Helicobacter pylori Up-regulates Cyclooxygenase-2 mRNA Expression and Prostaglandin E₂ Synthesis in MKN 28 Gastric Mucosal Cells in Vitro*

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Helicobacter pylori has been suggested to play a role in the development of gastric carcinoma in humans. Also, mounting evidence indicates that cyclooxygenase-2 overexpression is associated with gastrointestinal carcinogenesis. We studied the effect of H. pylori on the expression and activity of cyclooxygenase-1 and cyclooxygenase-2 in MKN 28 gastric mucosal cells. H. pylori did not affect cyclooxygenase-1 expression, whereas cyclooxygenase-2 mRNA levels increased by 5-fold at 24 h after incubation of MKN 28 cells with broth culture filtrates or bacterial suspensions from wild-type H. pylori strain. Also, H. pylori caused a 3-fold increase in the release of prostaglandin E₂, the main product of cyclooxygenase activity. This effect was specifically related to H. pylori because it was not observed with Escherichia coli and was independent of VacA, CagA, or ammonia. H. pylori isogenic mutants specifically lacking picA or picB, which are responsible for cytokine production by gastric cells, were less effective in the up-regulation of cyclooxygenase-2 mRNA levels.

Helicobacter pylori plays a central role in the etiology of chronic superficial gastritis and peptic ulcer disease and seems to increase the risk for development of gastric adenocarcinoma in humans (1–3). H. pylori-induced gastroduodenal disease depends on the inflammatory response of the host and on the release of a number of virulence factors such as urease, responsible for ammonia generation (4), a vacuolating cytotoxin (VacA) (5), and a cytotoxic-associated immunodominant protein (CagA) (6). In addition, multiple genes in the cag pathogenicity island have recently been described whose expression are necessary for cytokine production by gastric epithelial cells in vitro (7, 8). However, the mechanism whereby H. pylori contributes to gastric carcinogenesis is still unknown.

Prostaglandins (PGs) are arachidonic acid derivatives that protect the gastric mucosa against exogenous injury (9, 10). PGs synthesis depends on the activity of a constitutively expressed and an inducible PG endoperoxide synthase/cyclooxygenase (COX-1 and COX-2, respectively) (10, 11). Mounting evidence indicates that COX-2 is associated with colorectal carcinogenesis (12, 13), COX-2 being overexpressed in 80–90% of colorectal adenocarcinomas and in 40–50% of premalignant adenomas (13). Moreover, COX-2 overexpression has recently been reported in human gastric adenocarcinoma (14). Although the role of COX-2 in gastrointestinal carcinogenesis is still unclear, its up-regulation is probably an early event (13).

This study was designed to evaluate whether H. pylori affects COX-1 and COX-2 expression and PGE₂ synthesis in gastric mucosal cells (i.e. MKN 28 cells) (15, 16) in vitro and to study the role of H. pylori virulence factors in any such effect. We found that H. pylori time-dependently up-regulated COX-2 mRNA expression and significantly increased the release of PGE₂. This was partially related to the expression of two bacterial virulence factors (i.e. PicA and PicB) responsible for cytokine production by gastric cells (7). We postulate that COX-2-related events may contribute to development of gastric adenocarcinoma associated with H. pylori infection.

MATERIALS AND METHODS

Cell Culture—MKN 28 cells are derived from a human gastric tubular adenocarcinoma (15) and show gastric-type differentiation (16). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution (Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5% CO₂ in air, as described previously (17). Cells were grown in 100-mm dishes for the RNA extraction and in 60-mm dishes for the PGE₂ release studies.

Bacterial Strains and Growth Conditions—We have used the urease positive VacA^- CagA^- wild-type H. pylori 60190 strain (ATCC 49503) and isogenic mutants in which vacA, cagA, vacA and cagA, picA or picB genes were disrupted by insertional mutagenesis (5–7). We also used the VacA^- CagA^- H. pylori strain Bx2 U^- and its urease-negative mutant Bx2 U^- (provided by F Megraud, Bordeaux, France) and the CCUG 17874 (VacA^- CagA^-) strain (from the culture collection of the University of Göteborg, Göteborg, Sweden). Bacteria were grown in brucella broth supplemented with 5% fetal calf serum (Life Technologies, Inc.) for 24–56 h at 37 °C in a thermostatic shaker under microaerobic conditions. As described previously (17), when the bacterial suspensions reached 1.2 optical density units at 450 nm, bacteria were removed by centrifugation, and the supernatants were sterilized by passage through a 0.22-μm pore size cellulose acetate filter (Nalgé Co, Inc.).
H. pylori and COX-2 Expression in Gastric Mucosal Cells

RESULTS

To evaluate the effect of H. pylori on COX-1 and COX-2 mRNA expression, MKN 28 cells were incubated for up to 48 h with bacterial suspensions or broth culture filtrates derived from a H. pylori 60190 (wild-type) strain. We did not detect any basal level of COX-1 mRNA expression, nor did we find any increase in COX-1 mRNA expression following exposure of MKN 28 cells to H. pylori (data not shown). There was, however, a time-dependent increase in COX-2 mRNA content in these cells in response to H. pylori (Fig. 1). The increase in COX-2 mRNA expression was evident after 12 h of treatment, reached a peak at 24 h (5-fold increase versus control), and declined at 48 h (Fig. 1). To determine whether the increase in COX-2 mRNA expression was specific for H. pylori 60190, we evaluated the effect of a bacterial suspension or of a broth culture filtrate from wild-type H. pylori strain CCUG 17874 and obtained comparable results (data not shown). Because similar effects were obtained with bacterial suspensions or with broth culture filtrates from H. pylori 60190 or CCUG 17874 strains (data not shown), in the subsequent experiments, we used H. pylori broth culture filtrates only.

Fig. 1. Time course of H. pylori effect on COX-2 mRNA expression in MKN 28 cells. Semi-confluent monolayers were incubated with DMEM (control) or with a bacterial suspension (5 x 10^9 CFU/ml) from H. pylori 60190 (wild type) for up to 48 h. Total RNA was isolated. Northern blots were performed using 10 μg of total RNA/lane, and filters were sequentially hybridized to 32P-labeled cDNA probes for human COX-2 or GAPDH. The constitutively expressed GAPDH transcript was used to evaluate equivalence of RNA loading and transfer. A representative autoradiograph of four separate experiments is shown. C, control; Hp, H. pylori 60190.
**FIG. 2.** Role of *H. pylori* virulence factors in up-regulation of COX-2 mRNA expression in MKN 28 cells. Semi-confluent monolayers were incubated for 24 h with unincubated broth filtrate (control) or broth culture filtrates (each diluted 1:3 in culture medium, i.e. DMEM supplemented with 10% fetal calf serum) from *H. pylori* 60190 strain (wild-type) or its isogenic *vacA* (VacA*−*), *cagA* (CagA*−*), vacAcagA (VacA*−CagA*−*), *picA* (PicA*−*), or *picB* (PicB*−*) mutants, or *H. pylori* Bx2U*−* (Urease*−*) strain or its urease negative mutant (Bx2U*−*), or with 4 mM NH4Cl, or with *E. coli* (ATCC 25922 strain) broth culture filtrate. Isolation of total RNA, Northern blotting, and filter hybridization were as described in the legend to Fig. 1. A representative autoradiograph of four separate experiments is shown.

**Fig. 3.** Effect of *H. pylori* broth culture filtrates on PGE2 release by MKN 28 cells. Semi-confluent monolayers were incubated for 24 h with unincubated broth filtrate (control) or broth culture filtrates (each diluted 1:3 in serum-free DMEM) from *H. pylori* 60190 wild-type strain (wild-type) or its isogenic *vacA* (VacA*−*), *cagA* (CagA*−*), *picA* (PicA*−*), or *picB* (PicB*−*) mutants. Mean ± S.D. of four experiments run in triplicate. *p < 0.05 versus control; +, p < 0.05 versus wild-type, VacA*−*, or CagA*−*.

but this increase was significantly lower than that obtained with *H. pylori* 60190 wild-type strain (approximately 45% reduction; p < 0.05) (Fig. 3).

**DISCUSSION**

Several studies indicate that *H. pylori* is an important risk factor for adenocarcinoma of the distal stomach in humans (2, 3), but the mechanism whereby *H. pylori* might contribute to gastric carcinogenesis is still hypothetical. COX-2 has been suggested to be involved in the development of malignancies of the gastrointestinal tract (11–14). Although the role of COX-2 in the carcinogenesis of the gut is unknown, its up-regulation represents an early event (13). This study was therefore designed to evaluate COX-2 expression and activity in gastric mucosal cells exposed to *H. pylori*, in vitro.

Our data show that *H. pylori* up-regulates COX-2 mRNA expression and stimulates the release of PGE2 in MKN 28 gastric mucosal cells in vitro, effects not only observed with *H. pylori* suspensions but also with broth culture filtrates, suggesting that it might be mediated by a soluble product released from the bacterium. VacA, CagA, and urease-generated ammonia do not seem to play a role. That *H. pylori* 60190 isogenic *picA* and *picB* mutants are less effective than the parental wild-type strain in the induction of COX-2 expression and PGE2 release suggests that PicA and PicB may contribute to the increased COX-2 expression and activity, possibly through stimulation of cytokine production. In fact, the expression of *picA* and *picB* plays a major role in *H. pylori*-mediated induction of cytokine production by gastric mucosal cells in vitro (7). Moreover, cytokines are known to induce COX-2 expression (11).

Because *H. pylori* 60190 isogenic *picA* and *picB* mutants, even though to a lesser extent than the parental wild-type strain, still up-regulate COX-2 expression and activity, other factors may be involved. EGF-related growth factors are known to up-regulate COX-2 expression through activation of the EGF receptor (18). We have recently shown an increase in the expression of heparin-binding-EGF-like growth factor and amphiregulin, members of the EGF receptor ligand family, as early as 4 h following incubation of MKN 28 gastric mucosal cells with *H. pylori* broth culture filtrates or suspension (17). Because in the present study induction of COX-2 mRNA expression starts at 12 h of incubation, we hypothesize that *H. pylori*-induced up-regulation of COX-2 mRNA levels might be contributed to by EGF-related growth factors. In partial support of this hypothesis, we found that 24-h incubation with heparin-binding-EGF-like growth factor or amphiregulin (10 nM) up-regulated COX-2 mRNA expression in MKN 28 cells (data not shown).

MKN 28 cell line has been proven to be an appropriate model for the study of the response of gastric epithelial cells to *H. pylori* (4, 17, 19). Moreover, we previously have studied this cell line in comparison with human gastric cell monolayers obtained from normal gastric tissue to evaluate the response to cytotoxic drugs and to cytoprotective agents and obtained qualitatively similar results (16). However, because these cells are derived from an adenocarcinoma, the effects observed could reflect the biology of tumor cells more than that of normal, nontransformed cells. This might explain the lack of detection of COX-1 mRNA, which has been shown to be constitutively expressed in the normal gastric mucosa (14). Recent preliminary reports indicate that in human gastric mucosa, COX-1 is mainly expressed in parietal cells, endothelial cells, and lamina propria macrophages (20, 21). Therefore, COX-1 mRNA levels might be below the level of detection in MKN 28 cell line that consists of mucus producing cells (15, 16).

Even though epidemiological evidence of causality suggests that *H. pylori* is a human carcinogen (2, 3), mechanistic explanations of *H. pylori* carcinogenesis are still hypothetical. However, increased proliferative activity of epithelial cells in gastric mucosa colonized by *H. pylori* in the absence of a corresponding increase in apoptosis (22) and formation of reactive nitrogen derivatives that may cause DNA damage might contribute (23, 24). Because (i) COX-2 participates in activation and formation of carcinogenic (25) and (ii) COX-2 overexpression may facilitate tumor progression by increasing cell proliferation (25), by inhibiting apoptosis (25), and by stimulating the production of angiogenic agents in cancer cells (26), based on our findings, we postulate that development of carcinoma of the distal stomach associated with *H. pylori* infection may depend on the activation of COX-2-related events.

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