

Promoter Methylation Regulates *Helicobacter pylori*-stimulated Cyclooxygenase-2 Expression in Gastric Epithelial Cells¹

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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). COX³-2 is the rate-limiting enzyme for the production of prostanoids (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 proneoplastic 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,⁴ 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 KATOIII 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% 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 μ M on days 2 and 4. This was followed by stimulation with *H.*

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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.

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 \times 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 illumination. *COX-2* primer sequences were designed based on the published promoter sequence (30) as follows: unmethylated reaction, 5'-ATAGATTAGATATGGTGGTGGTGGT-3' (sense) and 5'-CACAATCTTACCCAAACTTCCA-3' (antisense), 171-bp product; and methylated reaction, 5'-TTAGATACGGCGGCGGGC-3' (sense) and 5'-TCTTTACCGAACGCTTCCG-3' (antisense), 161-bp product.

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 multiplex PCR reaction as an internal control for efficiency of reverse transcription and amount of RNA. Each PCR cycle consisted of a denaturation step (94°C for 1 min), an annealing step (60°C for 1 min), and an elongation step (72°C for 1.5 min). There was a total of 30 cycles, followed by an additional extension step (72°C for 7 min). The primer sequences and PCR product sizes were as follows: *COX-2*, 5'-CAGCACTTCACGCATCAGTT-3' (sense) and 5'-TCTGGTCAATGGAAGCCTGT-3' (antisense), 756 bp; and β -*actin*, 5'-CCAGAGCAAGAGAGGTATCC-3' (sense) and 5'-CTGTGGTGGTGAAGCTGTAG-3' (antisense), 436 bp. PCR products were run on 2% agarose gels with 0.5 $\mu\text{g}/\text{ml}$ of ethidium bromide, and stained bands were visualized under UV light, photographed, and analyzed with a digital gel documentation system and associated densitometry software (EDAS 290 and 1D software; Kodak Digital Science, Rochester, NY).

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 \times g soluble supernatants (Detergent Compatible protein assay kit; Bio-Rad Laboratories, Hercules, CA), 100 $\mu\text{g}/\text{lane}$ were separated by SDS-PAGE under reducing conditions and transferred onto Hybond-polyvinylidene difluoride membranes (Amersham, 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 antimouse secondary antibody conjugated to horseradish peroxidase and determination of enhanced chemiluminescence (Amersham) using exposure to Kodak BioMax MR film.

Results

Increased Basal and *H. pylori*-stimulated COX-2 Expression Correlates with Absence of COX-2 Promoter Demethylation. 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 or 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 10⁹ *H. pylori*/ml, a multiplicity of infection range of 2–2000 bacteria/cell. Peak PGE₂ production was observed at \sim 10⁸ bacteria/ml for 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 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).

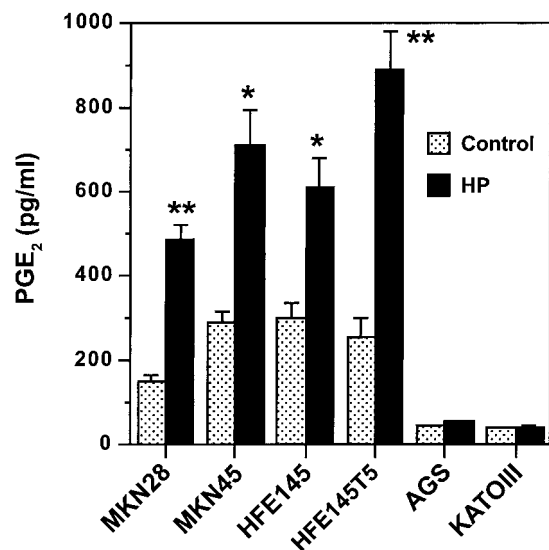


Fig. 1. PGE₂ production in gastric epithelial cell lines and response to *H. pylori*. Representative data are shown for 10⁸ lysed *H. pylori*/ml (media volume, 2 ml) in six-well plates to which 10⁶ cells/well were added, equivalent to 200 bacteria/cell. Media supernatants from the six cell lines shown were collected 24 h after stimulation for PGE₂ analysis. *, $P < 0.05$; **, $P < 0.01$ versus control by Student's *t* test. Bars, SE.

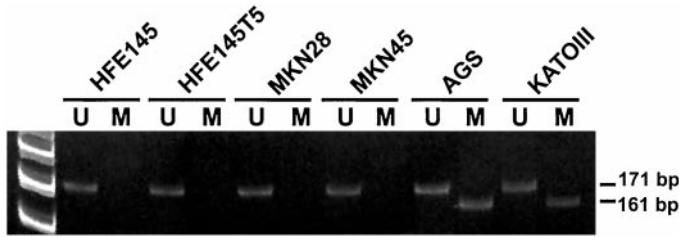


Fig. 2. Methylation status of the *COX-2* promoter in gastric epithelial cell lines. Methylation-specific PCR was performed after bisulfite modification of DNA as described in "Materials and Methods." U, the presence of PCR product indicates unmethylated *COX-2* product. M, the presence of PCR product 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, HFE145, and HFE145T5). 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

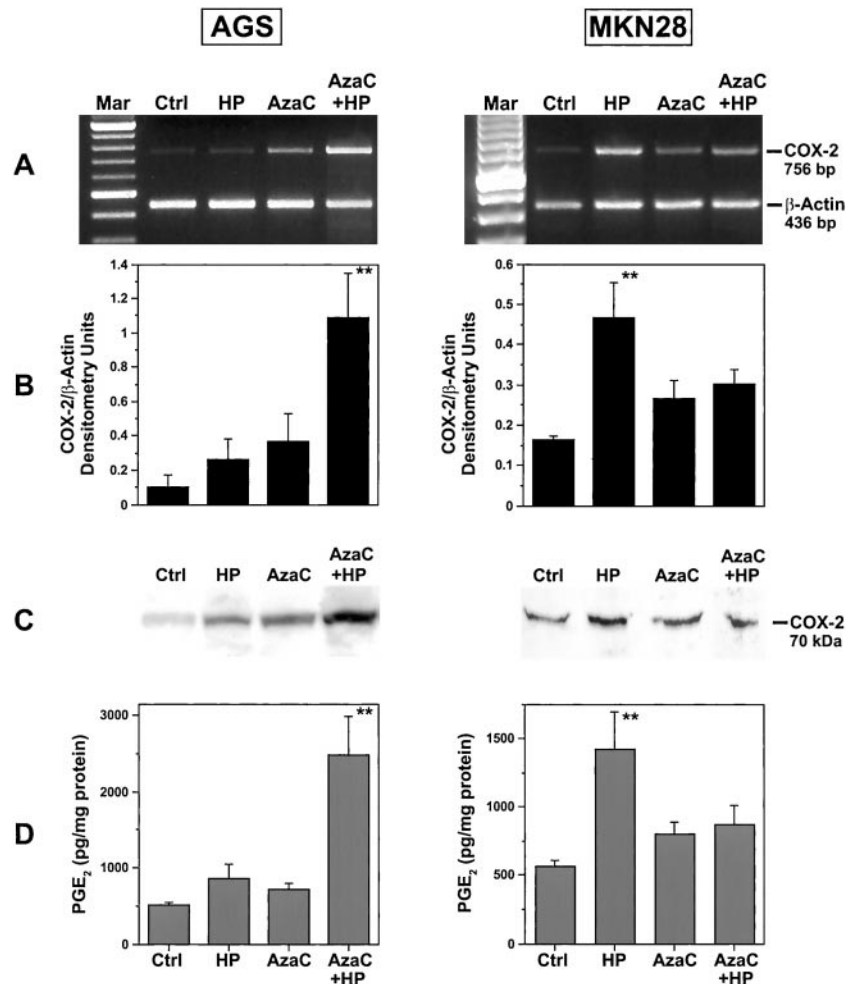


Fig. 3. Effect of *COX-2* promoter demethylation on *COX-2* expression in gastric epithelial cells. Left column, AGS cells that are methylated at the *COX-2* promoter. Right column, MKN28 cells that are unmethylated at the *COX-2* promoter. A, reverse transcription-PCR analysis of *COX-2* mRNA expression; representative gels of transcripts for *COX-2* and β -actin performed as a multiplex reaction are shown. Mar, DNA markers; Ctrl, control cells; HP, *H. pylori* lysates added at 10^8 bacteria/ml for 6 h; AzaC, 5-azacytidine, added for 3 days at $2 \mu\text{M}$; AzaC + HP, 5-azacytidine, added for 3 days at $2 \mu\text{M}$, followed by *H. pylori* lysate for 6 h. 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. Equal amounts of protein ($100 \mu\text{g}$) 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 $2 \mu\text{M}$ 5-azacytidine are shown; similar findings were observed with concentrations of 1, 4, and $5 \mu\text{M}$. 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.

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- κ B, 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.

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