Increased Gastric Epithelial Cell Apoptosis Associated with Colonization with cagA + Helicobacter pylori Strains

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INTRODUCTION

Carriage of Helicobacter pylori in the human stomach is associated with increased risk for peptic ulcer disease, distal gastric adenocarcinoma, and gastric B-cell mucosa-associated lymphoid tissue lymphoma (1). In developed countries, strains of H. pylori that carry the cag pathogenicity island, a 35–40-kb DNA fragment encoding a series of virulence-related genes associated with an extracellular secretory apparatus (2), are associated with a greater risk of peptic ulcer disease, distal gastric adenocarcinoma, and gastric B-cell mucosa-associated lymphoid tissue lymphoma. H. pylori colonization was assessed by histology, biopsy urease test, and serology. Proliferation was increased 2-fold in both antrum and corpus in H. pylori-positive patients, was not related to H. pylori cagA status, and was positively correlated with histological gastritis. Apoptosis was increased in the antrum and body only in patients with cagA-positive H. pylori strains. Antiparietal cell antibodies were not more prevalent in H. pylori colonization, and their presence was inversely related to epithelial apoptosis scores we therefore conclude that in patients with nonulcer dyspepsia, H. pylori carriage is associated with increased proliferation. Furthermore the cag pathogenicity island is associated with increased apoptosis. Our results do not support the hypothesis that there is a relative deficiency of gastric epithelial cell apoptosis associated with the carriage of cagA-positive strains. Host factors may be more important than bacterial products in determining the long-term outcome of H. pylori colonization.

ABSTRACT

Gastric colonization by Helicobacter pylori is a risk factor for noncardia gastric cancer. The association between H. pylori and cancer may be attributable to increased epithelial cell turnover, possibly related to anti-gastric antibodies. Two previous studies reported a disproportionate increase in proliferation relative to apoptosis in patients with H. pylori strains expressing the virulence-related cagA gene. This has led to the hypothesis that an abrogation of apoptosis by cagA-positive strains may promote neoplasia. We, therefore, examined the effect of H. pylori on gastric epithelial proliferation, apoptosis, and the presence of serum antiparietal cell antibodies in a large prospective study. Proliferation and apoptosis were evaluated “blindly” using validated immunohistochemical methods in two antral and two gastric corpus biopsies from 60 patients with nonulcer dyspepsia, and results were correlated with the presence of serum antiparietal cell antibodies. H. pylori colonization was assessed by histology, biopsy urease test, and serology. Proliferation was increased 2-fold in both antrum and corpus in H. pylori-positive patients, was not related to H. pylori cagA status, and was positively correlated with histological gastritis. Apoptosis was increased in the antrum and body only in patients with cagA-positive H. pylori strains. Antiparietal cell antibodies were not more prevalent in H. pylori colonization, and their presence was inversely related to epithelial apoptosis scores we therefore conclude that in patients with nonulcer dyspepsia, H. pylori carriage is associated with increased proliferation. Furthermore the cag pathogenicity island is associated with increased apoptosis. Our results do not support the hypothesis that there is a relative deficiency of gastric epithelial cell apoptosis associated with the carriage of cagA-positive strains. Host factors may be more important than bacterial products in determining the long-term outcome of H. pylori colonization.
MATERIALS AND METHODS

Patients and Biopsies. Sixty patients were recruited prospectively at St. Luke’s-Roosevelt Hospital in New York. Inclusion criteria were a clinical indication for diagnostic esophagogastroscopy, age ≥18 years, and ability to give informed consent. Exclusion criteria were evidence of active bleeding; previous gastric surgery; use of nonsteroidal anti-inflammatory drugs, proton pump inhibitors, Histamine (H2)-receptor antagonists, bismuth compounds or antibiotics within the previous 3 months; pregnancy; and endoscopic findings of ulcers, masses or bleeding lesions. The study was approved by the Investigational Review Board of St. Luke’s-Roosevelt Hospital Center.

Informed consent was obtained from each subject prior to the endoscopy, and 10 ml of blood was taken for ELISAs to measure total anti-H. pylori IgG and anti-CagA IgG, performed as described previously (3), and for autoantibodies (see “Autoantibodies”). The presence of serum antibodies to the CagA protein of H. pylori was used as a marker of H. pylori strains carrying the cag island. Five gastric biopsies were obtained during endoscopy using disposable forceps with a cup diameter of 9 mm (Microvasive, Watertown, MA); three were from the antrum [two fixed in formalin for histology and one for the biopsy urease test (CLOtes; Tri-Med Inc, Lenexa, KS)] and two were from the corpus (for histology). Histological examination of sections stained by H&E was performed by a single pathologist (Z. H.), who was blinded to the clinical data, serology, and biopsy urease test data. Biopsies from one patient were excluded because the formalin-fixed tissues could not be located. In three additional patients, there were too few well-oriented glands in the corpus biopsies for this site to be evaluated. Gastritis was scored according to the updated Sydney system (44).

Patients were considered positive for H. pylori if two or more of the following tests were positive: biopsy urease test, histology, total anti-H. pylori IgG, or anti-CagA IgG.

Measurement of Apoptosis. Apoptotic epithelial cells were identified in 4-μm thick formalin-fixed biopsy specimens by the use of TUNEL as described previously (31). Apoptotic cells were counted only in complete, well-oriented gastric glands for both antrum and corpus. The mean number of apoptotic cells per gland for each individual patient at each anatomical subsite was determined after evaluating all of the well-oriented glands in both biopsies taken at each anatomical subsite. The mean number of gastric glands scored in the antrum was 43.3 (range, 7–113) and for the corpus was 32 (range, 7–64). Assessment of the reproducibility of an observer to score apoptotic cells in this study was tested by randomly resoring 20% of the slides. Thus, in 11 cases, the block was recut, and sections were restained and counted on two separate occasions. For another 13 cases, the same stained slides were counted on two separate occasions. From these data, the intraclass correlation coefficients for cell counting reliability was calculated to be 0.922 using the method described by Shrout and Fleiss (45).

Measurement of Proliferation. Proliferating epithelial cells were identified by immunostaining for the Ki-67 antigen using the Mib-1 monoclonal antibody and a microwave accentuation technique (31). The method for counting cells was as described for apoptotic cells above, and the data were expressed as the average number of positively labeled cells per gland. The mean number of glands counted in the antrum was 14.3 (range, 4–22) and for the corpus 11.2 (range, 4–20). Proliferation reproducibility data were tested in 24 (20%) of the total number examined) randomly chosen slides from the antrum and corpus. The intraclass correlation coefficient for cell-counting reliability was 0.90.

Autoantibodies. Antiparietal cell antibodies were determined by a standardized, semiquantitative indirect fluorescent procedure (46) using mouse stomach/kidney substrate (Kallestad, Sanofi Pasteur), in comparison with positive and negative control sera (known patient samples) and commercial (Kallestad) controls. Sera were considered positive for antiparietal cell antibodies if fluorescent staining of the parietal cells, but not the cytoplasm and luminal border of the kidney tubules, was observed. Initial screening for reactivity was performed on sera diluted 1:20 in PBS. Sera that produced a specific pattern of fluorescence were considered positive, and serial 2-fold dilutions of each positive sample were restested to determine the autoantibody titer.

Statistical Analysis. Comparisons between groups were analyzed by the Mann-Whitney U test, and evaluation of correlation was assessed by Spearman’s ranked correlation. Differences between proportions were evaluated by χ2 with Yates correction. In all cases, a 2-tailed value of P < 0.05 was used to signify statistical significance.

RESULTS

Patient Characteristics. The mean age of the 60 patients was 56.4 years (range, 20–91) and 36 were female. Of the female patients, 22 were H. pylori-positive, and 16 of these carried cagA positive strains. Of the 24 male patients, 17 were H. pylori-positive and 13 carried cagA positive strains. The ethnic distribution was: Hispanic: 30 patients [23 H. pylori-positive (17 cagA positive)]; African-American: 23 patients [16 H. pylori-positive (12 cagA positive)]; Caucasian: 7 patients, all of whom were negative for H. pylori. Chronic gastritis was present in 38 (68%) of 56 evaluable patients. Eleven patients had chronic gastritis and both gastric atrophy and intestinal metaplasia, and two other patients had gastritis with intestinal metaplasia but no atrophy.

Proliferation. The proliferation labeling index was significantly increased in H. pylori-positive compared with H. pylori-negative patients (Figs. 1 and 2). In the antrum, the mean number of proliferating cells per gland in the negative patients (± SD) was 9.6 (± 3.9) compared with 15.9 (± 6.8) in patients with cagA + H. pylori, and 17.7 (± 5.0) in patients with cagA– H. pylori (P < 0.05 for each comparison). In the gastric corpus, the mean number of proliferating cells per gland in the negative patients was 8.6 (± 4.2) compared with 14.6 (± 9.5) in patients with cagA + H. pylori, and 15.7 (± 7.3) in patients with cagA– H. pylori (P < 0.05 for each comparison). Differences between the scores in persons carrying cagA-negative and cagA-positive strains were not statistically significant in either antrum or corpus. Scores for proliferation were positively correlated with the degree of histological gastritis (classified as none, mild, moderate, or severe) for both antrum (r2, 0.57; P < 0.0001) and corpus (r2, 0.37; P < 0.005).

Apoptosis. As for proliferation, H. pylori positive patients had increased numbers of apoptotic cells in both antrum and corpus compared with negative patients, but this increase was statistically significant only for those with cagA-positive strains (Figs. 3 and 4). The mean number of apoptotic cells per antral gland in H. pylori-negative patients was 1.5 (± 1.5), compared with 3.0 (± 3.3) in patients with cagA + H. pylori (P < 0.05), and 2.5 (± 3.0) in patients with cagA– H. pylori. Similar data were obtained for the gastric corpus. In the corpus, the mean number of apoptotic cells per gland in H. pylori-negative patients was 1.1 (± 1.3), compared with 1.8 (± 2.0) in patients with cagA + H. pylori (P < 0.05), and 1.3 (± 1.1) in patients with cagA– H. pylori. The difference between H. pylori-negative patients and patients with cagA– organisms was not statistically significant. Scores for apoptosis were not correlated with the grade of histological gastritis.

Proliferation:Apoptosis Ratio. For each patient at each anatomical subsite, a proliferation:apoptosis ratio was derived by dividing the mean number of proliferating cells per gland by the mean number of apoptotic cells per gland. The mean antral proliferation: apoptosis ratio was 7.5 (± 8.8) in the H. pylori-negative patients, 8.0 (± 10.2) in patients with cagA + H. pylori and 9.2 (± 7.7) in patients with cagA– H. pylori. In the gastric corpus, these values were 9.0 (± 9.0), 9.3 (± 10.0), and 9.3 (± 13.8), respectively. None of these differences were statistically significant.

Relationship between Autoantibodies, H. pylori Presence, and Parameters of Gastric Epithelial Cell Turnover. Classical antiparietal cell antibodies were found in the serum of three H. pylori-negative patients, of four patients with cagA + H. pylori, and of two patients with cagA– H. pylori strains. The proportion of patients with H. pylori (either cagA+ or cagA–) who had antiparietal antibodies...
was not significantly different from the proportion in the *H. pylori*-negative patients. To evaluate whether the presence of antiparietal cell antibodies was associated with increased cell turnover, apoptosis, proliferation, and the proliferation:apoptosis ratio were compared in patients with antiparietal cell antibodies and patients who did not have antibodies. Apoptosis in the antrum was statistically significantly lower, and the proliferation:apoptosis ratio in the antrum was significantly higher, in the 9 patients with antiparietal cell antibodies than in the 51 patients without such antibodies (Table 1).

**DISCUSSION**

Our results show that the presence of *H. pylori* in patients with nonulcer dyspepsia is associated with both increased gastric epithelial cell apoptosis and increased proliferation. Increased epithelial apoptosis and proliferation were observed in both antrum and corpus. Although proliferation was increased whether or not the colonizing *H. pylori* strain was *cagA*-positive, apoptosis was statistically significantly increased only in patients with *cagA*-positive strains, which suggests that there may be a role for genes within the *cag* pathogenicity island in the stimulation of gastric epithelial apoptosis. These findings are in accordance with recent studies in cell culture by some groups (25, 26) but not all (21). The involvement of genes within the *cag* pathogenicity island in apoptosis is consistent with the functions of several of these genes in forming a type IV bacterial secretion system, enabling the transfer of bacterial products, including the CagA protein itself, into eukaryotic cells (2). However, although *cagA* and its product, the CagA protein, are good markers for the presence of the *cag* island, the CagA protein does not appear important in apoptotic signaling to gastric epithelial cells, because *cagA*—isogenic mutants induce a similar amount of apoptosis in gastric epithelial cells in vitro compared with wild-type bacteria (22, 25).

Our results confirm previous positive associations between parameters of epithelial proliferation and gastric inflammation scores (16, 31). Previous studies have reported positive (31, 36, 37), inverse (39), or no (28, 30, 33, 34, 38) association between inflammation scores and apoptosis. These inconsistencies are not explained by study designs, nor by the endoscopic findings or clinical details of the patients studied. This lack of a clear correlation between inflammation and apoptosis, therefore, suggests either that the inflammatory response is not important in determining the extent of epithelial apoptosis in response to *H. pylori* in vivo or that the measurement of the inflammatory response to *H. pylori* by histological criteria alone does not adequately reflect proapoptotic inflammatory stimuli present in the *H. pylori*-colonized gastric mucosa.

Epithelial apoptosis is considered to be essential for the mainte-
nance of gastrointestinal homeostasis and health (47). It has been suggested that H. pylori colonization stimulates the production of autoreactive antigastic antibodies (42, 48), thus causing accelerated epithelial apoptosis and subsequently gastric atrophy (32). Such a model is supported by the increased frequency of serum antigastic antibodies in persons colonized by H. pylori (32, 42, 43, 49) and by their rapid disappearance after H. pylori eradication therapy (50). However, the control antigens for these studies have not been standardized nor described sufficiently to rule out artifactual autoreactivity. Nevertheless, even using conventional rodent antigens, Steininger and colleagues (32) reported antigastic antibodies more frequently in H. pylori-positive infected patients (6 of 84) than in controls (0 of 32).

Our failure to confirm an increased frequency of classical antiparietal cell autoantibodies could be attributable to the different ethnic background of our patients (African-American and Hispanic) compared with the northern Europeans, or to differences in assay methodology. Overall, however, our results show no evidence to support the involvement of autoimmune-mediated apoptosis in the pathogenesis of H. pylori-associated gastritis in African American and Hispanic patients with nonulcer dyspepsia.

In the present study, the presence of cagA-positive strains was associated with increased apoptosis. This result challenges the hypothesis initially suggested by our group (31) and supported by the results of Rokkas et al. (36), that cagA may play a role in abrogating the apoptotic response to H. pylori. Are these discrepancies regarding the effect of H. pylori cag status on gastric cell turnover attributable to methodological differences? All previous studies of apoptotic gastric epithelial cells have used the TUNEL stain, with similar protocols. However, several different methods have been used to assess proliferating cells including immunohistochemistry using antibodies directed against Ki-67 (Ref. 31 and the present study) or PCNA (33) and by the quantitation of silver-stained nucleolar organizing regions (36). Although there is no standardized methodology for the measurement of gastric epithelial proliferation, Ki-67 is superior to PCNA as an endogenous marker of cells in S phase in gastric mucosal biopsies (51) and in the human colon is more reproducible than PCNA (52).

Similarly, there is no standardized method of counting and quantifying positively stained cells, nor a minimum number of glands to evaluate (53). To minimize potential gland to gland variability, we examined the maximum number of glands cells possible, representing at least 3000 cells for apoptosis and 2000 for proliferation, numbers far in excess of the previous studies. Documentation of the high reproducibility of cell counts for each patient adds confidence to the...
findings of the present study. We consider that the methods used to measure cell turnover in this study are the currently available optimum for endoscopic biopsies. Another advantage of the present study is that we were able to examine apoptosis and proliferation in biopsies taken from both antrum and corpus. The corpus only has been examined once previously, in a study of patients with duodenal ulcers (35), in whom corpus involvement by H. pylori is generally mild.

What other reasons can explain the differences between the present results and those of our earlier collaborative study (31), in which carriage of cagA-positive strains was not associated with increased epithelial apoptosis? One major difference is the ethnicity and clinical status of the patient populations that were examined. The patients in the present study were mainly Hispanic and African-American, and had no ulcers at endoscopy. This contrasts with the population in the previous study who were recruited at a Veterans’ Affairs Medical Center (31) and were almost entirely Caucasian males, almost one-half of whom had peptic ulcers. In both human colonization and animal models, the epithelial and inflammatory responses to H. pylori depend not only on the type of infecting strain but also on the genetic background of the host (54–57). Thus, the most likely explanation for the discrepant results in these two clinical studies is the varying genetic compositions of the populations studied.

It should be emphasized that although cagA status is defined as positive or negative for any given individual, there is no widely used standard method to determine cag status. In the present study and a previous one (36), H. pylori cag status was determined by the presence of serum antibodies to the highly immunogenic CagA protein. In contrast, in our earlier study, the presence of the cagA gene was determined by PCR of bacteria cultured from gastric biopsies (31). Both methods are indirect compared with immediate PCR of an endoscopic biopsy (38). A further complexity is the coexistence of multiple H. pylori strains in a single host, including both cagA-negative and -positive variants (59). Because deletion of all or part of the cag island may occur in H. pylori subpopulations in vivo or in vitro, the detection of circulating antibodies to the CagA protein indicates colonization by cag-positive strains but does not rule out mixed infections. Because there is no widely accepted standard for the diagnosis of H. pylori infection, we defined patients as H. pylori-positive in this study if at least two of four tests were positive. Using these criteria, one patient was classified as H. pylori-positive based on serology alone but with negative rapid urease test and histology. Whether this single patient was truly H. pylori-positive (with false negative biopsy tests) or had undergone spontaneous or antibiotic-induced clearance of H. pylori is not known, but even if this patient were reclassified as H. pylori-negative, the overall conclusions of our study would not be altered.

In addition to genes within the cag pathogenicity island, several other bacterial factors may be markers of clinical outcome. These include expression of the vacuolating cytotoxin (VacA), products of the iceA gene, and the babA2 gene, which determines binding to the Lewis blood group antigen (4, 60–62). Although the possible relationship between these markers and epithelial cell turnover is worthy of further study, in practice it may be difficult to separate their effects from those of the cag pathogenicity island because there is often cosegregation of cag, babA2, and the vac sIa genotype in H. pylori strains in Western populations (60).

In conclusion, the evaluation of proliferation and apoptosis in this study has been the most extensive to date. We found that epithelial cell proliferation was higher in H. pylori-positive persons, irrespective of cagA status, and that genes within the cag pathogenicity island may play a role in the induction of apoptosis. Furthermore, we found no evidence for the involvement of antigastric antibodies in the stimulation of apoptosis. Because in some populations, cagA-related genes are associated with an abrogated apoptotic response, whereas in the current study they are associated with increased apoptosis, we speculate that host factors may be at least as important as bacterial factors in determining gastric mucosal responses to H. pylori.

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