

Effect of *Helicobacter pylori* on apoptosis and apoptosis related genes in gastric cancer cells

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Background/Aims: *Helicobacter pylori* induces the apoptosis of gastric epithelial cells in vivo and in vitro. However, the molecular mechanism has not been clarified. The aim of this study was to investigate the effect of *H pylori* on the apoptosis of gastric epithelial cells and the expression of apoptosis related genes in vitro.

Methods: Human gastric adenocarcinoma SGC-7901 cells were co-cultured with a cytotoxic *H pylori* strain, NCTC 11637, at various densities ranging from 3.2×10^4 to 1.0×10^8 colony forming units (CFU)/ml for 48 hours. Apoptosis in gastric cells was determined by transmission electron microscopy, Hoechst 33258 fluorochrome staining, and flow cytometry. The expression of apoptosis related proteins, Bcl-2, Bax, and c-Myc, was measured by an immunohistochemical method, and c-Myc mRNA expression was determined by the reverse transcription-polymerase chain reaction.

Results: *Helicobacter pylori* induces morphological changes typical of apoptosis. Both fluorochrome staining and flow cytometry showed that the apoptotic index began to increase when *H pylori* were at a density of $> 1.6 \times 10^4$ CFU/ml, and in a density dependent manner ($p < 0.01$; one way ANOVA). The expression of the Bax and c-Myc proteins and of c-Myc mRNA was increased, whereas Bcl-2 expression was decreased after co-culture for 48 hours.

Conclusions: *Helicobacter pylori* induced apoptosis in gastric epithelial cells is mediated by altered expression of the products of the Bcl-2, Bax, and c-Myc genes.

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Helicobacter pylori infection in the human stomach is common, particularly in developing countries, where most adults are infected.¹ Epidemiological data, clinical studies, and experimental studies have shown that *H pylori* is a major aetiological agent for gastritis, peptic ulcer disease, and gastric malignancy.^{2,3} However, the pathogenic mechanisms by which the organism causes the diseases have not been fully defined.

Gastric mucosal integrity is maintained by a balance between the rate of cell loss and the rate of epithelial cell regeneration.⁴ Cell proliferation and apoptosis (programmed cell death) are essential events involved in the cellular turnover of epithelial tissue.^{4,7} Apoptosis is a physiological suicide mechanism that occurs during normal tissue turnover and is involved in tissue homeostasis.^{4,5} In the gastric epithelium, apoptosis plays an essential role in maintaining tissue integrity. Normally, the rate of cell loss by apoptosis is matched by the rate of new cell production by proliferation.⁶ However, this balance may be affected by *H pylori* infection, leading to various gastroduodenal diseases. It has been shown that *H pylori* infection induces apoptosis in gastric epithelial cells,^{8–20} and subsequently results in an increase in cell proliferation as a host response to apoptosis.^{4,21,22} However, apoptosis induced by *H pylori* infection that is not accompanied by a matched increase in cell proliferation will result in the loss of mucosal integrity, leading to gastric erosion and ulceration, or the loss of gastric glands, leading to gastric atrophy.^{13,23,24} Alternatively, if the response to apoptosis is greatly increased cell proliferation, this will result in increased proliferation of the gastric mucosa, which is believed to increase the risk of the development of gastric neoplasia.^{4,7}

"In the gastric epithelium, apoptosis plays an essential role in maintaining tissue integrity"

Whether or not a cell undergoes apoptosis is controlled by the products of a large number of oncogenes and tumour sup-

pressor genes, some of which are aberrantly expressed in a proportion of gastric carcinomas.^{7,25} These products include proapoptotic proteins, such as c-Myc, p53, c-Fos, c-Jun, and some of the Bcl-2 family members (for example, Bak, Bad, Bcl-xs, etc), and antiapoptotic proteins, such as Mcl-1, c-Abl, retinoblastoma protein (pRb), and some of the Bcl-2 members (for example, Bcl-2, Bcl-xl, etc).⁷ However, the genetic mechanism that regulates *H pylori* induced apoptosis has not been fully elucidated. There are a few reports regarding an association between the Bcl-2 family and *H pylori* induced apoptosis,^{14,26,27} but most of these studies have been carried out in vivo. A recent study showed that Bcl-2 is associated with the release of mitochondrial cytochrome c, which is a major mediator of *H pylori* induced apoptosis.²⁸ Studies have shown that c-Myc has a dual role in regulating apoptosis and proliferation, but its role in *H pylori* induced apoptosis is unknown.²⁹ We hypothesise that *H pylori* infection induces apoptosis via pathways mediated by the proteins of the Bcl-2 family and/or the c-Myc gene. Therefore, this study was conducted to determine the effect of *H pylori* on cell apoptosis and the expression of apoptosis regulating proteins including Bcl-2, Bax, and c-Myc in an in vitro model.

MATERIALS AND METHODS

Helicobacter pylori culture

Experiments were performed with a cytotoxic (CagA+ and VacA+) reference strain of *H pylori*, NCTC 11637 (National Collection of Type Cultures, Public Health Laboratory, London, UK). *Helicobacter pylori* were grown for 48–72 hours at 37°C in an atmosphere with 5% CO₂ on nutrition agar plates (supplemented with 5% sheep's blood), harvested, and resuspended

Abbreviations: CFU, colony forming units; OD, optical density; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction

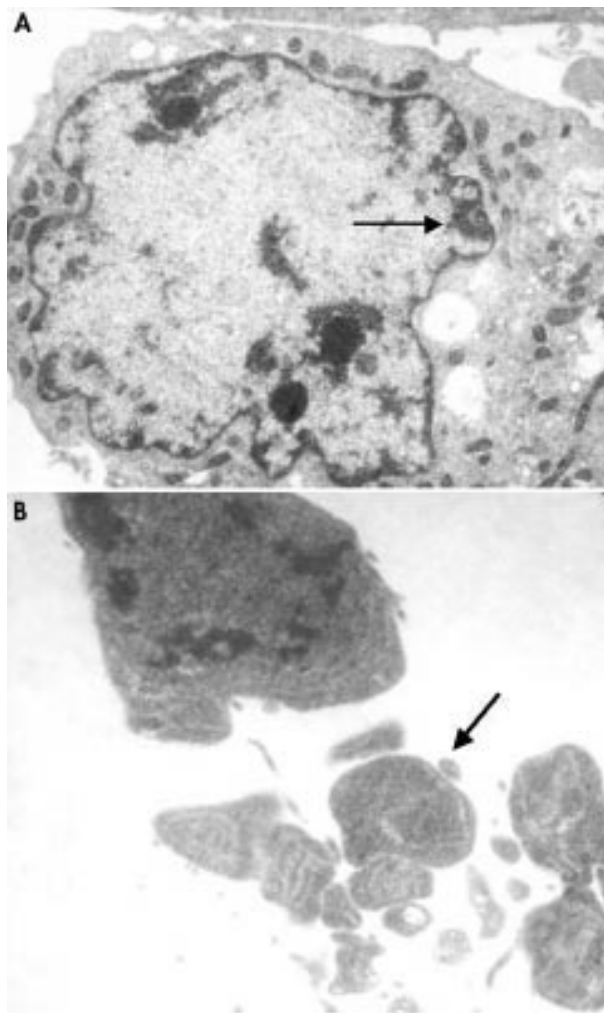


Figure 1 Transmission electron microscopy showing the ultrastructural apoptotic changes of SGC-7901 cells after co-culture with *Helicobacter pylori* for 48 hours: (A) condensation of irregular nuclear chromatin (arrow) (original magnification, $\times 10\,000$); and (B) formation of apoptotic bodies (arrow) (original magnification, $\times 20\,000$).

in RPMI 1640 (Gibco Company, New York, USA) medium, which was supplemented with 2% fetal calf serum (FCS). The bacterial densities were adjusted by the optical density (OD) measurement at 660 nm—that is, $1\text{ OD}_{660} = 10^8$ colony forming units (CFU)/ml.

Cell culture

The cell line SGC-7901, derived from a human gastric adenocarcinoma, was used in our study (Institute of Cytobiology of Chinese Academy of Sciences, Shanghai, China). Cells were grown in culture flasks containing RPMI 1640 culture medium supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO₂. For the evaluation of apoptosis and gene expression by fluorescence microscopy and immunohistochemistry, the cells were cultured on chamber slides in RPMI 1640 medium.

Co-culture of the gastric cells and *H pylori*

The gastric cells were seeded into flasks or the wells of microtitre plates at a density of 5×10^5 cells/ml or 5×10^4 cells/well, respectively. The flasks or microtitre plates were incubated overnight, and washed three times in RPMI 1640 medium. Then, medium containing intact, viable *H pylori* cells, ranging from 3.2×10^4 to 1.0×10^8 CFU/ml, was added, in quadruplicate, to corresponding flasks or wells. Co-culture of the gastric

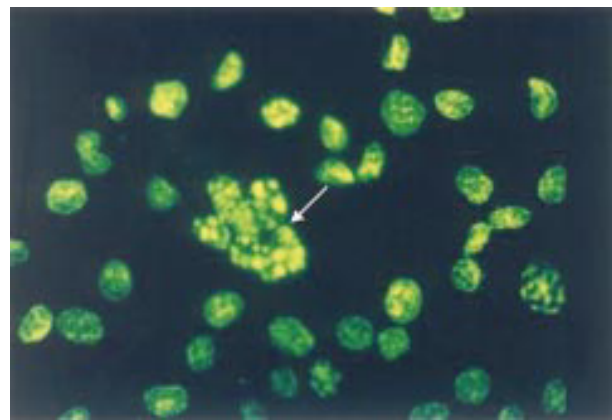


Figure 2 DNA specific fluorochrome staining showing condensation and fragmentation of nuclei of SGC-7901 cells (arrow) after co-culture with *Helicobacter pylori* (1.6×10^5 colony forming units/ml) for 48 hours (original magnification, $\times 400$).

Table 1 Apoptotic index (AI) of SGC-7901 cells after co-culture with increasing concentrations of *Helicobacter pylori* for 48 hours using DNA specific fluorochrome staining and flow cytometry

Concentration of <i>H pylori</i> (CFU/ml)	Mean apoptotic index (SD)	
	Fluorochrome staining	Flow cytometry
0 (control)	1.68 (0.13)	1.70 (0.12)
3.2×10^4	2.35 (0.44)	2.30 (0.27)
1.6×10^5	3.55 (0.24)*	3.56 (0.11)*
8.0×10^5	5.35 (0.37)*	5.31 (0.75)*
4.0×10^6	9.53 (0.15)*†	9.49 (0.17)*†

Apoptotic index of gastric cells was defined by the percentage of apoptotic cells over the total number of cells counted. * $p < 0.01$, compared with control; †one way ANOVA, $p < 0.01$. CFU, colony forming units.

cells and *H pylori* was performed for 48 hours before the determination of cellular apoptosis and the expression of apoptosis related proteins.^{26–30} The mean (SD) of the quadruplicate tests was used to assess the effect of *H pylori* on apoptosis and the expression of apoptosis related proteins at certain densities of *H pylori* cells.

Assessment of apoptosis

Apoptosis was assessed by three independent methods, namely: transmission electron microscopy, DNA specific fluorochrome staining, and flow cytometry.

Transmission electron microscopy

The gastric cells co-cultured with *H pylori* for 48 hours were harvested with a rubber policeman. The cell pellets were fixed with 2.5% glutaraldehyde for two hours, and then with 1% OsO₄ for 90 minutes. Next, the cells were dehydrated in an ethanol gradient, and embedded and polymerised at 60°C for 24 hours. Finally, ultrathin sections (40–80 nm) were prepared, double stained with 1% uranyl acetate and lead citrate, and examined by transmission electron microscopy (H-600; Hitachi Corporation, Tokyo, Japan).

DNA specific fluorochrome staining

The gastric cells co-cultured with *H pylori* on chamber slides for 48 hours were fixed with glacial acetic acid/methanol (3/1) for 10 minutes. Then the cells were incubated with Hoechst 33258 (Sigma Company, Saint Louis, Missouri, USA; 0.5 μg/ml) for one hour at room temperature. The slides were examined under fluorescence microscopy, and cells with condensed and fragmented chromatin in their nuclei were considered

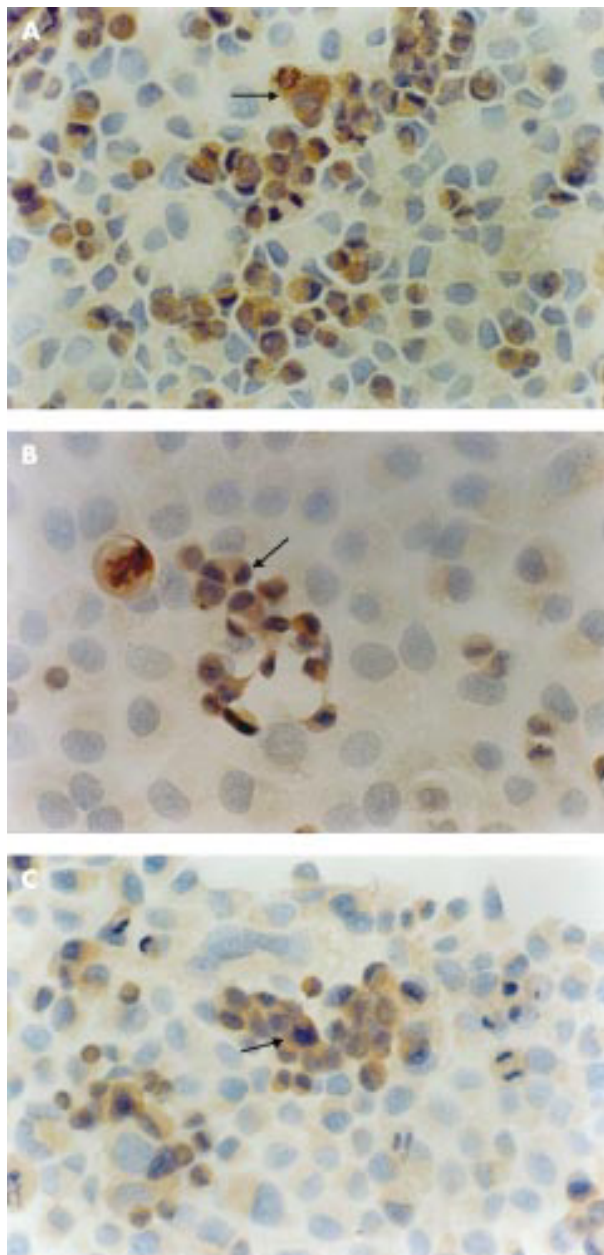


Figure 3 Positive immunohistochemical staining for (A) Bax [original magnification, $\times 200$], (B) c-Myc [original magnification, $\times 400$], and (C) Bcl-2 [original magnification, $\times 200$] proteins in the cytoplasm of SGC-7901 cells (arrows) after co-culture with *Helicobacter pylori* (1.6×10^5 colony forming units/ml) for 48 hours.

apoptotic. The apoptotic index of gastric cells was determined by the percentage of apoptotic cells over the total number of cells counted. At least 300 cells were counted in each experiment.

Flow cytometric analysis

The gastric cells co-cultured with *H. pylori* in flasks for 48 hours were harvested and fixed with 70% alcohol for 12 hours at -20°C . Next, the cells were incubated with propidium iodide (50 $\mu\text{g/ml}$) and RNase A (5 $\mu\text{g/ml}$) for 30 minutes at room temperature in the dark. The cells were analysed by flow cytometry (BD Company, New York, USA) for cell apoptosis.

Assessing the expression of the Bcl-2, Bax, and c-Myc proteins

Gastric cells co-cultured with *H. pylori* on chamber slides for 48 hours were fixed with glacial acetic acid/methanol (3/1), and

then used to detect the expression of the Bcl-2, Bax, and c-Myc proteins by means of immunohistochemistry. Briefly, the slides were treated with 3% hydrogen peroxide for 60 minutes at 37°C , and incubated with rabbit monoclonal antihuman Bax antibody for 30 minutes, or with mouse monoclonal antihuman Bcl-2 or c-Myc antibody for 90 minutes at 37°C , followed by the application of biotinylated goat antirabbit or antimouse immunoglobins for 10 minutes at 37°C . Next, the slides were stained with the avidin–biotin complex and developed with diaminobenzidine-hydrogen peroxidase substrate (DAB; Maxim Biotect, Fuzhou, China), and lightly counterstained with haematoxylin. All antibodies and the avidin–biotin complex were purchased from Maxim Biotect and were ready to use without further dilution.

For each slide, cells were counted for immunostaining in five randomly chosen fields with at least 100 cells in each field. The intensity of immunostaining was scored as follows: 0, negative; 1+, weak; 2+, moderate; and 3+, strong. Cells with moderate or strong immunostaining were defined as positively stained. The percentage of positively stained cells over the total number of cells counted was calculated to provide the positive rate.

Measurement of c-Myc mRNA

The gastric cells co-cultured with *H. pylori* in flasks for 48 hours were harvested. c-Myc mRNA in the cells was measured by the reverse transcription-polymerase chain reaction (RT-PCR). The primers specific for c-Myc were c1 (5'-GCATCCACGAAACTT-3') and c2 (5'-AACGTTGAGGGGCAT-3'). The primers specific for β actin (as an internal reference) were $\beta 1$ (5'-CTACAATGAGCTGCGTGTGGC-3') and $\beta 2$ (5'-CAGGTCCAGACGCAGGATGGC-3').

RNA was extracted from the cells using a total RNA extraction kit (SABC, Shanghai, China). Contaminated DNA was removed by treating the samples with RNase free DNase I (Promega, Madison, Wisconsin, USA). RT-PCR was performed using a reverse transcription system (Promega), according to the manufacturer's instructions. Briefly, the first strand cDNA was synthesised using the c2 and $\beta 2$ primers and avian myeloblastosis virus reverse transcriptase (Promega), followed by PCR amplification using the c1, c2, $\beta 1$, and $\beta 2$ primers. PCR amplification was performed for 30 cycles. The PCR products were electrophoresed on a 40% polyacrylamide gel and stained with AgNO_3 . The average grey density of the c-Myc RT-PCR products in the polyacrylamide gel electrophoresis (PAGE) bands, which reflects the extent of c-Myc mRNA expression, was analysed using an image analysis system (IAS-1000; Quality Engineering Associates, Burlington, USA).

Statistical analysis

The *t* test for independent samples was used to determine the difference in the apoptotic index, and the expression of the Bcl-2, Bax, and c-Myc proteins and c-Myc mRNA between each study group and the control. One way ANOVA was used to determine the effect of increasing numbers of *H. pylori* on apoptosis and the expression of the Bcl-2, Bax, and c-Myc proteins and c-Myc mRNA. All *p* values calculated were two tailed; the α level of significance was set at $p < 0.05$.

RESULTS

Effect of *H. pylori* on apoptosis

The ultrastructural changes associated with apoptosis in SGC-7901 cells after co-culture with *H. pylori* for 48 hours are shown in fig 1A and B. These changes included condensation beneath the nuclear membrane of irregular, crescent shaped, highly osmiophilic chromatin and the formation of membrane bound fragments (apoptotic bodies), as described previously.⁷ DNA specific fluorochrome staining also demonstrated the typical morphological changes of apoptosis in the gastric cells after

Table 2 Positive rate of Bcl-2, Bax, and c-Myc proteins of SGC-7901 cells after co-culture with increasing concentrations of *Helicobacter pylori* for 48 hours as determined using immunohistochemistry

Concentration of <i>H pylori</i> (CFU/ml)	Positive rate (% (SD))		
	Bcl-2	Bax	c-Myc
0 (control)	40.00 (2.94)	23.25 (1.26)	2.20 (0.65)
3.2×10 ⁴	36.75 (1.71)	24.25 (1.26)	2.78 (0.97)
1.6×10 ⁵	25.25 (1.71)*	34.50 (2.38)*	5.65 (1.07)*
8.0×10 ⁵	14.50 (2.65)*	43.75 (1.71)*	9.23 (0.95)*
4.0×10 ⁶	7.75 (1.71)*†	53.75 (2.63)*†	12.88 (0.74)*†

*p<0.01, compared with control; †one way ANOVA, p<0.01. CFU, colony forming units.

incubation with *H pylori* for 48 hours—that is, chromatin condensation and nuclear fragmentation (fig 2). There was no significant difference between the apoptotic index obtained with a bacterial density of 3.2 × 10⁴ CFU/ml and that seen in the control group (where no bacterial cells were added). However, the apoptotic index increased significantly when a bacterial density of 1.6 × 10⁵ CFU/ml or greater was used, and this occurred in a density dependent manner (table 1). Flow cytometric analysis showed similar results to the fluorochrome staining—the apoptotic index of the gastric cells started to increase at a bacterial density of 1.6 × 10⁵ CFU/ml, in a density dependent manner (table 1). However, a large number of necrotic cells were seen when the cells were co-cultured with *H pylori* at densities of 2 × 10⁷ and 1 × 10⁸ CFU/ml, so that results obtained at these densities were considered inaccurate, and not included in the analysis.

Effect of *H pylori* on the expression of Bcl-2, Bax, and c-Myc

SGC-7901 cells constitutively express Bcl-2, Bax, and c-Myc in their cytoplasm; 40%, 23.3%, and 2.2% of cells were immunostained for Bcl-2, Bax, and c-Myc, respectively, with moderate or strong intensity (table 2). The expression of the Bax and c-Myc proteins was increased (fig 3A, B), whereas the expression of the Bcl-2 protein was decreased after co-culture of the gastric cells with *H pylori* for 48 hours (fig 3C). These changes were also dependent on the density of *H pylori* (p < 0.01; one way ANOVA; table 2).

Effect of *H pylori* on the expression of c-Myc mRNA

The c1 and c2 primers amplified a 237 bp section of the c-Myc gene. The average grey density of the c-Myc RT-PCR product in PAGE bands was increased after co-culture of the gastric cells

Table 3 Mean grey density of c-Myc RT-PCR products of SGC-7901 cells in PAGE bands after co-culture with increasing concentrations of *Helicobacter pylori* for 48 hours

Concentration of <i>H pylori</i> (CFU/ml)	Mean (SD) grey density
0 (control)	156.2 (7.6)
3.2×10 ⁴	172.4 (4.2)*
1.6×10 ⁵	203.7 (3.7)*
8.0×10 ⁵	219.4 (1.4)*
4.0×10 ⁶	226.8 (3.2)*†

*p<0.01, compared with control; †one way ANOVA, p<0.01. CFU, colony forming units; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction.

with *H pylori* for 48 hours; these changes were also *H pylori* density dependent (p < 0.01; one way ANOVA; table 3).

DISCUSSION

Most previous studies evaluating the association between *H pylori* infection and apoptosis have used the terminal uridine deoxynucleotide nick end labelling method to identify apoptotic cells in gastric biopsy specimens.^{8–12,14–18–20} Although this technique is an important method for visualising apoptotic cells, it has the limitation of being unable to discriminate between apoptosis and necrosis.³¹ In our present study, three different techniques—namely, transmission electron microscopy, fluorochrome staining, and flow cytometry—were used to determine apoptosis. Our findings confirmed that *H pylori* induces apoptosis in gastric cells in vitro, consistently with previous studies carried out both in vitro and in vivo.^{8–20}

The mechanism by which *H pylori* induces apoptosis has yet to be elucidated. Both bacterial factors and the host response may be involved in the induction of apoptosis.⁴ The bacterial factors may include the VacA cytotoxin and lipopolysaccharide.^{12–14–16–18–21–27–32–35} In addition, the production of reactive oxygen metabolites, inducible nitric oxide synthase, and various cytokines such as tumour necrosis factor α , interferon γ , interleukin 2, and interleukin 1 during *H pylori* infection, as part of the host response, may also contribute to *H pylori* induced apoptosis.^{9–10–16–17–36–38} However, knowledge of the genetic background of *H pylori* induced apoptosis is scarce. Previous studies have reported that p53 is involved in *H pylori* associated apoptosis of gastric mucosa,^{11–15} although other studies suggest that *H pylori* induced apoptosis is mediated by multiple eukaryotic signalling cascades that are not dependent upon increased p53 concentrations.^{16–17–21–26–32–37} A few studies have investigated the role of the Bcl-2 family in *H pylori* induced apoptosis. It was reported that in cell culture, *H pylori* induced apoptosis was accompanied by an increased expression of Bak (Bcl-2 associated killer gene), with little change in the expression of other Bcl-2 family members, such as Bcl-2, Bcl-x_L, or Bax.³⁹ These findings were consistent with in vivo observations that the expression of Bak was increased in gastric epithelial cells in *H pylori* positive patients compared with *H pylori* negative patients, but there was no difference in the expression of Bcl-2.^{39–41} These data suggest that *H pylori* induced gastric epithelial cell apoptosis is at least partially mediated by a Bak dependent pathway.³⁹ In addition, in our present study, we showed that co-culture with *H pylori* resulted in the overexpression of Bax, and suppressed the expression of Bcl-2. These findings are in agreement with in vivo observations that *H pylori* infection induces apoptosis associated with an upregulation of Bax and downregulation of Bcl-2.¹⁴ In contrast, an in vitro study reported that both toxic and non-toxic *H pylori* strains induced the expression of the Bcl-2, Bax, and Bcl-x_s proteins in p53 deleted or mutated gastric epithelial cell lines at 24 hours when apoptosis did not occur.²⁶ However, the expression of Bcl-2 and Bax declined, whereas that of Bcl-x_s became raised at 48 hours when apoptosis increased significantly, implying that Bcl-x_s may be an important mediator in *H pylori* induced apoptosis.²⁶ Recently, Shibayama *et al* reported overexpression of two other proapoptotic Bcl-2 family members, Bad and Bid.²⁷ Overall, these observations suggest that the overexpression of proapoptotic proteins and the underexpression of antiapoptotic proteins among the Bcl-2 family may play an important part in *H pylori* induced apoptosis.

“Our findings are in agreement with in vivo observations that *H pylori* infection induces apoptosis associated with an upregulation of Bax and downregulation of Bcl-2”

In a previous study, Nardone *et al* detected the overexpression of the c-Myc protein in a substantial proportion (36%) of

Take home messages

- *Helicobacter pylori* induces morphological changes typical of apoptosis
- The apoptotic index began to increase when *H pylori* were at a density of $> 1.6 \times 10^4$ colony forming units/ml, in a density dependent manner
- The expression of the Bax and c-Myc proteins and of c-Myc mRNA was increased, whereas Bcl-2 expression was decreased after co-culture for 48 hours
- Thus, *H pylori* induced apoptosis in gastric epithelial cells is mediated by the altered expression of the Bcl-2, Bax, and c-Myc genes

patients with gastric carcinoma, and in some patients with *H pylori* associated atrophic gastritis (15%), whereas this protein was not found in normal controls.⁴² However, no data on apoptosis were given, and the association between c-Myc and *H pylori* induced apoptosis remains unknown. Therefore, our present study was the first, to our knowledge, to explore this issue. Our results clearly showed that co-culture of SGC-7901 cells with *H pylori* for 48 hours led to an increase in cell apoptosis, which was accompanied by the increased expression of the c-Myc protein and mRNA. This indicates that the c-Myc gene, in addition to the Bcl-2 family of genes, may be involved in regulating *H pylori* induced apoptosis, although in vivo studies are required to confirm this observation.

One limitation of our study was that the cells tested were derived from a human gastric cancer, which may not respond to *H pylori* in the same manner as intact gastric epithelial cells. It has been shown that cancer cell lines undergo about 50% less apoptosis in response to *H pylori* than cells in primary culture.¹⁶ However, non-transformed gastric cell lines are rarely available, and primary cultures are difficult to achieve. For these reasons, almost all studies evaluating the effect of *H pylori* on apoptosis of gastric epithelial cells in vitro have used gastric cancer cell lines. In our present study, a model of co-culture of *H pylori* with the gastric cancer cell line, SGC-7901, was established, which produced similar results obtained with other gastric cancer cells including AGS, a commonly used cell line.

In conclusion, *H pylori* induced apoptosis in gastric epithelial cells is mediated by the altered expression of the Bcl-2, Bax, and c-Myc genes.

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ECHO.....

Nuclear inclusions go missing from neurones in hereditary ataxias



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Mystery surrounding the events leading to degeneration of neurones in hereditary ataxias has deepened, as an immunohistochemical study discloses complete absence of nuclear inclusions in Purkinje cells in human brain stems. At least two opposing explanations are possible.

Nuclear inclusions are of interest because they are usually common in Purkinje cells in the brain stem in 10 or so inherited neurodegenerative diseases and contain products of the CAG repeat sequences in the disease gene. The authors chose four diseases with different degrees of cerebellar degeneration and compared the extent of nuclear inclusions, for evidence of a common pathogenesis.

The results were perplexing. Nuclear inclusions positive for either of two gene products—ubiquitin or polyglutamine—were restricted to the dentate nucleus in three diseases—spinocerebellar ataxia (SCA) 1, SCA3, and dentatorubral pallidoluysian atrophy (DRPLA)—and Golgi cells in SCA1, SCA2, and DRPLA. None occurred in Purkinje cells from any source.

A link between nuclear inclusions and neurodegeneration consistent with the results could be explained in two ways. If they were lethal early cell death could lead to their absence or if they were protective their absence would indicate impending cell death. Others have observed lack of nuclear inclusions in other diseases in this group. It seems that only more effort will unravel any link.

Brain stems were obtained from 13 patients with SC1, SC2, SC3, or DRPLA and three controls with neither neurological nor psychiatric conditions. Serial sections from each were stained for ubiquitin or polyglutamine with specific antisera.

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