

Helicobacter pylori Can Induce Heparin-Binding Epidermal Growth Factor Expression via Gastrin and Its Receptor

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Abstract

Both gastrin and *Helicobacter pylori* have been shown capable of up-regulating gene expression and protein shedding of heparin-binding epidermal growth factor (HB-EGF). Furthermore, the bacteria have previously been shown to induce serum hypergastrinemia in infected individuals. The aim of this work was to assess the extent to which the ability of *H. pylori* to up-regulate expression of HB-EGF can be attributed to its effect on gastrin. Gastric cells, transfected with either gastrin small interfering RNA or antisense plasmid or the gastrin/cholecystokinin-2 receptor (CCK-2R), were cultured for 24 hours with *H. pylori*^{+/−}, a CCK-2R antagonist. Gene expression levels were measured using reverse transcription-PCR, whereas protein changes were measured using ELISA, Western blotting, and immunofluorescence. *H. pylori* induced significantly higher levels of HB-EGF gene expression and ectodomain shedding in the CCK-2R-transfected cells than the vector control ($P < 0.01$). Addition of the CCK-2R inhibitor significantly decreased gene and shedding up-regulation. Gastrin down-regulation reduced the effect of the bacteria on HB-EGF gene and protein expression levels. Endogenous gastrin and CCK-2R expression were also found to be significantly up-regulated in all cell lines as a result of exposure to *H. pylori* ($P < 0.02$). Gastric mucosal tissue from *H. pylori*-infected individuals had significantly higher CCK-2R expression levels than noninfected ($P < 0.003$), and in hypergastrinemic mice, there was an increase in HB-EGF-expressing cells in the gastric mucosa and colocalization of HB-EGF with CCK-2R-positive enterochromaffin-like cells. In conclusion, gastrin and the CCK-2R play significant roles in the induction of HB-EGF gene and protein expression and ectodomain shedding by *H. pylori*. (Cancer Res 2006; 66(15): 7524-31)

Introduction

Distal gastric adenocarcinoma is the second leading cause of cancer-related deaths in the world, and long-term infection with the microaerophilic bacteria *Helicobacter pylori* significantly increases the risk of gastric carcinoma development (1, 2). However, despite the fact that approximately half of the population of the world is believed to be infected with *H. pylori*, only a relatively small percentage of cases ever progress to neoplasia. It has been suggested that this inconsistency may be due in part to bacterial strain-specific virulence factors (3, 4). The most important of these is the *cag* pathogenicity island (*cag* PAI).

The *cag* PAI is a group of ~30 genes inserted into the *H. pylori* glutamate racemase gene, which codes for a type IV secretion system that assists the injection of bacterial proteins into eukaryotic cells (5). CagA, the product of the terminal gene in *cag* PAI, is translocated into host epithelial cells following bacterial attachment and induces intracellular signaling pathways causing, among other effects, increased proliferation and decreased apoptosis (6). The presence of CagA leads to an increased risk of clinically significant sequelae (7, 8).

Infection with *H. pylori* is known to induce several conditions/factors, including, and most pertinent for this study, a pronounced serum hypergastrinemia, induction of matrix metalloproteinases (MMP), and enhanced expression of growth factors, such as heparin-binding epidermal growth factor (HB-EGF; refs. 9–12).

The actions of gastrin on cells are mediated via binding to the gastrin receptor, also known as the cholecystokinin receptor type 2 (CCK-2R). Gastrin binding to the CCK-2R is known to induce MMPs (13) and growth factors, such as EGF family of ligands (14). In cells expressing CCK-2R, gastrin has the facility to increase both HB-EGF gene and protein expression (15, 16). It has also been reported that gastrin may enhance *H. pylori* growth (17). These combined activities implicate gastrin in gastric malignancy (18).

HB-EGF is a member of the EGF family. It is synthesized primarily by macrophages but also by cultured cells, including vascular endothelial, smooth muscle, and tumor cells (19–21), as a membrane-anchored, propeptide of 208 amino acids, which is proteolytically processed to release a mature, soluble growth factor (22). HB-EGF processing occurs in two stages, the second of which releases a 75 to 86 residue peptide, including HB and EGF-like domains (23).

Although the proteases involved in this second stage have yet to be conclusively identified, it has been suggested that members of the MMP family are responsible (24).

Overexpression of HB-EGF has been identified in a large number of different cancer cell lines, including colon, breast, and bladder (25–29). Additionally, soluble HB-EGF has been identified as a potent inducer of tumor growth and angiogenesis (30).

As there seems to be a complex interplay between these three factors (gastrin, *H. pylori*, and HB-EGF), we analyzed the extent to which the effect of *H. pylori* on HB-EGF gene expression and protein shedding is mediated through both exogenous and endogenous gastrin acting on the CCK-2R.

Materials and Methods

Cell culture. AGS (European Collection of Animal Cell Cultures, Wiltshire United Kingdom), MGLVA1, and ST16 (Academic Unit of Cancer Studies, University of Nottingham, Nottingham, United Kingdom) human gastric adenocarcinoma cells were grown in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum and 2 mmol/L glutamine in an atmosphere of 5% CO₂ at 37°C.

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AGS cells had been stably transfected with either pcDNA1 containing the CCK-2R cDNA in the sense direction or an empty vector control (31). MGLVA1 and ST16 cells had been transfected with either pCR3.1 containing the entire gastrin cDNA in the antisense direction or an empty vector control (32). Individual clones with maximal expression from the vectors were isolated and used in this study. Geneticin (Sigma) was added to a final concentration of 200 µg/mL to the medium of genetically modified cell lines to ensure transfection.

AGS, MGLVA, and ST16 cells were transfected with a specific gastrin small interfering RNA (siRNA) provided by Dr. Anna Grabowska (Academic Unit of Cancer Studies). A scrambled version of the siRNA was used as a negative control for transfection. Transfections were carried out using siPORT NeoFX siRNA transfection reagent as per manufacturers' instructions. After transfection, cells were incubated at 37°C for 48 hours before further manipulation and analysis.

Bacterial coculture. Experiments were done with the *cag*⁺ *vacA* s1/m1 toxigenic *H. pylori* strain 60190 (American Type Culture Collection). The bacteria were routinely cultured on commercially prepared 5% horse blood agar plates (Oxoid, Buckinghamshire, United Kingdom).

Coculture experiments were done in 75-cm² 2-µm pore filter cap tissue culture flasks using *H. pylori* harvested 24 hours after plating and inoculated into serum-free RPMI 1640 to a multiplicity of infection (MOI) of 40. Bacteria numbers were measured by reading the absorbance at 600 nm. Cells were cocultured with the bacteria for 24 hours.

For certain coculture experiments, human gastrin-17 (G17; Sigma) or the specific CCK-2R antagonist (R)-1-[2,3-dihydro-1-(2'-methylphenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea (YM022), donated by the James Black Foundation (Dulwich, London, United Kingdom), was added concurrently with the bacteria to a final concentration of 10⁻⁸ mol/L.

Tissue specimens. Antral biopsy specimens were taken at endoscopy from six *H. pylori*-infected and six *H. pylori*-noninfected patients attending a routine upper gastrointestinal endoscopy list. Ethical permission was obtained from the Queen's Medical Centre Ethical Committee (Nottingham, United Kingdom). All patients gave informed consent for biopsy specimens to be used for this research.

Reverse transcription and PCR amplification. RNA extraction and reverse transcription were done as described previously (32). Briefly, PCRs were carried out in 96-well optical reaction plates using 1 µL cDNA and 20 µL reaction mix consisting of 1× reaction buffer, 1:2,000 SYBR Green, MgCl₂, deoxynucleotide triphosphate mix, 1 µL of primer and Hot GoldStar Taq (all from Eurogentec, Seraing, Belgium).

The samples were run on a GeneAmp 5700 Sequence Detector Real-time PCR machine (Applied Biosystems, Warrington, United Kingdom) using the following program: 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The level of the test gene was compared with the housekeeping gene *hypoxanthine phosphoribosyltransferase* (*HPRT*). Results are presented as relative gene expression compared with *HPRT* using 2^{-ΔΔCt} (33). The sequences of the specific primers used are available on request.

ELISA for detection of secreted HB-EGF protein. Following coculture of cells and bacteria, cells were washed with 2 mol/L NaCl and medium was collected, clarified by centrifugation, and concentrated to 150 µL using Centrplus-10 concentrators with a molecular weight cutoff of 10,000 (Amicon, Beverly, MA). As a control, medium alone was concentrated in the same way. Protein levels were normalized by seeding the same number of cells at the beginning of each experiment and concentrating the same amount of medium for each sample. Serial dilutions of human recombinant HB-EGF were used as a standard.

The concentrated conditioned media were added at 50 µL/well in 96-well plate and incubated overnight at 4°C. The plate was washed four times with PBS-Tween 20 (PBS-T) and then blocked with 5% bovine serum albumin (BSA) in PBS-T for 4 hours at 37°C. The plate was washed as before and incubated overnight at 4°C with 1 µg/mL goat anti-HB-EGF antibody (R&D Systems, Minneapolis, MN). The plate was probed with a biotinylated anti-goat secondary antibody and then incubated with horseradish peroxidase (HRP)-conjugated streptavidin (both from DAKO, Ely, United Kingdom).

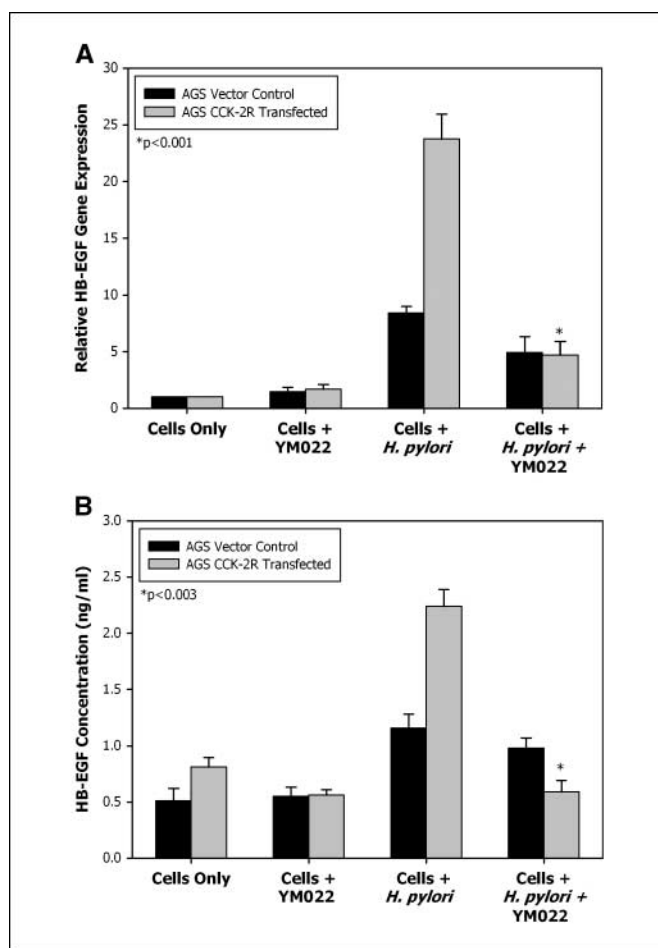


Figure 1. Role of CCK-2R in *H. pylori*-induced HB-EGF up-regulation. **A**, effect on gene expression. Mean relative HB-EGF gene expression for CCK-2R-transfected and vector control-transfected AGS cells following treatment with either 10⁻⁸ mol/L YM022 and/or *H. pylori* strain 60190 (MOI 40; *n* = 3). HB-EGF gene expression was significantly up-regulated in the CCK-2R-transfected cells compared with vector control with *H. pylori*. This up-regulation was significantly decreased in the presence of the inhibitor. Bars, SD. *, *P* < 0.001. **B**, effect on shed HB-EGF. Mean HB-EGF concentration detectable for AGS cell conditioned medium (CCK-2R and vector control transfected) following treatment with either 10⁻⁸ mol/L YM022 and/or *H. pylori* strains 60190 (MOI 40; *n* = 3). The concentration of HB-EGF shed into the medium was significantly greater in the CCK-2R-transfected cells compared with vector control with 60190 treatment. *, *P* < 0.003. The concentration of HB-EGF shed into the medium was significantly less in the presence of YM022. Bars, SD.

The plate was developed using 3,3',5,5'-tetramethylbenzidine substrate (Sigma), and the reaction was stopped with 0.5 mol/L H₂SO₄. The absorbance was then read at 450 nm. The cell conditioned medium absorbance values were converted to ng/mL values based on the linear regression transformation of the standard curve.

Western blot analysis. Cell lysates were mixed in a 1:1 ratio with sample buffer and heated at 90°C for 2 minutes. Each sample (20 µL) was loaded into the wells of 8% to 16% gradient Tris/glycine gels (Invitrogen, Paisley, United Kingdom). Equal loading was confirmed by reprobing the blots with a β-actin antibody (Sigma).

Protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% nonfat dry milk in TBS with 0.05% Tween 20 (TBST). They were then probed with a goat anti-HB-EGF antibody (C-18; Santa Cruz Biotechnology, Santa Cruz, CA). Negative controls were prepared using goat immunoglobulin G (IgG; Sigma). The membranes were washed in TBST and probed with a HRP-linked anti-goat secondary antibody.

Blots were washed as before, and immune complexes were visualized using enhanced chemiluminescence (Amersham Life Sciences, Inc., Buckinghamshire, England).

Animals. FVB mice were bred under sterile conditions within the *in vivo* unit of the Academic Unit of Cancer Studies at the University of Nottingham, under Home Office Project License No. 40/2323. Lansoprazole was given orally at a daily dose of 0.75 mg/mouse for 28 days. Lansoprazole (pharmacy stores, University of Nottingham) was prepared in 4.2% sodium bicarbonate vehicle. Proton pump inhibitor treatment at this dosage has previously been shown to increase murine serum gastrin levels 5- to 6-fold (34).

After 24 days of treatment, mice were anesthetized and sacrificed by cervical dislocation and stomachs were dissected out, washed in PBS, and

immersed in Tissue-Tek OCT compound (Bayer PLC, Berkshire, United Kingdom). Each stomach was cut in half along the greater curvature and placed in Tissue-Tek Cryomold (Bayer PLC) for freezing. The cryomold was then immersed in liquefied Richard-Allan Cytocool II (Apogent, Ipswich, United Kingdom) on dry ice for snap freezing. Frozen blocks were stored at -80°C until cut using a cryostat. Slides were then stored at -80°C .

Immunohistochemistry. Human gastric biopsy samples were sectioned at $0.4\ \mu\text{m}$ and mounted onto polylysine slides.

For staining of tumor cell lines, 1×10^4 cells were fixed for 30 minutes in 0.4% formalin before being cytospun onto slides. The slides were washed twice in PBS (pH 7.6) for 5 minutes each and incubated for 5 minutes in PBS with 0.5% Triton X-100.

After further PBS washes, slides were incubated for 30 minutes at room temperature in PBS with 3% BSA and 1% glycine. They were then incubated in PBS and 10% goat serum (DAKO; as appropriate to the secondary antibody) for 30 minutes. The serum was aspirated off, and the primary antibody was added overnight at 4°C .

The antibodies used were rabbit anti-N(6-14) progastrin (Apton Corp., Woodlands, CA) or rabbit anti-CCK-2R (S20; Abcam, Cambridge, United Kingdom). The slides were then washed thrice in PBS for 5 minutes each, and the secondary antibodies were applied in 10% of goat serum. The antibody used was Alexa Fluor 594 goat anti-rabbit (Molecular Probes, Leiden, the Netherlands). After a further wash step, the slides were counterstained with Hoechst and coverslipped using antifade mounting medium (DAKO). Slides were analyzed using a Leica Q550IW image analysis system. Immunofluorescence scoring was done based on absorbance.

Frozen murine gastric sections were air dried and then fixed in 4% paraformaldehyde. Sections were washed in PBS and blocked with 0.06% hydrogen peroxide for 15 minutes. Sections were then washed in PBS, blocked in 10% serum, and mounted with primary antibody at $10\ \mu\text{g}/\text{mL}$. For negative controls, concentration-matched IgG or serum was applied. The sections were washed in PBS, mounted with secondary antibody, and washed again in PBS. For HRP-based detection, staining was developed using liquid $3,3'$ -diaminobenzidine (DAKO Cytomation, Cambridgeshire, United Kingdom). The sections were washed in running water, counterstained with hematoxylin, washed in water, dehydrated in 98% alcohol washes, washed in xylene, and then coverslipped with DPX. For fluorescence-based detection, sections were blocked additionally with 1% BSA before the addition of primary antibody. At the end of the staining procedure, slides were counterstained with $4',6'$ -diamidino-2-phenylindole (Invitrogen) and coverslipped with glycerol.

Rabbit anti-histidine decarboxylase antibodies were obtained from Progen (Insight Biotechnology, Middlesex, United Kingdom). Goat

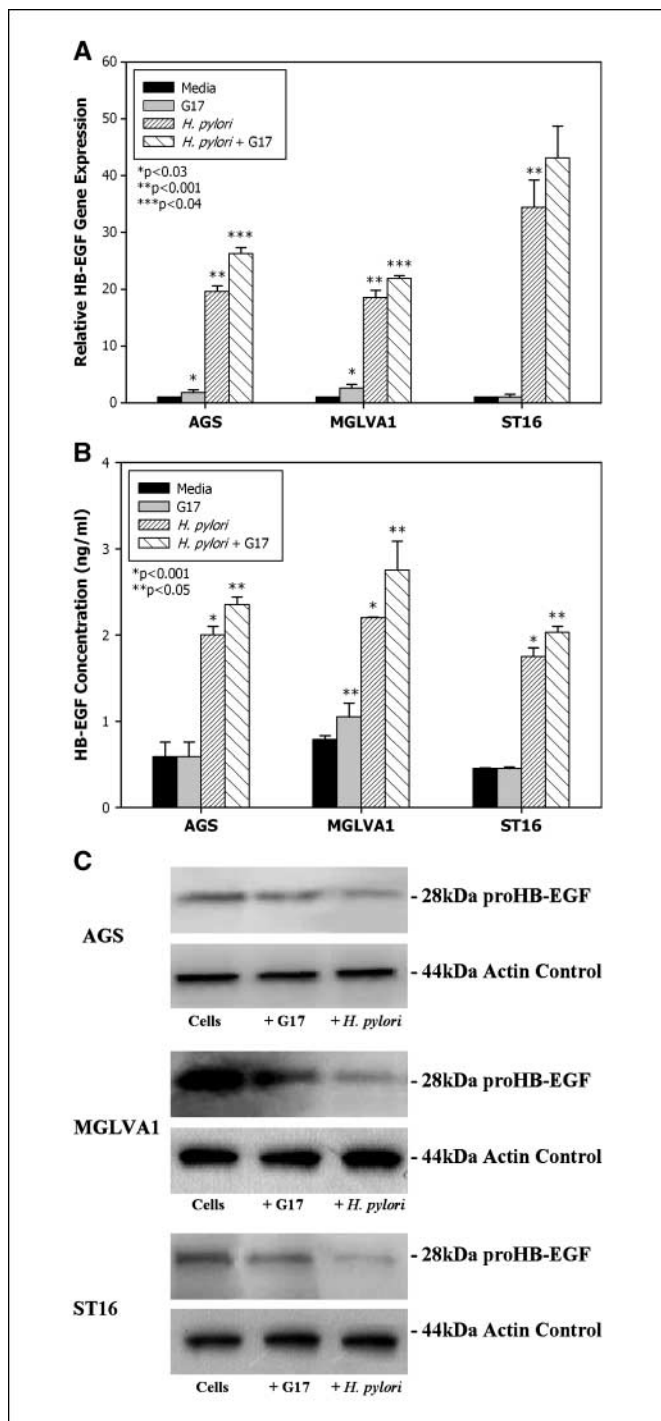


Figure 2. Role of exogenous gastrin in *H. pylori*-induced HB-EGF up-regulation. **A**, effect on gene expression. Mean relative HB-EGF gene expression for wild-type AGS, MGLVA1, and ST16 gastric adenocarcinoma cells following treatment with 10^{-8} mol/L G17 and/or *H. pylori* (MOI 40; $n = 3$). HB-EGF gene expression was significantly up-regulated in the AGS and MGLVA1 cell lines following gastrin treatment; however, there was no significant up-regulation in the ST16 cell line. **, $P < 0.03$. Treatment with *H. pylori* significantly up-regulated HB-EGF gene expression in all three of the cell lines tested. *, $P < 0.001$. HB-EGF gene expression was further increased with additional gastrin in the AGS and MGLVA1 cell lines compared with effect of the bacteria alone. *, $P < 0.04$. There was no effect in the ST16 cell line. **B**, effect on shed HB-EGF. Mean HB-EGF concentration detectable for cell conditioned medium (AGS, MGLVA1, and ST16) following treatment with 10^{-8} mol/L G17 and/or *H. pylori* strain 60190 (MOI 40; $n = 3$). The concentration of HB-EGF shed into the medium was significantly greater in all three cell lines following treatment with *H. pylori*. *, $P < 0.001$. Gastrin stimulation resulted in an increase in the concentration of HB-EGF in MGLVA1 cell-derived medium only. **, $P < 0.05$. In both the AGS and ST16 cell lines, there was no significant effect. The concentration of HB-EGF shed into the medium was significantly greater in all three cell lines following treatment with *H. pylori* with additional gastrin compared with *H. pylori* alone. Bars, SD. *, $P < 0.05$. **C**, Western blotting of HB-EGF in cell lysates. In total cell lysates prepared from AGS, MGLVA1, and ST16 cells, the intensity of an ~ 28 kDa band representing cell-associated HB-EGF seemed to decrease in all three cell lines following exposure to *H. pylori* strain 60190 (MOI 40). Representative of three experiments.

anti-HB-EGF was obtained from R&D Systems (Abingdon, United Kingdom).

Statistical methods. All results were analyzed using Student's *t* test, Mann-Whitney, or one-way ANOVA.

Results

Involvement of transfected CCK-2R in *H. pylori*-induced HB-EGF gene and protein up-regulation. It has been previously reported that *H. pylori* can induce an up-regulation in both HB-EGF gene expression and ectodomain shedding in human gastric cells (35, 36). In addition, it has been shown that gastrin stimulation results in the up-regulation of the *HB-EGF* gene in gastric cells transfected with CCK-2R (37). We speculated that the presence of CCK-2R might render gastric cells more susceptible to *H. pylori*-induced HB-EGF gene and protein up-regulation.

AGS cells were transfected with either the CCK-2R or an empty vector control and cultured for 24 hours with either 10^{-8} mol/L G17 or *H. pylori* (strain 60190). Following treatment with *H. pylori*, HB-EGF gene expression levels were significantly higher in the CCK-2R-transfected cells when compared with the vector control ($P < 0.01$). Additionally, treatment with the specific CCK-2R antagonist YMO22 resulted in a significant decrease in HB-EGF gene expression. Although the effect was much greater in those cells transfected with CCK-2R, there was also significant down-regulation with YMO22 treatment in the vector control-transfected cells (Fig. 1A).

The concentration of mature HB-EGF protein detectable in cell conditioned medium was significantly increased following *H. pylori* stimulation in CCK-2R-transfected cells (Fig. 1B). This indicates an increase in the rate of ectodomain shedding because the antibody used in the ELISA has been recognized as binding to the EGF-like domain of HB-EGF (38); thus, its use on conditioned medium identifies only HB-EGF that has been cleaved from the cell membrane.

In addition, YMO22 induced a significant decrease in *H. pylori*-induced HB-EGF shedding in the CCK-2R transfectants when compared with control cells.

Ability of exogenous gastrin to enhance HB-EGF expression concomitant to infection with *H. pylori* in representative human adenocarcinoma cell lines. To confirm that the results were not limited only to genetically modified cells, wild-type gastric adenocarcinoma cell lines AGS, ST16, and MGLVA1 were treated in the same manner as the AGS CCK-2R-transfected cells.

Infection with *H. pylori* has a well-documented association with serum hypergastrinemia. Consequently, the effect of additional

exogenous gastrin on *H. pylori*-induced effects was also examined. G17 was added simultaneously with the bacteria to better model the serum hypergastrinemia that can occur as a result of *H. pylori* infection (39).

H. pylori induced HB-EGF gene up-regulation in all three cell lines (Fig. 2A). Treatment with gastrin alone induced a small but significant HB-EGF gene up-regulation in the AGS and MGLVA1 cell lines only. Additional exogenous gastrin resulted in a further significant increase in HB-EGF gene expression only in the AGS and MGLVA1 cell lines.

The concentration of soluble HB-EGF detectable in the conditioned medium increased in all three cell lines in response to *H. pylori* (Fig. 2B). Western blotting on cell lysates using the HB-EGF (C-18) antibody also showed a corresponding decrease in expression of cell-associated HB-EGF in all three cell lines (Fig. 2C).

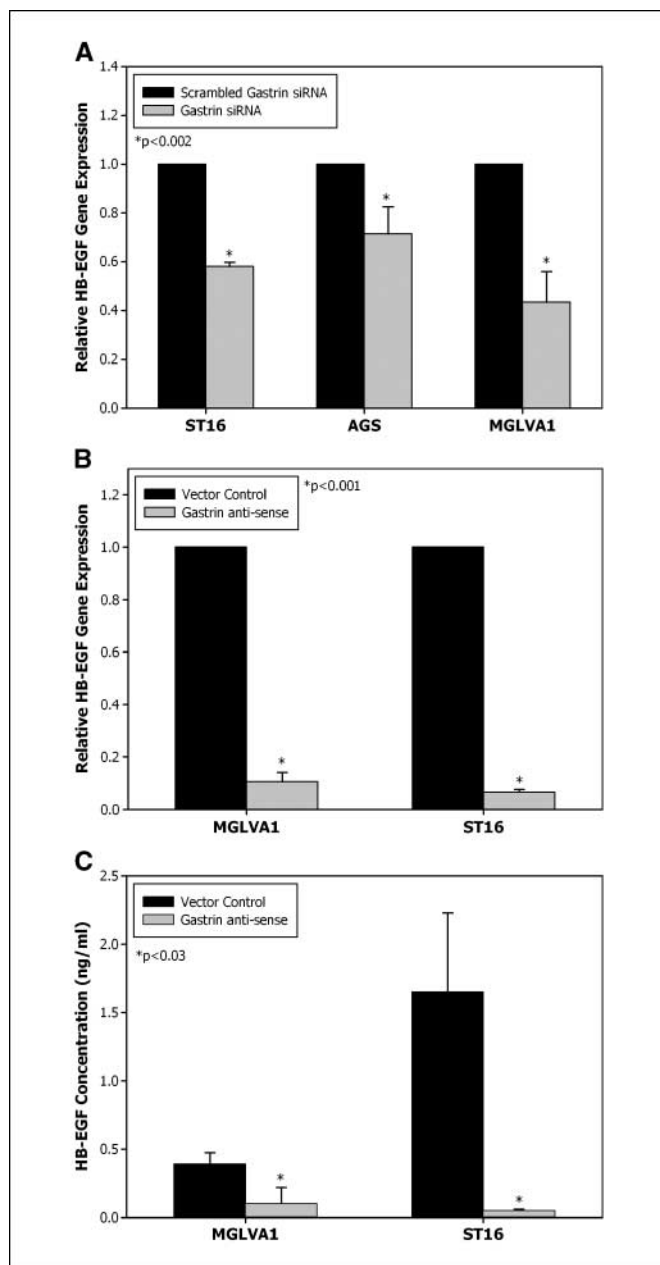


Figure 3. Role of endogenous gastrin in *H. pylori*-induced HB-EGF up-regulation. **A**, effect on gene expression by siRNA. Mean relative HB-EGF gene expression for AGS, MGLVA1, and ST16 gastric adenocarcinoma cells transfected with either a gastrin siRNA or a scrambled siRNA control following treatment with *H. pylori* strain 60190 (MOI 40; $n = 3$). The up-regulation in HB-EGF gene expression induced by the bacteria was significantly lower in the gastrin siRNA-treated cell lines compared with the scrambled siRNA-treated cells. Bars, SD. *, $P < 0.002$. **B**, effect on gene expression by stable transfection. Mean relative HB-EGF gene expression for MGLVA1 and ST16 gastric adenocarcinoma cells transfected with either a gastrin antisense plasmid or an empty vector control following treatment with *H. pylori* strain 60190 (MOI 40; $n = 3$). The up-regulation in HB-EGF gene expression induced by the bacteria was significantly lower in the gastrin antisense cell lines compared with vector control cells. Bars, SD. *, $P < 0.0001$. **C**, effect on shed HB-EGF by stable transfection. Mean HB-EGF concentration detectable for the cell conditioned medium from MGLVA1 and ST16 gastric adenocarcinoma cells transfected with either a gastrin antisense plasmid or an empty vector control following treatment with *H. pylori* strain 60190 (MOI 40; $n = 3$). The concentration of shed HB-EGF detectable in the cell conditioned medium was significantly lower in the gastrin antisense cells when compared with the vector control. Bars, SD. *, $P < 0.03$.

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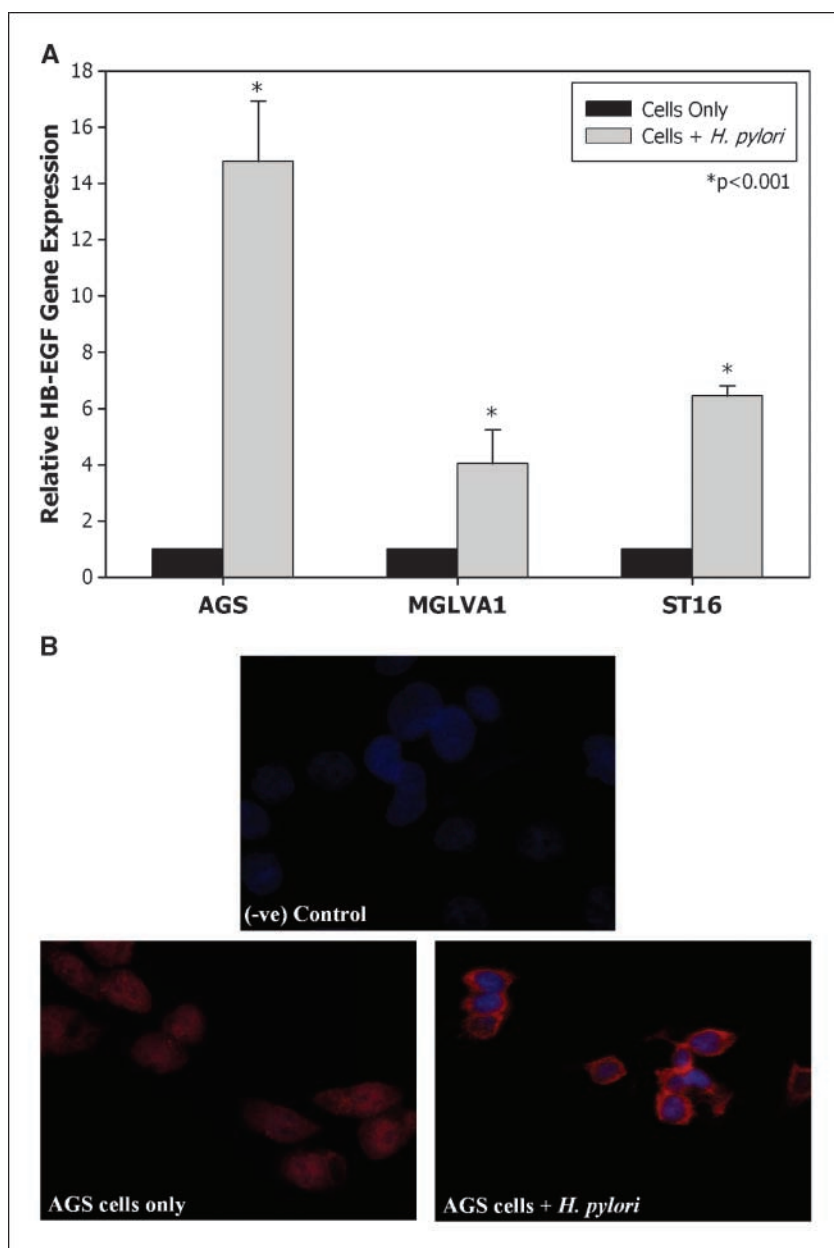


Figure 4. Effect of *H. pylori* on endogenous gastrin expression. **A**, effect on gastrin gene expression. Mean relative gastrin gene expression for AGS, MGLVA1, and ST16 gastric adenocarcinoma cells following exposure to *H. pylori* strain 60190 (MOI 40; $n = 3$). Gastrin gene expression was significantly increased in all cell lines following treatment with the bacteria. Bars, SD. *, $P < 0.001$. **B**, effect on progastrin protein expression in cocultured cells. Progastrin protein expression as determined by immunofluorescence seemed to be increased in those cells that had been exposed to *H. pylori* compared with noninfected control cells. Representative of multiple experiments.

In addition, all three cell lines had a significantly increased level of HB-EGF ectodomain shedding in response to additional exogenous gastrin.

Endogenous gastrin secretion by human gastric adenocarcinoma cells is involved in *H. pylori*-induced HB-EGF effects. AGS, MGLVA1, and ST16 gastric cells were transfected with either a gastrin antisense plasmid or empty vector control or treated with a gastrin siRNA or scrambled siRNA control and exposed to *H. pylori*. In all cell lines, the suppression of endogenous gastrin expression by either method resulted in a significant decrease in *H. pylori*-induced HB-EGF gene expression ($P < 0.002$; Fig. 3A and B). The *H. pylori*-induced increase in HB-EGF ectodomain shedding was also inhibited by transfection with a gastrin antisense plasmid with a significant reduction in MGLVA1 and ST16 cells of approximately 3- and 30-fold, respectively ($P < 0.001$; Fig. 3C).

***H. pylori* can induce gastrin and CCK-2R gene and protein expression from cultured human gastric adenocarcinoma cells and in *H. pylori*-infected gastric tissue.** Following exposure to *H. pylori*, gastrin gene expression in the AGS, MGLVA1, and ST16 cell lines and CCK-2R gene expression in MGLVA1 cells were significantly up-regulated ($P < 0.001$ and 0.008, respectively; Figs. 4A and 5A). Additionally, immunofluorescence staining confirmed that the level of progastrin and CCK-2R protein present in the cells was also increased (Figs. 4B and 5B, respectively). The pattern of CCK-2R staining was found to be different following *H. pylori* exposure, with the general diffuse staining of the noninfected cells being replaced with a tight membranous pattern in those cells that had been exposed to the bacteria (Fig. 5B).

Furthermore, to support these findings in a clinically relevant context, CCK-2R expression in human gastric biopsies was examined. Expression of CCK-2R in antral gastric tissue has

previously been established to be present on chromogranin A-positive neuroendocrine cells sparsely distributed within gastric glands (40). It was determined that CCK-2R staining in the epithelial cells of *H. pylori*-infected human gastric tissue was significantly higher than in similar *H. pylori*-noninfected tissue ($P < 0.04$; Fig. 5D) and was widely expressed, suggesting expression on additional cell types. CCK-2R gene expression was also found to be significantly higher in the *H. pylori*-positive tissue than in the uninfected samples ($P < 0.003$; Fig. 5C). These results may indicate that the observed effects are not limited to cultured cells but may extend to the *in vivo* situation.

Hypergastrinemia in a murine model increases expression of HB-EGF in CCK-2R-expressing enterochromaffin-like cells.

Figure 6 shows HB-EGF-expressing cells in normal (Fig. 6A) and hypergastrinemic (Fig. 6B) murine gastric mucosa. Along with the increase in mucosal width, there was an increase in expression of HB-EGF-positive cells. It was shown that one of the HB-EGF-expressing cell types was the enterochromaffin-like (ECL) cell, and

Fig. 6C shows colocalization of HB-EGF with the ECL marker histidine decarboxylase.

Discussion

The mechanisms by which *H. pylori* can induce neoplastic progression are multifactorial and poorly understood. However, it is clear that up-regulation of human growth factors, including HB-EGF, by the bacteria is a major contributory factor.

It has been previously reported that *H. pylori*-induced HB-EGF gene expression is due to the action of γ -glutamyltranspeptidase (GGT), a recently identified bacterial enzyme (35). It has been reported that there may be a direct interaction between the bacterial GGT and the HB-EGF promoter, possibly via an oxidative stress-mediated mechanism. Our work suggests that it may not be the only mechanism involved and that CCK-2R and endogenous gastrin expression may amplify this effect.

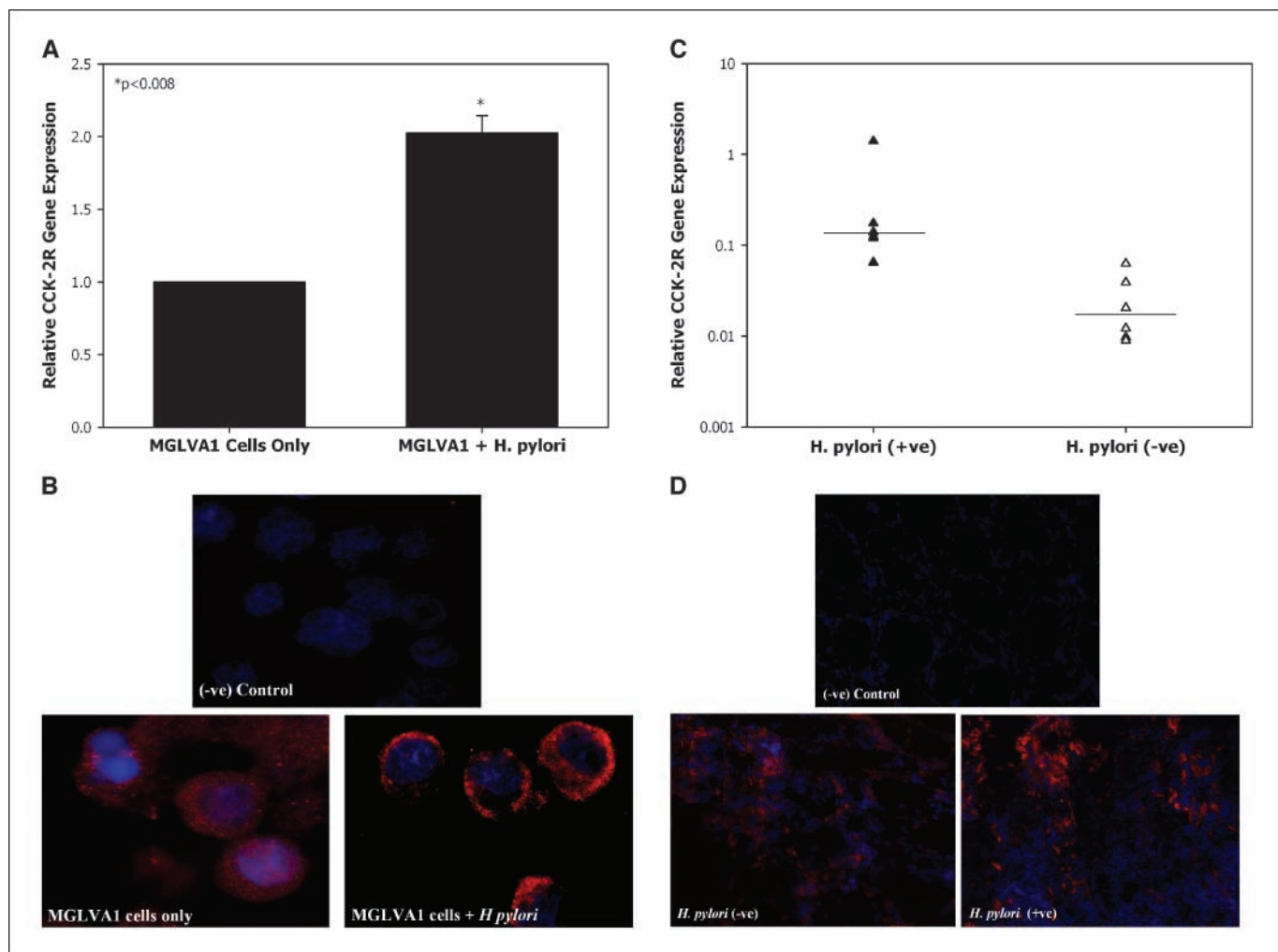


Figure 5. Effect of *H. pylori* on endogenous CCK-2R expression. **A**, effect on CCK-2R gene expression. Mean relative CCK-2R gene expression for MGLVA1 gastric adenocarcinoma cells following exposure to *H. pylori* strain 60190 (MOI 40; $n = 3$). CCK-2R gene expression was significantly increased following treatment with the bacteria. Bars, SD. *, $P < 0.008$. **B**, effect on CCK-2R protein expression in cocultured cells. CCK-2R protein expression as determined by immunofluorescence seemed to be increased in those cells that had been exposed to *H. pylori* compared with noninfected control cells. Representative of multiple experiments. **C**, CCK-2R gene expression in gastric biopsy tissue. The level of CCK-2R gene expression was found to be significantly higher in the *H. pylori*-positive biopsies compared with the *H. pylori*-negative tissue. *, $P < 0.003$. **D**, CCK-2R protein expression in gastric biopsy tissue. The intensity of CCK-2R staining present in the *H. pylori*-positive biopsies was found to be noticeably higher than in the *H. pylori*-negative tissue.

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Additionally, it has been shown that *H. pylori* can induce HB-EGF ectodomain shedding via stress-induced signaling pathways and through activation of MMPs and a disintegrin and metalloproteinases (36).

Gastrin exposure in cells transfected to overexpress CCK-2R has been shown to result in increased expression of the *HB-EGF* gene via a protein kinase C/Erk-dependent mechanism (16). It has also been

established that gastrin stimulation can induce proteolytic processing of membrane-tethered pro-HB-EGF to the secreted, mature form (38). Additionally, in an *in vivo* study by Wang et al. (41), HB-EGF was significantly up-regulated in the gastric mucosa of hypergastrinemic mice during the premalignant stages of cancer development.

The role of gastrin in the proliferation of gastrointestinal cancer cells is well established. In xenograft models of gastric, colonic, and pancreatic cancers, use of gastrin antisense or gastrin receptor antagonists was found to significantly reduce tumor growth (42–44).

We have expanded these findings by showing that gastric cancer cell up-regulation of HB-EGF gene expression and ectodomain shedding by *H. pylori* can be enhanced through overexpression of CCK-2R on exposed cells and can be decreased by use of a specific CCK-2R antagonist. We propose that this phenomenon is best explained by the effect of the bacteria on endogenous tumor cell expression levels of the gastric peptide hormone gastrin.

This was corroborated by showing enhanced HB-EGF expression in the gastric mucosa from hypergastrinemic mice with one of the cell types involved confirmed as the ECL cell, a cell type known to express the CCK-2R (45).

Several previous studies have shown that *H. pylori* can increase circulating gastrin levels in the blood (46). *H. pylori* has been reported to induce G-cell hyperfunction in antral gastric tissue, which could contribute to the onset of hypergastrinemia (47). The work presented here is the first to show that this effect also extends to an induction of gastrin and CCK-2R gene and protein expression from gastric adenocarcinoma cells. Initial pilot data have suggested cell types at the base of the antral gland express HB-EGF and CCK-2R. Further studies are under way to confirm whether similar HB-EGF paracrine-driven autocrine pathways regulate G-cell gastrin expression.

This increase in gastrin expression may help to explain why the supplementary increase in HB-EGF gene expression and ectodomain shedding resulting from the addition of exogenous gastrin into the *H. pylori* coculture system was significant but proportionally small compared with *H. pylori* alone. It is possible that the mechanism is already being saturated by the *H. pylori*-induced circulating serum and cell-associated gastrin, although at this time this is purely speculative. Furthermore, the increase in *H. pylori*-induced CCK-2R expression of gastric epithelial cells may enhance the sensitivity of the cells to raise serum gastrin levels as a consequence of *H. pylori* infection.

We have also shown that removal of endogenous gastrin by use of antisense and siRNA technology significantly inhibits the ability of the bacteria to up-regulate HB-EGF.

The correlation between *H. pylori* and gastrin is well known. In a previous study, gastric luminal concentrations of gastrin were found to be elevated by *H. pylori*, with no correlation to plasma gastrin levels. It was suggested that the luminal gastrin might originate from a different source than the plasma, possibly from the cancer cells themselves (48). Our work seems to confirm that cancer cells are capable of expressing gastrin in response to *H. pylori*.

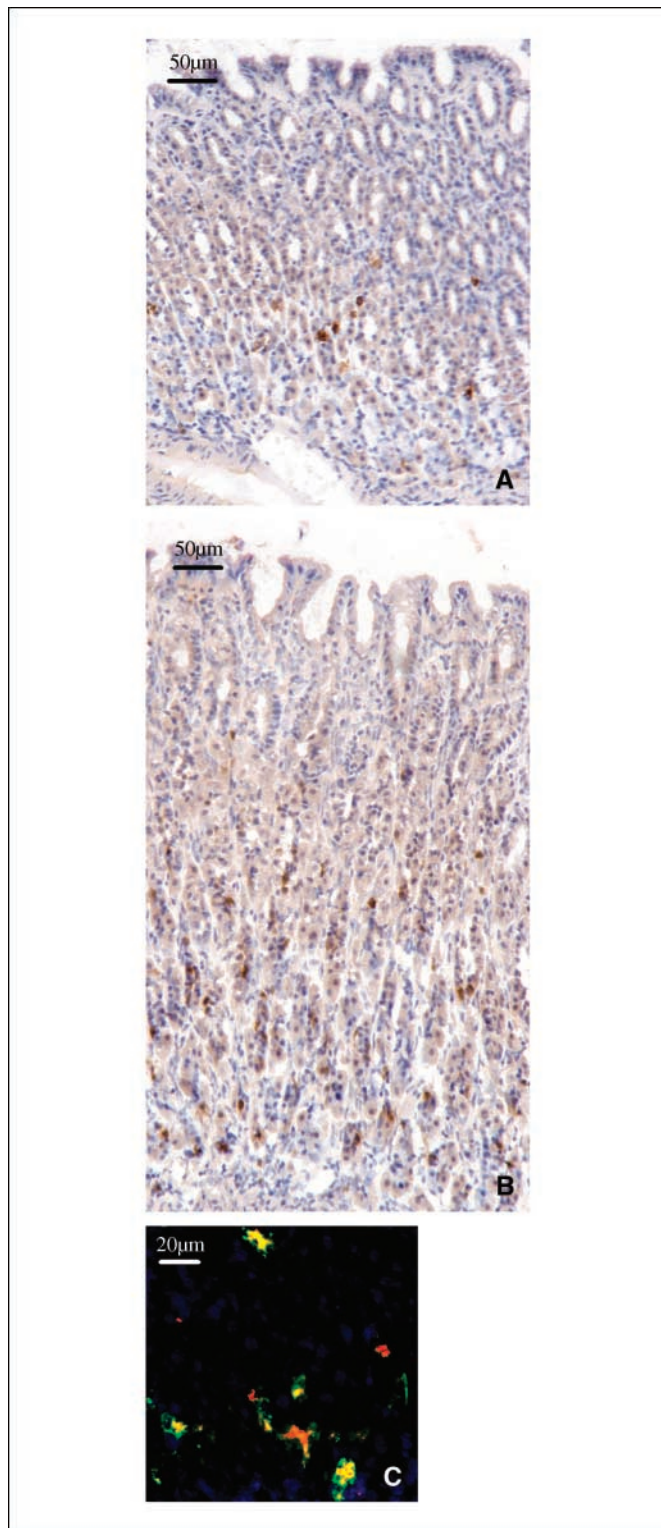


Figure 6. HB-EGF expression in gastric mucosa from normogastrinemic and hypergastrinemic mice. HB-EGF-expressing cells in normogastrinemic (A) and hypergastrinemic (B) FVB mice following treatment with lansoprazole. C, colocalization (yellow) of HB-EGF (green) and histidine (de)carboxylase (red) in ECL cells within the murine gastric mucosa.

We have also shown that exposure to *H. pylori* results in the increased gene and protein expression of the CCK-2R. This is evident both in a cocultured gastric cell line and in a human gastric mucosal tissue. Irrespective of the increase in HB-EGF, the effect of a significant increase in gastrin and its receptor may have substantial effects on cancer progression (49).

In conclusion, we report that *H. pylori* can up-regulate both gastrin and CCK-2R gene and protein expression. This, in concert

with endogenous gastrin, may act on the CCK-2R to induce the gene expression and ectodomain shedding of HB-EGF.

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