HELCOBACTER PYLORI

*Helicobacter pylori* induces apoptosis in gastric epithelial cells through inducible nitric oxide

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Abstract

**Background**: Gastric mucosal injury by *Helicobacter pylori* has been suggested to be mediated by various cytokines induced by this organism. Nitric oxide (NO) is an important effector molecule involved in immune regulation and defence. To clarify the mechanisms by which *H. pylori* induces gastric mucosal cell injury, we examined whether *H. pylori* induces gastric epithelial death via NO production.

**Methods**: Cytotoxic and non-cytotoxic strains of *H. pylori* were used. The death of MKN45 cells caused by *H. pylori* was examined by the 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Aminoguanidine was used to inhibit inducible nitric oxide synthase (iNOS) activity. Expression of iNOS mRNA was determined by the reverse transcriptase–polymerase chain reaction and the DNA fragmentation analysis was performed by using agarose gel electrophoresis.

**Results**: The MTT assay revealed that neither viable *H. pylori* nor other components of the microorganism induced cell death. Both preincubation of MKN45 cells with interferon-γ for 6 h and coculture with TNF-α significantly increased the cytotoxicity of *H. pylori*. Both cytotoxic and non-cytotoxic strains of *H. pylori* induced cell death. Expression of iNOS mRNA was observed in MKN45 cells at 6, 8 and 12 h after *H. pylori* inoculation. The cytotoxicity of *H. pylori* was inhibited by aminoguanidine and DNA fragmentation analysis showed that *H. pylori* induced apoptosis.

**Conclusions**: These findings suggested that viable *H. pylori* induces apoptosis of gastric epithelial cells via nitric oxide. Our study indicated that iNOS expression plays an important role in gastric cell injury. © 2000 Blackwell Science Asia Pty Ltd

**Key words**: apoptosis, *Helicobacter pylori*, inducible nitric oxide synthase.

INTRODUCTION

*Helicobacter pylori* infection is a major cause of gastro-duodenal inflammation, peptic ulceration and gastric carcinoma. 1–7 *Helicobacter pylori* infection induces immune responses in the lamina propria layer of the stomach and involves lymphocytes, plasma cells, macrophages, eosinophils and, often, neutrophils. 8,9 Neutrophil infiltration in the gastric epithelium tissue was the initial pathological abnormality described in *H. pylori* gastritis and remains the histological hallmark of active infection. 9 Furthermore, gastric mucosal inflammation by *H. pylori* has been suggested to be mediated by inflammatory cytokines such as interleukin (IL)-8,10,11 interferon (IFN)-γ12 and tumour necrosis factor (TNF)-α.13

Nitric oxide (NO), a simple gas with free radical chemical properties, is a bioregulator that plays an important role in diverse physiological processes including smooth muscle relaxation, platelet inhibition, immune responses and inflammation.14 Overproduction of NO seems to be an important factor in the pathology of inflammatory processes. Inhibition of the inducible isoform of NO synthase (iNOS) has been shown to be effective in reducing tissue damage in several models of inflammation.15 Increased iNOS
activity has been observed in patients with duodenal ulcers. Apoptosis, programmed cell death, has been characterized morphologically by cell shrinkage and chromatin condensation, and biochemically by DNA laddering. Nitric oxide-induced apoptotic cell death of mouse macrophages has also been described. Recently, *H. pylori* was reported to induce apoptosis of gastric epithelial cells. However, cytotoxic NO signalling and the apoptogenic action of NO are poorly understood.

To clarify the mechanism by which *H. pylori* induces gastric mucosal injury, we examined the relationship between the production of nitric oxide and apoptosis of gastric epithelial cells in the presence of *H. pylori* infection.

**METHODS**

**Bacterial strain and characterization of *Helicobacter pylori* constituents**

Four clinical strains of *H. pylori* were isolated from gastric biopsy specimens from patients with chronic gastritis at Tokai University Hospital and grown on blood agar plates as described previously. The presence of vacuolating cytotoxin (VacA) was assayed by cytotoxic assays using rabbit kidney (RK) cells. Two strains, TK1029 and TK130, were VacA positive, and two strains, TK1028 and TK1101, were VacA negative.

**Preparation of lipopolysaccharide and water extracts**

Lipopolysaccharides (LPS) from the two *H. pylori* strains were prepared by the hot phenol–water method of Westphal and Jann. Briefly, bacterial cells from agar plates were suspended in saline, centrifuged at 5000 g for 15 min and resuspended in distilled water followed by rinsing with an equal volume of 90% phenol at 60 °C for 15 min. After the mixture was cooled and centrifuged for 15 min and resuspended in distilled water followed by centrifugation at 3000 g for 25 min, the cell pellet was resuspended in an equal volume of 90% phenol at 60 °C for 15 min. After the mixture was cooled and centrifuged (10000 g for 20 min), the aqueous layer was removed. This extraction procedure was repeated twice and the pooled water-extracted layers were dialyzed for 48 h and lyophilized.

Water extract of *H. pylori* was prepared by the method of Mai et al. Cells were harvested in 0.1 mol/L NaCl and centrifuged at 3000 g for 25 min. The cell pellet was resuspended in an equal volume of sterile distilled water, vortex-mixed for 45 s, and again centrifuged at 3000 g. The supernatant was stored at −20°C until use.

Culture supernatant was prepared as follows: *H. pylori* was inoculated into Brucella broth containing 10% foetal bovine serum followed by incubation under microaerobic conditions at 37°C for 5 days. Samples were centrifuged at 3000 g and culture supernatants were used.

**Cell culture**

The human gastric cancer cell line MKN45 was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The MKN45 cells were grown in Roswell Park Memorial Institute 1640 medium (Life Technologies, Gaithersburg, MD, USA) containing 10% heat-inactivated foetal calf serum (Life Technologies). MKN45 cells were seeded at $8 \times 10^4$ cells/mL into 6- or 96-well tissue culture flasks and incubated for 5–7 days to confluency.

**Cytotoxicity assay**

The MKN45 cells were allowed to attach for 5–7 days in 96-well, flat-bottomed microtitre plates at a density of $8 \times 10^4$ cells/well. Recombinant human IFN-γ (RD Systems Inc, Minneapolis, MN, USA) was added at 0.4 ng/mL for 6 h, then the cells were washed twice and kept in fresh medium overnight. After the medium was changed to antibiotic-free medium, *H. pylori* (10^7 CFU/mL) was cocultured with or without recombinant human TNF-α (4 ng/mL; RD Systems) for 24 h. The death of MKN45 cells induced by *H. pylori* was studied using a cell proliferation kit 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Boehringer Mannheim, Mannheim, Germany). The MTT assay was used to measure cytotoxicity according to the manufacturer’s protocol. Briefly, after a 24-h incubation, 10 μL of the MTT labelling reagent (final concentration, 0.5 mg/mL) was added to each well and the microtitre plates were incubated for 4 h in a CO₂ incubator. An aliquot of 100 μL, solubilization solution was added to each well followed by incubation overnight in a CO₂ incubator. The spectrophotometric absorbance of the samples was measured at 550 nm. The reference wave length was 650 nm.

**Inhibition of inducible nitric oxide synthase activity**

To inhibit iNOS activity, aminoguanidine (Sigma Chemical Co., St Louis, MO, USA) was used.

**Expression of inducible nitric oxide synthase mRNA**

The time course of induction of iNOS mRNA expression was determined after stimulation with viable *H. pylori* (1 × 10^7 CFU/mL). The expression of iNOS mRNA was determined by the reverse transcriptase–polymerase chain reaction (RT-PCR). The mRNA of MKN45 cells was isolated by using a QuickPrep® Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). Complementary DNA was synthesized from mRNA using a first-strand cDNA synthesis kit (Pharmacia Biotech) and amplified using PCR with primers specific for human iNOS (5’ CTT CAA CCC CAA GGT TGT CTG CAT 3’, 5’ ATG TCA TGA GCA
AAG GCG CAG AAC 3′; Maxim Biotech Inc. San Francisco, CA, USA), and an internal control gene, human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (5′ TGA AGG TCG GAG TCA ACG GAT TTG GT 3′; Clontec, Palo Alto, CA, USA). The PCR was carried out in an Iwaki thermal cycler (Iwaki, Chiba, Japan) for 36 cycles consisting of denaturation at 94°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 85 s. The PCR products were analysed on ethidium bromide-stained 2% agarose gels.

DNA fragmentation analysis

DNA fragmentation was analysed using an apoptosis ladder detection kit (Wako, Osaka, Japan). MKN45 cells were exposed to IFN-γ (0.4 ng/mL) for 6 h, washed twice, and kept in fresh medium overnight. The following day, viable H. pylori and TNF-α (4 ng/mL) were added to the cells for 24 h. DNA extracts were electrophoresed in 2% agarose gels. After staining with 0.20 μg/mL ethidium bromide, DNA bands were visualized by UV fluorescence and photographed.

Statistical analysis

Multiple comparisons were performed by using a one-way factorial ANOVA followed by Fisher’s probability of least significant difference test. Differences were considered to be statistically significant when \( P < 0.05 \). Results are expressed as mean ± SD as percentages of cytotoxicity or viability.

RESULTS

Helicobacter pylori-induced gastric epithelial cell injury

To determine whether H. pylori alone or in combination with cytokines is cytotoxic to gastric epithelial cells, the MTT assay was used. It showed that viable H. pylori alone did not induce cell death. Furthermore, neither IFN-γ nor TNF-α induced cell death. However, preincubation of MKN45 cells with IFN-γ for 6 h and coculture of viable H. pylori with TNF-α significantly increased the incidence of cell death (Fig. 1). Helicobacter pylori culture supernatant, water extract or LPS did not induce cell death in the presence of inflammatory cytokines. The correlation between toxin-producing activity of H. pylori strains and cytotoxicity of MKN45 cells was examined. No differences in the induction of cell death were found between cytotoxic and non-cytotoxic strains (Fig. 2).

Coculture of viable Helicobacter pylori with interferon-γ and tumour necrosis factor-α induces apoptosis

DNA fragmentation assays were used to detect apoptosis of gastric epithelial cells and showed that coculture of viable H. pylori with IFN-γ and TNF-α induced DNA laddering of MKN45 cells for 24 h (Fig. 3). However, IFN-γ and TNF-α, alone or in combination did not induce DNA laddering. These results suggest that viable H. pylori induces apoptosis of MKN45 cells in the presence of inflammatory cytokines.

Helicobacter pylori stimulates expression of inducible nitric oxide synthase mRNA

The time course of induction of expression of iNOS mRNA was determined after stimulation with viable H. pylori (1 × 10^7 CFU/mL). As shown in Fig. 4, H. pylori
In the present study, we showed that *H. pylori* induced expression of iNOS mRNA in gastric epithelial cells, and also showed that aminoguanidine, a selective iNOS inhibitor, inhibited *H. pylori*-induced gastric epithelial injury. Although iNOS mRNA expression was observed, the production of nitrite/nitrate was undetectable (data not shown). The precise mechanism by which *H. pylori* induces epithelial cell death is unknown, but, *H. pylori* infection activates NFκ-B in gastric epithelial cells and NFκ-B is thought to be involved in apoptosis. As NFκ-B is activated by free radicals, NO induced by *H. pylori* may enhance cell death via activation of NFκ-B.

In the present study, we showed that *H. pylori*, IFN-γ or TNF-α alone did not have cytotoxic effects in MKN45 cells, but *H. pylori* induced cell death in combination with IFN-γ pretreatment and TNF-α. DNA fragmentation analysis showed that coculture of viable *H. pylori* with IFN-γ and TNF-α induced DNA laddering of MKN45 cells. Recent studies have shown that *H. pylori* alone induces apoptosis of AGS cells and HM02 cells. In contrast, the present study showed that *H. pylori* alone did not induce apoptosis, but that *H. pylori* in combination with IFN-γ and TNF-α resulted in apoptosis of MKN45 cells. The reasons for this discrepancy remain unclear. The differences may have been partially due to differences in cell lines and the experimental conditions used. T cell activation leads to local tissue injury by releasing cytotoxic cytokines. Several studies have suggested that TNF-α plays a central role in epithelial injury. Increased TNF-α levels and TNF-α mRNA expression have been reported in gastric mucosa with *H. pylori* infection. Recently, *H. pylori* urease has been shown to stimulate TNF-α production from mononuclear phagocytes. Interferon-γ, which is expressed in the gastric mucosa of patients with gastritis, is known to increase the expression of TNF-α receptors. The combination of IFN-γ and TNF-α synergistically causes cell death of colonic cell lines. The mechanism of their interaction has been postulated to be through up-regulation of the Fas receptor on gastric epithelial cells by IFN-γ, and the interaction of Fas with closely related TNF-α receptors. Our current findings indicate that IFN-γ and TNF-α play important roles in *H. pylori*-induced gastric epithelial injury.

Although *H. pylori* does not invade gastric epithelial cells, it induces a mucosal inflammatory reaction. The LPS of Gram-negative bacteria are capable of inducing cytokine expression. *In vivo* studies have suggested that *H. pylori* LPS plays a pathological role in gastric epithelial injury. In contrast, *H. pylori* LPS has been shown to have low biological activity. A previous study showed that soluble surface proteins from *H. pylori* activate monocytes/macrophages by an LPS-independent
Our present study shows that viable H. pylori induce cell death, while water extract and LPS do not. These results indicate that attachment of the viable bacteria is necessary for cell death.

Fifty to 60 per cent of H. pylori express VacA and CagA products, which are potential virulence factors that may influence the clinical outcome of H. pylori infection. The CagA* Tox* strains of H. pylori induce more IL-8 secretion than CagA* Tox* strains. In contrast, the present study showed that both VacA* and VacA* strains of H. pylori induce death of MKN45 cells. Moreover, neither supernatant of VacA* H. pylori nor that of VacA* H. pylori induced cell death. An in vivo study showed that apoptosis is observed more often in patients with CagA* H. pylori infection. Our current findings suggest that components other than cytotoxins play a role in cell death. Other potential priming signals from H. pylori or the inflammatory response to induce apoptosis have been reported, including ammonia, urease and oxygen radicals, but the mechanism remains unclear.

In summary, the results of this study suggest that viable H. pylori induces cell death through the production of nitric oxide in the presence of IFN-γ and TNF-α. Further studies are necessary to firmly establish the mechanism by which H. pylori induces cell death.

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iNOS expression in H. pylori infection


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