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


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 prostanoiads (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 proangiogenic 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 monocyes 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 KATOIIH 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 UMB41, 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% confluence) 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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To whom requests for reprints should be addressed, at Division of Gastroenterology, University of Maryland Medical Center, Room N3W62, 22 South Greene Street, Baltimore, MD 21201. Phone: (410) 706-1471; Fax: (410) 706-1573; E-mail: kwilson@umaryland.edu.

The abbreviations used are: COX, cyclooxygenase; HFE, human fundal epithelial; PGE2, prostaglandin E2;
**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 HFE145TS 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 10⁻⁶ and 10⁻⁴ *H. pylori* ml, a multiplicity of infection range of 2–2000 bacteria/cell. Peak PGE₂ production was observed at ~10⁶ bacteria/ml for the four cell lines MKN28, MKN45, HFE145, and HFE145TS. 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 HFE145TS 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 unmethylated 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 10⁶ lysed *H. pylori*/ml (media volume, 2 ml) in six-well plates to which 10⁶ cells/ml 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.
COX-2 METHYLATION IN GASTRIC EPITHELIAL CELLS

Fig. 2. Methylation status of the COX-2 promoter in gastric epithelial cell lines. Methylation-specific PCR was performed after bisulfite modification of DNA as described in “Materials and Methods.” U, the presence of PCR product indicates methylated COX-2. The DNA marker is shown in the first lane.

As shown in Fig. 2, all six cell lines examined exhibited an unmethylated product, which most likely represents loss of methylation in one allele. It is also possible that the unmethylated product could be attributable to a CpG that is never methylated or to heterogeneity in the cell cultures. In the same cell lines that exhibited COX-2 expression under basal conditions and increased expression with H. pylori, there was absence of the methylated band (MKN28, MKN45, HEFE145, and HEFE145T). In contrast, in the two cell lines that failed to express COX-2 in response to H. pylori or under basal conditions, there was a strongly methylated product.

Treatment with a Demethylating Agent Facilitates COX-2 Expression in Response to H. pylori. AGS cells, which exhibit COX-2 promoter methylation (Fig. 2), had minimal basal COX-2 mRNA (Fig. 3, A and B), protein (Fig. 3C), and PGE$_2$ production (Fig. 3D), which were not significantly induced by H. pylori preparations. Treatment of AGS cells with 5-azacytidine resulted in a modest increase in COX-2 mRNA and protein levels. However, pretreatment of these cells with 5-azacytidine, followed by stimulation with H. pylori, clearly resulted in a marked induction of COX-2 mRNA and protein levels (Fig. 3, A–C) and PGE$_2$ production (Fig. 3D). In contrast, the MKN28 cell line, lacking COX-2 promoter methylation (Fig. 2), had a significant induction of COX-2 mRNA (Fig. 3, A and B) and protein (Fig. 3C) expression as well as PGE$_2$ release (Fig. 3D) with H. pylori stimulation and had no enhancement of COX-2 expression or activity with 5-azacytidine treatment (Fig. 3, A–D). A similar lack of effect of 5-azacytidine was observed in experiments with MKN45 cells (data not shown).

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

![Image](https://example.com/image1.png)

Fig. 3. Effect of COX-2 promoter demethylation on COX-2 expression in gastric epithelial cells. Left column, AGS cells that are methylated at the COX-2 promoter. Right column, MKN28 cells that are unmethylated at the COX-2 promoter. A, reverse transcription-PCR analysis of COX-2 mRNA expression; representative gels of COX-2 expression performed on cell lysates harvested 24 h after treatments listed in A. Equal amounts of protein (100 µg) were loaded in each lane. B, densitometry of COX-2 transcripts, standardized to β-actin, for the conditions listed above in A; n = 8 for each condition. C, Western blot analysis of COX-2 expression performed on cell lysates harvested 24 h after treatments listed in A. Western blot analysis of COX-2 expression performed on cell lysates harvested 24 h after treatments listed in A. 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, the presence of PCR product indicates methylated COX-2. The DNA marker is shown in the first lane. **, p < 0.01 for HP versus control; Student-Newman-Keuls multiple comparisons procedure used in B and D for both cell lines. Bars, SE.
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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Mahmood Akhtar, Yulan Cheng, Romina M. Magno, et al.

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