Induction of gastric epithelial apoptosis by *Helicobacter pylori*

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Abstract

Background—*Helicobacter pylori* may contribute to gastric carcinogenesis by stimulating epithelial cell proliferation. How *H pylori* does so is unknown. Programmed, non-necrotic, cell death (apoptosis) occurs throughout the gut and is linked to proliferation. It was hypothesised that *H pylori* may induce hyperproliferation through increasing apoptosis.

Aim—To measure the effect of *H pylori* infection on gastric epithelial apoptosis in situ.

Patients—Patients with duodenal ulcers treated to eradicate *H pylori* and patients with *H pylori* negative non-ulcer dyspepsia.

Methods—Retrospective quantification of apoptotic epithelial cells in situ from formalin fixed biopsy specimens, counted after staining by terminal deoxynucleotidyl nick end-labelling.

Results—In the uninfected stomach, apoptotic cells were rare and situated in the most superficial portion of gastric glands (mean 2.9% of epithelial cells). In *H pylori* infection, they were more numerous and were located throughout the depth of gastric glands, comprising 16.8% of epithelial cells, falling to 3.1% after *H pylori* eradication, p=0.017. Apoptotic cell number did not correlate with the degree of histological gastritis.

Conclusions—These results suggest that *H pylori* induces epithelial apoptosis in vivo. Increased apoptosis may be the stimulus for a compensatory hyperproliferative and potentially preneoplastic response in chronic *H pylori* infection.

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Gastric *Helicobacter pylori* infection is associated with peptic ulceration, gastric lymphoma, and gastric carcinoma.1 How this organism interacts with the gastric epithelium to cause these diseases is still unclear.2 Although *H pylori* has recently been defined as a definite carcinogen,3 how it might promote neoplasia is largely speculative. Proposed mechanisms for the carcinogenic effect of *H pylori* infection include reduction of the gastric antioxidant ascorbate by *H pylori*4 and stimulation of epithelial proliferation5 because increased cell turnover predisposes to mutagenesis in the multistep model of gastric carcinogenesis.6 7

Mucosal epithelial hyperproliferation may be a response to *H pylori* induced cell damage or death, yet there is a notable lack of epithelial necrosis in chronic *H pylori* infection.6 8 Recently, however, it has been appreciated that necrosis is not the only form of cell death and that programmed cell death (apoptosis) is the physiological way in which cells die.9 10 Apoptosis, in contrast with necrosis, occurs in single cells, in response to the expression of specific cellular genes11 and may therefore be considered a type of cellular suicide. Apoptosis is an essential part of the cycle of cellular turnover in many tissues, including the gastrointestinal tract. Tissue integrity is maintained when the rate of cell loss by apoptosis is matched by the rate of new cell production by proliferation. Furthermore, changes in the rate of apoptotic cell loss may contribute to diseases characterised by abnormalities of tissue growth.12 In *H pylori* gastritis where there is increased epithelial proliferation and little or no necrotic cell death, we hypothesised that the increased cell proliferation in *H pylori* gastritis must be accompanied by increased programmed (apoptotic) cell death.

Apoptotic cells are very difficult to see by conventional light microscopy, especially in the presence of inflammation. One of the hallmarks of apoptosis, however, is the stepwise degradation of DNA by endonucleases, resulting in short fragments of double stranded DNA, enabling apoptotic cells to be identified in situ by incorporating labelled nucleotides onto the 3' free hydroxyl ends by terminal deoxynucleotidyl transferase.10 11 We therefore examined apoptosis in situ using the method of terminal uridine deoxynucleotide nick end labelling (TUNEL) in the ‘normal’ uninfected stomach and in the *H pylori* infected stomach of duodenal ulcer patients, in whom we could examine the effect of *H pylori* because biopsy specimens had been taken from these patients both before and after *H pylori* eradication therapy. Our findings show that *H pylori* infection is associated with increased epithelial apoptosis, suggesting that this is the stimulus that induces hyperproliferation and mutagenesis.

Methods

This study was a retrospective examination of formalin fixed paraffin wax embedded blocks of gastric antral biopsy specimens that had been collected for routine histopathology. Suitable cases were identified from the endoscopy clinic and medical records of Hammersmith Hospital, London and St Luke’s/Roosevelt Hospital Center in New York City. We specifically excluded patients...
who had undergone previous gastric surgery or were known to be taking non-steroidal anti-inflammatory drugs, corticosteroids or prostaglandin analogues, which may damage the gastric epithelium or impair the normal gastric cytoprotective response to injury. Patients were also excluded if they had taken antibiotics or other anti-\textit{H pylori} medication such as a bismuth compound or a proton pump inhibitor in the previous one month as these agents may have suppressed the numbers of \textit{H pylori} organisms. The study was approved by the research ethics committee of each hospital.

Patients

Controls – 12 patients who had undergone diagnostic endoscopy and biopsy in whom the final clinical diagnosis was non-ulcer dyspepsia and who were not infected with \textit{H pylori}. Seven were male, mean age 50 years, range 22 to 80.

Duodenal ulcer patients – 16 patients with active duodenal ulceration and \textit{H pylori} associated chronic superficial gastritis were treated with colloidal bismuth subcitrate, tetracycline, and metronidazole. They had a repeat endoscopy and biopsy to confirm ulcer healing and eradication one month after the end of treatment, as described previously. Ten were male, mean age 53 years, range 21 to 83.

Diagnosis of \textit{H pylori} infection

At the time of endoscopy three antral biopsy specimens were taken 2 cm from the pylorus along the greater curve for urease test, histological examination, and culture as previously described. If any test was positive, the patient was considered to be infected.

\textbf{TUNEL histochemistry}

TUNEL histochemistry was performed in our laboratory using a procedure modified from that described by Gavrieli et al. Four \(\mu\)m sections were cut from formalin fixed, paraffin wax embedded tissue blocks and mounted on ‘Superfrost-Plus’ coated microscope slides (Fisher Scientific, Pittsburgh, PA). The sections were deparaffinised through xylene and alcohol, washed, and then digested with proteinase K 20 \(\mu\)g/ml (Sigma, St Louis, MO) for 15 minutes at room temperature, followed by further washing and blocking of endogenous peroxidase with 2% hydrogen peroxide (Sigma) for five minutes. After preincubation with terminal transferase buffer containing 200 mM potassium cacodylate, 0.2 mM EDTA, 25 mM TRIS-HCl, bovine serum albumin 0.25 mg/ml, pH 6.6 (Boehringer Mannheim, Indianapolis, IN) for 10 minutes, the sections were incubated at 37°C for 90 minutes with the same buffer containing, in addition, 1 mM cobalt chloride, terminal transferase (0.5 U/\(\mu\)l), and 0.4 \(\mu\)M digoxigenin-11-deoxyuridine triphosphate (dUTP), all purchased from Boehringer. The reaction was terminated with a solution of 300 mM sodium chloride, 30 mM sodium citrate and the slides were washed in water, followed by 2% bovine serum albumin and then phosphate buffered saline. Detection of incorporated digoxigenin-11-dUTP was achieved by incubation with peroxidase conjugated Fab fragments of anti-digoxigenin (Boehringer) at a concentration of 1:300 in 100 mM TRIS-HCl, 150 mM sodium chloride, pH 7.5 at room temperature for 30 minutes. The slides were then washed in water and then 0.1 M aceta	

Assessment of gastritis

In each case an adjacent tissue section was stained by haematoxylin and eosin and the degree of histological gastritis was scored from a minimum of zero to a maximum of 10 using the scoring system developed by Rauws et al as used previously.

\textbf{Statistical analysis}

The Mann-Whitney U test was used to compare scores between clinicopathological groups. Differences between apoptotic indices in the same patients before and after eradication of \textit{H pylori} were compared by the Wilcoxon signed rank test.

\textbf{Results}

\textbf{Gastric apoptosis}

Apoptotic bodies were very rarely identified in the gastric epithelium in haematoxylin and eosin stained sections at 400\(\times\) magnification. In contrast, cells that had stained positively by TUNEL could be seen even at 100\(\times\) magnification. In the ‘normal’ stomach of non-ulcer dyspepsia patients without \textit{H pylori} infection, apoptotic epithelial cells were infrequently identified by TUNEL histochemistry. Positively stained cells were located in the most superficial part of the gastric gland, comprising a mean of 2-9% (median 2, range 0–9) of all gastric epithelial cells (Fig 1). In duodenal ulcer patients infected with \textit{H pylori}, apoptotic epithelial cells were more numerous and they were also seen deep in the glands (Fig 2). In addition, positively stained cells could be identified within the lamina propria. The mean
epithelial apoptotic index was 14.1% (median 6.5, range 0–44), p = 0.048 versus controls. After triple therapy, H. pylori was cured in 12 of 16 patients and in these patients the mean apoptotic index fell from 16.8% (median 7.5, range 0–44) to 3.1% (median 1.5, range 0–18), p = 0.017, Wilcoxon signed rank test (Fig 3). In the four patients in whom H. pylori was not cured, the apoptotic index fell from a mean of 6.8% (median 4.5, range 0–18) to 0.9% (median 1.0, range 0–1.5).

**Lack of correlation between apoptosis and inflammation**

The fall in apoptotic index in the duodenal ulcer patients may result from the associated improvement in gastritis when H. pylori is eradicated, particularly in view of the rapid resolution of neutrophil infiltration accompanying H. pylori eradication. We therefore examined the relation between apoptotic index and either neutrophil or total gastritis scores. In non-ulcer dyspeptic controls the mean total gastritis score was 0.36 (median 0, range 0–1) and the neutrophil score was 0 in all cases (Table). On eradication of H. pylori in the duodenal ulcer patients, the mean gastritis score decreased from 3.7 (median 3.5, range 1–8) to 1.9 (median 1, range 1–6), p = 0.024, and the neutrophil gastritis score fell from 1.5 (median 1, range 0–5) to 0.5 (median 0, range 0–2), p = 0.023. In contrast, in the four patients in whom H. pylori was not cured the mean total and neutrophil gastritis scores were 3.8 and 1.25 before and 3.5 and 0.8 after treatment respectively. The gastritis scores were in all cases significantly higher in the duodenal ulcer patient than in the controls, even after eradication (p < 0.001 in all cases, Table). Overall, there was no correlation between the apoptotic index and either the neutrophil score or the total gastritis score (p = 0.22 and p = 0.20 respectively).

**Discussion**

These results show that H. pylori infection is associated with increased epithelial apoptosis and that after treatment to eradicate H. pylori, apoptosis decreases to that found in normal controls. This implies that H. pylori induces gastric epithelial apoptosis and may explain how H. pylori promotes a hyperproliferative response. Although the increased proliferation may be considered an appropriate physiological response to maintain tissue mass, if hyperproliferation persists it may eventually produce cells that are more resistant to apoptosis, thereby leading to tissue growth and neoplasia, as has been found in colonic carcinogenesis. In support of this hypothesis, Lauwers et al. found that the expression of the anti-apoptotic protein bcl-2 was increased in precancerous gastric lesions, particularly in gastric epithelial dysplasia.

How H. pylori promotes apoptosis is not known. In attempting to understand the pathophysiological mechanisms of H. pylori it is important to separate the direct effect of H. pylori from the effect of the accompanying inflammatory infiltrate. Our results favour a direct bacterial effect as there was no correlation between the extent of inflammation, assessed histologically, and the apoptotic index. In addition, the control group had similar apoptotic indices to the duodenal ulcer patients in whom H. pylori had been cured although the duodenal ulcer patients had persistent inflammatory cells one month after the end of treatment. Furthermore, there was a reduction in apoptotic index in the few duodenal ulcer patients in whom H. pylori was eradicated although there was no change in inflammatory score. We suspect that the fall in apoptotic index in these patients may result from a reduction in bacterial load after unsuccessful H. pylori eradication therapy but cannot exclude the possibility that a component of the triple therapy may have been directly responsible for reducing the epithelial apoptotic index to normal, regardless of its effect on H. pylori.

Interestingly, the four patients with the highest apoptotic indices before treatment had no evidence of atrophy or intestinal metaplasia on antral biopsies, whereas seven of eight...
patients with pretreatment apoptotic scores less than 20% did. This may suggest that the induction of apoptosis occurs comparatively early in the natural history of \textit{H pylori} infection, followed later by a compensatory decrease in apoptotic activity during the progression of atrophic gastritis and intestinal metaplasia.

Precisely how \textit{H pylori} infection induces apoptosis is not apparent from our data. Several bacterial products may be directly responsible for the induction of apoptosis that we observed, including the CagA and VacA proteins. As our patients had duodenal ulcer they were probably infected with \textit{H pylori} strains expressing these products, but whether all strains promote apoptosis remains to be investigated. Another candidate bacterial virulence factor is ammonia, generated by the breakdown of urea by \textit{H pylori}'s urease, as ammonia has been shown to induce gastric epithelial apoptosis in vitro in rat cell lines. Whether the promotion of apoptosis is caused by the bacteria directly or by inflammatory infiltrate, the induction of apoptosis by \textit{H pylori} provides an explanation linking chronic infection by this organism with a compensatory hyperproliferative and potentially neoplastic response.

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