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Temporal increase in the gastric colonization of *Helicobacter pylori* after healing of acetic acid-induced gastric ulcer in a miniature pig

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While the eradication of *Helicobacter pylori* has been reported to reduce the frequency of ulcer relapse, the preventative mechanism remains unknown. We investigated the changes in the level of gastric colonization 140 days after inducing gastric ulcer by acetic acid in the antral mucosa of a miniature pig infected with *H. pylori*. The gastric ulcer was induced endoscopically with 1 mL of 40% acetic acid 12 days after inoculation of *H. pylori* in a 3-month-old miniature pig. Gastric ulcer was healed by 30 days after ulcer induction and the levels of *H. pylori* in cardiac and antral mucosa increased gradually from 30 to 71 days. The peak bacterial counts in the cardia and antrum were 6.1 and 6.6 $\log_{10}$ cfu/g, respectively, or about 100-fold higher than the initial levels. The levels of *H. pylori* in cardiac and antral mucosa steadily decreased until reaching the initial levels at 127 days, while that in the fundic mucosa remained constant throughout the observation period. No ulcer recurrence was detected by endoscopy. These results suggested that the levels of *H. pylori* colonization increased temporally after healing of the acetic acid-induced gastric ulcer in the miniature pig.

Key words: Gastric ulcer, *Helicobacter pylori*, miniature pig

Helicobacter pylori was first isolated from human gastric biopsy specimens in 1983 [1]. Since that time, several reports have indicated that *H. pylori* is the etiological agent of gastritis [2], a condition closely associated with peptic ulcers [3], mucosa-associated lymphoid tissue (MALT) lymphoma [4], and gastric cancer [5]. Among published papers on the relationship between *H. pylori* and various diseases, the first of universal significance medically, economically, and socially was a report demonstrating that the eradication of *H. pylori* reduced the frequency of ulcer recurrence [6]. The Consensus Conference of the National Institutes of Health held in 1994 concluded that "ulcer patients with *H. pylori* infection require treatment with antimicrobial agents in addition to antisecretory drugs, whether on first presentation or on recurrence" [7]. Although *H. pylori* is recognized as a cofactor in ulcerogenesis, the pathogenic mechanisms of ulcer recurrence remain unknown.

Studies on various aspects of *H. pylori* infection and gastric pathology have been compromised by the unavailability of convenient animal models that resemble the infection in humans. In a previous report, we described the suitability of a miniature pig model for clarifying how *H. pylori* colonizes and persists in the gastric mucosa [8]. In addition, the bacteria counts of *H. pylori* in the gastric mucosa could be easily followed within individual animals from this model and chronic gastric ulcers could be easily induced by direct endoscopic injection of acetic acid into the antral submucosa, permitting repeat endoscopic examination. In this model, *H. pylori* infection persisted for more than 22 months in a 3-month-old pig [9]. In the present study, we investigated the changes of gastric colonization of *H. pylori* after inducing gastric ulcer by acetic acid in a 3-month-old miniature pig infected with *H. pylori*.

Materials and Methods

Animals

Two male SPF CSK miniature pigs (CSK Research Park, Inc., Nagano, Japan) were used in this study. The pigs used were confirmed to be free from the following common porcine pathogens: *Mycoplasma* spp., *Bordetella* spp., *Haemophilus* spp., *Pasteurella multocida*, and *Salmonella* spp. This pig herd had also been previously determined to be free of gastric *Helicobacter* organisms [9]. The 2 11-week-old pigs

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(body weight, 10 kg) were housed individually in suitably adapted cages in a 12-h light-dark controlled room maintained at a constant temperature of 22°C to 25°C and humidity of about 55%, with free access to drinking water. After an acclimation period of 2 weeks, the pigs were inoculated with *H. pylori*. All animal experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co., Ltd. (Tokyo, Japan).

Diets

A mixed diet (NS: Lucerne Pellets = 4:1, Nisseiken Co., Ltd.), a commercial diet for pigs, was used and provided 3 times daily.

Bacterial inocula

H. pylori strain No. 9839, a cagA/VacA-positive strain of the pathogen, was used for the inoculation. Stock cultures were stored at –80°C in brucella broth (Becton Dickinson, Cockeysville, MD, US) supplemented with 2% fetal bovine serum (FBS brucella broth, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Bacteria were cultured in FBS brucella broth on a gyratory shaker at 110 rpm for 40 h at 37°C under microaerophilic conditions. The culture was harvested by centrifugation and the cells were suspended in FBS brucella broth to 1×10^9 cfu/mL. The bacterial cell concentration of all inocula were enumerated with brain heart infusion agar (Difco Laboratories, Detroit, US) supplemented with 5% horse blood. Plates were incubated at 37°C in a GasPak jar (Becton Dickinson) for 5 days with Campy Paks (Becton Dickinson).

Experimental infection

Two miniature pigs were fasted for 24 h before inoculation. The pigs were rendered temporally achlorhydric by intramuscular administration of famotidine (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) at 10 mg/kg, 0 and 3 h before inoculation. The pigs were pretreated with 0.05 mg/kg atropine (Tanabe Seiyaku Co., Ltd., Osaka, Japan), 0.08 mg/kg medetomidine (Meiji Seika Kaisha, Ltd., Tokyo, Japan), and 0.22 mg/kg butorphanol (Bristol-Myers Squibb K. K., Tokyo, Japan) plus an intramuscular injection of 5 mg/kg ketamine hydrochloride (Sankyo Co., Ltd.) for anesthesia just before inoculation. Under the anesthetized condition, 50 mL of FBS brucella agar was administered just before inoculation of the bacterial suspension, as previously described [9]. Twelve days after the inoculation of *H. pylori*, gastric ulcer was induced in one of the pigs.

Endoscopy and biopsy specimens

Prior to endoscopy, the inoculated pig was fasted for 18 h. Endoscopy was performed under anesthesia and a GIF XQ240 endoscope (Olympus Co., Ltd., Tokyo, Japan). Local *H. pylori* populations were sampled by taking one biopsy (about 2 mg) from each of 10 representative sites: 3 sites from the cardiac mucosa (sites 1-3 in Fig. 1), 3 from the fundic mucosa (sites 4-6), and 4 from the antral mucosa (sites 7-10). The biopsy specimens were homogenized in 2 mL of FBS brucella broth, and then diluted with the same broth. Aliquots (100 μL) of the dilutions were inoculated onto modified Skirrow's agar as previously described [9]. Plates were incubated at 37°C under microaerophilic conditions. Bacterial counts were expressed as cfu per gram of tissue. For this study we estimated the detection limit to be $10^{3.7}$ cfu, and used this in the calculations. The bacteria counts of *H. pylori* in the cardia, fundus, and antrum were calculated as the average of the 3 cardiac, 3 fundic, and 4 antral biopsy specimens, respectively, and were expressed as means ± standard errors.

Experimental gastric ulcer

One pig was examined endoscopically 12 days after inoculation with *H. pylori*, and then 1 mL of 40% acetic acid was injected into the antral submucosa at the lesser curvature (Fig. 1) with an injection needle (NM-8L-1; Olympus Co., Ltd.). Endoscopic examinations were

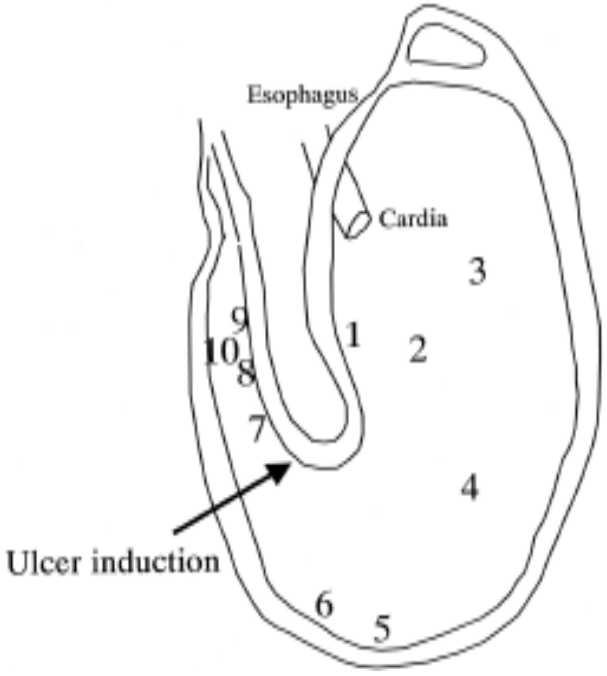

Fig. 1. Sites of biopsy for culture and ulcer induction. Sites 1 to 3, sites 4 to 6, and sites 7 to 10 were located in cardia, fundus, and antrum, respectively.

performed at 10, 21, 30, 43, 58, 63, 71, 85, 99, 113, 127, and 140 days after induction of the gastric ulcer to investigate the bacteria counts of *H. pylori* in the stomach.

Statistical analysis

To evaluate the differences between the *H. pylori* counts at the baseline with those at each measurement point, statistical analysis was performed by pairwise Dunnett's test using the logs of the cfu counts.

Results and Discussion

Figure 2 shows the endoscopic findings of ulcer healing. The ulcer lesions measured 10 x 6 mm and 6 x 3 mm in width at 10 and 21 days after ulcer induction, respectively. The ulcer had healed to a scar by Day 30. No recurrence of gastric ulcer was observed during the observation period.

Figure 3 shows the bacterial counts of *H. pylori* in the gastric mucosa after ulcer induction. The bacterial count in the cardiac mucosa was below the detection limit (<3.7 $\log_{10}$ cfu/g) at the time of ulcer induction. Thereafter, it increased gradually up to a peak of 6.1 $\log_{10}$ cfu/g at 71 days after ulcer induction, and then decreased until returning to the initial level at 127 days (Fig. 3A). The bacteria counts in the antral mucosa also increased gradually up to 71 days, after ulcer induction, reaching a peak of 6.6 $\log_{10}$ cfu/g. The bacteria count of the antral mucosa at 43, 63, 71, 85, and 113 days after ulcer induction were significantly higher than the initial level and decreased until returning to the initial level at 127 days (Fig. 3B). The bacterial count in the fundic mucosa did not change after ulcer induction (Fig. 3C).

In this investigation, the bacterial counts of *H. pylori* in the cardiac and antral mucosa increased temporally after healing of gastric ulcer in a miniature pig. Although the control pig with no gastric ulcer induction died, our previous studies have shown that the bacterial counts of *H. pylori* are constant in the antum of 3-month-old miniature pigs [8,9]. Our present results suggested that the gastric ulcer formation was responsible for the temporal increase in the bacteria counts of *H. pylori* in the cardia and antrum. *H. pylori* is reported to adhere to many host cell surfaces [10]. Much attention and research effort has focused on the adhesion and host receptor of *H. pylori*, but the exact mechanisms *in vivo* are still not fully understood. Host cell receptor expression may have increased temporally as a consequence of the ulcer formation in this study. In the fundus, low pH conditions prevent increases in

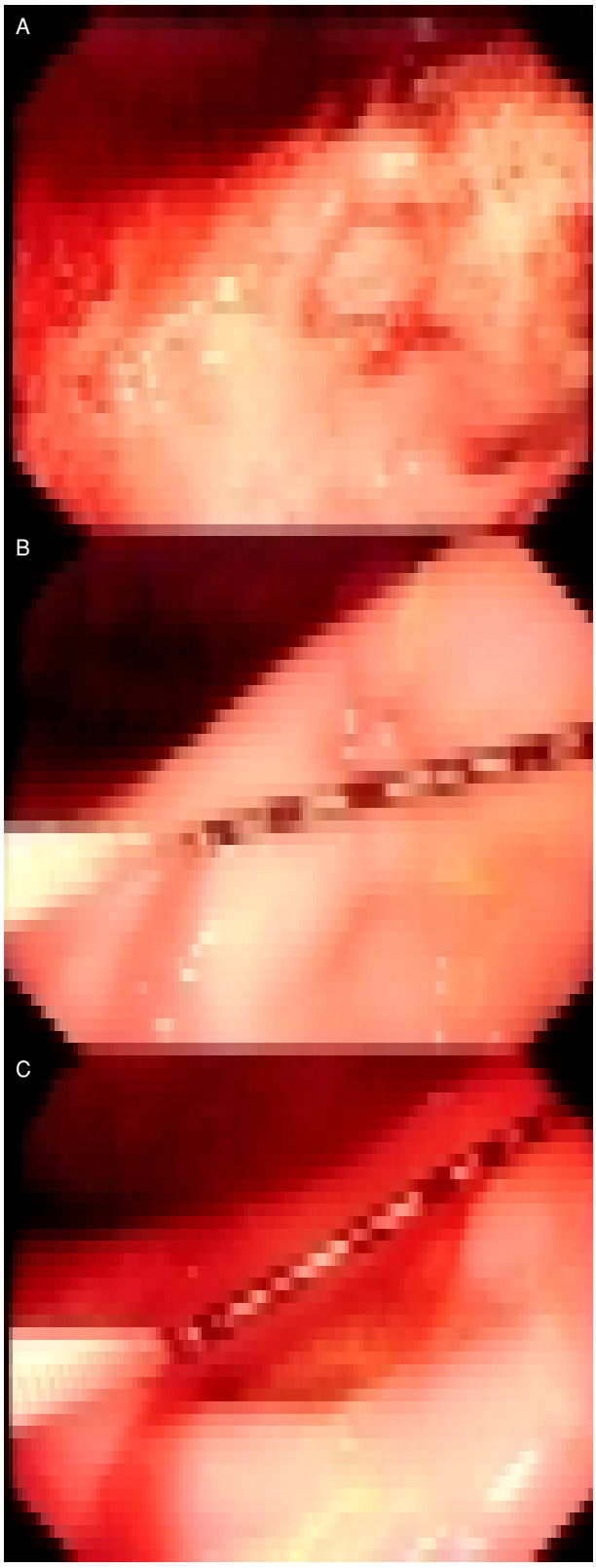

Fig. 2. Endoscopic findings of ulcer healing. Active ulcer in the *H. pylori* -infected miniature pig (A) 10 days, (B) 21 days, and (C) 30 days after ulcer induction. The scale of each section (each alternate black and silver bar) shown is 2 mm.

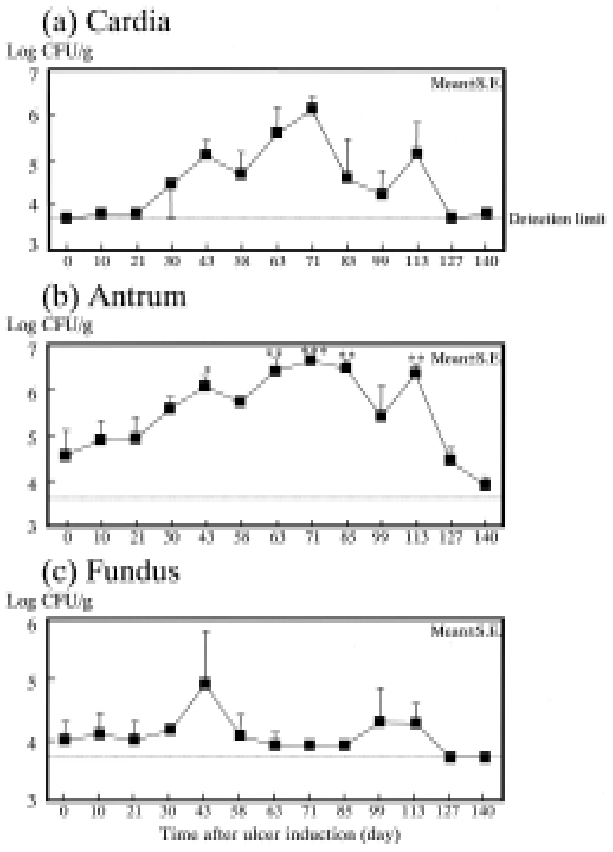

Fig. 3 .The bacterial counts of *H. pylori* in the (A) cardiac, (B) antral, and (C) fundic mucosa of a miniature pig infected with *H. pylori.* Values are given as the mean ± SE (*p<0.05, ** p< 0.01, *** p<0.001 versus initial levels).

the receptor expression and the growth of *H. pylori*.

Although the bacterial counts of *H. pylori* in the stomach increased after healing of ulcer formation, no ulcer recurrence was observed. The *H. pylori* strain No. 9839 used in this study was isolated from a patient with gastric ulcer [9]. In the miniature pig model, the colonization levels of *H. pylori* and the number of neutrophils present in the inflamed mucosa are much lower than those in humans [9]. These differences might account for the absence of ulcer recurrence.

Eradication of *H. pylori* is known to prevent the recurrence of ulcer [6], but the precise preventative mechanisms remain unclear. It may be that *H. pylori* disrupts the protective mucous layer directly and indirectly, leaving the underlying epithelium susceptible to injury by gastric acid [11]. In addition, the persistence of inflammation caused by *H. pylori* is presumed to lead to the "leaking roof" that permits the development or recurrence of ulcers [12]. Since not all patients with persistent *H. pylori* infection develop ulcers, host susceptibility [13], bacterial virulence [14], and environmental factors [15] are also cited as potential causal factors. It is interesting that the bacteria counts of *H. pylori* in the stomach increased temporally after the ulcer healed. Several clinical investigations found no significant difference in the ulcer relapse rate between patients treated by *H. pylori* eradication therapy and maintenance therapy with an H_2-receptor antagonist or proton pump inhibitor [16,17]. In addition to reducing acid production, H_2-receptor antagonists and PPI also decrease the bacterial counts of *H. pylori* in the stomach [8]. Moreover, maintenance therapy with colloidal bismuth subcitrate alone is just as effective in reducing duodenal ulcer relapse as maintenance therapy with H_2-receptor antagonists [18]. These results suggest that inhibition of the temporal increase in bacteria in the stomach may prevent ulcer relapses. Further studies will be needed to clarify the relationship between temporal increases in bacteria and ulcer relapses.

In conclusion, the colonization levels of *H. pylori* after healing of acetic acid-induced gastric ulcer increased temporally in a miniature pig and such a temporal increase could be one of the factors of ulcer relapse in humans.

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