BACTERIAL PATHOGENICITY

Association of gastric epithelial apoptosis with the ability of Helicobacter pylori to induce a neutrophil oxidative burst

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Both polymorphonuclear cell infiltration and increased epithelial apoptosis are seen in gastric mucosa in the presence of Helicobacter pylori infection. This study examined the association between bacterial ability to stimulate an oxidative burst in neutrophils and epithelial apoptosis. Biopsy specimens were obtained from 15 patients to detect apoptotic cells by the TUNEL method. H. pylori strains isolated from corresponding stomach biopsy samples were tested for the ability to stimulate an oxidative burst in human neutrophils. Neutrophils were isolated from healthy subjects without H. pylori infection and the oxidative burst was measured by flow cytometry with dichlorofluorescein diacetate. Stimulation with H. pylori increased both the percentage of activated cells and fluorescence intensity. There was a significant positive correlation between the number of epithelial apoptotic cells and fluorescence intensity. Increased neutrophil oxidative burst stimulated by H. pylori may play a role in enhanced gastric mucosal DNA damage and consequent atrophic gastritis and gastric cancer.

Introduction

Helicobacter pylori infection is associated with the development of atrophic gastritis and gastric carcinogenesis [1, 2]. H. pylori infection induces an acute gastritis which is followed by a chronic gastritis, both characterised by considerable polymorphonuclear leucocyte (PMNL) infiltration. The pathogenic role of reactive oxygen species (ROS) produced by PMNLs has been implicated in H. pylori-related gastritis. H. pylori activates neutrophil oxidative metabolism [3, 4] and increased oxidative DNA damage is observed in gastric mucosa in the presence of H. pylori infection [5]. High levels of DNA damage can lead to cell death, and increased apoptosis is seen in gastric mucosa infected with H. pylori [6, 7]. This mechanism could play an important role in the development of atrophic gastritis and gastric carcinogenesis in H. pylori-related chronic gastritis. On the other hand, certain H. pylori strains activate human neutrophils without opsonins [4]. Infection by H. pylori strains that are capable of activating neutrophils strongly seems to induce increased oxidative DNA damage in gastric mucosa. However, the relationship between the ability of H. pylori to activate a neutrophil oxidative burst and DNA damage of gastric mucosa is not clear. The aim of this study was to examine the association between the inducibility of a neutrophil oxidative burst by H. pylori and gastric epithelial apoptosis.

Materials and methods

Patients

Patients who were diagnosed as having atrophic gastritis by upper gastrointestinal endoscopy were enrolled prospectively into the study. Patients who had received anti-ulcer agents, antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs) in the two months before the examination were excluded. All patients provided informed consent before endoscopy. Biopsy specimens obtained from the antrum were used to evaluate the grades of PMNL infiltration and bacterial density according to the updated Sydney System [8], to detect apoptotic epithelial cells by staining (see below) and to isolate H. pylori strains. Patients were eligible for the present study if mild PMNL infiltration and mild bacterial density were observed in their gastric biopsy specimen and if H.
pylori was isolated from the corresponding biopsy specimen. This study was approved by the ethics committee of Hirotsuki University.

Bacterial culture and genotyping
Biopsy specimens were cultured for 3–5 days on Skirrow blood agar. The bacteria were identified as H. pylori by colony morphology and positive oxidase, catalase and urease reactions. The clone picked strains were each suspended in 1 ml of phosphate-buffered saline (PBS, pH 7.6).

Bacterial suspensions were centrifuged at 10000 g for 5 min. Bacterial DNA was extracted from the bacterial pellet with phenol-chloroform-isooamylalcohol after digestion with protease K. The presence of the cagA gene and the allelic variation of vacA signal sequence and mid-region were determined by PCR with the primers described previously [9, 10]. PCR products were electrophoresed in agarose 2% gel. The presence of the cagA gene and vacA genotype was determined when the product equivalent in size to the fragment described was found [9, 10].

The bacterial pellet was also resuspended in Hanks's Balanced Salts Solution (HBSS) to give an absorbance at 550 nm of 0.5 (c. 1.5×10^6 cfu/ml) for flow cytometry.

Flow cytometry
Neutrophils were isolated from two H. pylori seronegative healthy volunteers (A and B) by Histopaque (Sigma) density gradient separation. Briefly, peripheral blood samples were diluted two-fold in HBSS and decanted on equal volumes of Histopaque 1077 and 1119. The samples were centrifuged at 500 g for 30 min. The neutrophil fraction found at the 1077/1119 interphase was harvested and washed with HBSS. Neutrophils were suspended in HBSS to the required concentration.

The intracellular production of ROS in stimulated neutrophils was quantified in individual cells with a FACScan (Becton Dickinson, Heidelberg, Germany). Briefly, 100 μl of neutrophil suspension (2×10^7/μl) were incubated with 25 μl of 50 μM dichlorofluorescein diacetate (DCFH-DA) at 37°C for 15 min. Then 100 μl of bacterial suspension and 25 μl of HBSS were added and incubated at 37°C for 30 min. Samples were fixed with Lyse and Fix (Immunotech, Marseille, France) and were analysed within 1 h. Flow cytometric analysis was performed on 10,000 cells. Intracellular fluorescence was measured by the histogram analysis software of the FACScan and the mean channel number was calculated to express the fluorescence intensity.

Detection of apoptotic cells
Apoptic cells were stained by terminal uridine deoxyxynucleotide nick end labelling (TUNEL) with the in-situ Apoptosis Detection Kit (Oncor, Gaithersbrug, MD, USA). Briefly, paraffin-embedded gastric mucosal specimens were cut into 4-μm sections and were washed with xylene and graded concentrations of ethanol to remove paraffin. After digestion for 15 min with proteinase K (Sigma) 40 μg/ml and blocking of endogenous peroxidase with H2O2 3% for 30 min, the section was incubated with digoxigenin-dUTP at 37°C for 1 h. Incorporated digoxigenin was detected with anti-digoxigenin-peroxidase-conjugated antibody (Oncor). The sections were stained in dianinobenzidine hydrochloride 0.05% solution for 5 min and counterstained in haematoxylin for 5 s. The number of positive cells per 100 glands was counted in three sections and the mean number was considered to be the apoptotic index. The apoptotic index was determined by two experienced pathologists (M.T., Y.T.) without knowledge of the results of the flow cytometry.

Statistical analysis
Pearson’s correlation coefficient was determined to examine the correlation between mean channel number and apoptotic index. A probability (p) value of <0.05 was considered significant.

Results
Fifteen H. pylori strains were obtained from 15 patients with atrophic gastritis (mean age 53.7 SD 10.1, nine male and six female). All strains were cagA positive and vacA s1/m1 genotype. All strains stimulated intracellular ROS production in neutrophils from two volunteers. The mean channel number of control samples was 3.09 in volunteer A (Fig. 1) and 11.11 in volunteer B. The average mean channel number of activated neutrophils was 43.46 (SEM 3.83) and 48.32 (SEM 9.28) in volunteers A and B, respectively.

Histologically, PMNL infiltration and bacterial density were graded as mild in the gastric biopsy specimens from all the patients. Apoptotic cells were observed in 12 of 15 patients and the mean apoptotic index was 1.20 (SEM 0.27). The mean channel number of neutrophils stimulated by H. pylori correlated significantly with the apoptotic index in the corresponding gastric mucosa. The calculated correlation coefficient was 0.716 (p = 0.003) with PMNL from volunteer A (Fig. 2a) and 0.543 (p = 0.043) with PMNL from volunteer B (Fig. 2b).

Discussion
Activation of human neutrophils by H. pylori and substances produced by H. pylori has been shown in various studies. H. pylori strains capable of activating...
neutrophils strongly without opsonins are associated with peptic ulcer diseases [4]. It follows that individual *H. pylori* strains vary in their ability to activate human neutrophils. On the other hand, increased oxidative DNA damage has been shown in gastric mucosa infected with *H. pylori*. The oxidative DNA damage is more clearly apparent in gastric mucosa with severe disease than with simple chronic gastritis [11]. If the ability of *H. pylori* strains to activate neutrophils plays a major role in oxidative DNA damage of gastric mucosa, there could be a positive correlation between them.

In the present study, the apoptotic index varied in the 15 gastric mucosa samples investigated, whereas grades of PMNL infiltration and bacterial density were similar. *H. pylori* strains isolated from 15 corresponding biopsy specimens were tested for the ability to activate the human neutrophil oxidative burst by flow cytometry analysis with DCFH-DA. DCFH-DA has been reported to detect the generation of intracellular H$_2$O$_2$, which is a derivative of superoxide anion, and the DCFH oxidation assay is quantitatively related to the oxidative burst of PMNLs [12]. Interestingly, the ability of *H. pylori* strains to activate the human neutrophil oxidative burst correlated significantly with the degree of epithelial apoptosis in infected gastric mucosa. The results suggest that infection by *H. pylori* strains capable of inducing a strong neutrophil oxidative burst may affect the degree of atrophic gastritis.

ROS are known to interact with genomic DNA and have been implicated in carcinogenesis. Increased gastric epithelial DNA damage has been observed in patients with gastric cancer, particularly in gastric cancer precursor lesions such as atrophic gastritis and incomplete intestinal metaplasia [11, 14]. Thus, given the results of the present study, direct activation of neutrophils by *H. pylori* seems to be involved in gastric carcinogenesis. In other words, *H. pylori* acts as an initiator of gastric cancer.

In conclusion, the ability to stimulate the human neutrophil oxidative burst varies among *H. pylori* strains. The neutrophil oxidative burst stimulated by *H. pylori* may play a significant role in gastric mucosal DNA damage and consequent gastric epithelial apoptosis.

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**Fig. 1.** Profile of fluorescence in neutrophils (a) without stimulation (control); (b) stimulated by *H. pylori*. Intra-cellular fluorescence was measured by flow cytometry and is plotted on a log$_{10}$ scale from channel number $10^0$ to $10^9$ on the abscissa. The counts on the ordinate represent the number of cells in each channel. The region in plots represents the cells emitting fluorescence above that of unstimulated sample.

**Fig. 2.** Correlation between apoptotic index and mean channel number of neutrophils from (a) volunteer A; (b) volunteer B stimulated by corresponding *H. pylori* strains. Abscissa, apoptotic index; ordinate, mean channel number. Line represents regression line ($r = 0.716$, $p < 0.01$).

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We are grateful to Drs K. Suzuki and T. Yoshimura for their help with flow cytometry assay and TUNEL staining, and Professor Sugawara for his excellent advice.
References