Helicobacter pylori Induces Plasminogen Activator Inhibitor 2 in Gastric Epithelial Cells through Nuclear Factor-κB and RhoA: Implications for Invasion and Apoptosis

Andrea Varro, P. J. M. Noble, D. Mark Pritchard, Susan Kennedy, C. Anthony Hart, Rod Dimaline, and Graham J. Dockray

Physiological Laboratory, Departments of Medicine and Medical Microbiology, University of Liverpool, Liverpool, United Kingdom

ABSTRACT

The gastric pathogen Helicobacter pylori is associated with a progression to gastric cancer. The specific targets of H. pylori that might influence this progression are still unclear. Previous studies indicated that the gastric hormone gastrin, which may be increased in H. pylori infection, stimulates gastric expression of plasminogen activator inhibitor (PAI)-2, which is an inhibitor of the urokinase plasminogen activator and has previously been shown to be increased in gastric adenocarcinoma. Here, we report that H. pylori also increases PAI-2 expression. In gastric biopsies of H. pylori-positive subjects there was increased PAI-2, including subjects with plasma gastrin concentrations in the normal range, PAI-2 was expressed mainly in chief and mucous cells. In a gastric cancer cell line (AGS), H. pylori increased PAI-2 expression, which was associated with inhibition of H. pylori-stimulated cell invasion and apoptosis. The induction of PAI-2 by H. pylori was mediated by release of interleukin-8 and activation of cyclooxygenase-2, and interestingly, gastrin stimulated PAI-2 expression by similar paracrine pathways. The activation of NFκB was required for interleukin-8 and cyclooxygenase-2 activation but did not occur in cells responding to these paracrine mediators. The data suggest that induction of PAI-2 is a specific target in H. pylori infection, mediated at least partly by paracrine factors; induction of PAI-2 inhibits cell invasion and apoptosis and is a candidate for influencing the progression to gastric cancer.

INTRODUCTION

The gastric carcinogenic pathogen Helicobacter pylori infects a high proportion of the population in third-world countries and up to 50% in western countries. The clinical outcome of H. pylori infection depends on multiple variables including the environment, host factors, and bacterial virulence factors (1, 2). In some patients, there is an association with duodenal ulcer, and in others, with gastric cancer (3). The progression to intestinal-type gastric cancer is recognized to include the premalignant stages of active gastritis and chronic gastric atrophy (4). H. pylori infection induces a number of genes in host gastric epithelial cells that are potential determinants of the host response including genes encoding proteins involved in signal transduction, cytoskeletal organization, bioenergetics, cytokines, and extracellular matrix (5, 6).

It is well recognized that H. pylori is associated with an increase in the circulating concentration of the gastric hormone gastrin (7). Gastrin stimulates acid secretion and activates mechanisms that facilitate mucosal protection, including stimulation of epithelial cell migration, cell renewal, and release of growth factors of the epidermal growth factor family (8–10). We recently reported that gastrin also increases the expression in gastric epithelial cells of the gene encoding plasminogen activator inhibitor type 2 (PAI-2; Ref. 11). PAI-2 (also known as serpin B2) is a serine protease inhibitor with sequence homology to ovalbumin (12, 13). There is a relatively weak signal peptide sequence so that both secreted and nonsecreted forms occur. Secreted PAI-2 inhibits the activity of the urokinase-type plasminogen activator (uPA; Ref. 12). The latter catalyzes the production of plasmin from plasminogen leading to thrombolysis and to degradation of extracellular matrix and increased invasion of cancer cells. Activation of the uPA system is therefore implicated in metastasis (12). The nonsecreted form of PAI-2 confers an increased resistance of HeLa cells to apoptosis in response to tumor necrosis factor α (14).

Previous studies have established that uPA and the inhibitors PAI-1 and PAI-2 are expressed in gastric adenocarcinoma (15–17). In the present study, we asked whether H. pylori infection was associated with changes in gastric PAI-2 expression. We report here that H. pylori induces PAI-2 expression and that this restrains cell invasion and inhibits apoptosis; the data implicate both gastrin-dependent and gastrin-independent components in the response, and interestingly, both components include NFκB induction of interleukin-8 (IL-8) and cyclooxygenase-2 (COX-2) so that H. pylori-associated increases in PAI-2 occur not just in cells directly exposed to the bacterium but also in adjacent cells activated via paracrine signals.

MATERIALS AND METHODS

Cells, Plasmids, and Drugs. AGS cells were obtained from the American Type Culture Collection (Manassas, VA); AGS cells permanently transfected with the gastrin-CCKb receptor (AGS-Cck cells) or with green fluorescent protein (AGS-GFP cells; Ref. 18) were routinely cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and subcultured with 1% trypsin/versene (Life Technologies, Inc., Paisley, United Kingdom) as described previously (19). An expression vector for constitutively active-RhoA (L63RhoA) was a gift from Alan Hall (University College, London, United Kingdom), and an expression vector encoding PAI-2 and driven by the CMV promoter was a gift from Toni Antalis (Herston, Brisbane, Australia); C3 transferase and a dominant negative (DN) inhibitor of nuclear factor-κB (IκB) and control vectors were purchased from Upstate Biotechnology (Lake Placid, NY); a promoter-reporter vector consisting of 2.3-kb PAI-2 coupled to luciferase (PAI-2-luc) was described previously (11). An expression vector encoding NFκB p65 subunit conjugated to dsRed (p65-dsRed) was a gift from M. R. White (University of Liverpool, United Kingdom). BAY11-7082, NS-398, and mouse IL-8 neutralizing antibody were obtained from Calbiochem (Nottingham, United Kingdom). Antisense oligonucleotides (ASO) for PAI-2, both unlabeled and fluorescein labeled, and control oligonucleotide were obtained from Biognostik (Gottingen, Germany).

Patients. Endoscopic pinch biopsies of the gastric corpus were obtained from patients attending for routine gastroscopy for investigation of dyspepsia. None of the patients had neoplastic disease or peptic ulcer. H. pylori status was initially assessed by a rapid urease test (Prontody; Medical Instruments Corporation, Solothurn, Switzerland) and subsequently confirmed by histology and serology. The present report is based on studies of 23 patients (13 female, mean age 57.1 ± 2.1 years) who were H. pylori positive and 20 who were H. pylori negative (14 female, mean age 54.4 ± 3.5 years) and were used as controls. Samples were assigned randomly within the two groups either to extraction for Western blotting or to gastric gland culture. The study was...
approved by the Ethics Committee of Royal Liverpool and Broadgreen University Hospitals NHS Trust. All patients gave informed consent.

**Gastrin Radioimmunoassay.** The concentration of amidated gastrins in plasma was determined by radioimmunoassay using antibody L2 specific for the COOH-terminal amide sequence of gastrin, as described previously (20).

**Western Blotting.** Protein extracts were prepared in radioimmunoprecipitation assay or lysis buffer, and Western blotting was performed as described previously (11), using a goat anti-PAI-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were reprobed with a goat anti-β-actin antibody (Santa Cruz Biotechnology).

**Immunohistochemistry.** For immunohistochemistry (IHC) a goat anti-PAI-2 antibody (Santa Cruz Biotechnology) was used with fluorescein (FITC)-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA) as described previously (19). In colocalization studies, mouse anti-gastric mucin (Sigma, Poole, Dorset, United Kingdom), rabbit antipepsinogen (gift from Dr Mike Samloff, Center for Ulcer Research, Los Angeles, CA), rabbit anti-H/K^+^-ATPase (Calbiochem), and rabbit anti-VMAT-2 (21) antibodies were used by using Texas Red-labeled donkey antimouse or antirabbit IgG (Jackson ImmunoResearch) as appropriate.

**Human Primary Gland Culture.** Isolated human gastric glands were prepared from endoscopic biopsies as described previously (22). The isolated glands were routinely cultured in 6-well plates up to 72 h in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Sigma), at 37°C in 5% CO/H/O, and medium was changed every 24 h.

**Bacterial Infection of AGS Cells and Gastric Glands in Vitro.** We routinely used *H. pylori* strain 60190 (American Type Culture Collection). In a few experiments, isogenic mutants that were cag^−^ and vac^−^ were used (gifts from Richard Peek, Vanderbilt University, Nashville, TN; Ref. 23); a cag pathogenicity island-negative strain, G50, was a gift from Jean Crabtree (University of Leeds, Leeds, United Kingdom). Bacteria were grown in a microaerophilic atmosphere at 37°C on fresh chologelized Columbia blood agar for 3–9 days (Oxoid Ltd., Basingstoke, United Kingdom). Unless otherwise stated *H. pylori* were added to human gastric glands or AGS cells at a multiplicity of infection (MOI) of 100. In some experiments, bacteria were added either to human gastric glands or to AGS cells cultured on 0.2-μm Anapore filter inserts (Life Technologies, Inc.) that were then inserted over AGS cells transfected with PAI-2 luc (see below).

**AGS Cell Migration and Invasion Assays.** Transwell invasion assays were performed using AGS cells cultured in 24-well plates containing 8-μm pore BioCoat inserts coated with Matrigel, according to the manufacturer’s instructions (Becton Dickinson, Bedford, MA) either by direct counting or by reading the absorbance at 550 nm as reported earlier (11).

**Stable Expression of PAI-2 in AGS-G418 Cells.** AGS-G418 PAI-2-overexpressing cells were generated by stable transfection with a human PAI-2 expression vector, using TransFast reagent (Promega) as described previously (18), and clones resistant to G418 (Life Technologies, Inc.) and overexpressing PAI-2 were selected by Western blotting. Small interfering RNAs (siRNA) were generated by *H. pylori* and Cell Survival. Cells (3 × 10^5^, 5 ml) were plated in media containing 10% serum but no antibiotics in T25 flasks and allowed to adhere for 48 h. The medium was then changed, and cells treated with either 50 μM etoposide or *H. pylori* at MOI 1:400. After 24 h, the medium was removed, and attached cells were recovered with trypsin. Attached and floating cells were counted separately using a hemocytometer. Staining with Hoechst indicated that >95% of adherent cells exhibited normal morphology and >95% of nonadherent cells exhibited apoptotic morphology.

**Transient Transfection and Luciferase Assay.** Cells (2 × 10^5^) were plated in 6-well plates in full medium. The following day, medium was removed, and cells were transfected using TransFast (Promega, Madison) in serum-free medium of 1 h. Routinely, PAI-2-luc was used at 0.25–1.0 μg well. After transfection, 2 ml of full medium were added, and cells were incubated for 20–24 h. Medium was then replaced with 2 ml of serum-free medium, and cells were incubated with *H. pylori* and other compounds as indicated for 8 h. Luciferase activity was measured with Bright-Glo or Dual Glo (Promega) using a LumiCount PlateReader (Packard BioScience) according to the manufacturer’s protocol. Results are presented as fold increase over unstimulated control, so 1.0 signifies no change in luciferase activity. Protein concentration was determined when appropriate using Lowry protein assay kit (Sigma) to monitor plating efficiency and cell death, and in some experiments, cell viability in response to drug treatments was monitored by trypan blue exclusion.

**NFκB Nuclear Translocation.** AGS cells were transiently transfected as described above with p65-dsRed. After 48 h, the cells were transferred to serum-free medium. After an additional 18 h, the cells were transferred to the motorized stage of an inverted epifluorescence microscope (DMIRE; Leica; Ref. 8, 22). Samples, stage, and optics were enclosed in a heated chamber (Solent Scientific, Portsmouth, United Kingdom) and gassed with 5%CO/95% O. Images were captured from multiple fields using AQM software (Kinetic Imaging, Bromborough, United Kingdom). p65-dsRed fluorescence was excited with 525 nm of light, and emission at 575 nm was imaged. Nuclear translocation was defined as an increase in nuclear fluorescence to greater than or equal to cytoplasmic fluorescence. Cells were counted in 10 fields (×40 objective)/well over a 5-h period, discounting cells undergoing apoptosis or mitosis.

**Statistics.** Results are presented as means ± SE; comparisons were made using t tests and were considered significant at P < 0.05.

**RESULTS**

**H. pylori Increases Gastric PAI-2 Abundance.** In initial experiments, we compared the abundance of PAI-2 in biopsies of the gastric corpus of *H. pylori*-positive and *H. pylori*-negative subjects. In Western blots of *H. pylori*-negative subjects, a band corresponding to PAI-2 was detected in low abundance in keeping with our previous findings (11). The abundance of PAI-2 in samples from patients infected with *H. pylori* was significantly increased (Fig. 1). In view of
the tendency to increased plasma gastrin with \textit{H. pylori} (7) and because gastrin stimulates PAI-2 (11), we considered the possibility that the elevated PAI-2 in \textit{H. pylori}-infected subjects was attributable to circulating gastrin. In the group of \textit{H. pylori}-positive patients with plasma gastrin concentrations within the normal range for the assay used (<30 pm), PAI-2 abundance in gastric corpus biopsies was nevertheless significantly greater than in the control group. In subjects with plasma gastrin higher than the reference range, PAI-2 was further elevated. The data suggest then that \textit{H. pylori} increases PAI-2 in vivo by both gastrin-dependent and gastrin-independent mechanisms.

**Cellular Localization of PAI-2.** To examine the cellular expression of PAI-2 in epithelial cells, independent of inflammatory cells (24), we examined the localization of PAI-2 in cultured gastric epithelial gland cells from \textit{H. pylori}-positive and \textit{H. pylori}-negative subjects. Gastric corpus cells in \textit{H. pylori}-positive patients that expressed PAI-2 also commonly expressed either mucin or pepsinogen, indicating localization to mucous and chief cells (Fig. 2). Interestingly, in both mucin and pepsinogen-secreting cells, PAI-2 was localized to the same secretory vesicles as the primary secretary product. In addition, occasional parietal cells (identified by H\textsuperscript{+}/K\textsuperscript{+}ATPase) and enterochromaffin-like cells (identified by VMAT-2) also expressed PAI-2 (not shown). In \textit{H. pylori}-negative subjects, PAI-2 immunofluorescence was generally weaker, and there were fewer positive cells (Fig. 2), although as in \textit{H. pylori}-positive subjects, these tended to be mucous and chief cells.

**Induction of PAI-2 in AGS Cells by \textit{H. pylori} and Significance for Cell Invasion and Apoptosis.** To study the possible functional significance of \textit{H. pylori}-induced PAI-2, we first determined that addition of \textit{H. pylori} to a gastric cancer cell line (AGS cells) increased PAI-2 detected by Western blots (Fig. 3A). We then examined whether \textit{H. pylori} induction of PAI-2 influenced AGS cell invasion in Matrigel-coated Boyden chambers. There was a low invasive tendency of unstimulated AGS cells, but in the presence of \textit{H. pylori}, there was increased invasion that was stimulated further by treatment of cells with PAI-2 ASO (Fig. 3B), whereas control oligonucleotides had no effect on \textit{H. pylori}-induced invasion. Control experiments indicated that ASO treatment decreased PAI-2 abundance determined in Western blots by 46% (not shown).

We then asked whether increased expression of PAI-2 alone is sufficient to inhibit \textit{H. pylori}-stimulated invasion. We chose for these experiments to stably transfect AGS-G\textsubscript{R} cells with PAI-2, because the consequences of stimulating PAI-2 expression in these cells were already known (11). In AGS-G\textsubscript{R} cells stably overexpressing PAI-2,
there was decreased invasion in response to H. pylori (Fig. 3C). The difference was directly attributable to increased PAI-2, because treatment of cells with PAI-2 siRNAs reduced PAI-2 expression by 54% and reversed the depression in invasion (Fig. 3C).

To determine whether PAI-2 might influence AGS cell apoptosis in response to H. pylori, we incubated AGS cells and cells overexpressing PAI-2 with H. pylori at a high MOI (1:400). There was a significant increase in the number of nonadherent cells with an apoptotic morphology 24 h after treatment of cells with H. pylori (Fig. 3D). However, in clones constitutively overexpressing PAI-2, there was reduced apoptosis compared with wild-type cells. In contrast, there were no differences in apoptosis between the two clones in response to etoposide (Fig. 3D).

Mechanisms of H. pylori Induction of PAI-2 in AGS Cells. To study the mechanisms by which H. pylori induced PAI-2, we examined the response of AGS cells transiently transfected with 2.3 kb of the PAI-2 promoter linked to a luciferase reporter. The reference strain, 60190, which was CagA− and vacA−, increased by approximately 2-fold the expression of PAI-2-luc at an MOI of 1:100, and similar responses were obtained with isogenic mutant strains that were CagA− or vacA−. In contrast, a strain that lacked the Cag pathogenicity island (G50) did not stimulate PAI-2-luc expression (Fig. 4A).

Because in vivo, H. pylori infection is associated with moderate hypergastrinaemia, we then examined the effect on PAI-2-luc expression in AGS-G8R cells of a combination of a low concentration of gastrin (100 pM) and of H. pylori at a low MOI (1:50). The two stimuli alone each had small effects on PAI-2-luc expression, but together, the response was greater than the sum of the separate responses, suggesting a synergistic interaction (Fig. 4B).

Indirect Effects of H. pylori Mediated by COX-2 and IL-8. Previous studies have indicated that H. pylori induces epithelial cell release of various chemokine, cytokine, and paracrine mediators including IL-8 and the products of COX-2 induction (25, 26). We therefore considered the possibility that H. pylori stimulated PAI-2 via an indirect mechanism. When H. pylori were added to AGS cells cultured on Transwell filters suspended above AGS cells transfected with PAI-2-luc, the induction of PAI-2-luc was similar to that obtained by addition directly to transfected cells (Fig. 5, A and B). Moreover, when human gastric glands were cultured on Transwell filters, addition of H. pylori also significantly increased PAI-2-luc expression in transiently transfected AGS cells cocultured in the wells (Fig. 5, A and B). In contrast, application of H. pylori to filters without AGS cells had no effect on PAI-2-luc (not shown).

Consistent with the idea that a soluble factor was released from AGS cells and human gastric glands to induce PAI-2-luc expression, we
found that immunoneutralization of IL-8 and administration of COX-2 inhibitors both suppressed the induction of PAI-2-luc in response to *H. pylori* (Fig. 5E). Previous work has indicated that the induction of PAI-2 by gastrin was also in part dependent on a paracrine factor (11), and so we asked whether there might be similarities in paracrine mechanisms evoked by gastrin and *H. pylori*. Addition of gastrin to AGS-Ga cells or primary gastric glands cultured on Transwell filters over AGS cells transfected with PAI-2-luc induced luciferase expression in the latter cells (Fig. 5, C and D). Immunoneutralization of IL-8 and the COX-2 inhibitor NS-398 each reduced these responses in the coculture system (Fig. 5F). The data indicate therefore that both *H. pylori* and gastrin stimulate PAI-2 by activation of indirect mechanisms that include IL-8 and COX-2 activation.

**H. pylori** Stimulation of PAI-2 Is Mediated by RhoA. Because *H. pylori* activates small GTPases of the Rho family (27) and RhoA induces COX-2 (22, 26), we considered the possibility that *H. pylori* induced PAI-2 via RhoA. The *Clostridium botulinum* toxin, C3 transferase, which is a specific inhibitor of RhoA, inhibited induction of PAI-2-luc by *H. pylori* (Fig. 6A). We then asked whether activation of RhoA was sufficient to trigger the paracrine stimulation of PAI-2-luc via COX-2 and IL-8. Thus, AGS cells transfected with PAI-2-luc (reporter cells) were cultured in 6-well dishes, and AGS cells transfected with the constitutively active mutant L63RhoA (receptor cells) were cultured on Transwell filters inserted into the wells. Using this model, L63RhoA induced PAI-2-luc in the reporter cells by 3.9 ± 0.4-fold. The COX-2 inhibitor NS-398 suppressed the PAI-2-luc paracrine-mediated response to L63RhoA about 3-fold, and there was a smaller but still significant inhibition by neutralizing antibody to IL-8 (Fig. 6B).

**Putative Transcription Factors Mediating H. pylori Effects.** Induction of COX-2 and IL-8 occurs with activation of NFkB (28–30), which is also known to be downstream of *H. pylori*-induced RhoA stimulation (22). We therefore examined the effect of an inhibitor of IκB degradation on PAI-2-luc expression in response to *H. pylori* and gastrin. In both cases, there was inhibition of PAI-2-luc expression (Fig. 7, A and B). Activation of NFκB was shown to be upstream of paracrine signals, because cotransfection of DN-IκB and PAI-2-luc produced a partial inhibition of the response when *H. pylori* was applied directly to the cells (Fig. 7C), but in a coculture model in which reporter cells were transfected with DN-IκB, there was no effect on PAI-2-luc expression in response to *H. pylori* applied to AGS cells cocultured on Transwell inserts (Fig. 7D). The same experiment was then repeated using gastrin stimulation, and again cotransfection of receptor cells (i.e., AGS-Ga cells) with DN-IκB and PAI-2-luc partially reduced responses to gastrin (Fig. 7E). However, when reporter cells (i.e., AGS cells, lacking with gastrin receptor) were cotransfected with DN-IκB and PAI-2-luc and cocultured with untransfected AGS-Ga cells, there was no difference in the response to gastrin compared with cells not transfected with DN-IκB (Fig. 7F).
Kinetics of NFκB Translocation by H. pylori. To directly determine the kinetics of NFκB nuclear translocation, we used the NFκB p65 subunit tagged with dsRed in time lapse videomicroscopy studies (31). In unstimulated cells, p65-dsRed was located in the cytosol, and stimulation by H. pylori caused translocation to the nucleus (Fig. 8, A–C; Fig. 9). The precise kinetics of translocation varied between cells: nuclear translocation occurred from about 50 to 175 min after addition of H. pylori and was reversible; and in some cells, it was possible to identify oscillations in nuclear p65-dsRed (Fig. 9). Similar results were obtained with application of gastrin to AGS-GR cells transfected with p65-dsRed (Fig. 8, D–F). To determine whether p65-dsRed translocation occurred both in receptor and reporter cells i.e., upstream and downstream of paracrine signals, AGS cells were then transfected with p65-dsRed and cocultured with AGS cells on Transwell filters to which H. pylori was applied. In this system, there was minimal translocation of p65-dsRed in cells not directly exposed to H. pylori (Fig. 10A). Similarly, in a system in which receptor (AGS-Ga cells) and reporter cells (AGS-GFP cells) were cocultured and exposed to gastrin (8), there was nuclear translocation of p65-dsRed in the former but not the latter in response to gastrin (1–10 nm; Fig. 8, D–I; Fig. 10B). Finally, the nuclear translocation of p65-dsRed in response to H. pylori or gastrin was attributable to activation of RhoA, because in both cases, it was inhibited by treatment of cells with C3 transferase (Fig. 10, C and D).

DISCUSSION

The present study indicates that the oncogenic bacterium H. pylori induces PAI-2 in gastric epithelial cells. The gastric hormone gastrin, which is often increased in H. pylori infection (32), also stimulates PAI-2 expression (11). However, the association of H. pylori and increased gastric PAI-2 is seen even in those subjects with plasma gastrin concentrations within the normal range. Interestingly, both H. pylori and gastrin induce PAI-2 via IL-8 and COX-2. We show that there is RhoA-dependent activation of NFκB in cells exposed directly to H. pylori or gastrin but not in cells responding to the paracrine mediators (Fig. 11). We suggest that together these mechanisms direct expression of PAI-2 to multiple cell types in the gastric epithelium during H. pylori infection, including those not directly exposed to the bacterium. Expression of PAI-2 is associated with inhibition of cell invasion and apoptosis. The data indicate, therefore, that PAI-2 should be considered a determinant of the progression to gastric cancer in H. pylori infection.
We previously reported that gastrin induced PAI-2 in gastric epithelial cells via activation of PKC and RhoA, which in part triggered release of an unidentified paracrine mediator (11). The present findings therefore extend these studies both by identification of *H. pylori* as a regulator of PAI-2 in gastric epithelial cells and by demonstration of the role of NFκB and of IL-8 and COX-2 as mediators of the response. It is well recognized that NFκB is activated by *H. pylori* and in turn leads to induction of IL-8 and COX-2 (28, 33, 34). Less clear, however, are putative targets within the epithelial cell that might mediate the progression to cancer and the integrative mechanisms that might account for differential cell signaling in tissues as complex as the gastric mucosa. The present observations indicate that PAI-2 should now be considered a putative target of NFκB stimulation in tissues as complex as the gastric mucosa. The present observations indicate that PAI-2 should now be considered a putative target of NFκB activation by *H. pylori*. It seems, however, that the role of NFκB is limited to cells exposed directly to *H. pylori* and that other transcriptional mechanisms are activated in cells responding to paracrine mediators (Fig. 11).

Previous studies have reported that *H. pylori* induces PAI-2 expression in mononuclear cells (Ref. 24, 35). In part, the increase has been attributable to the effect of lipopolysaccharide, although *H. pylori* lipopolysaccharide is a relatively weak inducer of PAI-2 in mononuclear cells compared with other bacteria (35, 36). In addition, other virulence factors, including *H. pylori* neutrophil-activating protein, have been reported to induce PAI-2 in mononuclear cells (24). However, neutrophil-activating protein is not part of the Cag pathogenicity island that appears to be required for induction of PAI-2 in gastric epithelial cells. Moreover, it is thought that induction of NFκB by *H. pylori* in epithelial cells requires the type IV secretion system and pathogenicity island genes (37). It seems, then, that *H. pylori* induction of PAI-2 occurs through different mechanisms in gastric epithelial cells compared with mononuclear cells. There are, however, strain differences in lipopolysaccharide (38), and additional work is needed to define precisely the bacterial factors regulating PAI-2 expression.

Previous studies have shown that a nonclassical NFκB motif in the PAI-2 promoter plays a role in control of transcription (39). This may be a factor in cells that respond directly to *H. pylori* or to gastrin, but in cells that respond indirectly via paracrine mechanisms, this is not likely to be important. Thus using direct visualization of the nuclear translocation of the p65 subunit of NFκB, we show that there is translocation in cells responding directly to *H. pylori* or to gastrin but not in cells responding indirectly via paracrine stimulation. These observations are also compatible with the results of transfection of a DN-IκB plasmid, which inhibits PAI-2-luc expression in cells directly exposed to *H. pylori* or gastrin but not in cocultured cells. Serpins of the group that includes PAI-2 exhibit some homology with ovalbumin (13). The signal sequence in PAI-2 is weak, and it is thought that although some PAI-2 is secreted, there is also a distinct intracellular pool of protein. Secreted PAI-2 inhibits uPA and blocks uPA-induced invasion of cancer cells and uPA-mediated thrombolysis (12). We observed increased invasion of AGS cells in response to *H. pylori*. After inhibition of PAI-2 expression using ASO, the invasive phenotype was enhanced still further; moreover, overexpression of PAI-2 in stably transfected cells depressed the invasive phenotype in response to *H. pylori*. The data suggest that *H. pylori* induction of PAI-2 normally restrains the invasion of gastric cancer cells. We suggest that PAI-2 secretion can be considered as a protective response in at least two settings. First, in early or mild infection, inhibition of fibrinolysis is likely to promote the processes of wound healing. Second, in the progression to gastric cancer, inhibition of cell invasion by suppression of the uPA system would tend to restrain the oncogenic effects of *H. pylori* (Fig. 11).

However, the effects of nonsecreted PAI-2 may also be relevant in the progression to cancer. In particular, intracellular PAI-2 is reported to inhibit apoptosis in response to tumor necrosis factor-α (14). In the present study, we demonstrated that *H. pylori*-induced apoptosis of AGS cells (40, 41) is inhibited by PAI-2 overexpression. It is now recognized that activation of NFκB induces many different genes, some of which are associated with inhibition of apoptosis and others with innate immunity and inflammatory responses (42). The induction of PAI-2 by NFκB is consistent with this pattern. In the context of carcinogenesis in the stomach, the capacity of PAI-2 to inhibit apoptosis may be a predisposing factor in some subjects by promoting the
accumulation of cells with DNA damage, including cells that might not be directly exposed to the bacterium.

Previous studies have reported the expression of PAI-2 in gastric cancer. Some reports have indicated that PAI-2 expression in gastric tumors was associated with decreased metastasis (15) and was significantly higher in patients without lymph node involvement (15). In contrast, other reports have indicated either that PAI-2 expression was not correlated with prognosis (16) or was an indicator of recurrence (17). The reasons for these differences have been uncertain, and we now suggest that they reflect the balance between the effects of secreted and intracellular PAI-2. In particular, the secretion of PAI-2 by gastric epithelial cells in response to H. pylori may well be initially protective by decreasing fibrinolysis in areas of damaged mucosa; it may also be protective in adenocarcinoma by inhibiting cell invasion. However, in premalignant conditions e.g., H. pylori-associated chronic atrophic gastritis, increased PAI-2 may be oncogenic by inhibiting apoptosis and so preserving cells after DNA damage. In these circumstances, the induction of PAI-2 by H. pylori would accelerate the progression to gastric cancer.

ACKNOWLEDGMENTS

We are grateful to Debbie Sales and Cath McLean for technical help and to Dave Spiller for some help with confocal microscopy. We are also grateful to Mike White, Toni Antalis, and Alan Hall for donating plasmids and to Richard Peek and Jean Crabtree for strains of H. pylori.

REFERENCES

9. Kammori, M., Kamamin, M., Kobayashi, K., Oshara, T., Endo, H., Takubo, K., and Hashimoto, H. Immunohistochemical analysis of PAI-2 (plasminogen activator in

INDUCTION OF PAI-2 IN STOMACH BY H. PYLORI

accumulation of cells with DNA damage, including cells that might not be directly exposed to the bacterium.

Previous studies have reported the expression of PAI-2 in gastric cancer. Some reports have indicated that PAI-2 expression in gastric tumors was associated with decreased metastasis (15) and was significantly higher in patients without lymph node involvement (15). In contrast, other reports have indicated either that PAI-2 expression was not correlated with prognosis (16) or was an indicator of recurrence (17). The reasons for these differences have been uncertain, and we now suggest that they reflect the balance between the effects of secreted and intracellular PAI-2. In particular, the secretion of PAI-2 by gastric epithelial cells in response to H. pylori may well be initially protective by decreasing fibrinolysis in areas of damaged mucosa; it may also be protective in adenocarcinoma by inhibiting cell invasion. However, in premalignant conditions e.g., H. pylori-associated chronic atrophic gastritis, increased PAI-2 may be oncogenic by inhibiting apoptosis and so preserving cells after DNA damage. In these circumstances, the induction of PAI-2 by H. pylori would accelerate the progression to gastric cancer.

ACKNOWLEDGMENTS

We are grateful to Debbie Sales and Cath McLean for technical help and to Dave Spiller for some help with confocal microscopy. We are also grateful to Mike White, Toni Antalis, and Alan Hall for donating plasmids and to Richard Peek and Jean Crabtree for strains of H. pylori.

REFERENCES

17. Kamomori, M., Kamamin, M., Kobayashi, K., Oshara, T., Endo, H., Takubo, K., and Hashimoto, H. Immunohistochemical analysis of PAI-2 (plasminogen activator in

Downloaded from cancerres.aacrjournals.org on October 29, 2017. © 2004 American Association for Cancer Research.
*Helicobacter pylori* Induces Plasminogen Activator Inhibitor 2 in Gastric Epithelial Cells through Nuclear Factor-κB and RhoA: Implications for Invasion and Apoptosis


*Cancer Res* 2004;64:1695-1702.