**Helicobacter pylori** causes DNA damage in gastric epithelial cells

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**Helicobacter pylori** infection has been considered as a risk factor for gastric carcinoma. Strong evidence exists that reactive oxygen species (ROS) play an important role in carcinogenesis, and in vivo investigations have shown increased synthesis of ROS in the gastric mucosa of **H. pylori**-infected patients. In the present study the direct effects of **H. pylori** on ROS and DNA synthesis, induction of apoptosis and DNA repair were investigated in the gastric epithelial cell lines AGS and HM02. Incubation of gastric cells with **H. pylori** extract induced the synthesis of ROS, diminished the levels of reduced glutathione (GSH), induced DNA fragmentation and increased DNA synthesis in gastric cells. Poly(ADP-ribose) formation was increased in gastric cells exposed to **H. pylori** extract. FACS analysis of gastric cells exposed to **H. pylori** extract did not reveal any change in the percentage of cells in the G2/M phase of the cell cycle. The radical scavengers MnTBAP (a cell permeable superoxide dismutase mimic), ebselen (a GSH peroxidase mimic) and high doses of catalase completely blocked **H. pylori** extract-induced elevation in DNA synthesis. Our results indicate that **H. pylori** extract directly induces the synthesis of ROS in gastric epithelial cells and causes DNA damage.

**Introduction**

The Gram-negative bacterium **Helicobacter pylori** plays a causal role in the pathogenesis of chronic type B gastritis and peptic ulcer disease (1). Chronic type B gastritis has been shown to be sequentially followed by atrophic gastritis, intestinal metaplasia and carcinoma in a small group of patients, while the majority of infected subjects remain asymptomatic (1,2). Epidemiological studies have suggested an association between **H. pylori** and gastric cancer, and **H. pylori** has been classified as definitive carcinogenic by the WHO (3). However, the underlying pathogenic mechanisms are poorly defined. One characteristic event in inflammation is the infiltration of the affected tissue by neutrophils and macrophages, which produce large amounts of reactive oxygen species (ROS) in host defence reactions. Oxidative stress is supposed to be involved in tumor initiation (4) and, as ROS have been shown to enhance the expression of oncogenes and stimulate cell proliferation (5), in tumor promotion. In vivo, enhanced ROS levels due to neutrophil infiltration in **H. pylori** infected patients (6,7) as well as increased oxidative DNA damage in **H. pylori** infected mucosa (8) have been reported. Moreover, Bagchi et al. (9) have shown that **H. pylori** induces the synthesis of ROS in gastric cells in vitro.

Based on these observations we have investigated whether **H. pylori** directly causes DNA damage in gastric cells. We have studied the effects of **H. pylori** and a cytosolic extract of **H. pylori** on DNA synthesis, ROS formation and poly(ADP-ribose) formation in the gastric epithelial cell lines AGS and HM02.

**Materials and methods**

**H. pylori** strains and preparation of bacterial components

HP 87, a cagA-positive and cytotoxin-producing **H. pylori** strain (vacA s1m1 genotype) was used in all study protocols. Some comparative experiments were also conducted with the cagA-negative and non-cytotoxin-producing strain HP 85 (vacA s2m2 genotype) and with *Campylobacter jejuni* (ATCC 33560; American Type Culture Collection, Rockville, MD). Presence of cagA was analysed by RT–PCR as described (10). Presence of vacuolating toxin was determined by incubation of gastric HM02 cells with concentrated supernatants from **H. pylori** broth medium (10) and the vacA genotype was analysed by PCR as described by Atherton et al. (11). **H. pylori** was grown in brain–heart-infusion broth supplemented with 10% fetal calf serum (FCS) for 24 h at 37°C under microaerophilic conditions. Bacteria were harvested by centrifugation (11 000 g, 10 min). To estimate bacterial titers, optical density measurements were performed at 600 nm and were correlated to viable colony counts (c.f.u.). For preparation of cytosolic extracts, bacteria were sonicated for 15 min on ice and centrifuged twice (15 min at 13 000 g and 30 min at 40 000 g). The resultant supernatant was sterile filtered and used in the experiments on the basis of its protein content. An extract with a protein content of 20 µg was prepared from 106 bacteria.

**Characterization of the cytosolic extract**

Heat stability was determined by heating the **H. pylori** cytosolic extract to 95°C for 30 min. For digestion of protein, the bacterial extract was incubated with 0.1 µg/ml trypsin for 2 h at 37°C. The reaction was stopped with soy bean trypsin inhibitor. For preliminary sizing of the protein, the cytosolic extract was centrifuged through a Millipore filter containing a 100 kDa filtration membrane.

**Cell culture and co-cultivation experiments**

Two established human gastric epithelial tumor cell lines were used. HM02 cells are derived from a human, well-differentiated mucus-producing carcinoma (12), and AGS cells were obtained from ATCC. Gastric cells were plated at a density of 2.5 x 104 cells in 24-well plates or at 7.5 x 103 cells in 12-well plates in RPMI 1640 with 10% FCS and left to adhere for 24 h. Subsequently, cells were washed three times with RPMI 1640 and were incubated with indicated amounts of **H. pylori** or **H. pylori** extract for up to 24 h in serum-free RPMI.

**Assessment of DNA synthesis**

To assess DNA synthesis, cells were labelled during the final 4 h of the incubation period with 0.5 µCi [3H]thymidine. Incorporated radioactivity was quantified by harvesting cells on glass fiber filters, lysed by repeated washes with water, and incorporated radioactivity was detected by liquid scintillation counting.

**Assessment of cell protein**

Cell protein was measured according to the method of Skehan et al. (13). Cells were fixed with trichloroacetic acid (TCA) (final concentration 10%) and stained with 0.4% sulforhodamine (SRB) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid. Then the cultures were air dried, and bound SRB was solubilized with 10 mM Tris base. The optical density was measured at 550 nm.

**Abbreviations:** GSH, glutathione; ROS, reactive oxygen species.
Assessment of apoptosis

Apoptosis was assessed by two independent methods: (i) ELISA detection of cytosolic oligonucleosome-bound DNA and (ii) DNA-specific fluorochrome staining.

**ELISA method.** An ELISA kit (Boehringer Mannheim) that detects histone-associated DNA fragments in the cytoplasm was used for quantitative measurement of apoptosis (10). The test was performed according to the manufacturer’s instructions. Briefly, after incubation with *H. pylori* extract the cell plates were centrifuged for 10 min at 200 g. After removal of the supernatant cells were lysed, and the lysate was centrifuged for 10 min at 200 g. Supernatant (20 µl) was transferred to a streptavidin coated microtiter plate and 80 µl of the reaction mixture containing anti-histone-biotin and anti-DNA-POD was added. After 2 h of incubation at room temperature the reaction mixture was removed by subsequent washes with buffer. An aliquot of 100 µl of substrate solution was then added, and the plate was incubated for 10 min at room temperature. The optical density was measured at 405 nm.

**DNA-specific fluorochrome staining.** After cultivation with *H. pylori* extract cells were centrifuged at 2000 g for 5 min and stained at 37°C for 10 min in RPMI containing Hoechst 33342 (1 µg/ml). Cells were washed, mounted with PBS and examined for morphological changes using an Olympus fluorescent microscope (IMT-2). Nuclei with highly condensed and fragmented chromatin were considered apoptotic.

**Cell cycle analysis**

Cells were harvested by trypsination, washed with RPMI 1640 containing 1% fetal bovine serum and resuspended in 100 µl of a solution containing 5 µg propidium-iodide, 1% Triton X-100, 1% BSA and 4 mM sodium citrate-buffer, pH 7.4. After 15 min incubation at room temperature under light exclusion, the same volume of RNase A (10 mg/ml in 10 mM Tris and 15 mM NaCl, pH 7.4) was added and cells were incubated for an additional 30 min at room temperature. At the end of incubation period cells were analyzed using a Becton Dickinson FACScan and Lysis II software. Apoptotic cells were found in the sub-G1 fraction; this was confirmed using camptothecin (4 µg/ml), as a positive control.

**Assessment of ROS production**

ROS were determined with the cell-permeable fluorogenic probe 2',7'-dichlorofluorescin diacetate (DCF-DA). The assay is based on the fluorescence detection of dichlorofluorescin (DCF), which is formed by hydrogen peroxide oxidation of the non-fluorescent precursor dichlorofluorescin (14). Cell monolayers were incubated with 10 µg/ml DCF-DA and the *H. pylori* extract for 1 h at 37°C. After incubation, cells were harvested by trypsinization, washed with RPMI 1640 and resuspended in PBS. Intracellular DCF was analysed using a Becton Dickinson FACScan and Lysis II software.

**Assessment of glutathione (GSH) levels**

Cell monolayers were incubated with *H. pylori* extract for 24 h at 37°C. Cells were washed and lysed with 0.6 ml of 0.5% Triton X-100, and 60 µl of 50% TCA was added to the cell lysate. After centrifugation for 10 min at 14 000 g, GSH was determined in 20 µl aliquots of the supernatant with o-phthalaldehyde (15) by measuring fluorescence on a Shimadzu RF-5001-PC spectrofluorometer (excitation 350 nm; emission 420 nm).

**Assessment of poly(ADP-ribose) formation**

To detect poly(ADP-ribose) in gastric cells, we used the immunofluorescence technique described by Bürkle et al. (16). Gastric cells were grown on 96-well plates to confluency and treated as indicated. Cells were fixed with TCA (final concentration 10%) for 1 h, followed by successive washings in 70%, 90% and absolute ethanol at −20°C for 5 min each. The cells were dried, rehydrated in PBS and incubated for 45 min at 37°C with monoclonal antibody 10H directed against poly(ADP-ribose). After washing in PBS, fluorescein isothiocyanate–conjugated anti-mouse antibody was applied for 1 h at 37°C. Cells with labeled nuclei were counted and expressed as percentage of total cells.

**Statistics**

Results are expressed as means ± SEM of at least three independent experiments. For statistical analysis Student’s t-test and ANOVA were used. Probability values *P* < 0.05 were considered significant.

**Results**

**Effects of *H. pylori* on DNA synthesis of gastric epithelial cells**

Co-cultivation of AGS cells with increasing titres of *H. pylori* for 24 h was associated with an increase in [3H]thymidine incorporation. Bacterial counts of 5×10⁶ and 10⁷ c.f.u./ml significantly increased DNA synthesis, whereas a higher bacterial load decreased [3H]thymidine incorporation. In contrast, the *H. pylori* extract progressively enhanced DNA synthesis (Figure 1). Similar effects were observed in experiments using the gastric cell line HM02 (data not shown). In all further experiments investigating the stimulatory effects of *H. pylori* on DNA synthesis we used the *H. pylori* extract.

**Partial characterization of the DNA synthesis stimulatory factor of *H. pylori***

Increased DNA synthesis was observed with both *H. pylori* strains. HP 87 and HP 85 extracts (20 µg/ml) enhanced [3H]thymidine incorporation to 202 ± 11% and 203 ± 8% of the respective control values (n = three different experiments). To examine the specificity of the *H. pylori* effect, comparative experiments with *C. jejuni* extract (20 µg/ml) were performed. *Campylobacter jejuni* did not stimulate DNA synthesis (105 ± 2% of control value; n = three different experiments). To further characterize the nature of the stimulatory effect of *H. pylori*, the sensitivity of the extract to heat and enzymatic treatment was investigated. The effect on [3H]thymidine incorporation was sensitive to trypsin and partially sensitive to heat treatment. Filtration studies showed that the stimulatory activity on DNA synthesis was recovered within the 100 kDa retentate (Table I).
Table II. Comparison of the effects of the H. pylori extract and EGF on [3H]thymidine incorporation, cellular protein and DNA fragmentation

<table>
<thead>
<tr>
<th></th>
<th>[3H]thymidine uptakea</th>
<th>Cell proteinb</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>H. pylori extract</td>
<td>176 ± 24*</td>
<td>104 ± 2</td>
<td>149 ± 8*</td>
</tr>
<tr>
<td>EGF</td>
<td>189 ± 12*</td>
<td>134 ± 3*</td>
<td>38 ± 3*</td>
</tr>
</tbody>
</table>

AGS cells were incubated for 24 h in RPMI medium alone, with H. pylori extract (20 µg/ml) or EGF (1 ng/ml). Cell protein, [3H]thymidine incorporation and DNA fragmentation were determined as described in Materials and methods. Apoptosis was determined by an ELISA quantitating cytosolic oligonucleosome-bound DNA. Data were expressed as percentage of control. Values are mean ± SEM of three experiments.

*P < 0.05 versus control.

Table III. Cell cycle distribution of AGS cells treated with H. pylori extract or EGF

<table>
<thead>
<tr>
<th>Sub-G1</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>8.9 ± 2.9</td>
<td>62.4 ± 3</td>
<td>17.9 ± 1.5</td>
</tr>
<tr>
<td>H. pylori extract</td>
<td>8.4 ± 1.3</td>
<td>63.6 ± 2</td>
<td>18.1 ± 0.8</td>
</tr>
<tr>
<td>EGF</td>
<td>5.5 ± 1.5*</td>
<td>58.1 ± 1*</td>
<td>19.6 ± 1.3</td>
</tr>
</tbody>
</table>

Data represent percentage cells in each stage of the cell cycle. AGS cells were incubated for 24 h in RPMI medium alone, with H. pylori extract (20 µg/ml) or EGF (1 ng/ml). Cell cycle was analysed in dispersed cells stained with propidium-iodide by flow cytometry. Values are mean ± SEM of six experiments.

*P < 0.05 versus control.

Comparison of the effects of H. pylori extract and epidermal growth factor (EGF) on [3H]thymidine incorporation, cell count, apoptosis and cell cycle

Since increased [3H]thymidine incorporation is frequently taken as a marker for cell proliferation, the effect of H. pylori extract (20 µg/ml) on gastric cell count (SRB assay, a marker for total cellular protein), DNA synthesis, DNA fragmentation and cell cycle distribution was investigated and compared with those of the mitogen EGF. EGF (1 ng/ml) significantly increased [3H]thymidine incorporation, cell count, the number of cells in the G2/M phase and reduced the number of apoptotic cells. In contrast, the H. pylori extract had no effect on cell count and the cell cycle distribution detected by propidium-iodide FACScscan (Tables II and III), but increased DNA fragmentation measured by ELISA detection of cytosolic oligonucleosome-bound DNA (Table II) and DNA staining with Hoechst 33342 (Figure 2).

H. pylori induces poly(ADP-ribose) formation in gastric cells

One factor involved in the repair of DNA damage is poly(ADP-ribose)polymerase (17). The enzyme catalyses the synthesis of poly(ADP-ribose) from nicotinamide adenine dinucleotide. By using an immunofluorescence technique for the in situ detection of cellular poly(ADP-ribose) formation we found a 2-fold increase of poly(ADP-ribose)-positive AGS cells after treatment with H. pylori extract (20 µg/ml; Figure 3).

H. pylori induces oxygen radical production and reduces GSH levels in gastric cells

To investigate whether H. pylori extract is able to directly induce formation of oxygen free radicals in gastric cells, the production of oxygen radicals was determined in AGS cells loaded with DCF-DA. As shown in Figure 4, a significant increase in oxygen radical production was detected in AGS cells treated for 1 h with H. pylori extract. In addition, the H. pylori extract diminished GSH levels in a dose-dependent manner in whole-cell lysates from HM02 (Figure 5) and AGS cells (data not shown).
formation. These H. pylori cell growth.

There is ample evidence that ROS can induce cell proliferation, DNA damage and the infective load of H. pylori. It was suggested that the sources of ROS production are probably host neutrophils, which are activated by soluble product(s) of H. pylori (19). Our data provide evidence to support the hypothesis that H. pylori is able to directly induce ROS synthesis in gastric cells. Stimulation of ROS formation by H. pylori in vitro has also been observed by Bagchi et al. (9). However, these investigators did not address whether ROS formation is associated with DNA damage. Strong evidence exists that ROS play an important role in all stages of carcinogenesis (20,21). ROS cause formation of oxidized bases, single-strand breaks and crosslinking of DNA (4). One important type of DNA damage is the base modification 8-hydroxy-guanine (22). Baik et al. (8) reported increased levels of 8-hydroxy-guanine in gastric mucosal specimens of H. pylori infected individuals.

Enzymatic and non-enzymatic antioxidants provide the primary defence against ROS. H. pylori gastritis is associated with a significant decrease in the concentration of ascorbic acid in gastric juice (23). This effect is independent of plasma ascorbate concentration, and it has been postulated that secretion of ascorbic acid from the circulation is disrupted (24). Our in vitro study has shown a large depletion of cellular GSH levels during incubation with H. pylori. GSH acts at several levels of the antioxidant defence: it is a scavenger of free radicals, a substrate for the antioxidant enzyme GSH peroxidase and is involved in the direct repair of oxidative DNA lesions. GSH and ascorbate have some overlapping functions and spare each other (25). Any process that reduces the concentration of GSH potentiates the risk of ROS-induced DNA damage.

DNA damage induced by ROS can be repaired by specific and non-specific repair mechanisms (26). Since eukaryotic DNA is associated with histones, these proteins have to be removed before DNA repair takes place. The detachment of histones from DNA is supposed to be achieved by ADP-ribose polymers, which in the case of DNA damage are produced by the nuclear enzyme poly(ADP-ribose)polymerase (27). H. pylori extract markedly stimulated the formation of poly (ADP-ribose) in AGS cells suggesting a damaging effect of H. pylori extract followed by subsequent DNA repair.

There is ample evidence that ROS can induce cell proliferation, apoptosis and, at high doses, necrotic cell death (5). Recently we have shown that H. pylori induces apoptosis in the gastric cell line HM02 (10). Moss et al. (28) found a 5.8-fold increase of apoptotic cell number in gastric mucosa of H. pylori infected patients. The number of apoptotic cells did not correlate with the extent of gastritis, implying that a bacterial factor was directly responsible for induction of apoptosis. In this study we found that ROS formation was associated with apoptosis and increased DNA synthesis but not with an increase in cell numbers. These data suggest that...
the increased DNA synthesis is a compensatory mechanism to counteract DNA damage induced by \textit{H. pylori}. In contrast to this \textit{in vitro} situation, several \textit{in vivo} studies have shown increased gastric cell proliferation in \textit{H. pylori} infected patients (29,30). It has been postulated that \textit{H. pylori} induced apoptosis, and cell death causes a compensatory hyperproliferation of gastric mucosal stem cells (10).

In conclusion, we have shown that \textit{H. pylori} directly induces synthesis of ROS in gastric epithelial cells. Production of ROS was associated with DNA repair. These data suggest that \textit{H. pylori} itself has a genotoxic potential for gastric epithelial cells.

Acknowledgements

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