines are consistent with the recent report of COX-2 methylation in 15 of 33 cancer cell lines of nongastric origin (28). Toyota et al. (28) found that methylation of COX-2 was strongly associated with silencing of mRNA expression. Furthermore, exposure of colorectal and prostate cell lines with evidence of COX-2 promoter methylation to the demethylating agent, 5-deoxy-azacytidine, resulted in restoration of COX-2 gene expression (28). Our results also show the ability of 5-azacytidine to enhance expression of COX-2, but this effect was much more marked when cells were pretreated with 5-azacytidine and then stimulated with H. pylori. This synergistic effect may result from: (a) removal of the inhibitory effect of promoter methylation coupled with (b) transcriptional activation of COX-2 by pathways such as nuclear factor-κB, a major mediator of transcription in gastric epithelial cells exposed to H. pylori (33). We have observed a similar effect in colonic HCT116 cells. The COX-2 promoter in these cells is methylated, and the cells lack COX-2 expression. They also exhibit a synergistic increase in COX-2 expression with pretreatment with 5-azacytidine, followed by stimulation with phorbol myristic acid (data not shown). Altered COX-2 promoter methylation also occurs in vivo, having been clearly documented in 13% of primary colorectal carcinomas (28). In a series of 38 primary gastric cancers, we identified altered COX-2 promoter methylation in 45% of cases (4). Taken together, our data and that of Toyota et al. (28) indicate that there may be several pathways of gastrointestinal carcinogenesis, and that one of these does not involve COX-2. Loss of control of COX-2 expression in gastric epithelial cells, as may occur via altered methylation of the COX-2 promoter, may have important consequences in gastric carcinogenesis. COX-2 overexpression is strongly associated with loss of apoptosis (7, 9, 34) and enhancement of proliferation (8, 9, 34) in gastrointestinal epithelial cells. Consistent with the findings in the current study, we have found that COX-2 inhibitors increase H. pylori-stimulated apoptosis in gastric epithelial cells expressing COX-2 but not in those failing to express COX-2 (20). In addition, COX-2 inhibition has been shown to decrease cellular proliferation in gastric and intestinal cell lines constitutively expressing COX-2 (8, 9, 34, 35).

It is also important to note that in our study, in the cell lines with unmethylated COX-2, and after 5-azacytidine treatment in the cell lines with methylated COX-2, increased COX-2 mRNA expression in response to H. pylori is paralleled by increases in COX-2 protein and a representative product of the enzyme, PGE₂. This supports the primary importance of regulation of COX-2 transcription in the control of functional activity of the COX-2 enzyme. Production of PGE₂ in response to H. pylori is of fundamental importance. As an inhibitor of lymphocyte responses in tumors (36), PGE₂ may contribute to the immune escape of epithelial cells with DNA damage. In addition, we and others have found that PGE₂ itself inhibits apoptosis and stimulates proliferation in gastric (20) and colonic (9, 37) epithelial cells.

In conclusion, we suggest that COX-2 promoter methylation may be an important regulator of COX-2 expression in gastric epithelial cells, and that this control mechanism is especially relevant in the chronic exposure of gastric epithelial cells to H. pylori and its secreted products during infection with this organism. It is unlikely that H. pylori itself is a factor in directly causing alterations in methylation of epithelial DNA, because we exposed each of the six cell lines used in the current study to H. pylori for up to 2 weeks and found no effect on COX-2 promoter methylation status (data not shown). However, alterations in DNA methylation may occur as a consequence of chronic exposure of gastric epithelial cells to inflammatory mediators overproduced during H. pylori infection. For example, nitric oxide, produced in the host response to H. pylori infection (13, 29), has been shown recently to cause gene silencing and methylation of promoters containing CpG islands by activation of DNA methyltransferase (38).

Ultimately, loss of COX-2 promoter methylation in gastric epithelial cells may be a central event in H. pylori-associated gastric carcinogenesis, facilitating COX-2 expression and resulting in dysregulation of apoptosis and proliferation.

References
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