Chapter 10

Role of *COX-2* Promoter Methylation and *Helicobacter pylori* Infection in Impaired Gastric Ulcer Healing

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Abstract

Helicobacter pylori (*H. pylori*) infection causes aberrant DNA methylation of various genes in the gastric mucosa. Cyclooxygenases (COX) play a critical role in peptic ulcer development and healing. Human *COX-2* has CpG islands (CGIs) in its promoter region, suggesting a possible epigenetic regulation. Here, we evaluated *COX-2* promoter methylation in the gastric mucosa of patients with various gastric diseases and found that *COX-2* methylation levels in the gastric mucosa were significantly increased in patients with *H. pylori* infection. We further investigated the roles of COX-2 during the healing of acetic acid-induced gastric ulcers in *H. pylori*-infected Mongolian gerbils (MGs). While *COX-2* mRNA expression levels on the edges of acetic acid-induced gastric ulcers were significantly increased after ulcer induction in MGs in the absence of *H. pylori*, no such induction was observed in *H. pylori*-infected gastric mucosa. Cloning of the MG *COX-2* gene revealed abundant CGIs in the promoter region. *COX-2* mRNA expression in MG-derived gastric carcinoma MGC2 cells was significantly increased by addition of the demethylating agent 5-Aza-dC. Additionally, *COX-2* methylation levels were higher in *H. pylori*-infected MG gastric mucosa than in control mucosa. These results indicated that epigenetic inhibition of *COX-2* mRNA induced by *H. pylori* impairs gastric ulcer healing.

Keywords: COX-2, gastric ulcer healing, *Helicobacter pylori*, methylation, Mongolian gerbils, MGC2 cells

1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that selectively colonizes the gastric epithelium of humans and is the leading cause of peptic ulcers [1]. Although the majority of

individuals infected with *H. pylori* remain asymptomatic throughout their life, essentially all infected individuals develop chronic inflammation. Patients with antral-predominant gastritis are predisposed to duodenal ulcers, while patients with corpus-predominant gastritis and multifocal atrophy are more likely to have gastric ulcers. Eradication of *H. pylori* drastically lowers the recurrence of *H. pylori*-associated peptic ulcers. In addition, the observed delayed ulcer healing has been reported to involve *H. pylori*-induced inflammation, increased apoptosis of epithelial cells at the ulcer margin, overexpression of inflammatory cytokines, and reduced gastric microcirculation [2]. Cyclooxygenase (COX) is a membrane-bound glycoprotein that functions as the rate-limiting enzyme in prostaglandin (PG) synthesis. PGs increase the resistance of the gastric mucosa to injury by downregulating inflammatory responses. Two major COX isoforms have been identified, COX-1, which is constitutively expressed and considered a housekeeping enzyme, and COX-2, whose mRNA levels rise rapidly in response to inflammatory and mitogenic stimuli. There is a known synergism between *H. pylori* infection and nonsteroidal anti-inflammatory drug (NSAID) use in the development of peptic ulcers and ulcer bleeding [3]. These findings indicate possible interactions between *H. pylori* infection, COX activity, and ulcerogenesis. Aberrant methylation of 5′-CpG islands (CGIs) has been implicated in the transcriptional silencing of a wide range of genes involved in various diseases, such as cancer. Human *COX-2* has CG-rich CGIs in its promoter region, which suggests epigenetic regulation. Here, we report and discuss our recent results on the epigenetic regulation of *COX-2* activity in *H. pylori*-infected gastric mucosa of humans and Mongolian gerbils (MGs), and the possible relationship between *COX-2* methylation and delayed gastric ulcer healing.

2. COX and the gastric mucosal barrier

In 1971, Vane and colleagues first demonstrated that aspirin and other NSAIDs inhibited the synthesis of PGs by blocking COX activity [4]. COX plays pivotal roles in the gastric mucosal barrier [5, 6]. COX catalyzes the conversion of arachidonic acid to the common precursor prostanoids, prostaglandin (PG) H_2, and PGG_2. The major PGs produced by the human and rodent gastric mucosa are PGE_2 and PGI_2, with lesser amounts of PGF_2 and PGD_2. Each of these metabolites binds to a specific G protein-coupled receptor to trigger intracellular responses. PGs have been shown to accelerate ulcer healing in experimental models and humans [7]. COX exists in two isoforms commonly referred to as COX-1 and COX-2. COX-1 is constitutively expressed in various tissues. In the stomach, prostanoids synthesized via the COX-1 pathway are responsible for cytoprotection of the gastric mucosa and the production of thromboxane by platelets. Although COX-2 is generally expressed at very low levels in healthy tissues, including in the stomach, it is expressed at particularly high levels at sites of inflammation.

It was originally thought that only COX-1 was involved in the gastric mucosal defense system; however, several clinical trials have suggested that a COX-2 selective inhibitor produces lesser, but some, gastrointestinal toxicity compared to traditional NSAIDs [8, 9]. In accordance with this finding, animal studies have suggested that both COX-1 and COX-2 are necessary for gastric mucosal healing, and COX-1 inhibition alone, which can be induced pharmacologically

by specific inhibitors or genetically by gene targeting [10], does not cause gastric mucosal injury. It has been shown that a combination of selective COX-1 and COX-2 inhibitors is required to cause hemorrhagic erosion of the gastric mucosa, which is comparable to that observed with indomethacin [11]. Prostanoids produced by COX-2, especially PGE_2, enhance cell proliferation. The beneficial effects of PGE_2 on gastric ulcer healing in rodents appear to be mediated via the EP4 receptor [12]. In addition, COX-2-derived PG stimulates vascular endothelial growth factor (VEGF) release from gastric fibroblasts, which is an important contributor to ulcer healing [13, 14], likely via stimulation of new blood vessel growth. The increase in COX-2 immunoreactivity that is observed in monocytes, macrophages, fibroblasts, and endothelial cells at the ulcer margin is closely correlated, both temporally and spatially, with the increase in cell proliferation [15]. COX-2 appears to represent a second line of defense that is activated during ulcer healing to compensate for the temporary loss of COX-1 in the mucosa adjacent to the ulcer and assists COX-1 in protecting gastric mucosal integrity. The healing-impairment effect of NSAIDs is also observed with selective COX-2 inhibitors [16].

3. *H. pylori* infection and COX-2 in gastric mucosa

The pathophysiological roles of COX-2 in *H. pylori*-infected gastric mucosa are intriguing. *H. pylori* has been implicated as an inducer of COX-2 in the stomach [17–19]. In addition, COX-2 expression is elevated within *H. pylori*-induced gastritis and malignant lesions [20], and *H. pylori* induced COX-2 expression and enhanced PGE_2 production in a human gastric carcinoma cell line. Both *H. pylori* infection and NSAID use independently and significantly increase the risk of peptic ulcers and ulcer bleeding. While COX-2 is necessary for gastric mucosal healing [21], *H. pylori* infection is the leading cause of gastric ulceration. To understand the bimodal effects of *H. pylori* infection on COX-2 induction during ulcer healing, we explored the effects of epigenetic regulation and *H. pylori* infection on the induction of COX-2 *in vivo* and *in vitro.*

H. pylori infection causes aberrant DNA methylation of various genes in the gastric mucosa, including *COX-2* [22–25]. Human *COX-2* has CGIs in its promoter region. Thus, we compared *COX-2* promoter methylation levels in the gastric mucosa in *H. pylori*-positive and *H. pylori*-negative cases [26]. As mentioned above, in qualitative experiments, *COX-2* gene promoter methylation levels were significantly higher in *H. pylori*-positive cases than in *H. pylori*-negative cases (**Figure 1**). *COX-2* promoter methylation levels were significantly lower in patients with *H. pylori* eradication than in those with *H. pylori* infection. We then investigated the effects of *COX-2* promoter methylation on *COX-2* mRNA expression *in vitro* using the human gastric adenocarcinoma cell line Kato III, in which the *COX-2* promoter is densely methylated [22]. *COX-2* mRNA expression was not observed in these cells, despite the addition of the protein kinase C stimulator α-phorbol 12,13-dibutyrate (PDBu). However, *COX-2* expression was observed after the addition of the demethylating agent 5-Aza-dC, and expression was enhanced by adding PDBu (**Figure 2**) [26]. These results indicate that *H. pylori* infection causes reversible *COX-2* promoter methylation in the gastric mucosa, and that *COX-2* mRNA expression is regulated through an epigenetic mechanism.

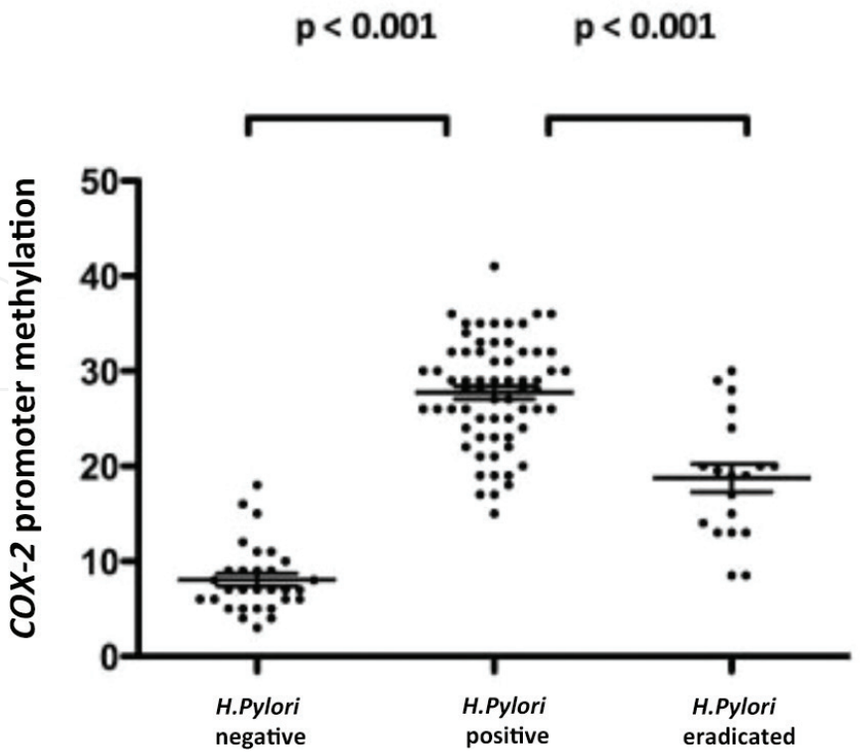

Figure 1. *COX-2* DNA methylation levels in patients with or without *H. pylori* infection, and patients previously with successfully eradicated *H. pylori* infection. The figure is modified from Ref. [26].

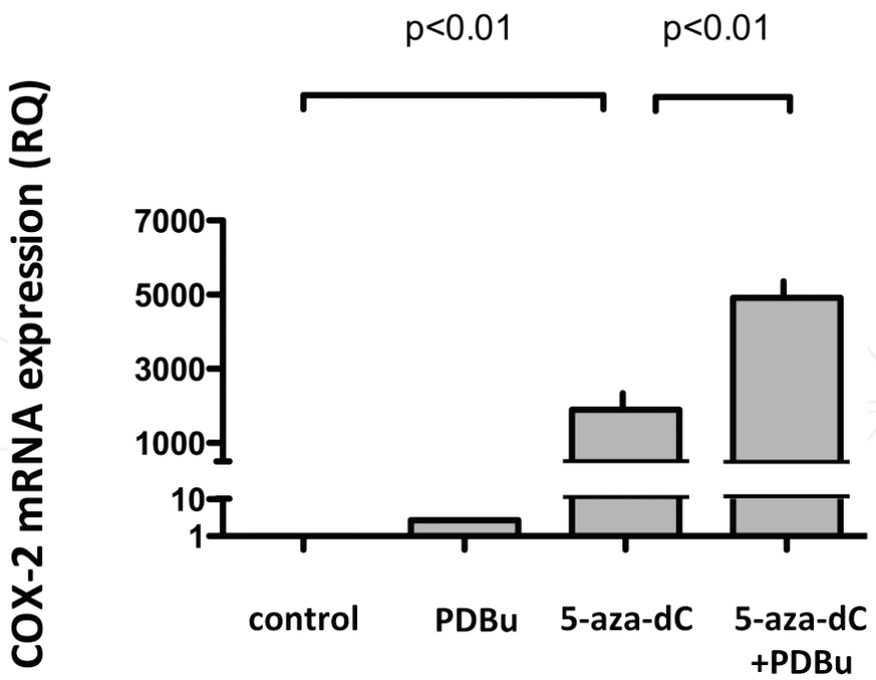

Figure 2. Effects of a PKC stimulator (α-phorbol 12,13-dibutyrate; PDBu) on COX-2 mRNA expression with or without 5-aza-dC in the human gastric adenocarcinoma cell line KATO-III. KATO-III cells were treated with vehicle (1 μmol/L) with or without 5-Aza-dC for 5 days. The figure is modified from Ref. [26].

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4. Acetic acid-induced gastric ulcer healing and *COX* mRNA levels in Mongolian gerbils with or without *H. pylori* infection

H. pylori infection in humans is best modeled in Mongolian gerbils (MGs), and chronic infection with *H. pylori* induces inflammatory cell infiltration in the gastric mucosa in MGs (**Figure 3A**). It was previously shown that *H. pylori* infection significantly delayed acetic acid-induced ulcer healing in mice and MGs [27–29]. *H. pylori* infection induces aberrant DNA methylation of several CGIs in MGs [30]. As described above, COX-2-derived PGs are important for gastric ulcer healing, and the *COX-2* promoter is densely methylated in the human gastric mucosa in the presence of *H. pylori* infection. To investigate the roles of *COX-2*

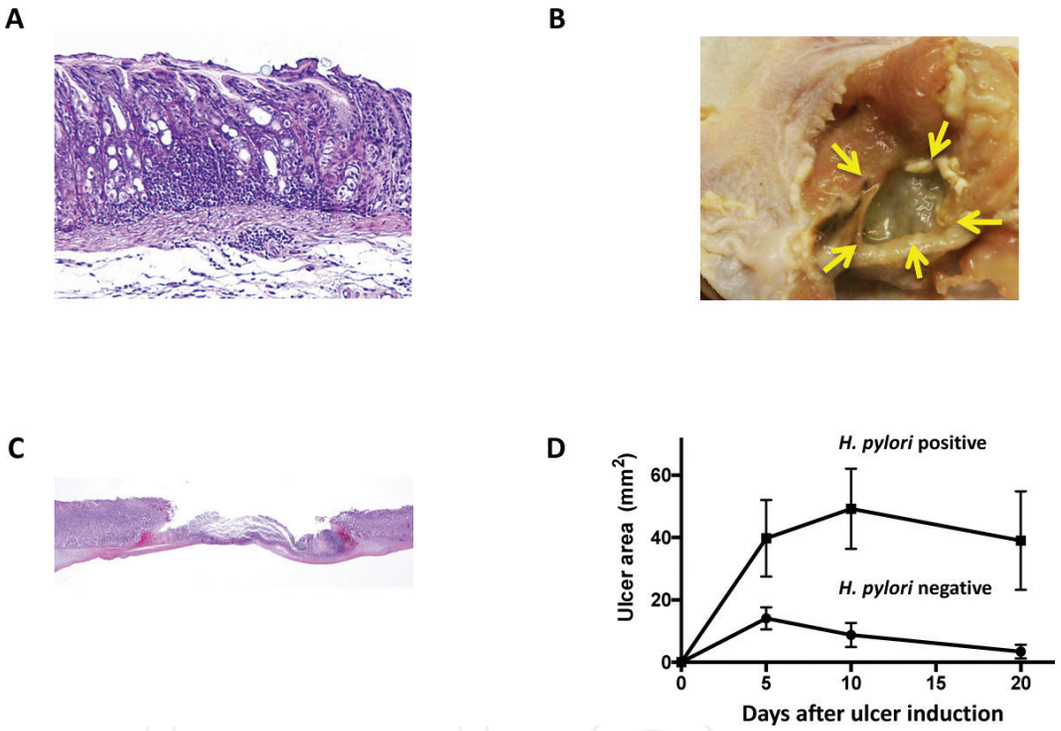

Figure 3. A. Microscopic features of *H. pylori*-infected gastric mucosa of Mongolian gerbils (MGs). Sections were stained with hematoxylin and eosin. B. Gross appearance 10 days after gastric ulcer induction of *H. pylori*-infected MGs. C. Microphotograph 10 days after gastric ulcer induction in *H. pylori*-infected MGs. D. Serial changes in acetic acid-induced gastric ulcer areas in MGs with or without *H. pylori* infection. *H. pylori* (ATCC43504; American Type Culture Collection, Rockville, MD) was grown in Brucella broth (Becton Dickinson, Cockeysville, MD) containing 10% v/v horse serum for 40 h at 37°C under microaerobic conditions (15% CO_2) and high humidity with shaking (150 rpm). Male MGs (MGs/Sea) were purchased from Kyudo (Saga, Japan). At 11 weeks of age, *H. pylori* (0.8 mL samples of Brucella broth containing 1.0×10^9 colony-forming units) was delivered intragastrically using an oral catheter after fasting for 24 h. Gastric ulcers were induced experimentally in MGs according to the method described by Wang et al. [40]. Briefly, after anesthetization with ketalar, the abdomen was opened through a midline incision, and 50 μL of 25% acetic acid was injected in the subserosa of the anterior wall of the stomach. The MGs were killed at5, 10, and 20 days after ulcer induction, and the stomach was dissected and removed. The maximum and minimum diameters of the ulcers were measured, and the ulcer area, which was approximately elliptical, was calculated and was compared between MGs with and without *H. pylori* infection. Values are the mean ± SE. The animal care committee of St. Marianna University approved the experimental design, and the animals were cared for in accordance with institutional guidelines.

methylation and *H. pylori* infection in gastric ulcer healing, *COX* mRNA levels in samples prepared from acetic acid-induced gastric ulcers in MGs were examined. Then, the effects of *COX-2* methylation on *COX-2* mRNA expression were also investigated *in vitro* using an *H. pylori*-infected MG stomach-derived cell line.

Gastric ulcers were produced by injecting 25% of acetic acid (0.03 mL) into the submucosal layer of the gastric wall of the antral-oxyntic border in MGs 48 weeks after inoculation with an *H. pylori* suspension in Brucella broth. The MGs were killed at 5, 10, and 20 days after ulcer induction, and the stomachs were dissected and removed. The maximum and minimum diameters of the ulcers were measured, and the area of each ulcer, which was approximately elliptical, was calculated and compared between MGs with and without *H. pylori* infection. The ulcer area was largest on day 5, and then gradually decreased. In accordance with previous reports [29], the gastric ulcer area was larger in *H. pylori*-infected MGs than in uninfected MGs (**Figure 3B–D**). While *COX-2* mRNA expression at the ulcer edge was increased 5 days after acetic acid injection in MGs without *H. pylori* infection, as was reported in rats and mice [31], no increases in *COX-2* mRNA levels were observed in *H. pylori*-infected MGs. In contrast, gastric ulceration was not associated with a change in *COX-1* mRNA levels in MGs with or without *H. pylori* infection (**Figure 4A,B**). Thus, *H. pylori* infection caused delayed ulcer healing and impaired COX-2 induction in MG stomachs.

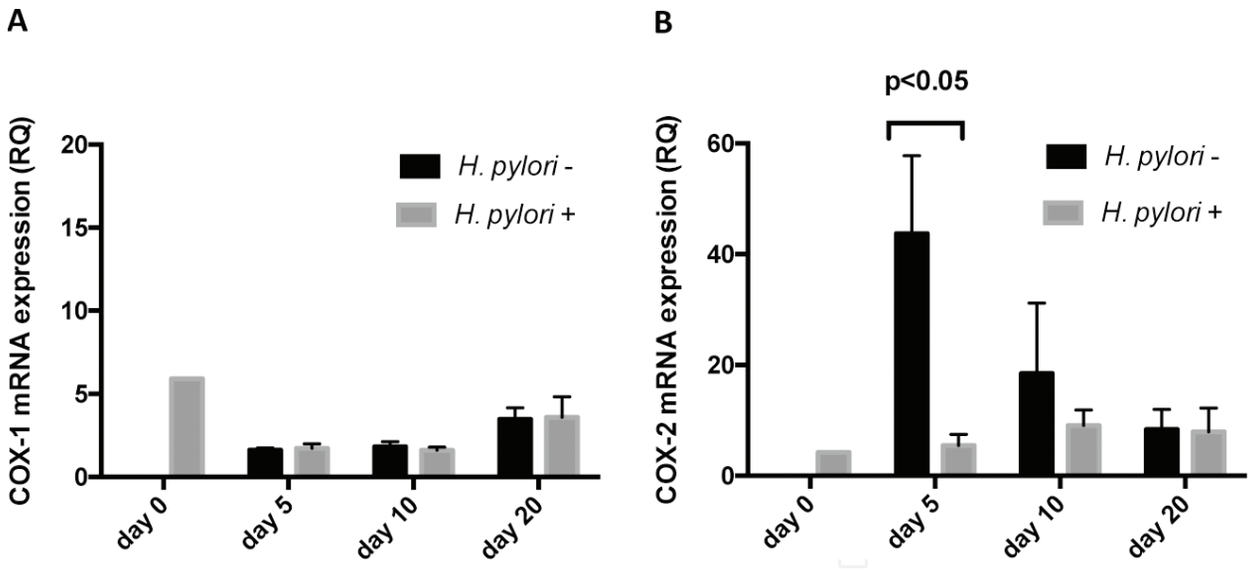

Figure 4. *COX* mRNA levels during healing of an acetic acid-induced gastric ulcer in MGs with or without *H. pylori* infection. *COX* mRNA levels in acetic-acid-induced gastric ulcers in MGs were measured by real-time PCR. First-strand cDNA was prepared by reverse transcription of 5 μg of total RNA using superscript III reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA). Real-time quantitative reverse transcription PCR was carried out using TaqMan Gene Expression Assays with a 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Primers for *COX-1*, *COX-2*, and *β-actin* were designed based on their cDNA sequences (GenBank accession nos. AB 044783, AB044784, and AB040445, respectively) and according to previous reports [33]. SDS2.1 software (Applied Biosystems) was used to perform the comparative Δ-Ct analysis. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control. Values are the mean ± SE. β-Actin left: gctacagcttcaccaccaca, right: ccatctcttgctcgaagtcc, 93 bp. COX-1 left: gtggctatttcctgcagctc, right: agtgggtgccagtggtagag, 112 bp. COX-2 left: tgggcgtgaaaggaaataag, right: ggggatcagggatgaacttt, 87 bp.

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5. Effects of *COX-2* methylation on its mRNA expression in MGs *in vitro*

As discussed above, *COX-2* mRNA expression is regulated by an epigenetic mechanism in KATO-III human gastric carcinoma cells in which *COX-2* is densely methylated [22]. To investigate the role of methylation in *COX-2* mRNA expression in *H. pylori*-infected gastric mucosa of MGs, we treated MGC2 cells with 5-aza-dC, a methyltransferase inhibitor, or trichostatin A (TSA), a histone deacetylase inhibitor. MGC2 is an adenocarcinoma cell line established from the gastric cancer tissue of a *H. pylori*-infected MG [32]. *COX-2* mRNA expression levels in these cells were restored after the addition of 5-aza-dC. In contrast, treatment with TSA did not induce *COX-2* mRNA expression (**Figure 5**). These results indicated that *COX-2* mRNA expression in MGs is regulated via both transcriptional and epigenetic mechanisms. Histone acetylation was not involved in silencing *COX-2* expression in these cells.

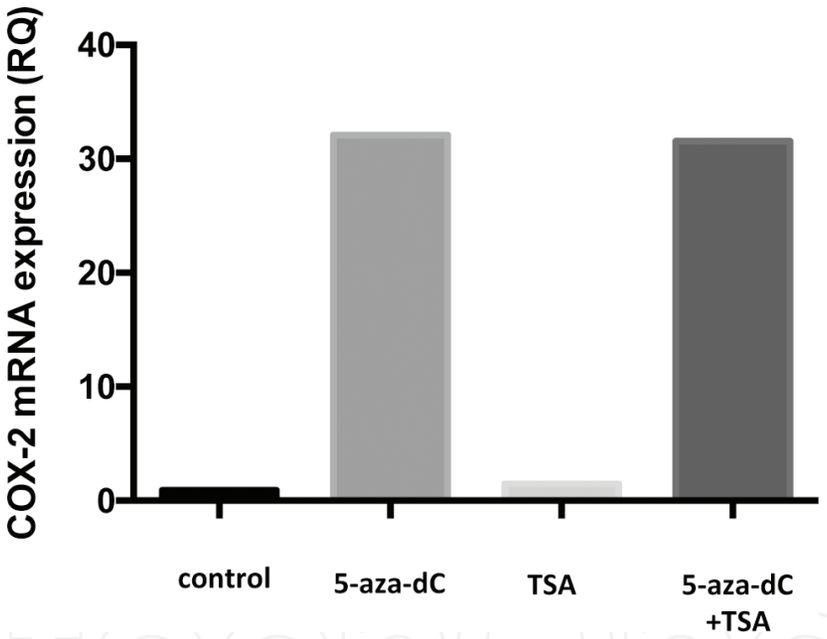

Figure 5. Effects of 5-aza-dC, a methyltransferase inhibitor, or trichostatin A (TSA), a histone deacetylase inhibitor, on *COX-2* mRNA expression in MG gastric adenocarcinoma MGC2 cells. The MGC2 cells [32] were a generous gift from Dr. Tatematsu and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) plus serum expander MITO (0.1%, Collaborative Biomedical Products Bedford, MA, USA) on a type I collagen-coated dish (Asahi Techno Glass, Japan). All the cultures were incubated at 37°C with 95% air and 5% CO_2. *COX-2* mRNA expression was measured by real-time PCR in the cell lines grown in the presence of vehicle with or without 5-Aza-dC (1 μmol/L) or TSA for 5 days. Values shown are the mean ± SE.

6. Cloning the MG *COX-2* promoter region

As mentioned above, human *COX-2* has abundant CGIs in the promoter region. To examine whether CGIs are present in the *COX-2* promoter region of MGs, we performed genomic

PCR with primers designed using as previously reported MG cDNA (Accession #: AB177842) [33]. The PCR product has an intron sequence in the promoter region. Then, gene walking was performed using a primer in this intron. The promoter region of the MG *COX-2* gene was obtained (**Figure 6**), and the sequence contains more CGIs than the mouse genome, and

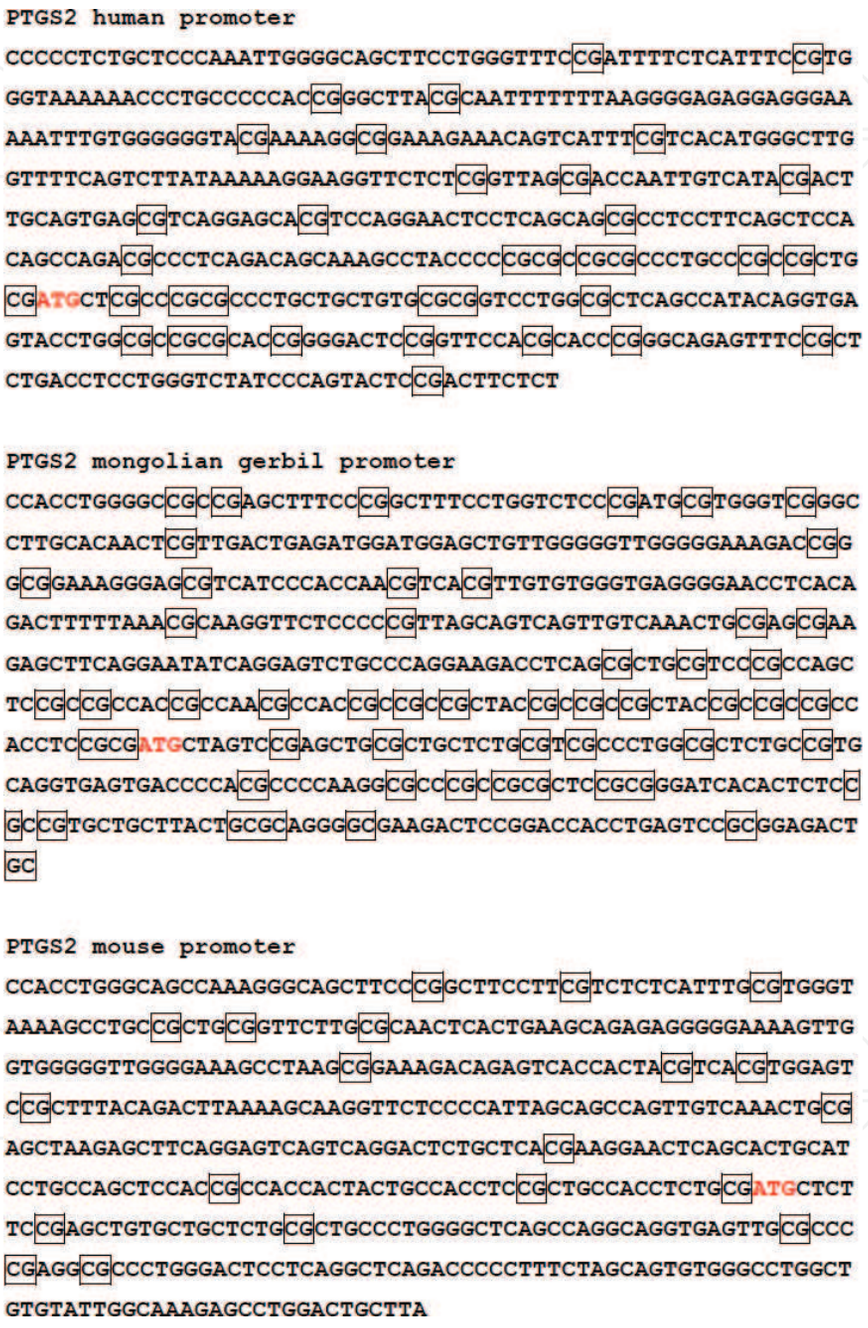

Figure 6. Human, MG, and mouse *COX-2* promoter regions. Genome walking to isolate genomic, the *COX-2* promoter region of MGs, was performed by using the Straight Walk Kit [41] according to the manufacturer's instructions (Bex, Tokyo, Japan). Amplified fragments were cloned into the pSTBlue-1 vector (Novagen, Madison, WI, USA) and sequenced. More abundant CGIs were observed in the MG genome when compared to the mouse genome, and the number was comparable to human genome.

is comparable to that of the human genome. Pyrosequencing showed several methylated CGIs (>15%) in the MGC2 cells. Treatment with 5-aza-dC decreased the methylation levels in these cells (**Figure 7**). As was observed in the human stomach, *COX-2* methylation levels were increased in *H. pylori*-infected gastric mucosa of MGs when compared to the levels in the control mucosa (**Figure 8**).

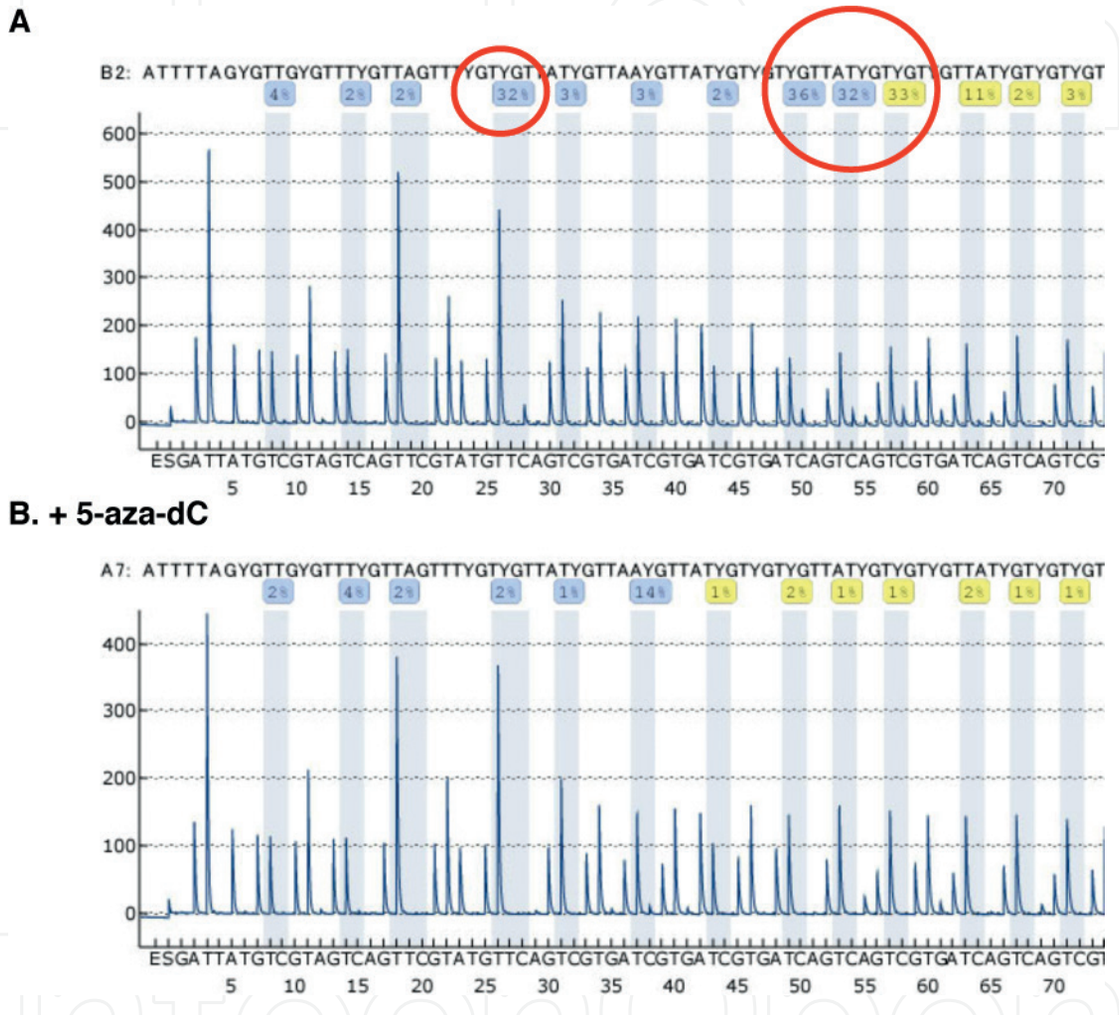

Figure 7. Effect of 5-aza-dC treatment on *COX-2* methylation levels in MGC2 cells. Cells were treated with vehicle (A) with 5-aza-dC (B, 1 μmol/L) for 5 days, and then methylation levels of *COX-2* were analyzed by quantitative bisulfite-pyrosequencing methods as previously reported [42]. Bisulfite treatment of gDNA was performed with the EpiTect bisulfite kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. Bisulfite-treated DNA (1 μL) was used as a template in subsequent PCR experiments. For most assays, we used touchdown PCR. All PCR assays included a denaturation step at 95°C for 30 s, followed by annealing at various temperatures for 30 s, and extension at 72°C for 30 s. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers. Pyrosequencing was performed using PSQ HS 96 Gold single-nucleotide polymorphism reagents on a PyroMark Q24 pyrosequencing machine (QIAGEN). The protocol for pyrosequencing was described in detail previously [42]. Pyrosequencing quantitatively measures the methylation status of CpG sites in a target region. Adjacent sites usually show highly concordant methylation. Therefore, the mean percent methylation in detected sites can be used as a representative value for each gene promoter. Cases with the methylation density > 15% were regarded as methylation positive. Forward primer: tgggtgaggggaattttataga, reverse primer: aaaccctaaccatccttacaa; and sequencing primer: aggagtttgtttaggaag.

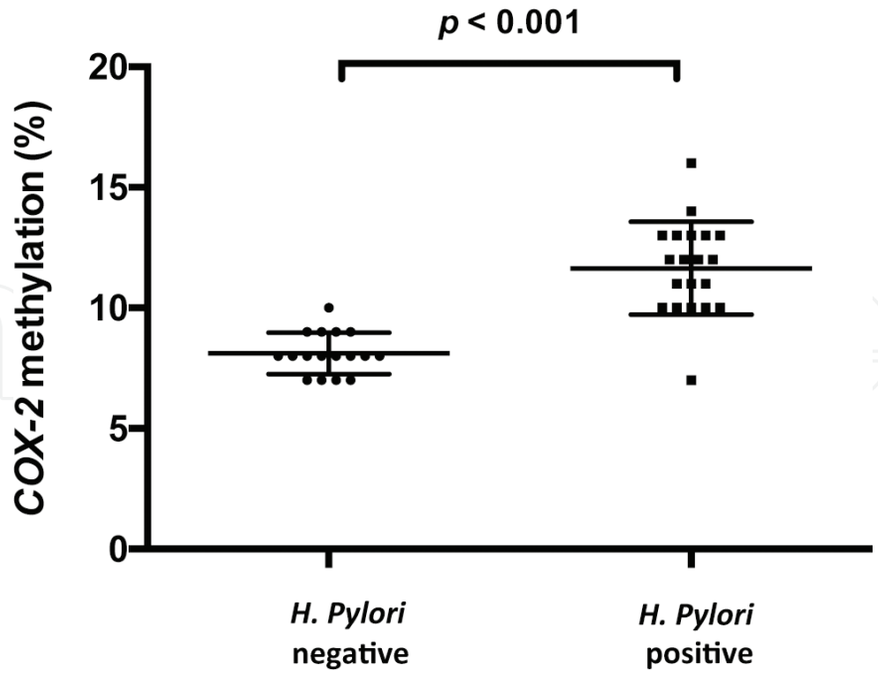

Figure 8. DNA methylation levels at *COX-2* in the MG gastric mucosa in the presence or absences of *H. pylori* infection. Methylation levels at *COX-2* in the MG gastric mucosa were analyzed by quantitative bisulfite pyrosequencing.

7. Role of *COX-2* methylation and *H. pylori* infection in gastric mucosal healing

Nonselective COX inhibitors damage the gastrointestinal mucosa, and gastrointestinal injury represents the most significant side effect of chronic nonsteroidal anti-inflammatory drug (NSAID) use [34]. Thus, selective COX-2 inhibitors have been developed as ideal anti-inflammatory drugs, devoid of GI toxicity, and clinical trials have suggested that selective COX-2 inhibitors produce less gastrointestinal injury than conventional NSAIDs [8, 9]. In support of this, animal studies have also suggested that, in contrast to the initial concept, the importance of COX-2 in the repair of gastric mucosal damage has been recognized [31, 35]. In rat gastric mucosa, markedly elevated levels of *COX-2* mRNA were observed after induction of damage by ischemia-reperfusion [35, 36]. COX-2 mRNA and protein expression increase during repair of gastric mucosal lesions, and selective COX-2 inhibitors delay mucosal healing in mice [31]. PGs derived from ulceration-induced COX-2 at the ulcer margin enhanced epithelial cell proliferation and increased the expression of growth factors, including hepatocyte growth factor, epidermal growth factor, transforming growth factor-α, and VEGF [16].

The release of inflammatory cytokines and recruitment of inflammatory cells have been considered the potential factors for delayed ulcer healing in *H. pylori* infection [2, 28]. Chronic inflammation, including *H. pylori* infection, is known to cause aberrant DNA methylation [37, 38]. Here, we focused on *COX-2* methylation in *H. pylori*-infected gastric mucosa. Human *COX-2* has CGIs in the promoter region, and methylation levels in this region are increased in

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H. pylori-infected gastric mucosa. In addition, *COX-2* promoter methylation levels in patients with successfully eradicated *H. pylori* infection are lower than the levels in *H. pylori*-positive cases. As mentioned above, experiments using rodents have shown that *COX-2* mRNA expression is enhanced during ulcer healing. Thus, we investigated the influence of *H. pylori* infection on COX-2 expression during stomach ulcer healing in MGs. Acetic acid-induced ulcer healing was delayed in *H. pylori*-infected MG stomachs when compared to the healing of ulcers without *H. pylori* infection [29]. The *COX-2* promoter region of MGs also has abundant CGIs, comparable to human *COX-2*. Thus, MGs are good models for investigating the role of *COX-2* methylation in gastric mucosal healing. While *COX-2* mRNA expression at the ulcer edge was increased 5 days after acetic acid injection in MG stomachs without *H. pylori* infection, as was observed in the mouse and rat models, such increases in *COX-2* mRNA expression were not observed in *H. pylori*-infected MG gastric mucosa. However, a demethylating agent restored COX-2 mRNA expression in both human and MG gastric carcinoma cell lines in which COX-2 is densely methylated. Accordingly, we demonstrated *in vitro* and *in vivo* that *COX-2* mRNA expression is regulated through an epigenetic mechanism in human and MG gastric mucosa. Aberrant DNA methylation has been extensively investigated in the context of the pathogenesis of various cancers, including stomach cancer. In addition, the involvement of epigenetic modifications has been reported in the pathogenesis of various chronic diseases, such as essential hypertension and cardiovascular disease [39]. Taken together, the epigenetic inhibition of COX-2 expression due to chronic inflammation induced by *H. pylori* infection seems to interfere with ulcer healing and increase the vulnerability of the gastric mucosa. The decrease in *COX-2* methylation levels after *H. pylori* eradication may restore gastric mucosal defense.

8. Conclusions

H. pylori infection, the leading cause of peptic ulcer disease, induces sustained inflammation in the gastric mucosa. This chronic inflammation causes aberrant DNA methylation in various genes. The *COX-2* promoter regions in both MGs and humans contain abundant CGIs. COX plays critical roles in peptic ulcer development and healing, and both COX-1 and COX-2 are necessary for the gastric mucosal defense system. COX-1 is a housekeeping enzyme, which maintains microcirculation and mucous production. During ulcer healing, COX-2 is expressed at the margin of an ulcer, and COX-2-derived PGs induce various growth factors to promote mucosal healing and angiogenesis. Ulcer healing is delayed in *H. pylori* infected-MG stomachs, which is the best model of *H. pylori* infection in humans. We showed that *COX-2* mRNA induction during ulcer healing was impaired in *H. pylori*-infected-MG stomachs. The *COX-2* promoter region is methylated in both *H. pylori*-infected human and MG gastric mucosa. These CGIs are also methylated in human Kato III cells and MGC2 gastric carcinoma cells. *COX-2* mRNA expression in these cells is restored by treatment with a demethylating agent. In *H. pylori*-infected gastric mucosa, *COX-2* promoter methylation appears to be involved in the impaired *COX-2* mRNA induction typically observed during ulcer healing, which leads to delayed ulcer healing.

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Conflict of interest

There is no conflict of interest to disclose.

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