Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 44, Issue of October 30, pp. 28560–28563, 1998

Helicobacter pylori Up-regulates Cyclooxygenase-2 mRNA Expression and Prostaglandin E_2 Synthesis in MKN 28 Gastric Mucosal Cells *in Vitro**

(Received for publication, August 10, 1998, and in revised form, September 8, 1998)

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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 E_2, 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 expression and in the stimulation of prostaglandin E_2 release compared with the parental wild-type strain. This study suggests that development of gastric carcinoma associated with *H. pylori* infection may depend on the activation of cyclooxygenase-2-related events.**

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 cytotoxin-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)[1] 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 PGE_2 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 PGE_2. 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% CO_2 in air, as described previously (17). Cells were grown in 100-mm dishes for the RNA extraction and in 60-mm dishes for the PGE_2 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 Goteborg, Goteborg, Sweden). Bacteria were grown in brucella broth supplemented with 5% fetal calf serum (Life Technologies, Inc.) for 24–36 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 (Nalge Co,

* This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro, Comitato Nazionale Biotecnologie e Biologia Molecolare from Consiglio Nazionale delle Ricerche, and Ministero dell' Università per la Ricerca Scientifica e Tecnologica. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; CFU, colony forming units.

Rochester, NY) to obtain the broth culture filtrates. Broth culture filtrates from different bacterial strains and different preparations from the same strain were standardized by growing the bacterial suspensions until they reached an optical density of 1.2 units at 450 nm, which corresponds to a bacterial concentration of 5×10^8 CFU/ml. The presence or absence of VacA and/or CagA in *H. pylori* cells or filtrate was verified by means of SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-VacA or anti-CagA polyclonal antiserum, as described previously (17). In the experiments with bacterial suspensions, cells were incubated with bacterial preparations at the concentration of 5×10^7 CFU/ml in DMEM supplemented with 10% fetal calf serum.

RNA Isolation and Northern Analysis—Total RNA was isolated from MKN 28 cells by the guanidinium thiocyanate acid-phenol procedure and subjected to Northern analysis as described previously (17). COX-1 mRNA expression was analyzed on poly(A)$^+$ RNA from untreated (control) and *H. pylori*-treated cells. Poly(A)$^+$ RNA was isolated from total RNA using the Amersham poly(A)$^+$ RNA kit (Amersham Italia Srl, Milano, Italy) according to the manufacturer's procedures. In brief, 10 μg of total or poly(A)$^+$ RNA/lane was separated by electrophoresis in 1% agarose-formaldehyde gels. RNA was transferred to Hybond-N$^+$ (Amersham Italia Srl), cross-linked (UV Stratalinker-1800, Stratagene, La Jolla, CA), and hybridized to ^{32}P-labeled cDNA probes. ^{32}P-Labeled isotopes were from Amersham Corp. The COX-1 probe was a *Hin*dIII/*Pst*I 514-base pair fragment corresponding to the 3′ end of the human COX-1 cDNA (11). The COX-2 probe was a 276-base pair *Eco*RI/*Eco*RI fragment of the human COX-2 cDNA (11). COX-2 mRNA levels were normalized to mRNA levels of the constitutively expressed GAPDH gene. mRNA levels were quantitated by densitometric scanning of the autoradiograph using a Howtek Scanmaster-3 densitometer with RFL Print-TM software (Pharmacia Biotech Inc., Cologno Monzese, Italy).

PGE_2 Assay—Semi-confluent monolayers were washed three times with DMEM (37 °C) and incubated for 24 h with uninoculated broth filtrate (control) or with broth culture filtrates from *H. pylori* 60190 wild-type strain or its isogenic *vacA*, *cagA*, *picA*, or *picB* mutants. Uninoculated broth filtrate and broth culture filtrates were diluted 1:3 in DMEM not supplemented with fetal calf serum. The conditioned media were collected and centrifuged for 5 min at 2,000 rpm at 4 °C. The pellets were combined with corresponding monolayers, and protein was determined with the Bradford's dye binding test. PGE_2 concentration in the conditioned media was measured directly in triplicate with a highly sensitive ^{125}I radioimmunoassay kit according to the procedure indicated by the manufacturer (Amersham Italia Srl). Briefly, conditioned media were subjected to solid phase extraction, and extracted PGE_2 was converted into its methyl oximate derivative using the methyl oximation reagent provided by the manufacturer. Samples were then stored at −70 °C in N_2 atmosphere before analysis. Standards in the range of 1.25–160 pg PGE_2/tube were treated in the same way as the samples, and the curve was calculated by regression analysis. The limit of detection was 1.0 pg of PGE_2 in 0.1 ml volume/assay tube. For PGE_2, cross-reactivity with PGE_1 was 5% and with all other PGs was less than 0.01%. Cross-reactivity between uninoculated broth filtrate and broth culture filtrates and antiserum to PGE_2 was evaluated in each assay experiment and found to be not significant. Results were expressed as pg/mg protein.

Statistical Analysis—Significance of differences was assessed by one-way analysis of variance and, when the F value was significant, by Duncan's multiple range test. Differences were considered significant if $p < 0.05$.

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.

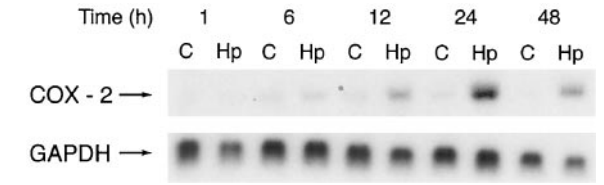

FIG. 1. **Time course of *H. pylori* effect on COX-2 mRNA expression in MKN 28 cells.** Semi-confluent monolayers were incubated with DMEM (control) or with a bacterial suspension (5×10^7 CFU/ml) from *H. pylori* 60190 (wild type) for up to 48 h. Total RNA was isolated, Northern blots were performed using 10 μg of total RNA/lane, and filters were sequentially hybridized to ^{32}P-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.

To identify potential *H. pylori* virulence factors responsible for induction of COX-2 mRNA expression, MKN 28 cells were incubated for 24 h with uninoculated broth filtrate (control) or with broth culture filtrates from *H. pylori* 60190 (wild-type) strain or its isogenic mutants lacking *vacA, cagA, vacA*/*cagA, picA,* or *picB*. Fig. 2 shows that a comparable increase in COX-2 mRNA expression was observed independently of the *vacA* or *cagA* status. On the contrary, *H. pylori* strains specifically lacking *picA* or *picB*, which are genes in the *cag* pathogenicity island whose expression are necessary for the induction of cytokine production (7, 8), were less efficient in the induction of COX-2 expression compared with the wild-type strain (approximately 2-fold increase compared with 5-fold increase, respectively). We also investigated whether urease-dependent ammonia generation might play a role in the up-regulation of COX-2. Broth culture filtrates from a urease positive (Bx2U$^+$) strain and its urease negative mutant (Bx2U$^-$) did not differ in the ability to induce COX-2 mRNA expression, nor did NH_4Cl (4 mM) exert any effect on COX-2 mRNA expression (Fig. 2). This concentration of NH_4Cl was similar to the ammonia concentration present when MKN 28 cells were incubated with broth culture filtrates from all the urease-positive *H. pylori* strains used (4). To determine whether similar effects were induced by other Gram-negative bacteria, we studied the effect of *Escherichia coli*. That incubation of cells with broth culture filtrate or cell suspension from a clinical *E. coli* isolate (ATCC 25922) did not have any effect on COX-2 mRNA expression compared with control (Fig. 2 and data not shown) suggests that the observed effects were due to unique properties of *H. pylori*.

We also studied whether induction of COX-2 mRNA expression was associated with an increased release of PGE_2 in the conditioned medium by MNK 28 cells. To address this issue, MKN 28 cells were incubated for 24 h with broth culture filtrates from *H. pylori* 60190 wild-type strain or from its isogenic *vacA*, *cagA*, *picA*, or *picB* mutants. *H. pylori* 60190 wild-type strain caused an approximately 3-fold increase in PGE_2 release by MKN 28 cells compared with control cells (478 ± 126 pg/mg protein *versus* 143 ± 42 pg/mg protein, respectively; $p < 0.05$) (Fig. 3). Comparable effects were observed using isogenic *vacA* or *cagA* mutants, thus confirming that VacA and CagA do not play a role in the up-regulation of COX-2 expression and activity. Incubation with isogenic *picA* or *picB* mutants still caused a significant increase in PGE_2 release by MKN 28 cells (271 ± 65 pg/mg protein and 246 ± 55 pg/mg protein, respectively, *versus* 143 ± 42 pg/mg protein in control cells; $p < 0.05$),

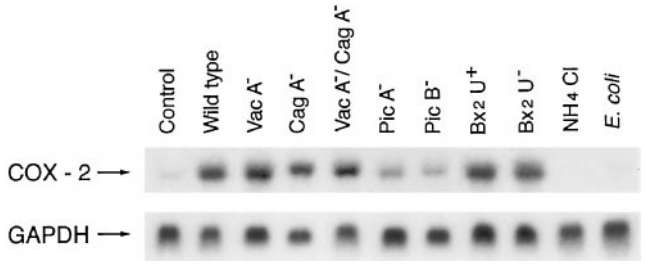

FIG. 2. **Role of *H. pylori* virulence factors in up-regulation of COX-2 mRNA expression in MKN 28 cells.** Semi-confluent monolayers were incubated for 24 h with uninoculated broth filtrate (control) or broth culture filtrates (each diluted 1:3 in culture medium, *i.e.* DMEM supplemented with 10% fetal calf serum) from *H. pylori* 60190 strain (*wild type*) or its isogenic *vacA* (VacA$^-$), *cagA* (CagA$^-$), *vacA/cagA* (VacA$^-$/CagA$^-$), *picA* (PicA$^-$), or *picB* (PicB$^-$) mutants, or *H. pylori* Bx2U$^+$ (Urease$^+$) strain or its urease negative mutant (Bx2U$^-$), or with 4 mM NH_4Cl, 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.

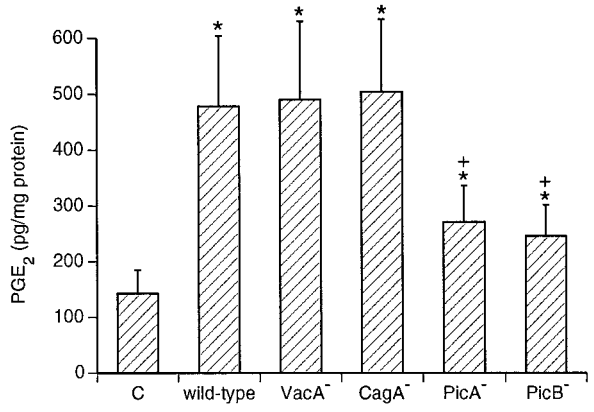

FIG. 3. **Effect of *H. pylori* broth culture filtrates on PGE_2 release by MKN 28 cells.** Semi-confluent monolayers were incubated for 24 h with uninoculated broth filtrate (control) or broth culture filtrates (each diluted 1:3 in serum-free DMEM) from *H. pylori* 60190 wild-type strain (*wild-type*) 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 PGE_2 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 PGE_2 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 carcinogens (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.

Acknowledgments—We thank Dr R. N. DuBois (Vanderbilt University, Nashville, TN) for kindly providing the cDNA probes for COX-1 and COX-2. We also thank M. Berardone and F. D'Agnello for the artwork.

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J. Biol. Chem. 1998, 273:28560-28563.
doi: 10.1074/jbc.273.44.28560
