Increase in Proliferation and Apoptosis of Gastric Epithelial Cells Early in the Natural History of Helicobacter pylori Infection

Nicola Lorraine Jones,* Patrick Thomas Shannon,† Ernest Cutz,† Herman Yeger,† and Philip Martin Sherman*
From the Division of Gastroenterology and Nutrition,* Research Institute, The Hospital for Sick Children, and the Departments of Pediatrics,* Microbiology,* and Pathology,† University of Toronto, Toronto, Ontario

Childhood acquisition of Helicobacter pylori is a critical risk factor for gastric cancer. Since tumorigenesis involves deregulation of proliferation and apoptosis, we examined gastric epithelial cell proliferation and apoptosis in H. pylori-infected children. Apoptosis and proliferation of gastric antral epithelial cells in biopsy specimens from patients with H. pylori-induced gastritis, secondary gastritis, and noninflamed controls were compared. p53 protein expression was examined immunohistochemically. Apoptotic cells were identified in the surface epithelium in each group. The apoptotic index was higher in specimens from patients with H. pylori gastritis (120 ± 10) than secondary gastritis (50 ± 10) and noninflamed controls (40 ± 10, analysis of variance P < 0.005). Apoptosis decreased following H. pylori eradication and resolution of gastritis (P < 0.02). An expanded proliferative compartment was identified in H. pylori-induced gastritis (32.4 ± 3.5; proliferative labeling index ± SE) compared with secondary gastritis (18.9 ± 2.8) and noninflamed controls (13.7 ± 3.1, analysis of variance P < 0.01). The accelerated cell turnover was associated with p53 overexpression (analysis of variance P < 0.005). Accumulation of p53 was not associated with expression of the cyclin-dependent kinase inhibitor p21. The occurrence of altered cell turnover early in the natural history of chronic infection provides an explanation for the increased risk of gastric cancer development associated with childhood acquisition of infection. (Am J Pathol 1997, 151:1695–1703)

The gastric pathogen Helicobacter pylori is now recognized as an important causal agent in both chronic-active gastritis and peptic ulcer disease.1 In addition, epidemiological studies have consistently identified an association between chronic infection with H. pylori and the subsequent development of gastric cancers2 including carcinoma, lymphoma, and mucosa-associated lymphoid tissue lymphomas. In 1994, a working group of the WHO International Agency for Research on Cancer concluded that H. pylori is a group 1 carcinogen in humans and plays a causal role in the development of gastric cancer.3

It is widely accepted that tumor progression is a multistep process in which regulation of both cell proliferation and programmed cell death are disturbed.4 Programmed cell death, or apoptosis, is a distinct form of cell death that can be distinguished morphologically by condensation and margination of nuclear chromatin with the later formation of apoptotic bodies.5 During apoptosis, DNA characteristically undergoes fragmentation into oligonucleosome fragments that can be detected by a variety of complementary techniques.6 The identification of these fragments in situ is widely used to distinguish cells undergoing apoptosis.

Acquisition of H. pylori infection during childhood appears to be a critical risk factor for the later development of gastric cancers.7 The examination of alterations in cell turnover early in the natural history of infection should further our understanding of the relationship between H. pylori and the development of gastric cancers. Therefore, both the degree of apoptosis and the proliferation of gastric antral epithelial cells were assessed in this study using paraffin-embedded biopsy samples obtained from pediatric patients infected with H. pylori and compared with specimens from patients with secondary gastritis and those with noninflamed gastric mucosa.

Mutation of the p53 gene is the most frequent genetic abnormality encountered in human malignancies8 including gastric cancers. A protective role for p53 expression has been demonstrated in normal tissues with rapid cell turnover9,10 or following exposure to DNA damaging...
agents or mutagens. Here, p53-mediated cell cycle arrest or apoptosis may protect the organism from propagation of cells with genetic damage. We hypothesized that overexpression of wild-type p53 in gastric epithelial cells may occur in response to infection with *H. pylori*. The p53 protein mediates cell cycle arrest by transcriptionally activating the cyclin-dependent kinase inhibitor p21<sup>WAF1/CIP1</sup>. Therefore, to investigate the role of the tumor suppressor p53 protein during infection with *H. pylori*, an immunohistochemical analysis of both p53 and p21 expression was used.

**Materials and Methods**

**Tissue Samples (Table 1)**

Gastric biopsy specimens were obtained from the antrum of pediatric patients who underwent diagnostic upper gastrointestinal tract endoscopy at The Hospital for Sick Children (Toronto, Ontario, Canada) between 1985 and 1996. The histopathology had previously identified four patient populations: 1) primary gastritis infected with *H. pylori* before (N = 17) and after (N = 6) eradication therapy; 2) secondary gastritis with inflammation caused by other conditions including Crohn’s disease, eosinophilic gastroenteritis, and nonsteroidal anti-inflammatory medication (N = 12); 3) noninflamed mucosa (N = 12); and 4) graft-versus-host disease (N = 3). Eradication therapy consisted of a 2 to 4 week course of bismuth subsalicylate and amoxicillin, with or without metronidazole. After eradication therapy, gastric biopsies were obtained following a mean of 3.1 ± 0.9 months from the time of the initial endoscopies. *H. pylori* status was assessed as described previously by either a modified Steiner silver stain alone (N = 6) or by both a modified Steiner silver stain and culture of gastric biopsy specimens (N = 11). Informed written consent was obtained for upper endoscopy and biopsy procedures.

**Biochemical Assessment of Apoptosis**

The terminal transferase mediated biotinylated dUTP nick end-labeling (TUNEL) assay was performed as described previously with minor modifications on sections of paraffin-embedded biopsy specimens. Briefly, the sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol at room temperature. After blocking endogenous peroxidase by incubation in 1.2% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes, sections were microwaved in 0.01 mol/L citrate buffer for 1 minute. The sections were then incubated with proteinase K (20 µg/ml, Boehringer Mannheim, Laval, Quebec) for 15 minutes at room temperature and washed three times in distilled water. Sections were then preincubated with terminal transferase buffer containing 200 mmol/L potassium cacodylate, 25 mmol/L Tris-HCl, pH 6.6, 0.2 mmol/L ethylenediaminetetraacetic acid, and 0.25 mg/ml bovine serum albumin for 5 minutes at room temperature. The sections were then incubated for 60 minutes at 37°C with a reaction mixture containing the terminal transferase buffer with 1 mmol/L cobalt chloride, 0.01 mmol/L biotin 16-dUTP, and 0.5 U/µl of terminal transferase (Boehringer Mannheim). The reaction was terminated using a solution of sodium chloride (300 mmol/L) and sodium citrate (30 mmol/L). The sections were incubated with avidin-conjugated peroxidase (Vector, Burlingame, CA) at a concentration of 1:1000 in PBS at 37°C for 30 minutes followed by reaction in diaminobenzidine (Vector Laboratories, Burlingame, CA) as described previously. After light counterstaining with hematoxylin (Fisher Scientific, Fairlawn, NJ), the sections were mounted with Permount (Fisher Scientific). The biopsy specimens obtained from patients with graft-versus-host disease served as positive controls. For negative controls, terminal transferase was omitted from the reaction mixture.

Apoptotic cells identified by the TUNEL assay were quantitated under brightfield microscopy. The apoptotic index was determined by enumerating the number of positively labeled cells per crypt-epithelial cell unit in at least three well-oriented crypt epithelial cell units and expressing the mean number multiplied by a factor of 100.

**Immunohistochemistry**

Serial sections from paraffin-embedded antral biopsy specimens were assessed immunohistochemically using the microwave antigen retrieval method, as reported by Shi et al., with slight modifications noted below. Briefly, sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. After blocking endogenous peroxidase with H<sub>2</sub>O<sub>2</sub>, the sections were heated in 0.01 mol/L citrate buffer in a microwave pressure cooker for 20 minutes. The slides were allowed to cool to room temperature, and nonspecific binding was blocked with normal horse serum (Vectastain Elite ABC kit, Vector Laboratories) for 20 minutes at room temperature. The MIB-1 monoclonal antibody (Oncogene Science, Cambridge, MA) was used for detection of nuclear Ki-67, a marker of proliferating cells (dilution 1:40). For p53 detection, the monoclonal antibody NCL-p53-DO7 (NovoCastra, Newcastle, UK), which recognizes both wild type and mutant p53, was used (dilution 1:50). The optimal concentration for p53 detection was determined.
by employing a series of two fold antibody dilutions on consecutive sections of gastric mucosa. For detection of p21Waf1/Cip1, the WAF1 (Ab-1) antibody (Oncogene Research Products, Cambridge, MA) was used (dilution 1:20). The antibodies were incubated overnight at 4°C in a humidified chamber. The specimens were then stained using the avidin-biotin complex (ABC) immunoperoxidase technique employing commercially available reagents (Vectorstain Elite ABC kit, Vector Laboratories, Burlingame, CA). The sections were counterstained with hematoxylin (Fisher Scientific) and mounted with Permount (Fisher Scientific). Sections from human tonsil and skin served as positive controls for the MIB-1 and p53 antibodies, respectively. Colonic tissue sections served as a positive control for p21.

Confirmation of p53 expression was obtained using an additional monoclonal antibody NCL-p53-BP (NovoCastra), which recognizes both wild-type and mutant p53 but recognizes a different epitope of the p53 protein.

p53 and Ki-67 expression were evaluated under light microscopy. The proliferative labeling index was determined by enumerating the number of MIB-1 positive cells in at least three well-oriented crypt epithelial cell units and expressing the mean percentage of the total number of cells counted in each crypt epithelial cell unit. Similarly, the p53 expression index was determined by enumerating p53-positive epithelial cells in at least three well-oriented crypt epithelial units and expressing the mean percentage of the total number of cells counted in each crypt epithelial unit.

Gastritis Score

Sections stained with hematoxylin and eosin for light microscopy were graded for the severity of gastritis as we have done previously. The presence of an increased number of mononuclear cells, the presence and severity of mucus depletion, and the presence of polymorphonuclear leukocytes were each assessed separately and graded from 0 to 3. The presence and number of H. pylori present in sections were excluded from this analysis.

Statistics

Results are expressed as means ± SEM. Comparison of results between groups was performed using analysis of variance (ANOVA) followed by posthoc comparisons with the Newman-Keuls test. For comparisons between two groups, an unpaired Student's t-test was performed. Correlation among data was determined by the linear least-squares regression method.

Results

Effect of H. pylori Infection on Gastric Epithelial Cell Apoptosis

Apoptotic epithelial cells, identified by the presence of characteristic DNA fragmentation demonstrated using the TUNEL assay, were observed primarily in the superficial gastric mucosa (Figure 1) in all groups. In addition, apoptotic cells were present within lymphoid follicles that were present only in the lamina propria of H. pylori-infected mucosa. For epithelial cells, the apoptotic index was highest in gastric biopsy specimens obtained from patients with graft-versus-host disease (280 ± 40, mean ± SE). Patients infected with H. pylori (120 ± 10) had a higher apoptotic index compared with both biopsies from patients with secondary gastritis (50 ± 10) and histologically normal controls (40 ± 10; ANOVA, P < 0.005) (Figure 2). The apoptotic index decreased following therapy for H. pylori only when the bacterium was successfully eradicated and gastritis resolved (13 ± 13, N = 3; P < 0.02).

There was no correlation between the degree of gastric epithelial cell apoptosis in H. pylori-infected patients and the severity of gastroduodenal disease identified during endoscopy. The presence of both duodenal or gastric ulceration and gastritis (100 ± 20, N = 7) was not associated with a higher degree of apoptosis compared with the presence of gastritis alone (150 ± 20, N = 9; P = 0.07). In addition, the degree of gastritis did not correlate with the apoptotic index (Figure 3).

Proliferative Activity of Epithelial Cells Determined by Ki-67 Immunohistochemistry

In gastric antral sections obtained from noninflamed mucosa and secondary gastritis (Figure 4), proliferating epithelial cells were limited to the neck region of the crypts. The proliferative compartment extended beyond the neck region toward the surface epithelium in antral sections obtained from children infected with H. pylori. As shown in Figure 5, the proliferative index increased approximately twofold in patients with H. pylori-induced gastritis (33.1 ± 3.4) compared with both secondary gastritis (18.9 ± 2.8) and noninfamed controls (13.7 ± 3.1; ANOVA P < 0.01). No correlation was found between the proliferative index and the degree of gastritis (Figure 3). The proliferative index did not differ between patients with peptic ulceration (32.1 ± 5.5) compared with those with gastritis alone (31.5 ± 4.8; P = 0.9). Following anti-helicobacter eradication therapy, the proliferative index returned toward normal values (16.3 ± 0.7, N = 4; P = 0.08).

p53 Expression during H. pylori Infection

In noninflamed antral mucosa, nuclear p53 expression was identified in occasional epithelial cells concentrated in the neck region (Figure 6). Sections from patients with secondary gastritis showed similar epithelial cell expression of p53. In contrast, sections from subjects with H. pylori infection showed increased nuclear expression of p53 corresponding to the proliferative regions identified by the Ki-67 immunohistochemistry in serial sections (Figure 6). As shown in Figure 7, quantitation of p53 expression demonstrated comparable results in sections from patients with noninflamed mucosa (3.7 ± 0.9, N = 10) and secondary gastritis (2.4 ± 0.9, N = 11), whereas
sections from patients infected with H. pylori demonstrated overexpression of p53 (19.9 ± 3.7; N = 16, ANOVA, P < 0.005).

**Figure 2.** Quantification of apoptotic epithelial cells in patients with graft-versus-host disease (GVHD, N = 5), H. pylori-induced gastritis (HP+ gastritis, N = 16), secondary gastritis (HP- gastritis, N = 12), and noninflamed mucosa (normal, N = 12). Results are expressed as the mean apoptotic index (100 times the mean number of apoptotic cells per crypt epithelial unit). Error bars represent SE. *P < 0.005, ANOVA; †P < 0.005, ANOVA.

**p21 Expression during H. pylori Infection**

Wild type p53 can transcriptionally transactivate genes involved in cell cycle arrest including the cyclin-dependent kinase inhibitor p21. Therefore, to investigate the relationship between p53-mediated cell cycle arrest and apoptosis during infection with H. pylori, we examined p21 expression. Staining for p21 was observed in the superficial epithelium and the gastric pits, as previously identified in normal gastric tissues. In serial sections of H. pylori-infected gastric biopsies, p21 staining was localized to regions where there was no evidence of p53 expression (Figure 8).

**Discussion**

The pathogenic mechanisms responsible for the association of chronic infection with H. pylori and gastric cancers are yet to be determined. During the multistep process of carcinogenesis, the regulation of cell proliferation and programmed cell death are disturbed. Therefore, we sought to investigate whether there were alterations in gastric epithelial cell turnover during infection with H. pylori. This is the first study that examines the balance...
H. pylori Induces Gastric Epithelial Apoptosis and Proliferation

H. pylori

Induces

Gastric

Epithelial

Apoptosis

and

Proliferation

1699

AJP

December 1997, Vol. 151, No. 6

U U

UU

U UU

o U

U U

U O

U

0 2

5 8

10

Gastritis Score

Figure 3. Lack of correlation among gastritis score and A: apoptotic index in secondary gastritis (open squares, \( r = 0.221 \)) and H. pylori-induced gastritis (closed squares, \( r = 0.095 \)) and B: proliferative index in secondary gastritis (open squares, \( r = 0.308 \)) and H. pylori-induced gastritis (closed squares, \( r = 0.073 \)).

between proliferation and apoptosis in the same patients during infection with H. pylori; the importance of which has recently been emphasized. In addition, this study investigates these effects among children in whom acquisition of infection with H. pylori is reported to be associated with a higher risk of developing neoplastic complications.

Our results demonstrate that infection with H. pylori induces apoptosis and increased proliferation of gastric epithelial cells in children. The findings of increased epithelial cell apoptosis during H. pylori infection are supported by two recent reports that examined gastric antral biopsy specimens from adults before and after eradication therapy for H. pylori. The presence of a compa-

Figure 4. Photomicrograph of MIB-1 labeling of proliferating cells in noninflamed controls (A) and H. pylori-induced gastritis (B). An increase in brown stained proliferating cells is identified in B (arrows). Original approximate magnification, \( \times 400 \).

Figure 5. Comparison of MIB-1 positive epithelial cells among noninflamed control mucosa (\( N = 9 \)), secondary gastritis (\( N = 9 \)), and H. pylori-induced gastritis (\( N = 10 \)). Results are expressed as the mean proliferative index (mean percentage of positively labeled cells per crypt epithelial unit). Error bars represent the SE. * \( P < 0.01 \), ANOVA.
The induction of apoptosis during infection in childhood was reversible, as demonstrated among patients with successful eradication of *H. pylori* infection. This finding provides additional evidence for the specificity of involvement of the bacterium and allows for speculation regarding the merits of eradication of *H. pylori* infection. These data imply that eradication of the organism may well result in a reduction in the risk of gastric cancers. Indeed, preliminary studies indicate that eradication of *H. pylori* infection in patients undergoing endoscopic resection for gastric cancer results in a decreased risk for the subsequent development of metachronous gastric cancers.\(^3\)

The degree of induction of apoptosis or proliferation during infection with the bacterium was not associated with disease severity. No correlation could be demonstrated among the presence of peptic ulcer disease and gastritis or gastritis alone and the apoptotic or proliferative indices. These results suggest that increased cell turnover may not be directly involved in ulcerogenesis. Alternatively, since the biopsy specimens were not obtained at the sight of the ulcer crater they may not directly represent cell turnover at this location.\(^3\)

The mechanism for the induction of apoptosis and resultant-increased proliferation during infection with *H. pylori* is unknown. Infection with strains possessing the cytotoxin-associated gene (cagA) has been associated with more severe disease and with an increased risk for the development of gastric cancers.\(^3\) One study in adult patients with *H. pylori* suggests that infection with cagA positive strains results in enhanced proliferation of gastric epithelial cells but is not associated with increased induction of apoptosis.\(^3\) During the natural history of chronic infection to adulthood with cagA positive strains, a selection of cells resistant to apoptosis may occur and thereby provide a potential explanation for the association of these strains with gastric malignancies. However, the presence of the cagA gene in 33 of 33 *H. pylori* isolates tested from our institution\(^3\) suggests that cagA status probably is not an important determinant for the induction of apoptosis, at least in children. Among a subgroup of patients in which the expression of vacuolating cytotoxin activity in infecting strains has been determined,\(^3\) the degree of epithelial cell apoptosis did not correlate with the presence or absence of the cytotoxin (data not shown).

A lack of correlation between the degree of gastritis and alterations in cell turnover suggest that mucosal inflammation does not play a significant role in the induction of apoptosis and proliferation during infection with *H. pylori*. Fan et al.\(^3\) demonstrated that the rate of gastric cell proliferation *in vitro* increases in response to both *H. pylori* infection and the immune/inflammatory responses.
to the bacterium. In addition, T-cell-mediated epithelial cell proliferation and apoptosis have been described. An increase in the population of CD8+ T cells has been demonstrated both within the epithelium and the lamina propria in H. pylori positive gastritis. Moreover, granzyme B-expressing T-cell lymphomas are predominantly localized in mucosa-associated lymphoid tissue. This is of considerable interest since mucosa-associated lymphoid tissue carcinomas are associated with infection with H. pylori. Ongoing studies aimed at specifically examining the involvement of cytotoxic T cells in the induction of gastric epithelial-programmed cell death and proliferation should clarify the potential role of these T lymphocytes during infection with the bacterium.

In this study, an increase in the expression of p53 was demonstrated in gastric antral epithelial cells in children infected with H. pylori. This may appear in contrast with previous studies in which an increase in gastric epithelial cell expression of p53 has been demonstrated only in areas of intestinal metaplasia or dysplasia. However, previous studies have not routinely used the microwave antigen retrieval technique that is necessary for optimal p53 staining. Several lines of evidence suggest that the overexpression of p53 demonstrated in these studies is of wild-type. A low level of p53 expression was evident in proliferating gastric epithelial cells in biopsy specimens obtained from noninflamed mucosa. Prior studies examining p53 mutations in gastric tissues have demonstrated enhanced expression of mutant p53 in advanced precancerous lesions such as intestinal metaplasia or dysplasia. In the present study, precancerous lesions were not identified histologically in any of the biopsy specimens obtained from any of the children studied. Expression of the cell cycle dependent kinase inhibitor p21 in cells of the nonproliferative compartment of the gastric epithelium is also indicative of wild-type p53 status. An inverse relationship between proliferating cells and p21 was found in gastric biopsies from each of the patient groups. Taken together, these findings indicate that the p53 expression identified in our study is of the wild-type.

The tumor suppressor protein p53 accumulates in normal tissues in response to DNA damage or increased proliferation as it is involved in both cell cycle arrest for DNA repair and the induction of apoptosis. The ability of p53 to induce cell cycle arrest is due to the transcriptional activation of the cyclin-dependent kinase inhibitor p21. To investigate the relationship between p53 expression and either cell cycle arrest or apoptosis during H. pylori infection in children, we assessed the expression of p21 in gastric tissue biopsies. Staining for p21 was identified in the superficial epithelium and gastric pits. The accumulation of p53 was not associated with expression of p21 suggesting that p53 does not cause cell cycle arrest during H. pylori infection. Therefore, these findings support the contention that the p53 protein could function to induce apoptosis during infection with H. pylori.

The mechanism for the stimulation of p53 accumulation is not known. H. pylori stimulates the production of antral mucosal reactive oxygen species. Recent find-
ings demonstrate increased oxidative DNA damage in H. pylorī-infected mucosa from children.47 H. pylorī infection is also associated with enhanced nitric oxide synthase activity both in vitro48 and in vivo.31 Nitric oxide induces DNA strand breakage in human cells.49 The accumulation of p53 and resultant induction of apoptosis could occur in response to DNA damage induced by bacterial infection.

Evidence from a variety of experimental systems suggests that p53 mutation and inactivation is firmly associated with tumor progression in many human malignancies50 including gastric cancers.44,51 Mutations in p53 have been demonstrated in gastric precancerous lesions such as intestinal metaplasia.44 Loss of p53-mediated cell cycle checkpoints could result in enhanced genomic instability. In addition, loss of p53-mediated apoptosis during H. pylorī infection would be expected to provide enhanced cell survival effects.1 Indeed, although frank neoplasia did not develop, the loss of one copy of the p53 gene in an animal model of Helicobacter infection conferred a growth advantage to gastric epithelial cells as demonstrated by an enhanced epithelial cell proliferation compared with infected wild-type animals.52

In summary, this study demonstrates a reversible alteration in gastric epithelial cell turnover, including increased apoptosis and proliferation, associated with accumulation of p53 induced specifically by infection with H. pylorī in children. This alteration in cell turnover occurring early in the natural history of chronic infection provides an explanation for the association between early acquisition of H. pylorī infection and the initiation of gastric cancers. The reversibility of these changes implies the potential for a reduction in the risk of gastric cancers following eradication of H. pylorī. Additional epidemiological studies should help to elucidate if prevention of gastric cancer is possible with the eradication of H. pylorī.

References
eradication inhibits the growth of intestinal type of gastric cancer in initial stage. Gastroenterology 1996, 110:A282


