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The Effect of the *cag* Pathogenicity Island on Binding of *Helicobacter pylori* to Gastric Epithelial Cells and the Subsequent Induction of Apoptosis

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Abstract

Background—*Helicobacter pylori* infection leads to gastritis, peptic ulcer, and gastric cancer, in part due to epithelial damage following bacteria binding to the epithelium. Infection with *cag* pathogenicity island (PAI) bearing strains of *H. pylori* is associated with increased gastric inflammation and a higher incidence of gastroduodenal diseases. It is now known that various effector molecules are injected into host epithelial cells via a type IV secretion apparatus, resulting in cytoskeletal changes and chemokine secretion. Whether binding of bacteria and subsequent apoptosis of gastric epithelial cells are altered by *cag* PAI status was examined in this study.

Methods—AGS, Kato III, and N87 human gastric epithelial cell lines were incubated with *cag* PAI-positive or *cag* PAI-negative strains of *H. pylori* in the presence or absence of clarithromycin. Binding was evaluated by flow cytometry and scanning electron microscopy. Apoptosis was assessed by detection of DNA degradation and ELISA detection of exposed histone residues.

Results—*cag* PAI-negative strains bound to gastric epithelial cells to the same extent as *cag* PAI-positive strains. Both *cag* PAI-positive and *cag* PAI-negative strains induced apoptosis. However, *cag* PAI-positive strains induced higher levels of DNA degradation. Incubation with clarithromycin inactivated *H. pylori* but did not affect binding. However, pretreatment with clarithromycin decreased infection-induced apoptosis.

Conclusions—*cag* PAI status did not affect binding of bacteria to gastric epithelial cells but *cag* PAI-positive *H. pylori* induced apoptosis more rapidly than *cag* PAI-negative mutant strains, suggesting that *H. pylori* binding and subsequent apoptosis are differentially regulated with regard to bacterial properties.

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Keywords

Bacterial binding; gastric epithelial cells; apoptosis; *cag* PAI

Colonization of the gastric mucosa by *Helicobacter pylori* gives rise to chronic gastritis, which may be complicated by peptic ulcer disease [1] or gastric adenocarcinoma [2] and mucosa-associated lymphoid tissue lymphoma [3]. Ulcers and gastric cancer may represent divergent results of the repair process arising from disruption of the balance between cellular proliferation and apoptosis in the gastric epithelium, resulting in part from binding of bacteria to epithelial cells [4]. The genotype of the infecting strain, specifically expression of the *cag* pathogenicity island (*cag* PAI), is one of many factors that has been found to impact gastric epithelial apoptosis. Expression of the 40 kb *cag* pathogenicity island (*cag* PAI), present within 50–70% of *H. pylori* strains [5], has been linked to increased overall disease severity [6] and a greater likelihood of progression to gastric cancer [7,8]. *cag* PAI-positive strains have been shown to enhance apoptosis in T lymphocytes [9], but the specific impact of the *cag* PAI on the induction of apoptosis in gastric epithelial cells remains in contention [10–14].

Expression of cytotoxin-associated gene A (*cagA*), located within the *cag* PAI, plays a key role in the apparent enhanced virulence of *cag* PAI-positive strains of *H. pylori*. Several genes encoded by the *cag* PAI are devoted to the development of a type four (IV) secretion system [15]. Once bacteria have bound to the cell surface, the type IV secretion system enables delivery of gene products, such as CagA, directly into gastric epithelial cells, dramatically altering cell behavior [16]. Some consequences of the delivery of CagA into epithelial cells are the secretion of pro-inflammatory cytokines, changes in cell structure, and altered cellular proliferation [17,18]. Whether these intracellular events resulting from infection with *cag* PAI-bearing strains are directly related to programmed cell death and whether the resultant apoptosis is affected by bacterial binding and bacterial viability are not clear. To examine this further we evaluated the effect of *cag* PAI expression in *H. pylori* on binding of bacteria to gastric epithelial cells and also apoptosis using cultured human gastric epithelial cell lines.

Methods

Cell Culture

Kato III cells [19] and AGS cells, both derived from human gastric adenocarcinoma, were obtained from American Type Culture Collection (Manassas, VA, USA). Kato III cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum in polystyrene tissue culture flask at 37 °C. Non-adherent as well as adherent cells were used in the assay; nonadherent cells were collected by gentle scraping with rubber scraper and centrifuged at 200 ×g for 5 minutes. Cells were washed and resuspended in medium.

AGS cells were grown as monolayers in F-12 medium (Invitrogen) containing 10% fetal bovine serum in a polystyrene tissue culture flask at 37 °C. For use in assays, adherent cells were detached using 2% EDTA and centrifuged at 200 ×g for 5 minutes. N87 cells, derived from gastric carcinoma cells metastasized to the liver, were also obtained from ATCC. They were grown as monolayers under conditions similar to Kato III. For use in assays, adherent cells were detached using 2% EDTA and centrifuged at 200 ×g for 5 minutes. Cells were resuspended in medium.

Three different gastric epithelial cell lines were employed to ensure that findings were not unique to a specific cell line and could also be related to our prior studies as well as others employing these three cell lines [20–25].

Bacteria

H. pylori strain LC11 [26] has been determined to express the *cag* PAI. Strain AH244 does not express the *cag* PAI, as it has a natural mutation in the *cag* PAI region. Strain 84-183 [27] has been determined to express the *cag* PAI and was used to generate *cag* PAI deletion strain 2-1. Strain no. 9 has been determined to express *cag* PAI and was used to generate *cag* PAI deletion strain no. 9 acoush (Table 1).

These strains and the *cag* PAI-positive strain 26695 [28] were grown on blood agar base (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C under microaerobic conditions and harvested on day 3. Fifty to 70 colonies from blood agar were transferred into Brucella broth (Becton Dickinson) containing 10% FBS. The broth was incubated at 37 °C under microaerobic conditions for 16–24 hours. The optical density (OD) of *H. pylori* broth cultures was measured by Spectronic 20 (Baush and Lomb, Rochester, NY, USA) at 530 nm. For washing, broth-cultured bacteria were centrifuged at 700 ×g for 15 minutes three times with phosphate-buffered saline (PBS).

Broth-cultured bacteria were grown in blood agar at 37 °C under microaerobic condition for 4 days. Colonies were counted and colony-forming units (CFU) to inoculate 10 µL containing 10⁴ CFU were confirmed. Viability of *H. pylori* was examined by acridine orange staining. Acridine orange was prepared using Hanks Balanced Salt Solution (HBSS). To stain *H. pylori* with acridine orange, bacteria were exposed to stain for 1 minute. After washing gently, staining was confirmed using a UV fluorescence microscope (Zeiss, Thornwood, NY, USA).

Bacterial Treatments

Clarithromycin was provided by Abbott Laboratories (Chicago, IL, USA). Its concentration was adjusted to 20 µg/mL or 200 µg/mL for incubation with *H. pylori*. *H. pylori* 26695 was cultured on TSA II 10% SB for 2 days. Colonies were mixed in RPMI1640 with 10% FBS, and the OD was adjusted to 0.1. Clarithromycin was added to bacterial suspensions. The suspensions were incubated for 1 or 24 hours at 37 °C in 5% CO₂ and then inoculated on agar plates for quantitative culture.

Bacteria were fixed in 2% paraformaldehyde for 3 minutes, then were triple washed with excessive PBS.

Electron Microscopy

1 × 10⁶ N87 cells were seeded onto culture plate inserts (Millipore, Billerica, MA, USA) and were cultured for 7–10 days. Resistance was measured using Millicell-ERS to determine appropriate times to use this polarized cell line for electron microscopy. Our preliminary studies established that at 150Ω, N87 cells had formed a polarized monolayer suitable for evaluation by electron microscopy. Polarized N87 cells were incubated with *H. pylori* for 1 hour at room temperature and washed three times with HBSS. They were fixed 2% with glutaraldehyde, and binding was analyzed by scanning electron microscopy at 12,500× magnification.

Flow Cytometry

Binding of *H. pylori* to surfaces of epithelial cells was evaluated by flow cytometry using a modification of technique described elsewhere [22]. Briefly, *H. pylori* were stained by

PKH26 (Sigma-Aldrich, St. Louis, MO, USA). Kato III or N87 cells were incubated with stained *H. pylori* for 1 hour at room temperature and washed three times with PBS. Subsequently, the cells were resuspended in 400 μ L 1% paraformaldehyde and analyzed by flow cytometry (FACScan, Becton Dickinson). Relative mean fluorescent intensity (MFI) was calculated by [MFI of *cag* PAI(-) $\div$ MFI of *cag* PAI(+)].

Apoptosis ELISA

2×10^6 Kato III cells were incubated with *H. pylori* for 48 hours. They were harvested in RPMI 1640 containing FBS without antibiotics. Harvested cells were counted by hemocytometer into aliquots of 5×10^5 cells. Cells were then assayed for the presence of DNA fragmentation using a commercially available ELISA assay (Cell Death Detection Kit, Roche, Basel, Switzerland) for small-molecular-weight nucleosome fragments in the cytoplasmic fractions of affected cells that arise during apoptosis. DNA selectively cleaved at the junction of nucleosomes exposes histone proteins on the ends of DNA fragments.

The ELISA plate was coated with antibodies to histones and a secondary anti-DNA antibody was added, and then the reaction was developed. The absorbance was measured at 405/492 nm by Titertek Multiskan MCC/340 (Titertek, Huntsville, AL) and compared to substrate solution as a blank. The apoptosis index was calculated according to manufacture's instructions by dividing the absorbance of stimulated cells by the absorbance for control cells [(OD_{405nm-492nm} of stimulated cells) $\div$ (OD_{405nm-492nm} of control cells)] [23].

Gel Electrophoresis

1×10^6 Kato III cells infected with live *H. pylori* or *H. pylori* previously treated with clarithromycin or paraformaldehyde for 48 hours were washed with PBS, then pelleted by centrifugation at 400 $\times g$ for 5 minutes. Pelleted cells were resuspended in sample buffer comprised of Tris-buffered glycerol with 1.4 mg/mL ZnSO₄, bromophenol blue, and RNase A (Sigma-Aldrich) at 10 mg/mL. Nucleosomal ladders were visualized in 2% agarose gels in TBE buffer (0.09 mol/L Tris, 0.09 mmol/L boric acid, 0.24 mol/L EDTA, pH 8.0). Cells were lysed by 2% SDS and 53 μ g/mL of proteinase K (Invitrogen) contained within a 1% agarose gel slice above the wells. The gel was run 10–14 hours at 30 V, stained with 2 μ g/mL ethidium bromide for 1 hour, and washed overnight. Gels were illuminated with UV light to visualize DNA fragments and their migration compared to a 100 bp DNA ladder (Invitrogen).

Statistical Analysis

Results are expressed as means with standard deviations. Statistical differences were determined by Friedman Repeated Measures Analysis of Variance on Ranks for multiple groups. Student's *t*-test was performed for comparisons of two groups. Statistical significance was determined at $P < 0.05$.

Results

Deletion of the *cag* PAI Does not Affect Bacterial Growth or Viability

H. pylori counted by light microscopy showed no statistically significant differences in bacterial count, CFU, or viability between parent strains and *cag* PAI-deleted strains (Table 2). This suggests that the deletion of the *cag* PAI in these strains of *H. pylori* had little or no effect on bacterial growth and viability.

Deletion of the *cag* PAI Does not Affect Binding of *H. pylori* to Gastric Epithelial Cells In Vitro

N87 cells were grown in a subconfluent monolayer on culture plate insert, incubated with either PAI(+) or PAI(−) strains of *H. pylori*, and visualized by scanning electron microscopy at 12,500× magnification (Fig. 1). All strains tested (Table 1) exhibited binding to N87 cells.

Both Kato III (Fig. 2A) and N87 cells (Fig. 2B) were incubated with equal numbers of *H. pylori* stained with PKH 26. MFI reflected numbers of stained bacteria. None of the strains tested showed any significant differences in binding based on *cag* PAI status.

Bacterial binding to gastric epithelial cells was strongly dependent on bacteria:cell ratio. When the ratio increased, MFI (and thus bacterial binding) increased for both Kato III (Fig. 3A) and N87 cells (Fig. 3B). Relative MFIs for each bacteria:cell ratio were calculated by dividing the MFI of the PAI(−) strain by the MFI of the corresponding PAI(+) strain. There were no significant differences in relative MFI among the three strain pairs. These data suggest that deletion of the *cag* PAI has no significant effect on binding efficiency of *H. pylori* to target cells and that binding is strongly correlated with numbers of bacteria per cell.

Binding of *H. pylori* to Gastric Epithelial Cells is Dependent on the Bacteria:Epithelial Cell Ratio and not on Bacterial Viability

Kato III and AGS cells incubated with *H. pylori* were stained with PKH 26. Binding was confirmed by flow cytometry (data not shown). Higher bacteria:epithelial cell ratios displayed increased MFI indicating that bacterial binding to gastric epithelial cells was dependent on the ratio of epithelial cells to bacteria. There was no significant difference in MFI between cells incubated with or without clarithromycin (data not shown), indicating that binding is unaffected by pretreatment with clarithromycin. Suspended *H. pylori* 26695 were killed within 1 hour of incubation with 20 µg/mL of clarithromycin ($2.27 \pm 1.5 \times 10^7$ CFU/mL at baseline, 0 CFU/mL at 1 hour and 24 hours, mean ± SEM, n = 3).

cag PAI(+) *H. pylori* Strains Induce Greater Apoptosis Than *cag* PAI(−) Strains in Kato III Cells In Vitro

An ELISA to detect histone-containing nucleosomes generated during apoptosis was performed to ascertain the relative ability of *cag* PAI(+) strains and *cag* PAI(−) strains to induce apoptosis in Kato III cells over a 48-hour incubation period. After incubation, apoptotic indices of all strains, but AH244, showed a significant difference from the uninfected control (Fig. 4). No significant differences in apoptotic index were detected between the three *cag* PAI(+)/*cag* PAI(−) strain pairs. However, at 24 hours, the apoptotic index with strain 26695 was significantly greater than uninfected cells (1.69 ± 0.66 , mean ± SEM, n = 13, $P < 0.05$), while there was no significant difference between infection with strain 8-1 and control cells (1.19 ± 0.40 , mean ± SEM, n = 13). Furthermore, at 12 hours but not at 24 or 48 hours, the apoptotic index in Kato III cells infected with the *cag* PAI bearing strain 84-183 was significantly greater than uninfected control cells (1.80 ± 1.06 , mean ± SEM, n = 8, $p < .05$), while the isogenic mutant, 2-1, was not different from control cells (1.15 ± 0.19 , mean ± SEM, n = 8, $p = \text{NS}$).

Agarose gel electrophoresis is a standard procedure for assaying fragmentation in total genomic DNA [29]. We analyzed the induction of apoptosis in Kato III cells by two pairs of *cag* PAI(+)/*cag* PAI(−) bacterial strains (84-183/2-1 and no. 9/no. 9 acoush) via gel electrophoresis. Both *cag* PAI(+) strains and *cag* PAI(−) strains induced apoptosis to a greater degree than the media control. Within each pair of strains, cells incubated with the *cag* PAI(+) strains showed greater fragmentation than cells incubated with the *cag* PAI(−) strains over identical time periods (Fig. 5).

Taken together, these data indicate that, while *cag* PAI(+) and *cag* PAI(−) strains induced similar degrees of apoptosis in Kato III cells, *cag* PAI(+) strains induced apoptosis more rapidly than their *cag* PAI(−) mutants.

Bacterial Killing Attenuates Induction of Apoptosis in Kato III Cells by *H. pylori*

To determine whether bacterial killing affected apoptosis of host epithelial cells we analyzed the effect of clarithromycin or paraformaldehyde treatment of *H. pylori* on *H. pylori*-induced DNA fragmentation in Kato III cells. After 48 hours, the untreated *H. pylori* induced apoptosis to a greater degree than paraformaldehyde-fixed or clarithromycin-treated *H. pylori*. There was no significant difference between rates of apoptosis induced by paraformaldehyde-fixed *H. pylori* and those induced by clarithromycin-treated *H. pylori* (Fig. 6). These data suggest that live bacteria are necessary to induce apoptosis in gastric epithelial cells.

Discussion

In this study we have shown that while both *cag* PAI-positive and *cag* PAI-negative strains are capable of inducing apoptosis in gastric epithelial cells, *cag* PAI-positive strains induced apoptosis more rapidly than their *cag* PAI-negative counterparts. In contrast, *H. pylori* binding to gastric epithelial cells was unaffected by *cag* PAI expression and binding was, as expected, strongly correlated with bacteria:epithelial cell ratio. Viable bacteria were necessary to induce apoptosis.

Apoptosis is a normal process regulating epithelial cell growth. Healthy maintenance of tissue requires a balance between cell proliferation and apoptosis, as hyperproliferation of tissue carries an increased risk of malignant transformation and reduced apoptosis may fail to remove damaged cells. We found that both groups of bacteria could bind to and induce apoptosis in gastric epithelial cells. Our studies showed that *cag* PAI(+) strains induced apoptosis in Kato III cells more rapidly than their deletion mutants, suggesting that gene products of the *cag* PAI play a role in apoptotic induction. Peek et al. and others have reported that CagA(+) patients had greater rates of proliferation of gastric epithelium than CagA(−) patients [10,12], yet Moss et al. found that CagA(−) strains, more so than CagA(−) strains, biasing the apoptosis/proliferation ratio toward apoptosis [11].

Other studies in gastric epithelial cell lines as well as gastric tissues have reported evidence that supports a link between *cag* PAI expression and increased epithelial apoptosis [30–32]. Cabral et al. demonstrated a significant association between *cag* PAI-positive *H. pylori* infection and over-expression of pro-apoptotic proteins in the gastric mucosa [1e3]. Higashi et al. found that in vitro, CagA protein expression may contribute to apoptotic transformation of *H. pylori*-bound gastric epithelial cells by binding and activating SHP-2, an SH2 domain-containing protein-tyrosine phosphatase [33,34]. A negative-feedback mechanism for this process has been identified, wherein CagA binds to C-terminal Src kinase to inhibit formation of the CagA-SHP-2 complex [35].

The apoptotic effects of *cag* PAI expression in the gastric mucosa may also be mediated through its ability to enhance inflammatory responses in epithelial host cells. A variety of cytokines released from immune cells during infection, such as tumor necrosis factor- α and interferon- γ , have been shown to play a role in programmed cell death.

Binding activity of *H. pylori* depends on expression of several bacterial adhesins and their interactions with epithelial cell-surface antigens, which can vary widely among bacterial strains [36]. *H. pylori* has been shown to interact with the gastric epithelial cell via class II receptors [23], Lewis antigens [37], and sialylated/fucosylated glycans [38]. We found that

killed bacteria bind to gastric epithelial cells and that clarithromycin-treated *H. pylori* display binding activity similar to that of untreated *H. pylori*. Clarithromycin inhibits 23S rRNA-dependent protein synthesis in bacteria, which is considered metabolic level inhibition [39], and paraformaldehyde-fixed *H. pylori* also undergo metabolic level inhibition. Based on our findings, clarithromycin may be involved in mechanisms leading to inhibition of apoptosis in gastric epithelial cells in addition to its direct effects on killing of bacteria.

We have shown that expression of the *cag* PAI by *H. pylori* can result in increased DNA fragmentation in gastric epithelial cell lines in vitro. Our results suggest that the genotype of infecting bacteria may impact the rate of apoptosis experienced by epithelial cells in infected gastric mucosa. Additional studies are needed in order to further elucidate the effect of *cag* PAI expression on the apoptotic regulation of gastric epithelium in response to *H. pylori* infection. Such studies may broaden our understanding of the pathogenesis of *H. pylori* infection and its role in gastric carcinogenesis.

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Figure 1. Representative electron micrographs demonstrating binding of *cag* PAI(+) *H. pylori* no. 9 (A) and *cag* PAI(-) no. 9 acoush (B) to human gastric epithelial cell line N87 after incubation for 1 hour. All tested strains (LC11, AH244, 84-183, 2-1, no. 9, and no. 9 acoush) bound to N87 cells. Bars at bottom of images indicate 10 μm lengths.

Figure 2.

Flow cytometric analysis of *H. pylori* binding to gastric epithelial cells. The first peak (filled) represents mean fluorescent intensity (MFI) of cells not co-cultured with *H. pylori* (control). The second peak (open) represents MFI of cells cocultured with *H. pylori*. The left column of histograms shows binding of *cag* PAI(+) strains. The right column of histograms shows binding of *cag* PAI(-) strains. Ratio of epithelial cells:bacteria was 1 : 30,000. (A) Representative histogram analysis of *H. pylori* binding to Kato III cells. (B) Representative histogram analysis of *H. pylori* binding to N87 cells.

Figure 3. Mean fluorescent intensity (representing binding bacteria binding) as a result of increasing cell:bacteria ratio. Unbroken lines represent *cag* PAI(+) strains. Dashed lined represent *cag* PAI(-) strains. Data are the result of three separate experiments. (A) *H. pylori* binding to Kato III cells in a suspension. (B) *H. pylori* binding to N87 cells in a monolayer.

Figure 4.

Comparison of the effects of different *H. pylori* strains on DNA fragmentation, assayed by ELISA for histones. Bacterial strains are indicated on the x-axis. The y-axis indicates apoptotic index: $[(OD_{405nm-492nm} \text{ of stimulated cells}) \div (OD_{405nm-492nm} \text{ of control cells})]$. *cag* PAI(+) strains are represented by grey bars, *cag* PAI(-) by black bars. All strains but AH244 elicited an apoptotic index significantly different from that of the control. There were no significant differences between *cag* PAI(+) and *cag* PAI(-) strains. The figure represents data from seven different experiments shown as mean $\pm$ SEM, * $p < .05$.

Figure 5.

Comparison of the abilities of different *H. pylori* strains to induce DNA fragmentation in Kato III cells after 48 hours, assayed by gel electrophoresis. A 100 bp DNA ladder was loaded into lane M. Lane 1 is the media control, lane 2 is strain 84-183, lane 3 is strain 2-1, lane 4 is strain no. 9, and lane 5 is strain no. 9 acoush. Cells incubated with *cag* PAI(+) strains displayed greater fragmentation than cells incubated with the *cag* PAI(-) strains over identical time periods.

Figure 6.

Comparison of the effect of live, untreated and paraformaldehyde- or clarithromycin-treated *H. pylori* on DNA fragmentation in Kato III cells at 48 hours, assayed by gel electrophoresis. Lane M is a 100-bp DNA ladder, lane 1 is the media control, lane 2 is cells incubated with clarithromycin, lane 3 is cells incubated with *H. pylori*, lane 4 is cells incubated with *H. pylori* with clarithromycin, and lane 5 is cells incubated with paraformaldehyde-fixed *H. pylori*.

Table 1*cag* PAI status of *H. pylori* strains

Strain	<i>cag</i> PAI status	Notes
LC11	+	
AH244	–	Natural mutant
84-183	+	Parent strain of 2-1
2-1	–	<i>cag</i> PAI deleted
No. 9	+	Parent strain of no. 9 acoush
No. 9 acoush	–	<i>cag</i> PAI deleted
26695	+	

Table 2Quantification and viability of *H. pylori*

Strain	Count by microscopy ($\times 10^{10}/\text{mL}$)	Count by CFU ($\times 10^7$ CFU/mL)	Viability (%)
LC11	1.2 ± 0.4	4.5 ± 0.7	99.3 ± 0.6
AH244	1.2 ± 0.4	5.4 ± 1.6	99.3 ± 1.2
84-183	1.6 ± 0.8	3.6 ± 2.2	97.8 ± 3.8
2-1	1.6 ± 0.6	3.5 ± 2.5	99.3 ± 1.2
No. 9	1.1 ± 0.3	3.7 ± 4.7	99.0 ± 1.0
No. 9 acoush	1.1 ± 0.2	1.2 ± 0.8	98.3 ± 2.1

CFU, colony-forming units.