Full Paper

Effects of *Helicobacter pylori* Infection on Gastric Parietal Cells and E-cadherin in Mongolian Gerbils

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Abstract. Atrophic gastritis caused by infection with *Helicobacter pylori* is characterized by parietal cell loss, which is a main risk factor for gastric cancer. Parietal cells play a crucial role in the regulation of cell lineage maturation and proliferation in the gastric units. Among the classical cadherins, E-cadherin plays an important role not only in epithelial cell–cell connections, but also in the maintenance of epithelial polarity and gastric glandular architecture and regulation of cell proliferation. The aim of this study is to elucidate how parietal cells and E-cadherin are altered in gastritis with *Helicobacter pylori* infection. We studied the effects of *Helicobacter pylori* on gastric mucosal E-cadherin 2 weeks after inoculation and investigated the relationship between parietal cell loss and the amount of E-cadherin on parietal cells in Mongolian gerbils. The number of parietal cells and amount of staining of E-cadherin below the isthmus were investigated by immunohistochemistry. It was shown that a reduction in intercellular E-cadherin preceded the disappearance of parietal cells. The gastric glands where parietal cells were lost were replaced by mucus secreting cells without E-cadherin. These results suggest that *Helicobacter pylori* damaged E-cadherin on parietal cells and caused massive parietal cell loss, leading to the deregulation of gastric morphology.

Keywords: *Helicobacter pylori*, E-cadherin, gastritis, parietal cell, gastric

Introduction

Helicobacter pylori (*H. pylori*) is a pathogen of gastric diseases, such as atrophic gastritis, peptic ulcer, gastric adenocarcinoma, and MALT lymphoma in humans (1, 2). Atrophic gastritis caused by *H. pylori* is characterized by loss of parietal cells, which play a role not only in gastric acid secretion but also in maintenance of the normal structure and function of gastric mucosa. Gastric atrophy, endocrine cell hyperplasia, pseudopyloric metaplasia, and intestinal metaplasia could be regarded as the result of altered morphogenesis in gastric mucosa due to parietal cell loss (3 – 5). However, it remains unclear how parietal cells are lost. The mechanism and sequence

in which parietal cells are lost by *H. pylori* infection is important for not only the understanding of atrophic gastritis but that of gastric carcinogenesis as well.

Adhesion molecules play a crucial role in the integrity and tumorigenesis of the epithelium. Cadherins are key molecules in the morphogenesis, regulation of cell proliferation, and differentiation. They include E-cadherin (epithelial), N-cadherin (neuronal), VE-cadherin (vascular endothelial), and so forth. They mediate Ca²⁺-dependent homophilic interaction with their extracellular domains. Among them, E-cadherin is a major constituent of adherens junction and essential for not only in epithelial cell–cell connections, but also maintenance of epithelial polarity and mucosal architecture and the barrier function of epithelial cells (6 – 8). The adherens junction is composed of transmembrane cell adhesion molecules of E-cadherin and cytoplasmic proteins such as p120, β -catenin, and α -catenin, which link the actin

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cytoskeleton with cadherin. The E-cadherin catenin complex functions as an important molecule in regulating not only cell adhesion, but also differentiation, proliferation, migration, and survival of epithelial cells (9). E-cadherin up-regulation may be a potential therapeutic strategy to suppress cancer progression and metastasis that could significantly enhance the treatment of cancer and other diseases (10–12). The loss of E-cadherin leads to sloughing or apoptosis of cells due to the loss of the cell-to-cell adherence apparatus between cells (10). Mizuno et al. demonstrated that E-cadherin plays an important role in barrier function in epithelial cells (13).

In the present study, we investigated the effect of gastric *H. pylori* infection on E-cadherin on parietal cells in Mongolian gerbils 2 weeks after inoculation with *H. pylori*. The model has been developed by Hirayama et al. and has provided an important tool for the study of gastric mucosal diseases (14). We showed that the loss of E-cadherin preceded the disappearance of parietal cells as early as 2 weeks after infection, suggesting that disruption of E-cadherin, which physically connects gastric parietal cells to neighboring cells, plays an important role in the pathogenesis of parietal cell loss, leading to the gastric mucosal disorganization observed in atrophic gastritis.

Materials and Methods

General procedures

Mongolian gerbils infected with *H. pylori* were used in the present study. The number of parietal cells and staining of E-cadherin in the stomach were investigated by immunohistochemistry with a monoclonal antibody against H^+/K^+ -ATPase and E-cadherin, respectively, 2 weeks after inoculation.

Animals

Male Mongolian gerbils (6-week-old, 40–50 g), purchased from Kyudo (Saga), were maintained in an isolated clean room with regulated temperature (20°C–22°C), humidity (approximately 55%), and a 12/12-h light/dark cycle. Gerbils were fasted for 24 h before *H. pylori* inoculation, and drinking water was also withheld after inoculation. Both food and water were then freely available to animals from 4 h after inoculation. Animal maintenance and experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

H. pylori infection to Mongolian gerbils

CagA- and VacA-positive strains of *H. pylori* (TN2GF4)

Fig. 1. A photograph and schematic representation of macroscopic corpus gastric mucosa of forestomach (f), anterior wall of corpus (c-a), and posterior wall of corpus (c-p) in a Mongolian gerbil. The dashed line indicates the lesser curvature of the stomach and the white line shows the location of the specimen used for histological investigation.

were incubated in a brain-heart infusion broth (Difco, Detroit, MI, USA) containing 10% fetal bovine serum at 37°C overnight under a microaerophilic atmosphere and allowed to grow to a density of 2×10^8 colony-forming units (CFU)/mL. *H. pylori* (2×10^8 CFU, 1.0 mL) was orally inoculated to each animal ($n = 4$). Normal control animals ($n = 3$) received 1.0 mL of the medium alone. Two weeks after inoculation, animals were killed by deep ether anesthesia and stomachs were incised along the greater curvature and spread out with pins on a silicon-coated 6-cm dish. Gastric specimens were cut out from anterior wall of the corpus mucosa, from the lesser curvature side to the greater curvature, and fixed in 4% paraformaldehyde in PBS. The location of the specimen cut out is indicated as a white line (Fig. 1).

Evaluation of gastric mucosa after *H. pylori* infection

Paraffin sections (5 μ m in thickness) were prepared and PAS-pH2.5-Alcian Blue staining and immunoperoxidase staining for H^+/K^+ -ATPase and E-cadherin were carried out. To confirm the presence of *H. pylori* and gastritis in the stomach, Giemsa staining for identification of *H. pylori* was performed. Giemsa-stained sections and PAS-pH2.5-Alcian Blue staining were examined independently by a pathologist and a gastroenterologist for the presence of gastritis and for *H. pylori* as curved or spiral-shaped microbes. Gastric mucosal thickness, the number of parietal cells, and E-cadherin expression were evaluated.

Immunostaining for H^+/K^+ -ATPase and E-cadherin of gastric mucosa

Paraffin-embedded sections were deparaffinized and endogenous peroxidase was inactivated with 3% H_2O_2 for 30 min. Sections were washed and blocked with 3% normal donkey serum in TBS for 1 h at room temperature. Sections were incubated with mouse monoclonal

Fig. 2. Schematic illustration for the measurement of the amount of parietal cells and E-cadherin along the longitudinal axis of specimens taken from *H. pylori*-infected gerbils using photomicrographs (magnification $\times 100$). Dots: parietal cells, cross stripes: E-cadherin. Longitudinal axis (x): one unit expresses mean width of the sum of 10 glands, t: total mucosal thickness, p: vertical width of parietal cells, e: E-cadherin.

antibody, which recognizes the beta-subunit of H^+/K^+ -ATPase (ab2866; Abcam, Cambridge, UK), for 1 h at room temperature or with rabbit polyclonal antibody raised against E-cadherin (sc-7870; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C . Tissue sections were incubated with biotinylated goat anti-mouse or anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) secondary antibodies for 60 min. Sections were incubated with streptavidin labeled with horseradish peroxidase and visualized with a metal enhanced diaminobenzidine substrate kit (Thermo Fisher Scientific, Rockford, IL, USA). Negative controls were achieved by omission of the primary antibody and incubation with the non-immune control immunoglobulin fraction from the same species, keeping all the other steps the same.

Quantitative evaluation of the amounts of parietal cells and E-cadherin

The amount of parietal cells was expressed by the ratio p/t and that of E-cadherin by the ratio e/t , in Fig. 2, where t indicates the width of the vertical mucosal section; p , the width of remaining parietal cells; and e , width of the preserved E-cadherin observed under microscopy. Measurements were undertaken by the microphotographs observed by $100\times$ magnification.

Statistics

Results obtained are presented as the mean $\pm$ S.E.M. Results were analyzed by the two-tailed Student's t -test and values of $P < 0.05$ were regarded as significant.

Results

Giemsa staining for identification of *H. pylori* and the PAS staining to identify stomach pathology

Giemsa-stained sections of the stomach showed *H. pylori* in all inoculated groups examined for the presence of *H. pylori* as curved or spiral-shaped microbes (Fig. 3). Histological examination of all animals inoculated with *H. pylori* showed the diffuse loss of parietal cells from the lesser curvature of the stomach, showing the beginning of chronic gastritis (data are not shown). The atrophic border zone was diffuse and wide, not distinct at this stage of infection. Figure 4 shows the gastric glands where parietal cells are lost and replaced by PAS-positive mucus secreting cells.

Effects of *H. pylori* infection on H^+/K^+ -ATPase and E-cadherin of parietal cells

The distribution of parietal cells was investigated by immunohistochemistry with a monoclonal antibody against H^+/K^+ -ATPase 2 weeks after *H. pylori* infection. Parietal cells (Fig. 5: A, C) and E-cadherin (Fig. 5: E, G) in the non-infected gerbils were not lost, whereas a marked decrease in parietal cells (Fig. 5: B, D) and E-cadherin (Fig. 5: F, H) was observed in infected gerbils. Parietal cell loss was revealed at this time of 2 weeks in the lesser curvature side of the stomach. In contrast to non-infected animals, in which E-cadherin was observed from the apical side to the base, E-cadherin was lost not only in mucosa where parietal cells were lost, but also in mucosa where parietal cells still existed or were damaged in infected gerbils.

Relationship between loss of E-cadherin and parietal cells

The relationship between parietal cells and staining of E-cadherin in the stomach was investigated with monoclonal antibodies against H^+/K^+ -ATPase and E-cadherin, respectively. The amounts of parietal cells and E-cadherin were expressed by the vertical length of stained H^+/K^+ -ATPase and E-cadherin divided by the length of whole gastric mucosal thickness, respectively, along the longitudinal specimen from the lesser curvature to greater curvature (Fig. 6). It was shown that the loss of intercellular staining of E-cadherin preceded parietal cells loss (Fig. 6, left) and the rate of decrease in E-cadherin was faster than that of parietal cells (Fig. 6, right) in the stomach of *H. pylori*-infected gerbils. Such decreases in E-cadherin and parietal cells were not observed in the stomach of non-infected animals.

Fig. 3. This microphotograph shows small clusters of *H. pylori* (arrows) at the greater curvature side of the specimen where parietal cells still existed but were being damaged. Spotty small *H. pylori* clusters were observed from the lesser curvature to greater curvature side. The presence of *H. pylori* is spotty and in the mucus.

Fig. 4. Infiltration of inflammatory cells in the stroma at the basal side of the glandular units is observed. Parietal cells are damaged, lost, and replaced by mucus-secreting PAS-positive cells. Bar: 50 μ m.

Fig. 5. Alteration of parietal cells and E-cadherin in *H. pylori* infected gerbils. A – D: parietal cells (H^+/K^+ -ATPase staining), E – H: E-cadherin staining. Parietal cells and E-cadherin were present in the intact mucosa in non-infected gerbils (A, C, E, and G) in contrast to that in infected gerbils (B, D, F, and H). Parietal cell shedding into lumen or degeneration and almost no E-cadherin staining was observed in the mucosa in infected gerbils. Bars: 50 μ m.

Fig. 6. Relationship between loss of E-cadherin and parietal cells 2 weeks after *H. pylori* infection. Left panel shows the change in the amounts of parietal cells and E-cadherin along the longitudinal axis of a specimen from the greater curvature to lesser curvature in infected gerbils. Measurements were performed in the transitional zone where parietal cells were being lost. The amounts of E-cadherin and parietal cells in each specimen were expressed as their vertical width divided by the vertical width of total mucosal thickness at the same site, respectively. The unit of the horizontal longitudinal axis in the left panel represents the mean length of the width of 10 glandular units. Amounts of parietal cells and E-cadherin were expressed by grade 0–4. Grades 0, 1, 2, 3, and 4 were defined when percentages (Y) of the width of parietal cells or E-cadherin per total mucosal thickness were 0%, $0% < Y \leq 25\%$, $25\% < Y \leq 50\%$, $50\% < Y \leq 75\%$, and $75\% < Y \leq 100\%$, respectively. Significantly larger extent of the decreases in E-cadherin was observed compared to the decreases in parietal cells, suggesting that the loss of E-cadherin preceded parietal cell loss. Each value represents the mean $\pm$ S.E.M. for 4 animals. Asterisks indicate a significant difference between the ratios of decrease of parietal cells and E-cadherin ($P < 0.05$). From the data shown in left panel, the relationship between decreases in parietal cells and that of E-cadherin were depicted (right panel). The distribution pattern of the dots shows that the loss of E-cadherin was faster than that of parietal cells ($n = 28$; 7 points in each specimen, 4 gerbils).

Discussion

Gastric cancer is associated with chronic gastritis and arises from mucosa of oxyntic atrophy (parietal cell loss), hyperproliferation of mucus cell lineages, and metaplastic changes (15–17).

Aoyama et al. demonstrated that the natural death of pit-parietal cells was observed under normal conditions, resulting from autophagy, protrusion, and exfoliation from basal lamina, followed by sealing on the basal side by adjacent surface mucous cells concomitant with the change in immunoreactivity of E-cadherin of parietal cells (18). E-cadherin functions as an important molecule in the regulation of cell adhesion, differentiation, and proliferation as well as morphogenesis. In the present study, we investigated the loss of parietal cell and E-cadherin in Mongolian gerbils infected with *H. pylori* by immunohistochemistry and examined longitudinal specimens of corpus gastric mucosa from the lesser curvature to greater curvature. This examination of infected mucosa allowed observation of the progression of *H. pylori* induced gastric atrophy because it is well known that gastric atrophy proceeds from the lesser curvature to greater curvature. Parietal cells and E-cadherin were spared in the greater curvature side of the corpus mucosa at this early stage of *H. pylori* infection. The mechanism for why parietal cell loss occurs from the lesser curvature side and proceeds to the greater

curvature remains to be clarified.

In the present study, it was shown that parietal cell damage was not due to the pervasive aggressive force from the luminal surface but was targeted to parietal cells per se in the process of mucosal inflammation. Indeed, histological observations revealed that parietal cell loss occurs in the presence of surface epithelial cells in the pits. Quantitative investigation demonstrated the decrease in total parietal cells and E-cadherin in glandular units and further showed that E-cadherin decreased faster than parietal cells in infected gerbils. E-cadherin was absent not only in mucosa where all parietal cells were lost, but also in mucosa where some parietal cells were damaged, showing that the loss of E-cadherin preceded parietal cells loss.

Parietal cells in gastric mucosa are highly polarized cells, and they are connected to one another near the apical surface both at the tight junction and adherence junction. The tight junction separates the apical and basolateral surface of the cell and has a fence or barrier function, which hampers paracellular permeability. Localization of *H. pylori* is in the mucus, close to the intercellular adhesion junction in the lateral side or in the apical side of the epithelium, and it has tropism in the area adjacent to the atrophic border (19, 20). Wroblewski et al. reported that *H. pylori* deregulated gastric epithelial tight junctions by urease-mediated myosin II activation, which is independent of the *cag* pathogenicity island

Fig. 7. Schematic illustration showing the loss of E-cadherin on the epithelium after *H. pylori* infection causes shedding and damage of parietal cells, leading to the depletion of them in the glandular unit.

or VacA in MNK 28 gastric epithelial cells (21). The adherens junction provides reinforcement and strong binding below the tight junction. The integrity of the epithelium depends on lateral adhesion of cells with one another at the adherence junction to resist separation. Disruption of the zonula adherens results in impairment of gastric mucosal barrier function at this site, leading to sloughing of parietal cells (Fig. 7). The zonula occludens in the tight junction plays an important role in the fusion of adjoining cell membranes, a barrier to the paracellular pathway of hydrogen ions, but their resistance to mechanical stress is limited.

As to the pathophysiological mechanism by which E-cadherin is lost, recent studies demonstrated several causes for reductions in E-cadherin such as abrogating E-cadherin expression (22, 23), disruption of the E-cadherin and catenin complex (24), deregulated proteases (25), exposure to chelates (26), ischemia/ATP depletion (27), and endocytosis from the intracellular junction (28, 29).

Among the toxic factors encoded by *H. pylori*, Cag A is most closely linked with the loss of E-cadherin, the severity of gastritis, and the increased risk of carcinogenesis. *H. pylori* injects CagA directly into the host cytoplasm using a specialized type IV secretion system

(T4SS) encoded by CagPAI (30). Injected CagA induces cell migration and changes in morphology and cell-to-cell and cell-to-matrix adhesion through CagA tyrosine phosphorylation by the protein kinase Src and Abl families (31, 32). On the other hand, a CagA-independent loss of the adherence junction was demonstrated (33). It includes shedding of E-cadherin by proteases specific for extracellular E-cadherin cleavage such as matrix-metalloproteinase (34) and ADAM-10 (24), regulation by transcriptional repressors of the expression of E-cadherin (35), promoter CpG hypermethylation (36), and endocytosis of cadherin in bovine kidney epithelial cells cultured in a low Ca^{2+} ion medium (37). The mechanisms by which E-cadherin was lost in the present study need to be further investigated.

Taken together, our results suggest that the disruption in E-cadherin that physically links gastric parietal cells together leads to the loosening of E-cadherin-mediated adherens junctions of parietal cells and by so doing induces their loss. These results may indicate that *H. pylori* targets the E-cadherin of parietal cells and cause destruction of parietal cells to produce their niche, leading to the resultant oxyntic atrophy, a possible initiating factor in the sequence of gastric carcinogenesis.

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