Influence of *Helicobacter pylori* on reactive oxygen-induced gastric epithelial cell injury

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Introduction

Infection of the gastric mucosa with *Helicobacter pylori* is strongly associated with chronic gastritis, peptic ulcer disease and gastric cancer. Multivariate logistical regression analysis of data implicating *H.pylori* as an etiological agent in the development of gastric cancer supports the hypothesis that this organism is an independent risk factor for gastric cancer (1). The World Health Organization has classified *H.pylori* as a group I carcinogen, with an attributable risk for gastric cancer of 50–60% (2). Epidemiological studies have also suggested that gastric cancer may be caused by reactive oxygen species (ROS) and that dietary antioxidants can prevent stomach cancer (3–6).

The gastric epithelium is continuously exposed to toxic ROS generated within the gastric lumen (ingested food, cigarette smoke, etc.). In addition, the gastritis associated with *H.pylori* infection stimulates the generation of ROS by the inflammatory cells present in the mucosa (7–9). Zhang et al. (10) showed an increase in ROS in the gastric mucosa in persons with *H.pylori*-associated gastritis and showed that the amount of reactive oxygen directly correlated with bacterial load. Protection of cells against ROS is accomplished through the activation of oxygen-scavenging enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. However, the effects of *H.pylori* bacterial products on the ability of gastric cells to protect themselves from damage by ROS is not known. Impairment in this important host cell defense mechanism would greatly reduce the ability of the gastric epithelial cells to tolerate an environment high in ROS, such as would be present with the chronic gastritis associated with *H.pylori* infection. Disturbance of the oxidant–antioxidant balance in the stomach might greatly increase the risk of cell death or DNA damage from ROS.

In the following experiments we evaluated the ability of gastric epithelial cells to defend themselves from ROS-induced cell injury after exposure to cagA+ and cagA- *H.pylori* strains, as well as evaluating the effects of bacterial exposure on the activity of ROS-scavenging enzymes such as catalase, glutathione peroxidase and SOD.

Materials and methods

**Chemicals**

Xanthine (Sigma Chemical Co., St Louis, MO) and xanthine oxidase (Sigma) dissolved in Hensleit buffer (11) were used to generate ROS. A soluble tetrazolium compound (MTS) was purchased from Promega Corp. (Madison, WI).

**Gastric epithelial cell culture**

A human gastric epithelial cell line (AGS), purchased from American Type Culture Collection (Manassas, VA), was used in these experiments. AGS...
cells were cultured in Dulbecco’s minimum essential medium (DMEM; Life Technologies, Grand Island, New York, NY) supplemented with 10% fetal bovine serum (Life Technologies), without antibiotics. Experiments were performed on AGS cells grown in 96-well tissue culture clusters (Corning Costar Corp., Cambridge, MA).

**H. pylori culture**

CagA- strains J117, J166, J254, J291 and cagA+ strains J68, J75, J110, J154 (a gift from Drs Timothy Cover and Richard Peek, Vanderbilt Medical Center, Nashville, TN) were used in these experiments. Each of these four cagA- strains was tested and found to have vacuolating cytotoxin activity (tox+). Also, we used *H. pylori* 60190 (ATCC 49503) a wild-type tox+ strain, and *H. pylori* 60190-v1, an isogenic mutant strain in which the vacA gene has been disrupted by insertional mutagenesis with loss of cytotoxin activity and absence of detectable protein (12). *H. pylori* bacteria were cultured on Trypticase Soy Agar with 10% sheep’s blood (Fisher Scientific, Gaithersburg, MD) for 72 h. *H. pylori* was also cultured in Brucella broth (Fisher Scientific) supplemented with 5% fetal bovine serum (Life Technologies). The bacteria were removed from the Brucella broth by centrifugation; the broth supernatant was concentrated 40-fold, using a Centricon-100 filter (Amicon, Inc., Beverly, MA), and then passed through a 0.2 µm filter. The concentrated *H. pylori* broth culture supernatants were then stored frozen at −70°C. Broth supernatant from *H. pylori* strain 60190 shows vacuolating cytotoxin activity when tested using HeLa and AGS cells, while broth from strain 60190-v1 does not display vacuolating cytotoxin activity. Experiments were performed by suspending whole bacteria in DMEM to a final concentration of 1 × 10^7 bacterial/ml. *H. pylori* broth culture supernatants were diluted in DMEM to a final concentration of 4 mg/ml of total bacterial protein.

**Gastric epithelial cell viability**

AGS cells grown in 96-well cell culture clusters were incubated for 24 h in culture medium containing cagA+ or cagA- *H. pylori* strains. Afterwards the cell medium was removed from AGS cells and the cells were washed twice with phosphate-buffered saline. AGS cells were then incubated in Heinslet buffer containing 100 µM xanthine. Xanthine oxidase (25 µg/ml) was added for 1 h to generate ROS. Negative controls were incubated in Heinslet buffer without xanthine oxidase. After 1 h, the Heinslet buffer was removed and the cells were incubated for 2 h in culture medium containing MTS. Gastric cell viability was determined through metabolism of tetrazolium, with a kit from Promega Corp., using a Beckman MR700 microplate reader.

**ROS-scavenging enzyme activity**

The activities of SOD, catalase and glutathione peroxidase were measured after incubating AGS cells for 24 h with cagA+ and cagA- *H. pylori* strains. Cultures were not exposed to ROS before measuring enzyme activities. Experiments were performed on AGS cells grown on 75 cm® flasks. Soluble AGS cell protein was extracted and frozen in aliquots, after determining the total protein concentration by the Bradford method (13).

Glutathione peroxidase activity was measured using the method of Suttrop et al. (14). The specific activity was calculated based on one unit of glutathione peroxidase converting 1 µmol glutathione/min which is equal to 0.5 µmol NADPH oxidized/min. Catalase activity was measured using the method of Woodbury et al. (15). The specific activity of catalase was calculated using the following equation: units of catalase/mg protein = (A∆A240nm/20 min) × 1000 (43.6 nmol/min of reaction solution). Total SOD activity was measured using hypoxanthine, xanthine oxidase and oxidized cytochrome c by the method of Loven et al. (16). Total SOD, Mn-SOD and CuZn-SOD protein levels were determined by enzyme-linked immunosorbent assay (ELISA) using the methods of Gotz et al. (17). No cross-reactivity between human and bacterial SOD occurred in these ELISAs.

**Preparation of AGS cell lysates for enzyme assays**

AGS cells grown to 60% confluence on tissue culture plates and subsequently exposed to *H. pylori* and or ROS were washed twice with Hank’s balanced salt solution and then harvested by scraping in 1.0 ml of lysis buffer [0.05 M HEPES (pH 7.4), 0.5% Triton X-100, 1 mM sodium orthovanadate and a 1:20 protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN)]. Lysates were centrifuged at 14 000 × g for 20 min at 4°C. Supernatant was stored in 0.5 ml aliquots at −70°C until used.

**Detection of 8-hydroxy-2-deoxyguanosine adducts**

DNA was isolated from AGS cells using a genomic DNA isolation kit (Sigma). DNA was hydrolyzed by digestion in nucleosome P1 (Sigma) followed by digestion with alkaline phosphatase (Boehringer) using a modification of the method of Fiala et al. (18). Proteins were removed by separation through a 30 000 mol. wt cut-off filter (Millipore Inc., Bedford, MA) and the resulting nucleosides were analyzed by HPLC with an electrochemical detector as previously described by Conway et al. (19). The nucleotides were injected into a Waters HPLC pump system using a Rhodyne injector. Nucleosides were detected by UV absorbance at 280 nm with a Beckman 160 absorbance detector. Signals were analyzed using Millennium Software (Millipore Inc.). Amounts of 8-hydroxy-2-deoxyguanosine (8-OH-dG)/10^7 dG were calculated from the areas under the curves of the peaks recorded in uV s. Converted to concentrations based on standard curves of authentic dG (detected by UV) and 8-OH-dG (detected by ECD) as follows:

\[
8\text{-OH-dG}/10^7 \text{dG} = (\text{picomoles 8-OH-dG/nanomoles dG}) \times 100
\]

**Statistical analysis**

Statistical analysis was performed for each experiment using general linear models (GLM) procedure. The analysis was performed using PROC GLM in the SAS 6.12 (SAS Institute Inc., Cary, NC) to determine whether the means were equal among three groups, i.e. cagA-, cagA+ and controls. The Student–Newman–Keuls test (multiple comparison test) was used to find which means differed from each other means. A P value of <0.05 was considered statistically significant.

**Results**

Exposure of AGS cells to *H. pylori* strains for 24 h reduced cell viability on average to 91% (there was no significant difference between cagA+ and cagA- strains). Exposure of AGS cells to ROS (in the absence of *H. pylori*) reduced cell viability to 84%. Exposure of AGS cells to ROS after incubating overnight with either cagA+ or cagA- *H. pylori* strains resulted in a mean cell viability of 73.7% and 39.5% of controls, respectively (Figure 1). AGS cells were exposed overnight to broth culture supernatants from isogenic *H. pylori* strain 60190 with VacA activity or *H. pylori* strain 60190-v1, without VacA activity or Brucella broth (control). The viability of AGS cells exposed to ROS was 97.7%, 70.5% and 63.5% after overnight
H. pylori enhances ROS cytotoxicity

**Fig. 2.** ROS-induced cell injury after exposure to *H. pylori* broth culture supernatant. AGS cells were incubated overnight with broth culture supernatant from cagA− *H. pylori* strain 60190 with vacuolating cytotoxin (vacA) activity (‘Tox-positive’), *H. pylori* strain 60190-v1, without vacA activity (‘Tox-negative’) and *Brucella* broth (‘ Controls’). Afterwards the AGS cells were exposed to ROS for 1 h. Viability was then assessed by metabolism of MTS. These data represent the mean (+ SD) of three experiments. Pre-incubation with *H. pylori* broth culture supernatant significantly reduced (P < 0.0001) AGS cell viability after exposure to superoxide. There was no significant difference in viability between AGS cells exposed to broth from *H. pylori* strain 60190 and 60190-v1. Statistical analysis was performed using ANOVA.

**Table 1.** Superoxide dismutase levels in AGS cells exposed to *H. pylori* strains

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>cagA−</th>
<th>cagA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZn-SOD (ng/mg protein)</td>
<td>693</td>
<td>674</td>
<td>681</td>
</tr>
<tr>
<td>Mn-SOD (ng/mg protein)</td>
<td>50</td>
<td>81.0*</td>
<td>85.6*</td>
</tr>
<tr>
<td>Total SOD activity (U/mg)</td>
<td>22.7</td>
<td>34.4</td>
<td>58.13*</td>
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*Significantly different from controls (P < 0.001).

incubation with *Brucella* broth, VacA− broth and VacA+ broth, respectively (Figure 2). The mean viability of AGS cells exposed overnight to VacA− broth or VacA+ broth alone was 94.8 and 94.0%, respectively, which was not significantly different from that of cells exposed to control broth. VacA activity does not appear to be the primary reason for the reduction in oxidant-associated cell injury between cagA+ and cagA− strains.

The activity of ROS-scavenging enzymes was also assessed after incubating cagA− and cagA+ *H. pylori* strains with AGS cells for 24 h. The total activity of SOD, which converts superoxide to hydrogen peroxide, increased significantly (156%) in AGS cells after incubation with cagA+ strains and only marginally (52%) after incubation with cagA− *H. pylori* strains (Table I). CuZn-SOD activity was not changed by exposure to *H. pylori*, whereas Mn-SOD activity increased 71.2% and 62.0% following exposure to cagA+ and cagA− *H. pylori* strains, respectively (Table I). Hydrogen peroxide within the cell is converted to water by either catalase or glutathione peroxidase. When AGS cells were incubated with cagA+ *H. pylori* strains, catalase activity increased on average by 51% and glutathione peroxidase activity increased on average by 240% (Figures 3 and 4). However, incubating AGS cells with cagA− *H. pylori* strains resulted in a mild reduction in catalase activity and a small increase in glutathione peroxidase activity (Figures 3 and 4).

We then sought to determine if the alteration in levels of
ROS-scavenging enzymes following exposure to H. pylori was associated with formation of DNA adducts associated with presence of ROS (8-OH-dG) in gastric epithelial cells. After exposing AGS cells to cagA⁻ or cagA⁺ strains for 24 h, DNA was extracted from AGS cells and analyzed for 8-OH-dG adducts. AGS cells that had not been exposed to H. pylori strains showed no evidence of 8-OH-dG adducts (background levels in epithelial cells can range from 0 to 25 8-OH-dG/10⁶ dG). AGS cells incubated with cagA⁻ and cagA⁺ strains displayed on average 99.0 and 44.5 8-OH-dG/10⁶ dG, respectively. These data show that exposure to H. pylori can induce ROS-related DNA adducts to levels at least two to four times higher than background.

Discussion

H. pylori infection of the gastric mucosa is associated with an abundant inflammatory response. Studies have shown that this bacterium is capable of stimulating oxidative bursts from neutrophils (20). Gastric tissue from H. pylori-infected persons contains more ROS than normal tissue and there is a direct correlation between bacterial load and the amount of ROS in the gastric mucosa (9,10). Also, mucosal damage quantitatively correlates with bacterial load and ROS production (9). Our data support the direct correlation between ROS and gastric mucosal damage in persons infected with H. pylori. These data also demonstrate that H. pylori can increase the susceptibility of gastric epithelial cells to ROS-associated cell injury.

The increase in catalase, glutathione peroxidase and SOD activity in AGS cells after exposure to cagA⁺ H. pylori strains is probably responsible for the increased survival of these cells after subsequent exposure to ROS. The generation of intracellular ROS by exposing AGS cells to cagA⁺ H. pylori strains is one possible explanation for the increases in activity of ROS-scavenging enzymes. One study has shown that exposure of gastric cells to H. pylori induces the production of intracellular ROS (21). This increase in ROS in gastric cells was enhanced by increasing the concentration of H. pylori and inhibited by use of antioxidants (21). Zhang and colleagues (20) have demonstrated that vacuolating cytotoxin-positive strains (tox⁺) induced a stronger oxidative burst in neutrophils than tox⁻ strains. All of our cagA⁻ strains are also tox⁺. Even though there are more ROS in the gastric mucosa of people infected with cagA⁻ strains, our data would suggest that gastric epithelial cells will be better able to avoid lethal injury in people infected with cagA⁺ strains than in people infected with cagA⁻ strains. H. pylori has been shown to produce catalase and SOD, but the reported amounts of these enzymes secreted would not be sufficient to scavenge extracellular oxidants (22). Gotz and colleagues have shown that gastric tissue from persons infected with H. pylori is higher in activity and content of the cytokine-inducible Mn-SOD, with little change in the constitutive CuZn-SOD. These changes in Mn-SOD could be reversed by successful treatment of H. pylori infection (23). Our studies also show an increase in Mn-SOD; however, the increase is much smaller in the absence of inflammatory cytokines in our system. Also, our study has shown a significant increase in total SOD activity (Table 1) in AGS cells exposed to cagA⁺ H. pylori strains as compared with AGS cells exposed to cagA⁻ H. pylori strains, without any increase in CuZn-SOD and only a slight increase in MnSOD. Superoxide and other oxygen radicals have been shown to inhibit SOD activity (24,25).

Gastric cells exposed to cagA⁺ H. pylori strains have significantly higher catalase and glutathione peroxidase activity, so these cells will more rapidly convert superoxide to hydrogen peroxide and reduce inhibition of SOD by superoxide, which translates into higher SOD activity. Superoxide has been shown to be produced in the cytoplasm of hepatocytes within the livers of mice infected with H. hepaticus (26). The increase in glutathione peroxidase and catalase activity, caused by overnight exposure of gastric cells to cagA⁺ H. pylori, is likely to have helped to protect these cells from sudden exposure to superoxide, giving them a survival advantage over gastric cells pre-incubated with cagA⁻ strains before exposure to superoxide. However, in the case of DNA damage from ROS, increased cell survival may ultimately be detrimental to the cell.

Studies have shown that the production of ROS is increased in the gastric mucosa of persons infected with H. pylori (8–10). In H. pylori-associated gastritis there are increased levels of 8-OH-dG adducts (27), which is supportive of a mechanism link between ROS, H. pylori and gastric cancer. This is also supported by data from studies on H. hepaticus, which causes liver cancer in mice. H. hepaticus infection of the liver in mice significantly increases the levels of 8-OH-dG adducts (28). The accumulation of DNA oxidative damage increases with progression of the liver disease (28). Our data show that H. pylori directly contributes to formation of 8-OH-dG adducts in gastric epithelial cells. The presence of exogenous ROS along with the reduction of antioxidants, such as vitamin C, in the gastric mucosa of people infected with H. pylori potentially increases the risk of oxidant-related cellular injury and DNA damage. Changes in the levels of cellular ROS-scavenging enzymes induced by H. pylori may further increase this risk of developing gastric cancer from ROS in people infected with H. pylori.

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References


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