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Relationship of *Helicobacter pylori* CagA Status to Gastric Cell Proliferation and Apoptosis

THEODORE ROKKAS, MD, PhD, SPIROS LADAS, MD, CHRISTOS LIATSOS, MD, EVANGELIA PETRIDOU, MD, GEORGE PAPATHEODOROU, STAMATIS THEOCHARIS, MD, ANDREAS KARAMERIS, MD, and SOTIRIOS RAPTIS, MD

Despite the fact that the association of *Helicobacter pylori* with an increased risk of gastric cancer is well documented, the exact mechanisms of this association have not been elucidated. Our aim was to shed some light on these mechanisms by studying the relationship of *H. pylori* CagA status to gastric cell proliferation and apoptosis, since both play an important role in gastrointestinal epithelial cell turnover and carcinogenesis. We studied fifty patients [32 men, 18 women, median age 39.5 years (range 18–67)], referred for upper gastrointestinal endoscopy, from whom antral biopsies were taken. On biopsy specimens gastritis was estimated by scoring the severity of inflammatory infiltrate, and the presence of atrophy and intestinal metaplasia were also noted. The gastric cell proliferation index (PI) was estimated by AgNOR staining, the epithelial apoptotic index (AI) was measured by special staining for apoptosis, and CagA status was determined serologically by immunoblotting the sera of patients against *H. pylori* antigens. Thirty-eight (76%) of the 50 patients were *H. pylori* (positive) and 12 (24%) *H. pylori* (negative). Among the 38 *H. pylori*(+) patients, 28 (73.6%) were CagA(+) and 10 (24.6%) CagA(−). In the *H. pylori* CagA(+) and CagA(−) groups, the PI values [median (ranges)] were 5 (4–7) and 3.7 (3.5–5.5), respectively ($P < 0.05$). In addition the difference in PI between the *H. pylori* CagA(+) and *H. pylori*(−) groups was highly significant ($P < 0.001$). Concerning apoptosis, in the *H. pylori* CagA(+) and CagA(−) groups, the values for AI were 1 (1–30) and 5.5 (1–35), respectively ($P < 0.05$). In addition, the difference in AI between the *H. pylori* CagA(−) and *H. pylori*(−) groups, was significant ($P < 0.05$). We conclude that *H. pylori* CagA(+) strains induce increased gastric cell proliferation, which is not accompanied by a parallel increase in apoptosis. This might explain the increased risk for gastric carcinoma that is associated with infection by *H. pylori* CagA(+) strains.

KEY WORDS: *Helicobacter pylori* infection; CagA status; proliferation; apoptosis; gastric carcinogenesis.

In recent years a close relationship between *Helicobacter pylori* infection and gastroduodenal pathology has been found. Thus *H. pylori* has been recognized as the principle cause of type B gastritis and peptic ulcer disease (1–5), and, in addition, a close association between *H. pylori* and gastric malignancy has been found (6–13), mainly on the basis of seroepidemiological data. Furthermore, *H. pylori* has been classified as a type I carcinogen for gastric cancer (14) by the International Agency for Research on Cancer

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From the Gastroenterology Unit, Immunology Laboratory, and Histopathology Department, 401 Army General Hospital, Athens, Greece; and 2nd Department of Internal Medicine, Evangelismos Hospital, Medical School, Athens University, Athens, Greece.

Address for reprint requests: Dr. Theodore Rokkas, 192B Alexandras Ave., Athens 115 21, Greece.

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(IARC). This association has been found significantly greater in *H. pylori* strains that possess the cytotoxin-associated gene *cagA* (15–18), a key gene of the so-called "pathogenicity island" (19). However, despite the well-documented association of *H. pylori* with an increased risk of gastric malignancy, the mechanisms by which it affects gastric mucosa have not been fully elucidated as yet and more research is needed. One possible assumption is that *H. pylori* may affect the normal balance between gastric epithelial cell proliferation and cell death, thus interfering with the maintenance of gastric mucosal homeostasis. Increased cellular proliferation rates characterize malignant tissue and, indeed, increased gastric epithelial cell proliferation in *H. pylori* infection has been reported (20–26). On the other hand, in chronic *H. pylori* infection, there is a notable lack of epithelial necrosis (27, 28), suggesting that other forms of cell death, such as apoptosis (the programmed cell death), may be involved.

From all the above it is apparent that an investigation of the relationship of *H. pylori* CagA status to cell proliferation and apoptosis could contribute to the elucidation of the mechanisms that regulate the development of gastric malignancy in *H. pylori* infection. The aim of the present prospective study, therefore, was to examine this relationship.

MATERIALS AND METHODS

Subjects

For the purposes of this study we prospectively studied 50 dyspeptic patients [32 men, 18 women, median age 39.5 (range 18–67)], referred for upper gastrointestinal endoscopy. None of the subjects studied had undergone upper gastrointestinal surgery, and none had used antibiotics, bismuth, PPIs, or NSAIDs during the previous eight weeks. Patients on NSAIDs were excluded because these drugs may influence apoptosis in gastric mucosa (29). During endoscopy antral biopsies were taken from each patient for *H. pylori* detection and histological assessments. Immediately after endoscopy, a blood sample was drawn from each patient and, after centrifugation at 3000*g* for 10 min, sera were collected and stored at −20°C before use for serological assay to determine the *H. pylori* CagA status.

***Helicobacter pylori* Detection.** In each subject *H. pylori* was sought in two ways, ie, the rapid urease test (30) and histology. For histological detection, slides were stained with Giemsa, modified for *H. pylori,* and then the presence of *H. pylori* was microscopically evaluated (31). Patients were considered to be *H. pylori*(+) when the bacterium was identified in both tests and negative when the bacterium was not identified in either test. For the purposes of this study, patients with only one test positive were not included.

Histological Assessments

Antral mucosal biopsy specimens were immediately fixed in buffered neutral formalin and embedded in paraffin. Sections were then stained with hematoxylin and eosin for histological evaluation of the severity of gastritis, with modified Giemsa staining for *H. pylori* detection, as developed above, AgNOR staining for proliferation assessment, and special staining for apoptosis measurement.

Gastritis Evaluation. In hematoxylin-eosin sections, the diagnosis and evaluation of gastritis, atrophy, and intestinal metaplasia was based on accepted criteria (32). Severity was estimated separately for acute and chronic gastritis by scoring the inflammatory infiltrate in the lamina propria. In addition, the presence of atrophy and intestinal metaplasia was also reported. The inflammatory infiltrate was semiquantitively scored for polymorphonuclear leukocytes (acute gastritis) and mononuclear cells (chronic gastritis) as follows: (a) acute gastritis: 0 = absence of any infiltration, 1 = mild infiltration (polymorphonuclear leukocytes occasionally infiltrate glandular structures), 2 = the above in moderate degree, and 3 = severe infiltration (polymorphonuclear leukocytes infiltrating the glandular lumen); and (b) chronic gastritis: 0 = absence of any infiltration, 1 = mild infiltration (sparse mononuclear cells detected through the lamina propria), 2 = the above in moderate degree, and 3 = severe (diffuse and severe mononuclear cell infiltrate). All histological slides were reviewed by the same experienced pathologist.

Cell Proliferation. Cell proliferation was assessed by AgNOR counting, a method that has been used successfully in previous studies (33, 34). Briefly, paraffin sections were dewaxed and hydrated using graded ethanol and distilled water. The silver colloid solution was prepared by dissolving gelatin in 1% aqueous formic acid in a concentration of 2%. This solution was mixed with 50% aqueous silver nitrate to obtain the final working solution. This was dropped onto the sections and left for 30 min at room temperature. Then the sections were washed with deionized water, treated with xylene, and mounted in a synthetic medium. For the AgNOR counting procedure, the slides were examined using a 100× oil immersion lens at a final magnification of 1000. AgNORs appeared as black dots either dispersed in the nucleus of the cells or clustered within one or two nucleoli. AgNOR counting was focused in the glandular neck region, in nonmetaplastic areas, which best correspond to the region of cell proliferation (34). Two hundred cells, where available, were randomly selected from each specimen, and the AgNOR dots were counted individually, carefully focused, even when occuring in large aggregates. The results were then expressed as the mean number of AgNORs per nucleus, and this was named the proliferation index (PI). All AgNOR measurements were confirmed by image analysis (Digital Image Systems, DIS-200, Hellas) and were performed blindly by the same experienced pathologist.

Apoptosis. Epithelial apoptosis was quantitated in situ by using the ApopDETEK Cell Death Assay System (Enzo Diagnostics, Inc., Farmingdale, New York), based on a method, that has been shown to give excellent results on formalin-fixed, paraffin-embedded tissue sections (35, 36). The number of positive cells per 100 epithelial cells was expressed as the apoptotic index (AI). All apoptotic evalu-

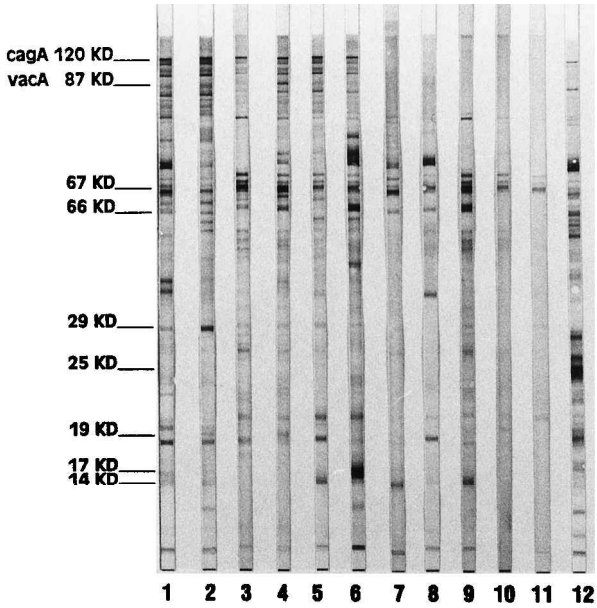

Fig 1. Immunoblot patterns obtained with sera from a sample of 12 patients studied. The 120-kD band is the *cagA* gene product. The remaining bands are: 87 kD, *vacA* gene product; 67 kD, flagellin protein; 66 and 29 kD, subunits of urease enzyme; 25, 19, 17, and 14, *H. pylori* proteins of which little is known. Numbers on the horizontal axis represent patients. Patients 1–6 and 12 were CagA(+), and patients 7–11 were CagA(−).

ations were made by the same experienced examiner unaware of the infection status, gastritis, and proliferation results.

H. pylori CagA Status

H. pylori CagA status was determined serologically by immunoblotting the sera of patients against *H. pylori* antigens (37). This was performed by using the commercial immunoblot kit manufactured by AID GmbH, Strasburg, Germany. According to this method, antigens of *H. pylori* are electrophoretically separated by SDS-PAGE and migrate through the gel as fine bands, according to their molecular weights. After electrophoresis, the bands are transferred to nitrocellulose membranes and the band pattern is analyzed with the kit-specific template. Antibodies against *H. pylori* antigens of 120, 87, 67, 66, 29, 25, 19, 17, and 14 kD can be observed (Figure 1).

Statistical Analysis

All statistics were computed using a suitable program (GraphPad, Prism, Version 2.0). The results are represented graphically as boxes and whiskers, whereas data in the text are expressed as median values with ranges. As most data showed skewing, comparisons between the multiple groups were performed using nonparametric Kruskal-Wallis analysis of variance. If the result of this was significant, then simple comparisons between pairs of groups were performed with the nonparametric Mann-Whitney U test (38). Correlation coefficients were calculated using the nonparametric Spearman rank test. Comparisons between proportions were made by the chi-square and Fisher's exact test. $P < 0.05$ was considered significant.

RESULTS

Among the 50 patients studied, 38 (76%) were *H. pylori*(+) and 12 (24%) *H. pylori*(−). There were no differences between the two groups of patients concerning age, sex and other demographic parameters (Table 1). Among *H. pylori*(+) patients, 28 (73.6%) were CagA(+) and 10 (24.6%) CagA(−), and similarly there were no significant differences between these groups as far as demographic data were concerned (Table 1). According to endoscopic findings, patients were divided into two groups, ie, the duodenal ulcer group ($N = 21$) and the nonulcer group ($N = 17$). All ulcer patients belonged to the *H. pylori*(+) group. The relationship of the endoscopic picture to CagA status is shown in Table 2. The median acute gastritis score in the *H. pylori* CagA(+) group was 2 (range 1–3) and was statistically higher ($P = 0.0029$) than in the *H. pylori* CagA(−) group [1 (1–3)]. A significant difference was also noted for chronic gastritis [2 (1–2) in the *H. pylori* CagA(+) group vs 1 (0–2) in the CagA(−) group, $P < 0.05$]. Atrophy was noted in 15/28 (53.6%) subjects in the *H. pylori* CagA(+) group in comparison to 1/10 (10%) subjects in the *H. pylori* CagA(−) group ($P = 0.025$). The corresponding numbers for intestinal metaplasia were 10/28 (35.7%) and 0/10 (0%) respectively ($P = 0.038$) (Table 2).

TABLE 1. DEMOGRAPHIC DATA IN VARIOUS GROUPS OF PATIENTS STUDIED

	H. pylori(−) (N = 12)	H. pylori(+) (N = 38)	CagA(+) (N = 28)	CagA(−) (N = 10)
Age (yrs), median (range)	38.5 (19–66)	39.5 (18–67)	38.5 (18–67)	41 (20–63)
Sex (M/F)	8/4	24/14	18/10	6/4
Smoking habits	8/12 (66.6%)	20/38 (52.6%)	15/28 (53.5%)	5/10 (50%)
Daily alcohol consumption	3/12 (25%)	10/38 (26.3%)	8/28 (28.5%)	2/10 (20%)

TABLE 2. DUODENAL ULCER, ATROPHY, AND INTESTINAL METAPLASIA IN *H. PYLORI* CAGA(+) AND CAGA(−) GROUPS OF PATIENTS

	H. pylori *CagA(+)*	H. pylori *CagA(−)*	P
DU ulcer	20/28 (71.4%)	1/10 (10%)	0.002
Atrophy	15/28 (53.6%)	1/10 (10%)	0.025
Intestinal metaplasia	10/28 (35.7%)	0/10 (0%)	0.038

Cell Proliferation. In the *H. pylori* CagA(+) and CagA(−) groups, the PI values [median (ranges)] were 5 (4–7) and 3.7 (3.5–5.5), respectively ($P < 0.05$). In addition, the difference between the *H. pylori* CagA(+) and *H. pylori*(−) groups, was highly significant ($P < 0.001$), whereas the comparison between the *H. pylori* CagA(−) and *H. pylori*(−) groups yielded no significant results (Figure 2).

Apoptosis. Concerning apoptosis, in the *H. pylori* CagA(+) and CagA(−) groups, the corresponding AI values [median (ranges)] were 1 (1–30) and 5.5 (1–35), respectively ($P < 0.05$). The difference between the *H. pylori* CagA(−) and *H. pylori*(−) groups was also significant ($P < 0.05$), whereas the difference between the *H. pylori* CagA(+) and *H. pylori*(−) groups was not significant (Figure 3).

Correlation of Gastritis Score with Proliferation and Apoptosis. The correlation of gastritis score (both for acute and chronic gastritis) with proliferation and apoptosis indices in the groups of *H. pylori* CagA(+) and CagA(−) patients showed significant results for gastritis, both acute and chronic, only with proliferation and not apoptosis in the *H. pylori* CagA(+) group. None of these correlations in the *H. pylori* CagA(−) group were significant (Table 3).

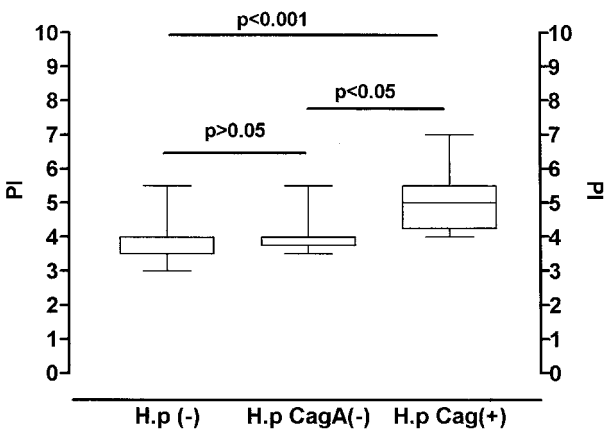

Fig 2. Proliferation indices (PI) in groups studied. Results are expressed as boxes and whiskers. Boxes indicate 25–75% range and central vertical lines indicate median values. Whiskers represent upper and lower extremes.

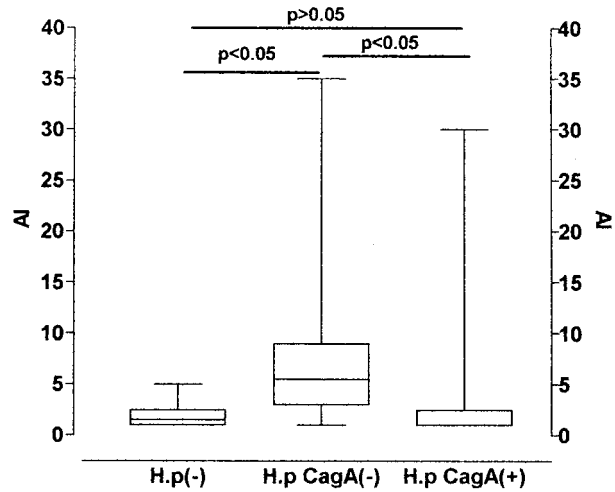

Fig 3. Apoptotic indices (AI) in groups studied. Results are expressed as boxes and whiskers. Boxes indicate 25–75% range and central vertical lines indicate median values. Whiskers represent upper and lower extremes.

DISCUSSION

Epithelial cell proliferation and apoptosis (programmed cell death) are essential events in tissue homeostasis, and in a healthy stomach there is a balance between the rates of proliferation and apoptosis (39). Our findings demonstrate that subjects harboring *H. pylori* CagA(+) strains have significantly higher gastric cell proliferation rates than subjects infected with *H. pylori* CagA(−) strains or uninfected persons. However, apoptotic indices in subjects infected with CagA(+) *H. pylori* strains were significantly lower than in subjects infected with CagA(−) *H. pylori* strains and similar to uninfected persons. In other words, we found that in *H. pylori* CagA(+) infection the observed increase in gastric cell proliferation rates was not accompanied by a parallel increase in apoptosis. Similar observations were made

TABLE 3. CORRELATION BETWEEN GASTRITIS SCORE (BOTH ACUTE AND CHRONIC GASTRITIS) AND INDICES OF PROLIFERATION (PI) AND APOPTOSIS (AI)

	Acute gastritis		*Chronic gastritis*	
H. pylori *group*	*PI*	*AI*	*PI*	*AI*
CagA(+)				
Number of XY pairs	28	28	28	28
Spearman *r*	0.713	0.071	0.470	0.086
P (two tailed)	<0.0001	0.717	0.011	0.662
CagA(−)				
Number of XY pairs	10	10	10	10
Spearman *r*	0.093	0.6	0.439	0.232
P (two tailed)	0.797	0.086	0.204	0.517

recently by Peek et al (40) in the United States, using a different methodology, for estimation of epithelial cell proliferation and apoptosis. They found that in subjects infected with *H. pylori* CagA(+) strains there was a dissociation of gastric epithelial cell proliferation from apoptosis. In addition, in their study, when antral proliferation and apoptosis were examined in relation to *vacA* status, the results for *s1a* genotype paralleled those for *cagA*($^+$) isolates. It is notable therefore that on this important subject of current interest the conclusions of two independent studies from two different parts of the world are in agreement. The possible implication of these findings is that the increased cell proliferation in the absence of a corresponding increase in apoptosis may explain the increased risk for gastric carcinoma that is associated with infection by CagA(+) strains of *H. pylori*, as occurs in other types of gastrointestinal neoplasia, such as colonic neoplasia (41, 42). Furthermore we could speculate that *H. pylori* CagA(+) strains act as tumor promoters by making the gastric epithelium, via increased proliferation, more susceptible to various genotoxic carcinogens. If, then, genetically damaged cells are not expelled via apoptosis, malignancy ensues.

On dealing with proliferation and apoptosis separately, as far as the relationship of *H. pylori* infection and gastric cell proliferation rates is concerned, past studies, using various methodologies have shown increased (20–26) or unaltered rates (43). Although the reason for these conflicting results is not clear, a possible explanation is that among *H. pylori* strains there is heterogeneity, and they may differ in their ability to induce proliferative responses. The results of our study are consistent with this notion since we found increased proliferation rates only in subjects infected with *H. pylori* CagA(+) in comparison to *H. pylori* CagA(−) strains. Furthermore only in subjects infected with *H. pylori* CagA(+) strains was there a significant positive correlation between proliferation rates and gastritis scores. Although the ability of *H. pylori* CagA(+) strains to induce more severe mucosal inflammation is the possible explanation for this observation, other as yet unknown factors that may stimulate cell proliferation, such as reactive oxygen metabolites produced by polymorphonuclear cells or other bacterial factors, may also be involved.

Earlier studies concerning apoptosis in *H. pylori* infection in adults (27) and children (44) found that there was a significant increase in apoptotic index only in infected subjects, in comparison to uninfected subjects. In support of this, Wagner et al (39), using a differentiated human gastric cell line, provided evidence that *H. pylori* induced apoptosis *in vitro*. However, these studies did not examine the effects of *H. pylori* CagA(+) and CagA(−) strains separately, although Wagner et al (39) did group strains by toxin production. It is known that toxigenicity and presence of *cagA* are tightly linked within *H. pylori* strains, and thus it is unlikely that a difference would have been found when strains were stratified on the basis of *cagA*. In our study, when we compared the entire group of *H. pylori*-positive patients to uninfected subjects, we found no difference in apoptotic index. On first interpretation one might suppose that our data are in contrast to those of Wagner et al (39). However, on careful analysis of the apoptotic results of the subgroups of *H. pylori*(+) patients, we found that only the *H. pylori* CagA(−) and not the CagA(+) group had a significantly increased apoptotic index in comparison to uninfected subjects. It is apparent, then, that our results are in partial agreement with the above-mentioned studies. The reduced apoptotic results for the *H. pylori* CagA(+) group were unexpected; however, two facts might explain these results. First, CagA(+) strains made up 73.6% of the entire *H. pylori*(+) group, and it has been found that they are stronger inducers of the apoptosis-suppressing nuclear factor-kappa B (NF-κB), than *H. pylori* CagA(−) strains (45–47). Second, in the *H. pylori* CagA(+) group, in comparison to CagA(−) group, there was a significantly higher percentage of intestinal metaplasia, which is in agreement with previous observations (18, 48). There is recent evidence (49) that apoptosis is significantly reduced in *H. pylori*-related intestinal metaplasia, which is further strengthened by the fact that reduced apoptosis was found in early intestinal-type gastric carcinoma (50), a situation closely related to intestinal metaplasia (51).

In this study we used western blotting to determine CagA status, but we did not determine *cagA* genotype of the *H. pylori* isolates. Cover et al (52), studying the relationship between CagA seropositivity and *cagA* genotype of *H. pylori* isolates, described anti-CagA antibodies in 26.7% of their patients from whom a strain lacking *cagA* was isolated and, in contrast, no antibodies in 7.6% of patients with *cagA*$^+$ isolates. Based on this, it could be suggested that serology alone may not be the most appropriate test in determining differences in virulence among *H. pylori* strains. However, *H. pylori* CagA status, as determined by immunoblotting, has increasingly been used in recent papers in the literature (53, 54) and this test

has been found to have greater sensitivity than other relevant serological tests, such as ELISA (55).

In conclusion we showed that subjects infected with *H. pylori* CagA(+) strains had increased gastric cell proliferation and decreased apoptosis indices in comparison to *H. pylori* CagA(−) strains. According to these findings, it is plausible that dissociation of proliferation from apoptosis may be an important mechanism of increased malignant potential related to infection with *H. pylori* CagA(+) strains.

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