Helicobacter pylori Strain-specific Genotypes and Modulation of the Gastric Epithelial Cell Cycle

Richard M. Peek, Jr., Martin J. Blaser, Deborah J. Mays, Mark H. Forsyth, Timothy L. Cover, Si Young Song, Uma Krishna, and Jennifer A. Pietenpol

Division of Gastroenterology [R. M. P., U. K.] and Division of Infectious Diseases [M. J. B., M. H. F., T. L. C.], Department of Medicine, and Departments of Biochemistry [D. J. M., J. A. P.] and Surgery [S. T. S.], Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2279; and Department of Veterans Affairs Medical Center, Nashville, Tennessee 37212 [R. M. P., M. J. B., T. L. C.]

ABSTRACT

Helicobacter pylori cag+ strains enhance gastric epithelial cell proliferation and attenuate apoptosis in vivo, which may partially explain the increased risk of gastric cancer associated with these strains. The goals of this study were to identify specific H. pylori genes that regulate epithelial cell cycle events and determine whether these effects were dependent upon cagA+ strains. AGS gastric epithelial cells were cultured alone or in the presence of 21 clinical H. pylori isolates, H. pylori reference strain 60190, or its isogenic cagA−, picB−, vacA−, or picB+/vacA− derivatives. Coculture of H. pylori with AGS cells significantly increased proliferation of AGS cells from G1 into G2/M at 6 h and enhanced apoptosis by 72 h. Compared with the parental 60190 strain, the picB− mutant attenuated cell cycle progression at 6 h (P ≤ 0.05), and decreased apoptosis with enhanced AGS cell viability at 24 h (P ≤ 0.04). The vacA− mutant decreased apoptosis and enhanced viability at later (48–72 h) time points (P ≤ 0.05). Compared with the wild-type strain, the picB−/vacA− double mutant markedly attenuated apoptosis and increased cell viability at all time points (P ≤ 0.05). Furthermore, coculture with H. pylori had no significant effect on expression of p53, p21, and MDM2. The diminished AGS cell viability, progression to G2/M, and apoptosis associated with cagA+ H. pylori strains were dependent upon expression of vacA and genes within the cag pathogenicity island. These results may explain heterogeneity in levels of gastric epithelial cell proliferation and apoptosis found within H. pylori-colonized mucosa.

INTRODUCTION

Gastric colonization with Helicobacter pylori induces local inflammation in essentially all hosts, a persistent process that increases the risk of developing atrophic gastritis, intestinal metaplasia, and non-cardia gastric adenocarcinoma (1–4). However, only a small percentage of persons carrying H. pylori develop neoplasia; enhanced cancer risk may be related either to differences in expression of specific bacterial products, to differences in host response to the bacteria, or to the interaction between host and microbe (5). The first strain-specific gene identified in H. pylori was cagA (6, 7), a component of the cag pathogenicity island (8, 9). Several genes contained within this island, such as picB (cagE), encode products that are homologues of type IV bacterial secretory proteins (8), and previous studies have shown that mutation in picB markedly reduces nuclear factor-kB induction (10) and interleukin 8 secretion (11) by gastric epithelial cells. Persons colonized with H. pylori strains that possess cagA (and thus the cag island) are at increased risk for developing severe gastritis, atrophic gastritis, and distal gastric cancer compared with persons harboring cagA− strains (12–16). The gene vacA, which encodes a secreted vacuolating cytotoxin, represents a second locus of heterogeneity among H. pylori strains. H. pylori strains of the vacA s1 subtype are usually strongly toxigenic in vitro cell culture assays, and strains of this subtype almost always are cagA+ (17); this strong association has been observed (18) despite the relative distance of vacA and the cag pathogenicity island on the H. pylori chromosome (19).

Host responses to H. pylori also may be important in affecting the threshold for carcinogenesis. Exposure of epithelial cells to H. pylori alters cell replication and apoptosis in vitro and in vivo (20–33). H. pylori colonization increases gastric mucosal apoptosis (20, 21, 23), but epithelial cell proliferation rates in gastric tissue from persons carrying H. pylori have been either reduced or enhanced in various studies (22, 25, 26, 27). One explanation for this variation is that induction of cell growth or death may be affected by specific bacterial characteristics (34). For example, we have reported recently that cagA− strains selectively enhance proliferation and attenuate apoptosis compared with cagA+ strains (22); however, in another study, apoptosis was not related to strain variation (24).

Coincubation of gastric cells with H. pylori and either tumor necrosis factor α, IFN-γ, or receptor-activating Fas antibodies increases apoptosis compared with coincubation with H. pylori alone (28, 35). Coincubation with H. pylori also stimulates gastric epithelial cell apoptosis in vitro by binding to class II MHC receptors (36), suggesting that several signal transduction cascades may be affected by bacterial contact. One pathway that affects both cell growth and death is mediated by p53, which regulates cell cycle checkpoint function (37). Activation of p53 and subsequent induction of the cyclin-dependent kinase inhibitor p21 may either inhibit cell growth or stimulate apoptosis, depending on the specific cell type and/or growth conditions (37).

Therefore, we investigated the relationship of H. pylori genotype and epithelial cell cycle events. Our goals were to identify specific H. pylori constituents that regulate gastric epithelial cell cycle events and to determine whether these effects were dependent upon p53-mediated pathways. For these experiments, we used the AGS gastric epithelial cell line as our model system because the interaction between H. pylori and this cell line induces transcription of both specific bacterial (38) and host cell genes (10) relevant to inflammation. We report here that VacA and products of genes within the cag pathogenicity island have independent effects on cell viability and apoptosis, and that these events are not mediated by elevations in p53 or p21.

MATERIALS AND METHODS

H. pylori Clinical Isolates and Isogenic Mutant Strains

Experiments were performed with the cagA− vacA s1a H. pylori reference strain 60190 (ATCC 49503), as well as with 21 well-characterized clinical H. pylori strains (Table 1) isolated from patients undergoing upper endoscopy at the Nashville Department of Veterans Affairs Gastroenterology Clinic. The 21 clinical strains (10 cag−, 11 cag+) used in this study were selected from a larger population of isolates that have been described previously as part...
allelic exchange using these plasmids, as described previously (39).
inactivated in a similar fashion using either pCTB8:km (41) or pCTB5:CAT, H. pylori
pGem (Promega), and . The resultant PCR product was then cloned into
CATGCATTTTTTCC-3

\[ \text{cat} \]

null mutants were constructed by insertional mutagenesis, using either
cag viability and apoptosis experiments represented a subset of the total
reported linkage dysequilibrium between
5s1a (60190 and 178), and two were
vacA signal sequence genotype. Two of the
strains used in
cagA and vacA genotype, facilitating identification of specific bacterial components
that regulate gastric epithelial cell cycle events. The four cag+ strains used in
viability and apoptosis experiments represented a subset of the total cag+ population (n = 11) and varied in vacA signal sequence genotype. Two of the
strains were vacA s1a (60190 and 178), and two were vacA s1b (166 and 291). For uniformity, the same four cag+ strains used in survival experiments also
were used in apoptosis studies. This allowed us to assess the influence of genes
within the cag pathogenicity island, as well as differing vacA types, on epithelial
cell cycle events. Procedures were approved by the Vanderbilt University and Nashville Department of Veterans Affairs Institutional Review
Boards. All clinical strains underwent a maximum of five in vitro passages prior to incubation with gastric epithelial cells. To genotype
H. pylori clinical isolates, genomic DNA was prepared (12), and PCR for cagA and vacA signal and midregion type was performed as described previously (12, 17).

Isogenic vacA and picB mutants were generated within H. pylori reference
strain 60190 harvested at the same in vitro passage level. Two different picB
null mutants were constructed by insertion mutagenesis, using either cat3
(39) or aphA (confering km; Ref. 40) genes. Briefly, the 5' region of picB,
containing a unique BglII site, was amplified from strain 60190 using the
primers 5'-GAGCGAAGAGGTCTAAAAGGCC-3' and 5'-CACCACCGC-
ATGCTATTTTCC-3. The resultant PCR product was then cloned into
pGem (Promega), and cat or aphA was subsequently cloned into the BglII site.
H. pylori isogenic picB mutants were generated by natural transformation and
allelic exchange using these plasmids, as described previously (39). vacA was
inactivated in a similar fashion using either pCTB8:km (41) or pCTB5:CAT, which
contains cat cloned into the unique BglII site of vacA in pCTB5 (41). Isogenic picB and vacA mutants were selected on Brucella agar with either
chloramphenicol (10 µg/ml) or kanamycin (25 µg/ml). Isogenic cagA mutants
of strain 60190 have been reported previously (42). To inactivate both picB and
vacA in a single strain, the picB::km H. pylori isogenic mutant was transformed with pCTB5:CAT, whereas the vacA::km mutant was transformed with the plasmid containing picB::CAT. Double mutants were selected on Brucella agar
containing kanamycin (25 µg/ml) and chloramphenicol (10 µg/ml). The presence
of the cat or aphA cassettes within vacA and/or picB was confirmed by
PCR-amplifying products of the expected size from bacterial chromosomal
DNA, using specific primers.

H. pylori Culture

For coculture experiments with gastric epithelial cells, H. pylori were grown in
Brucella broth with 5% FBS for 48 h, harvested by centrifugation
(2000 × g), and resuspended in antibiotic-free RPMI 1640 with 10% FBS to a
concentration of 1 × 10^8 cfu/ml. For all experiments, H. pylori were added to
cells at a bacteria:cell concentration of 100:1, based on previous reports that
H. pylori reproducibly induce apoptosis in AGS cells at this ratio (28, 29). For
bacterial viability studies, serial 10-fold dilutions of supernatants remained
from H. pylori:AGS cell cocultures were plated onto Trypticase soy agar with
5% sheep blood and incubated for 72 h under microaerobic conditions, as
described previously (12). Bacteria were identified as H. pylori by urease and
oxidase activity as well as by Gram’s stain morphology, and colony counts
were determined by an observer blinded to AGS cell cycle results.

Cell Culture

AGS human gastric epithelial cells (ATCC CRL 1739) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS and 20 µg/ml
gentamicin in an atmosphere of 5% CO₂ at 37°C. Experiments were performed in
antibiotic-free media with 10% FBS using T-150 flasks (Corning Costar,
Cambridge, MA), 24- or 96-well polypropylene tissue culture plates (Nunc,
Roskilde, Denmark), and chamber slides (four-well/slide; Lab-Tek; Nunc). AGS
cells were studied because they possess wild-type p53 (29), in contrast to
KATO III and other gastric cell lines that have p53 deletions and/or rearrange-
ments, which may affect the response to signals that regulate cell cycle events
(43). AGS cells were not serum starved and remained subconfluent during each
assay, because we attempted to recapitulate events that occur in the native
actively replicating gastric mucosa.

Table 1 Characteristics of 22 H. pylori isolates and relationship to cell cycle distribution after coculture with AGS gastric epithelial cells

<table>
<thead>
<tr>
<th>H. pylori strain designation</th>
<th>6-h % G2M</th>
<th>72-h % sub-G1</th>
<th>cagA status</th>
<th>vacA alleled</th>
<th>Signal Region</th>
<th>Midregion</th>
<th>Toxb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control AGS cells alone</td>
<td>42.3 ± 5</td>
<td>3.0 ± 2</td>
<td>NAa</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>H. pylori strains that significantly increased proportion of cells in both 6-h G2M and 72-h sub-G1 compared with controlsd</td>
<td>166 56.3</td>
<td>16.2</td>
<td>+</td>
<td>s1b</td>
<td>m1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>291 56.8</td>
<td>27.5</td>
<td>+</td>
<td>s1b</td>
<td>m1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9365 53.8</td>
<td>18.5</td>
<td>+</td>
<td>s1b</td>
<td>m1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>178 57.8</td>
<td>23.1</td>
<td>+</td>
<td>s1a</td>
<td>m1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>238 48.4</td>
<td>14.0</td>
<td>+</td>
<td>s1a</td>
<td>m1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60190 48.2</td>
<td>14.2</td>
<td>+</td>
<td>s1a</td>
<td>m1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. pylori strains that significantly increased proportion of cells in either 6-h G2M or 72-h sub-G1 compared with controlsd</td>
<td>198 50.0</td>
<td>4.2</td>
<td>+</td>
<td>s1a</td>
<td>m2</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>117 53.1</td>
<td>13.1</td>
<td>+</td>
<td>s1a</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. pylori strains that did not increase cells in either 6-h G2M or 72-h sub-G1 compared with controlsd</td>
<td>54 40.9</td>
<td>5.5</td>
<td>+</td>
<td>s1a</td>
<td>m1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>939 44.6</td>
<td>2.5</td>
<td>+</td>
<td>s1a</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>104 41.7</td>
<td>4.1</td>
<td>+</td>
<td>s1a</td>
<td>m1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9366 42.6</td>
<td>2.6</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>68 46.3</td>
<td>2.0</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>190 44.5</td>
<td>7.0</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>102 45.2</td>
<td>6.5</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>107 47.1</td>
<td>1.9</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>63 42.1</td>
<td>2.6</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 42.0</td>
<td>2.6</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>188 45.5</td>
<td>6.3</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>195 45.2</td>
<td>0.8</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>262 37.1</td>
<td>3.6</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>116 40.1</td>
<td>3.0</td>
<td>–</td>
<td>s2</td>
<td>m2</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a cagA and vacA allele types determined by PCR of genomic DNA, as described previously.
b Toxicity determined by neutral red assay for HeLa cells, as described previously.
c NA, not applicable.
d P < 0.05 versus controls.

The abbreviations used are: cat or CAT, chloramphenicol acetyltransferase; km, kanamycin resistance; FBS, fetal bovine serum; cftu, colony-forming unit(s).
Assessment of AGS Cell Viability

AGS cells were seeded to a subconfluent density of 5 × 10^5 cells/well in 24-well plates, incubated overnight, and washed with sterile PBS before inoculation with H. pylori (5 × 10^5 cfu/well; total volume 1.0 ml). Control cells were inoculated with RPMI 1640/10% FBS alone, and coincubation was performed up to 72 h in triplicate. At the end of each incubation, cell viability was determined in a hemacytometer by trypan blue exclusion (0.04% trypan blue; Life Technologies, Inc.) using phase-contrast microscopy.

Flow Cytometry

AGS cells were seeded into T-150 flasks at a subconfluent density of 0.6–1.2 × 10^6 cells/flask, incubated overnight, washed with PBS, and inoculated with either H. pylori at a concentration of 100:1 bacteria to cells or with RPMI/10% FBS alone. Coculture experiments were performed for 6–72 h, at which times control and treated cells were trypsinized, and 1.0 × 10^6 cells were aliquoted for flow cytometry. The remaining cells were processed for protein analysis by the Bradford assay (Bio-Rad). Cells were incubated with 20 μg/ml propidium iodide (Sigma), and DNA content was measured using a FACSCaliber (Becton Dickinson); 15,000 events were analyzed for each sample.

Assessment of Apoptosis

DNA-specific Fluorochrome Staining. AGS cells were cultured on chamber slides with or without H. pylori for 24–72 h, fixed with ice-cold methanol for 10 min, incubated with 1 μg/ml propidium iodide for 5 min, and evaluated by fluorescence microscopy. Nuclei with highly condensed and fragmented chromatin were considered apoptotic.

DNA Fragmentation ELISA. DNA fragmentation was quantified using a commercially available ELISA (Boehringer Mannheim Biochemicals, Indianapolis, IN) that detects nucleosomal fragments in cytoplasmic fractions of cells undergoing apoptosis but not necrosis. For these experiments, 5 × 10^5 AGS cells/well in 96-well plates were incubated subconfluently in triplicate with H. pylori (5 × 10^5 cfu/well) or media alone for 24 to 48 h and lysed, and supernatants after centrifugation were used for ELISA. Absorbance measured at 405 nm was compared between controls and H. pylori-cultured samples.

Western Analysis

Cells were lysed in Kinase Lysis Buffer [KLB; 50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 0.1% NP40, 4 mM EDTA, 50 mM NaF, 0.1 mM NaV, 1 mM DTT, and the protease inhibitors: 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 50 μg/ml phenylmethylsulfonyl fluoride (Sigma), and 200 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride] (Calbiochem-Novabiochem Corp.). Total cell protein extracts were normalized for concentration by the Bradford assay (Bio-Rad). Cells were incubated with 20 μg/ml propidium iodide for 5 min, and DNA content was measured using a FACSCaliber (Becton Dickinson). Data were plotted using Cell Quest software (Becton Dickinson); 15,000 events were analyzed for each sample.

Statistics

Results are expressed as mean ± SE. The Mann-Whitney U test was used for statistical analyses of intergroup comparisons, whereas vacA subtypes, toxin production, and clinical outcome within the group of cag+ strains were compared using the Mantel-Haenszel test. Ps ≤ 0.05 were considered significant.

RESULTS

Viability of AGS Gastric Epithelial Cells Is Decreased after Coculture with H. pylori Cells. In various studies, H. pylori has been shown to either reduce or enhance gastric epithelial cell viability in vitro (28, 30–33). To determine whether H. pylori strain variation may account for the discordant results, we incubated AGS gastric epithelial cells with six well-characterized, minimally passaged clinical H. pylori isolates (three cag+; 166, 291, and 178; three cag-; 68, 9366, and 107) or a cag- toxigenic H. pylori reference strain (60190) and quantified cell viability at 24, 48, and 72 h after inoculation. AGS cells cocultured with H. pylori had significantly (P < 0.05) reduced viability compared with control AGS cells at each time point (Fig. 1). When survival was compared for cells cocultured with cag+ or cag– strains, cag+ isolates significantly decreased AGS cell viability at each time point (P < 0.001 at 24, 48, and 72 h; Fig. 1). Because differences in bacterial viability among cag+ compared with cag– strains during the 72-h time course could have contributed to differences in AGS cell survival, we also quantitated bacterial viability at 24, 48, and 72 h using the seven H. pylori strains described above. There were no significant differences in H. pylori viability at any time point during AGS cell coculture among cag+ and cag– strains, and viable bacteria persisted for 72 h (data not shown). These findings indicate that coculture with H. pylori, particularly cag+ strains, significantly reduces AGS cell viability and raise the hypothesis that differences in cell survival may be attributable to bacterial strain-specific alterations in gastric epithelial cell cycle events.

H. pylori Strains Differentially Alter Cell Cycle Events in Gastric Epithelial Cells in Vitro. We next examined whether the H. pylori-induced reductions in AGS cell viability were associated with arrest of cells at a specific phase of the cell cycle or with increased apoptosis and whether these changes correlated with particular H. pylori genotypes. For these experiments, 21 minimally passaged clinical isolates (10 cag+ and 11 cag–; Table 1), as well as strain 60190, were incubated with AGS cells for 6–72 h, and the AGS cells were analyzed by flow cytometry. In preliminary experiments examining a smaller number of clinical strains (5 cag+ and 5 cag–), the effects of H. pylori on cell cycle distribution in this system were most prominent at 6 and 72 h (data not shown); therefore, we focused our analysis on events occurring between these time points.

Six h after incubating AGS cells with cag+ strains, progression of cells from G1 into G2-M (50 ± 6%) increased compared with controls (42 ± 5%; P = 0.07; Fig. 2A). For AGS cells cocultured with cag–
strains, the proportion at 6 h that were in G2-M did not differ from controls (44 ± 3% versus 42 ± 5%; P = 0.37; Fig. 2B). By 72 h, coincubation with cag+ strains significantly increased the sub-G1 AGS cell population compared with controls (13 ± 3% versus 3 ± 0.5%; P = 0.03; Fig. 2A). The sub-G1 population among AGS cells coincubated with cag+ strains was no different than for AGS cells alone (P = 0.4). Similarly, the proportion of AGS cells in sub-G1 at 72 h was significantly greater in samples coincubated with cag+ than with cag− strains (P = 0.002; Fig. 2).

However, although some cag+ strains clearly affected cell cycle events, this property was not conserved among all isolates examined (Fig. 2A). Within the cag+ group, 7 of 11 strains accelerated progression from G1 into G2-M at 6 h, and 6 (86%) of these 7 strains significantly enhanced movement into sub-G1 by 72 h (Table 1). One additional cag+ strain (117) was associated with the 72-h increase in sub-G1 but failed to induce early (6 h) entry into G2-M. The remaining 3 cag+ strains as well as the 11 cag− strains were not associated with these changes, thus demonstrating a strong (91%) concordance between the early H. pylori-induced progression of cells into G2-M and a later increase in the sub-G1 population. Of interest, a higher percentage of AGS cells cocultured with cag− strains were in G1 at 72 h (50 ± 5.6%) compared with cag+ isolates (41 ± 9%; P = 0.009), suggesting that H. pylori cag− strains may preferentially induce G1 growth arrest. These findings indicate that coincubation with certain cag+ but not cag− strains increases progression of AGS cells into G2-M at 6 h and sub-G1 by 72 h, thereby resulting in the appearance of an enhanced population of cells with subdiploid DNA content.

To determine whether the enhanced sub-G1 population represented apoptotic cells, we examined nuclear morphology in propidium iodide-stained AGS cells that were grown alone or in the presence of H. pylori. Coincubation with H. pylori strain 60190 induced morphological features consistent with apoptosis, including chromatin condensation, as well as nuclear segmentation compared with controls (data not shown).

To independently assess as well as quantitate apoptosis, we also measured the extent of DNA fragmentation present in cytoplasmic fractions of AGS cells after coincubation with H. pylori. Incubation of AGS cells with H. pylori cells significantly increased cytoplasmic oligonucleosomal fragments at both 24 and 48 h (P < 0.001 for each time point; Fig. 3), compared with AGS cells alone. Coculture with cag+ isolates significantly increased nucleosomal release at both 24 and 48 h compared with cag− strains (P < 0.001 for each time point; Fig. 3). To determine whether induction of apoptosis might represent a nonspecific response to bacterial coculture, AGS cells also were incubated with equivalent numbers of Escherichia coli, Shigella flexneri, or Campylobacter jejuni for 24 h, and nucleosomal release was quantitated by ELISA. DNA fragmentation was no different in AGS cells coincubated with these bacterial species compared with AGS cells alone (data not shown), suggesting that in this system, H. pylori specifically stimulated apoptosis. In total, these data indicate that both cag+ and cag− H. pylori strains can induce apoptotic changes in the epithelial cell, but that the effect is more pronounced in the presence of cag+ strains.

H. pylori-induced Cell Cycle Progression and Apoptosis Is Not Mediated by Induction of p53, p21, or MDM2. Next, we sought to determine whether the strain-specific H. pylori-induced modulation of AGS cell cycle correlated with activation of p53 signaling. To verify that the p53 signaling pathway was intact in AGS cells as reported previously (29), we treated cells with 5-fluorouracil or Adriamycin

![Fig. 3. Apoptosis in AGS cells after incubation with different H. pylori strains assessed by DNA fragmentation analysis. AGS cells (5 × 10^4/well) were incubated alone or in the presence of H. pylori (5 × 10^3/well) for 24–48 h, and DNA fragmentation was quantitated by ELISA. H. pylori strains examined included cag+ strains 60190, 166, 291, and 178 (filled symbols); and cag− strains 68, 9366, and 107 (open symbols), and results are expressed as the levels of nucleosomal release relative to controls. Bars, SE.](https://cancerres.aacrjournals.org)
and examined p53 and p21 protein levels. Exposure of AGS cells to either drug markedly elevated p53 and p21 protein levels in a time-dependent manner (data not shown), indicating that our model in vitro system could be used to analyze p53-mediated pathways.

AGS cells were cultured alone or in the presence of five clinical \textit{H. pylori} strains, and expression of p53 and its downstream targets p21 and MDM2 was analyzed at sequential time points. The five strains examined were chosen from among the seven isolates studied in survival (Fig. 1) and apoptosis (Fig. 3) experiments and included strains that were \textit{cagA}+ \textit{vacA} A1 (60190), \textit{cagA}+ \textit{vacA} s1b (291, 166), and \textit{cagA}+ \textit{vacA} s2 (68, 9366). As shown in Fig. 4, expression of p53, p21, and MDM2 was not increased 6–72 h after incubation with either the \textit{cagA}+ \textit{vacA} s1b strain 291 or the \textit{cagA}+ \textit{vacA} s2 strain 9366, and similar effects were seen in AGS cells after incubation with strains 60190, 166, and 68 (data not shown). In fact, a slight time-dependent decrease in levels of protein expression were seen after incubation with all of the strains. Because these findings are opposite to the corresponding alterations in cell cycle induced by these strains (apoptosis from \textit{cag}+ strains and G1 arrest from \textit{cag}− strains), these data indicate that the eukaryotic signaling pathways responsible for \textit{H. pylori}-induced effects on AGS cell cycle events are not mediated by elevations in p53.

**Correlation of \textit{H. pylori} \textit{vacA} Genotypes with Differential Cell Cycle Effects.** Heterogeneity among \textit{cag}− \textit{H. pylori} isolates in their ability to alter cell cycle events suggests that strain-specific traits in addition to the presence of the \textit{cag} island may be important in inducing these responses. We therefore examined AGS cell cycle distribution after coinoculation with 11 \textit{cag}− wild-type \textit{H. pylori} strains that differed in \textit{vacA} allelic types and toxigenicity (Table 1). All six \textit{cag}− strains that significantly induced both G2-M progression and apoptosis possessed a type s1/m1 \textit{vacA} allele, whereas three of the remaining five \textit{cag}− strains possessed a type s1/m2 \textit{vacA} allele (Table 1; \(P = 0.03\)). The six \textit{cag}− strains that increased both G2-M progression and apoptosis also were more likely to be toxigenic for HeLa cells (83%), compared with \textit{cag}+ strains that lacked these effects (40%; \(P = 0.1\); Table 1). These findings raised the hypothesis that particular \textit{vacA} genotypes, as well as genes within the \textit{cag} island (i.e., \textit{picB}), may contribute to strain-specific effects on cell cycle events in vitro.

**Effects of \textit{vacA} and \textit{picB} Inactivation on Gastric Epithelial Cell Cycle Events.** To examine the effects of two known \textit{H. pylori} virulence-related genes on cell cycle progression in gastric epithelial cells, we next cocultured AGS cells with the \textit{cag}− toxigenic \textit{H. pylori} reference strain 60190 or its isogenic \textit{vacA}− null mutant or \textit{picB}− null mutant derivatives. Analyzing cells by flow cytometry, as expected, incubation with strain 60190 induced significant AGS cell accumulation at G2-M at 6 h (\(P = 0.04\); versus controls), followed by a significant increase in the sub-G1 population at 24–72 h (\(P = 0.04\) for each time point versus controls; Fig. 5A). AGS cells incubated with \textit{aphA} (km)-derived and \textit{cat}-derived \textit{H. pylori} mutants demonstrated nearly identical flow patterns (data not shown); therefore, results for isogenic \textit{H. pylori} \textit{vacA}− and \textit{picB}− derivatives at each time point were combined. The parental 60190 strain and \textit{vacA}− null mutant strains did not differ in effects on cell cycle progression up to 48 h (Fig. 5A). However, the parental strain induced enhanced progression of AGS cells into sub-G1 at 48 and 72 h compared with the \textit{vacA}− null mutant (\(P = 0.04\); Fig. 5A). Filtered supernatant containing VacA (10 \(\mu\text{g/ml}\)) from wild-type strain 60190 did not significantly increase apoptosis in the AGS cells (data not shown). Because mutation in \textit{vacA} decreased the sub-G1 population of AGS cells at 48–72 h (Fig. 5A), we incubated AGS cells with strain 60190 \textit{vacA}− in the presence...
of 60190 supernatant containing VacA to determine whether adding the toxin reconstituted the increased sub-G₁ AGS cell population found after coincubation with the wild-type 60190 strain. No additional alterations in cell cycle were found when supernatants were incubated with the vacA⁻ isogenic derivative (data not shown), suggesting that VacA must be presented by H. pylori cells to increase apoptosis.

Coculture of AGS cells with the cagA⁻ derivative and the wild-type strain did not result in significant differences in cell cycle events (data not shown), consistent with previous reports (29). In contrast to the parental 60190 strain, deletion of picB did not induce any significant alterations in $G_1$ to $G_2$-M transition at 6 h (Fig. 5A) and decreased the sub-G₁ AGS cell population at 24 h (Fig. 5A). Nucleosomal release in AGS cell lysates at 24 h was significantly lower in samples incubated with the picB⁻ strains (absorbance, $0.40 \pm 0.04$) compared with the wild-type strain (0.67 $\pm$ 0.08, $P = 0.005$), confirming the decrease in apoptosis. At the 48- and 72-h time points, the parental strain and its picB⁻ mutant induced similar effects (Fig. 5A). In total, these findings indicate that vacA and picB products contribute to cell cycle progression and apoptosis in AGS cells.

**Effect of Inactivating Both vacA and picB in the Same Strain on AGS Cell Cycle Events.** The results described above indicate that loss of vacA and picB may have independent effects on cell cycle progression or apoptosis. To analyze the effect of simultaneous loss of both genes, reciprocal double H. pylori mutants (vacA::km/picB::cat and vacA::cat/picB::km) were created in the strain 60190 background, and their ability to modulate AGS cell cycle distribution was determined. In preliminary studies, we cocultured each vacA⁻/picB⁻ strain with AGS cells and measured toxicogenicity of supernatants for HeLa cells to confirm that vacA expression had been inactivated. The vacA⁻/picB⁻ derivatives failed to induce cytoplasmic vacuolation, in contrast to the wild-type strain and the isogenic picB⁻ mutants (data not shown), indicating that toxin production was successfully interrupted. Data from flow cytometry and DNA fragmentation experiments were similar for experiments involving both double mutants; therefore, results for both mutants were combined. The vacA⁻/picB⁻ derivatives did not induce a significant transition into $G_2$-M at 6 h and significantly ($P = 0.01$ at $24-72$ h) decreased the sub-G₁ population of AGS cells compared with the wild-type strain (Fig. 5A). To confirm that the reduced population of sub-G₁ cells reflected cells undergoing apoptosis, DNA fragmentation was quantitated in AGS cell lysates at 24 h (Fig. 5A). Nucleosomal release in AGS cell lysates at 24 h was significantly lower in samples incubated with the picB⁻ strains (absorbance, $0.40 \pm 0.04$) compared with the wild-type strain (0.67 $\pm$ 0.08, $P = 0.005$), confirming the decrease in apoptosis. At the 48- and 72-h time points, the parental strain and its picB⁻ mutant induced similar effects (Fig. 5A). In total, these findings indicate that vacA and picB products contribute to cell cycle progression and apoptosis in AGS cells.

**Effect of Inactivating Both vacA and picB In the Same Strain on AGS Cell Viability.** Because vacA and picB mutants have independent effects on cell cycle progression and/or apoptosis, we next examined the effect of strains harboring either or both of these mutations on the viability of AGS cells (Fig. 5B). As expected, coincubation with strain 60190 significantly reduced AGS cell survival at all time points compared with AGS cells in medium alone ($P \leq 0.002$ for each time point), a pattern consistent with our earlier results (Fig. 1). At 24 and 48 h, the parental strain caused significantly greater loss of viability than the picB⁻ null mutant ($P \leq 0.05$), but these differences disappeared by 72 h. The parental strain caused significantly greater loss of viability at 48 and 72 h compared with the vacA⁻ null mutant ($P \leq 0.02$ versus 60190). The vacA⁻/picB⁻ double mutant caused significantly less cell death than the parental strain at each time point ($P \leq 0.0001$). In total, these data indicate that expression of vacA and picB together are necessary for the maximal H. pylori-induced modulation of AGS cell cycle progression, consistent with the flow cytometry and DNA fragmentation results.

**DISCUSSION**

Apoptosis is a normal component of epithelial cell turnover in the gastrointestinal tract, but the effect of H. pylori on gastric cell cycle events is not uniform. Carriage of H. pylori is associated with either increased or reduced rates of gastric mucosal proliferation and apoptosis, depending on the study (20–27). H. pylori strain-specific characteristics may contribute to this heterogeneity, as we have reported that strains that possess the cag island and produce a functional cytotoxin are associated with increased epithelial cell proliferation but reduced apoptosis (22). However, other studies have found no differences in levels of epithelial cell growth and loss among H. pylori-colonized persons harboring cag⁺ or cag⁻ strains (24). One explanation for these discrepant findings is that host differences may contribute to cell turnover variation in persons carrying H. pylori.

Binding of class II MHC molecules by H. pylori induces gastric epithelial cell apoptosis (36). Signaling pathways differ after engagement of bacterial superantigens to class II MHC molecules, depending on host haplotype (36), and thus variations in the apoptotic response to H. pylori may reflect heterogeneity of MHC genotypes. Inflammatory mediators present in H. pylori-colonized mucosa also may influence rates of epithelial cell growth and loss. For example, tumor necrosis factor α, a proinflammatory cytokine present in H. pylori-infected mucosa, can inhibit apoptosis in certain cell types (44–46). Another possible mechanism is that certain H. pylori strains express proteins that can directly affect cell cycle events. By using an in vitro model system, we have identified specific components that may vary the ability of H. pylori strains to modulate gastric epithelial cell cycle events, which may explain in part the heterogeneity in cell growth and death rates reported previously in H. pylori-colonized persons (20–27).

Because cag⁺ and cag⁻ H. pylori strains are associated with differing risks for development of distal gastric cancer (14–16), we first compared the ability of clinical cag⁺ and cag⁻ isolates to alter gastric epithelial cell cycle events in vitro. Our results demonstrate that a majority of cag⁺, but not cag⁻, strains reduce AGS cell viability, induce a transient accumulation of cells in $G_2$-M, and increase apoptosis by 72 h. These effects were not seen when cells were incubated with E. coli, S. flexneri, or C. jejuni, which is consistent with the previous demonstration that C. jejuni does not alter gastric epithelial cell viability or apoptosis in vitro (28, 47), indicating that the response of AGS cells to H. pylori strains was specific. In contrast, Wagner et al. (28) reported no difference in the ability of H. pylori to alter proliferation or apoptosis in vitro when strains were analyzed by cagA status and toxin production. These discordant findings may be attributable to differences in study design. Wagner et al. (28) used different gastric epithelial cell lines to assess apoptosis and proliferation, and the number of in vitro passages for the H. pylori strains examined was not reported. We cocultured minimally passaged H. pylori strains with asynchronously grown AGS cells so as to recapitulate events occurring within actively replicating gastric mucosa. We examined a larger number ($n = 21$) of clinical isolates and analyzed the effect on cell cycle, not only by segregating strains on the basis of cagA genotype and toxigenicity, but also by vacA genotype. This characterization of strain-specific modulation of cell cycle events and apoptosis facilitated the identification of specific bacterial components that regulated these events. We also found that H. pylori-
induced apoptosis occurred relatively late after coculture (by 72 h), in contrast to previous studies examining the effects of *H. pylori* on epithelial cell cycle events *in vitro* (29, 33). However, we studied *in vivo*-adapted *H. pylori* clinical strains that may differ from *in vitro*-passaged reference strains used by other investigators (29, 33). Consistent with this hypothesis, apoptosis was more rapidly induced in our system when reference strain 60190 was used, indicating that *in vivo* selection may attenuate or delay the apoptotic response to *H. pylori* *in vitro*.

Analysis of a.picB− null mutant *H. pylori* strain indicated that expression of the *picB* product contributes to AGS cell cycle progression and to an apoptotic response. The predicted protein product of *picB* shares homology with the *Bordetella pertussis* toxin secretion protein (PtlC) and the VirB4 protein from *Agrobacterium tumefaciens*, both of which are involved in bacterial type IV secretion pathways (8). The current finding that *picB* is a necessary component in the *H. pylori*-induced apoptotic response is consistent with earlier data that demonstrated that *picB*, as well as other genes within the cag island, were necessary for induction of interleukin 8 from gastric epithelial cells with the *cag* island or of *vacA* expression of the *picB* derivative was incubated with supernatant containing VacA derived from the wild-type 60190 strain. Similarly, purified supernatant from wild-type 60190 alone only slightly increased apoptosis in AGS cells. It is possible that higher concentrations of VacA-containing supernatant than those used in our study (10 μg/ml) might have significantly increased apoptosis, similar to results reported by Rudi et al. (35). However, the absence of apoptosis in the setting of vacuolation also suggests that cell vacuolation may not be related to enhanced apoptosis. A potential explanation for these findings is that VacA may need to be specifically presented by the *H. pylori* cell to interact with a signal transduction pathway that stimulates apoptosis. Another possibility is that induction of apoptosis is dependent on the amount of VacA present intracellularly. For example, *Staphylococcus aureus* α-toxin only induces apoptosis at low concentrations (<300 nm; Ref. 51). Finally, the ability of VacA to stimulate apoptosis may depend on environmental conditions, because pH variations can alter VacA structure (52).

The results of the experiments focusing on *picB* and *vacA* raise the hypothesis that their gene products may have complementary effects on AGS cell cycle events, because inactivation of both genes together reconstituted the phenotype observed when AGS cells were cocultured with clinical *cag*− strains. In addition, inactivation of *picB* predominantly modulated cell cycle events early after coincubation with *H. pylori*, whereas loss of *vacA* had effects later during the time course. These complementary effects are consistent with the linkage disequilibrium reported previously for *vacA* alleles and presence of the *cag* island (18) and may indicate natural selection based on related interactions with host epithelial cells. However, cocoinfusion of AGS cells with the *vacA*/*picB*− double mutant strains still resulted in apoptosis, at levels similar to those produced by *cag*− wild-type isolates, suggesting that *H. pylori* determinants independent of the *cag* island or of *vacA* may affect epithelial cell cycle events. The recent observation that urease, a highly conserved *H. pylori* constituent, can induce gastric epithelial cell apoptosis *in vitro* by binding to MHC class II molecules (53) is consistent with this hypothesis. Thus, *H. pylori* appears to possess a repertoire of both conserved and nonconserved activation determinants that are capable of affecting epithelial cell growth and death *in vitro*.

Expression of *p53* can inhibit cell growth and/or induce apoptosis, depending on the type and cell conditions and, *p53* overexpression has been demonstrated in gastric tissue from *H. pylori*-colonized children compared with children without the organism (23). Because the majority of *cag*+ strains in the current study increased apoptosis, whereas *cag*− strains induced G1 arrest, we investigated whether these alterations were mediated by *p53*. However, levels of *p53*, *p21*, and MD2 were essentially unchanged, and even slightly reduced, after coculture with either *cag*+ or *cag*− strains, findings that are consistent with previous reports (33). The uniformity of responses among all strains tested, combined with the fact that these findings are opposite to the corresponding alterations in cell cycle induced by the same strains (apoptosis from *cag*− strains and G1 arrest from *cag*+ strains), indicates that modulation of the epithelial cell cycle by *H. pylori* is not dependent upon induction of *p53*. Furthermore, these results suggest that *in vivo* elevations of *p53* previously demonstrated (23) may result from mediators present in concomitantly inflamed gastric tissue, and not from a direct *H. pylori* effect. Consistent with the current data, other investigators have reported that signaling pathways independent of *p53* may be involved in *H. pylori*-induced apoptosis (28, 29, 35, 47). For example, *H. pylori*-induced apoptosis in certain gastric cells appears to be mediated by activation of the CD95 (APO-1/Fas) receptor and ligand system (28, 35, 47). In addition, induction of apoptosis in AGS cells after incubation with *H. pylori* is accompanied by increased expression of the proapoptotic protein Bak (29). In total, the literature and our current data suggest that *H. pylori*-induced apoptosis is mediated by multiple eukaryotic signaling cascades that are not dependent upon increased *p53* levels.

In conclusion, we have demonstrated that the diminished AGS cell viability, progression to G2-M, and apoptosis associated with coincubation with particular *cag*+ *H. pylori* strains are mediated in part by expression of *vacA* and by genes in the *cag* island. These results suggest that differential expression of *H. pylori* genes *in vivo* may in part explain heterogeneity in levels of gastric mucosal cell proliferation and apoptosis.

ACKNOWLEDGMENTS

We thank Tirsha Dukes and Tyler Richmond for excellent technical assistance.

REFERENCES


Helicobacter pylori Strain-specific Genotypes and Modulation of the Gastric Epithelial Cell Cycle

Richard M. Peek, Jr., Martin J. Blaser, Deborah J. Mays, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/24/6124

Cited articles
This article cites 53 articles, 29 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/24/6124.full.html#ref-list-1

Citing articles
This article has been cited by 54 HighWire-hosted articles. Access the articles at:
/content/59/24/6124.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.