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<td><strong>Author(s)</strong></td>
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Effect of Helicobacter pylori on apoptosis and apoptosis related genes in gastric cancer cells

Y Yang, C S Deng, J Z Peng, B C-Y Wong, S K Lam, H H-X Xia

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 × 10^6 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 × 10^5 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.
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 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% CO$_2$. 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 $5 \times 10^5$ cells/ml or $5 \times 10^4$ 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 $3.2 \times 10^4$ to $1.0 \times 10^5$ CFU/ml, was added, in quadruplicate, to corresponding flasks or wells. Co-culture of the gastric 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% OsO$_4$ 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 Hoescht 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.

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

<table>
<thead>
<tr>
<th>Concentration of H pylori (CFU/ml)</th>
<th>Mean apoptotic index (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorochrome staining</td>
</tr>
<tr>
<td>0 [control]</td>
<td>1.68 (0.13)</td>
</tr>
<tr>
<td>3.2×10$^4$</td>
<td>2.35 (0.44)</td>
</tr>
<tr>
<td>1.6×10$^5$</td>
<td>3.35 (0.24)*</td>
</tr>
<tr>
<td>8.0×10$^5$</td>
<td>5.35 (0.37)*</td>
</tr>
<tr>
<td>4.0×10$^6$</td>
<td>9.53 (0.15)*†</td>
</tr>
</tbody>
</table>

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.
hours were fixed with glacial acetic acid/methanol (3/1), and gastric cells co-cultured with proteins Assessing 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 anti-human Bcl-2 or c-Myc antibody for 30 minutes, or with mouse monoclonal anti-H. pylori and apoptosis 21

Flow cytometric analysis
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′-GCATCCACGAAACTF-3′) and c2 (5′-AAGTTGAGGGCCAF-3′). The primers specific for β-actin (as an internal reference) were β1 (5′-CTCAATGAGCTGCTGCTGGC-3′) and β2 (5′-CAGTCCAGCCCGAGGATGCC-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 β2 primers and avian myeloblastosis virus reverse transcriptase (Promega), followed by PCR amplification using the c1, c2, β1, and β2 primers. PCR amplification was performed for 30 cycles. The PCR products were electrophoresed on a 40% polyacrylamide gel and stained with AgNO3. 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).

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′-GCATCCACGAAACTF-3′) and c2 (5′-AAGTTGAGGGCCAF-3′). The primers specific for β-actin (as an internal reference) were β1 (5′-CTCAATGAGCTGCTGCTGGC-3′) and β2 (5′-CAGTCCAGCCCGAGGATGCC-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 β2 primers and avian myeloblastosis virus reverse transcriptase (Promega), followed by PCR amplification using the c1, c2, β1, and β2 primers. PCR amplification was performed for 30 cycles. The PCR products were electrophoresed on a 40% polyacrylamide gel and stained with AgNO3. 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. DNA specific fluorochrome staining also demonstrated the typical morphological changes of apoptosis in the gastric cells after

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 μg/ml) and RNase A (5 μg/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 anti-human Bcl-2 or c-Myc antibody for 30 minutes, or with mouse monoclonal anti-
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 × 10^6 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 × 10^7 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 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 × 10^6 and 1 × 10^7 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 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.\(^{8–20}\) Although this technique is an important method for visualising apoptotic cells, it has the limitation of being unable to discriminate between apoptosis and necrosis.\(^{9–11}\) 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, consistent with previous studies carried out both in vitro and in vivo.\(^{9–20}\)

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.\(^{9–20}\) The bacterial factors may include the VacA cytotoxin and lipopolysaccharide.\(^{12–14}\) Moreover, 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.\(^{8–20}\) 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,\(^{21–23}\) although other studies suggest that *H. pylori* induced apoptosis is mediated by multiple eukaryotic signalling cascades that are not dependent upon increased p53 concentrations.\(^{13–18}\) A few studies have investigated the role of the Bcl-2 family in *H. pylori* induced apoptosis. It was reported that *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-xL, or Bax.\(^{26}\) 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.\(^{24–26}\) These data suggest that *H. pylori* induced gastric epithelial cell apoptosis is at least partially mediated by a Bak dependent pathway.\(^{26}\) 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.\(^{26}\) 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-xL proteins in p53 deleted or mutated gastric epithelial cell lines at 24 hours when apoptosis did not occur.\(^{27}\) However, the expression of Bcl-2 and Bax declined, whereas that of Bcl-xL became raised at 48 hours when apoptosis increased significantly, implying that Bcl-xL may be an important mediator in *H. pylori* induced apoptosis.\(^{27}\) 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

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**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.

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

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

**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.

<table>
<thead>
<tr>
<th>Concentration of <em>H. pylori</em> (CFU/ml)</th>
<th>Mean (SD) grey density</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 [control]</td>
<td>156.2 (4.6)</td>
</tr>
<tr>
<td>3.2 × 10^4</td>
<td>172.4 (4.2)*</td>
</tr>
<tr>
<td>1.6 × 10^5</td>
<td>203.7 (3.7)*</td>
</tr>
<tr>
<td>8.0 × 10^5</td>
<td>219.4 (1.4)*</td>
</tr>
<tr>
<td>4.0 × 10^6</td>
<td>228.6 (3.2)*†</td>
</tr>
</tbody>
</table>

* p<0.01, compared with control; † t one way ANOVA, p<0.01. CFU, colony forming units; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction.
patients with gastric carcinoma, and in some patients with H pylori associated atrophic gastritis (15%), whereas this protein was not found in normal controls. 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. 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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REFERENCES

ECHO..................................................................................................................

Nuclear inclusions go missing from neurones in hereditary ataxias

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.