STOMACH

Helicobacter pylori eradication induces marked increase in H^+/K^+ -adenosine triphosphatase expression without altering parietal cell number in human gastric mucosa

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Gut 2006;55:152-157. doi: 10.1136/gut.2005.066464

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Revised version received 13 April 2005
Accepted for publication 28 April 2005
Published online first 4 May 2005

Background and aims: Gastric acid secretion is downregulated by *Helicobacter pylori* infection and upregulated after its eradication, but the mechanisms are still unclear. We examined the effects of *H pylori* eradication on the number of parietal cells and on expression of molecules functioning in acid secretion in the human gastric mucosa.

Methods: We enrolled 111 consecutive men with chronic gastritis induced by *H pylori*. Biopsy specimens were endoscopically obtained before and 12 weeks after successful eradication of *H pylori* and parietal cell numbers were counted. mRNA expression levels of H^+/K^+ -adenosine triphosphatase (H^+/K^+ -ATPase), anion exchanger 2, M3 muscarinic receptor, intrinsic factor, and interleukin 1 β were determined with a real time reverse transcriptase-polymerase chain reaction method. The severity of gastric atrophy was evaluated using the serum pepsinogen I/II ratio.

Results: No significant difference was observed in parietal cell numbers before and after *H pylori* eradication. Median mRNA expression levels of H^+/K^+ -ATPase in the gastric mucosa increased 250-fold after *H pylori* eradication accompanied by attenuation of interleukin 1 β . A large increase in H^+/K^+ -ATPase expression was observed even in patients with severe atrophic gastritis. In contrast, fold increases in mRNA expression levels, including intrinsic factor, anion exchanger 2, and M3 muscarinic receptor, after eradication therapy, were limited to 1.4, 2.3, and 2.5 times, respectively.

Conclusions: In the absence of alteration of parietal cell number, gastric H^+/K^+ -ATPase mRNA expression was markedly restored after successful *H pylori* eradication, suggesting a central role for the restoration of H^+/K^+ -ATPase expression in gastric acid secretion recovery after *H pylori* eradication.

Helicobacter pylori infection is a major cause of chronic gastritis and gastroduodenal ulcers.^{1,2} Furthermore, *H pylori* infection also affects gastric acid secretion. In the stomach infected with *H pylori*, maximum acid output was considerably attenuated.³⁻⁶ The effects of *H pylori* on gastric pH have been studied in association with administration of proton pump inhibitors (PPIs). Higher gastric pH is observed during administration of PPIs in *H pylori* positive than in *H pylori* negative subjects, and two plausible explanations have been reported. Bercik *et al* reported that a higher pH is predominantly ascribed to neutralisation by *H pylori* derived ammonia⁷ whereas Gillen *et al* reported that it is associated with marked inflammation of the oxyntic mucosa.⁸

Gastric acid secretion returns to normal after eradication of *H pylori* and this is thought to be responsible for gastro-oesophageal reflux disease seen after *H pylori* cure.^{9,10} Although these observations indicate a close association between *H pylori* infection and gastric acid secretion, the precise mechanisms by which *H pylori* eradication restores gastric acid secretion are still unclear. In this respect, there are two opposing hypotheses: *H pylori* eradication may increase the number of parietal cells in the gastric mucosa as a result of recovery from atrophy or, alternatively, it may restore the acid secretory ability of parietal cells.

Thus we conducted this study to compare the number of parietal cells in the human gastric mucosa before and 12 weeks after *H pylori* eradication. We also simultaneously compared mRNA expression levels of molecules involved in

acid secretion by parietal cells, including H^+/K^+ -adenosine triphosphatase (H^+/K^+ -ATPase), anion exchanger 2, and M3 muscarinic receptor. We report here that *H pylori* eradication did not increase parietal cell number in the early period after *H pylori* cure but strikingly enhanced mRNA expression of H^+/K^+ -ATPase in the stomach, even in patients with severe atrophic gastritis.

MATERIALS AND METHODS

Patients and samples

Of those who participated in the Gastric Cancer Surveillance Program from June 2001 to the end of 2002 at Tochigi, Japan, 115 consecutive men with *H pylori* infection had received eradication therapy, and eradication was successful in 111 subjects (mean age 49.2 (SEM 0.5) years). These 111 subjects were enrolled in the present study. Indications for eradication therapy in these patients included chronic gastritis alone (14 patients), chronic gastritis accompanied by adenoma, a family history of gastric cancer, gastric hyperplastic polyp, severe atrophic gastritis, or gastric ulcer (67 patients), and duodenal ulcer (30 patients). All subjects were clinically stable at the time of evaluation and had no history of eradication therapy before the study. Written informed

Abbreviations: H^+/K^+ -ATPase, H^+/K^+ -adenosine triphosphatase; RT-PCR, reverse transcriptase-polymerase chain reaction; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPI, proton pump inhibitor

Table 1 Primer pairs and TaqMan probes used in real time reverse transcriptase-polymerase chain reaction

	Sense (5' to 3') and antisense (5' to 3')	TaqMan probe (5' to 3')
H ⁺ /K ⁺ -ATPase	CTTTGCCATCCAGGCTAGTGA GGTGACGACAACCACAGCAAT	ACCACCGACGACAATCTGTACCTGGC
Intrinsic factor	CACTGCTCTTGGCTGTTGGA GGGTCAATATCAAAGTCACTAAATGTG	CCCCACAAGGGCACCACCATCACTC
Anion exchanger 2	CGGAGCAGATCCCCGATA CAACGCGCTCAC TGT CATG	CCCCTGCTCCTTGACTTCTGAATCT
M3 receptor	CCCTGGCAGAGGGAACCT CCAAAATATGTGCCATTA CTGTTTCAT	CCCAGTCCATTGCTGCTGCTACA

Amounts of IL-1 β mRNA were measured using the probe and primers in TaqMan gene expression assays by Applied Biosystems.

consent was obtained from the participants in accordance with the Declaration of Helsinki and its later revision. The ethics committee of Jichi Medical School, Japan, approved the study.

Eradication therapy for *H pylori* infected subjects and data collection

A triple regimen (lansoprazole 30 mg twice daily, clarithromycin 200 mg twice daily, and amoxicillin 750 mg twice daily) was given for seven days after endoscopic examination. Eradication was confirmed when *H pylori* was not detected at 12 weeks after the end of therapy in any of the Giemsa stainings or culture of biopsy specimens. Venous blood samples were obtained after fasting overnight before and after eradication of *H pylori* for determination of serum levels of pepsinogen I and II.

Specimens

Five adjacent biopsy specimens from the greater curvature at the midcorpus of the stomach as well as five from the antrum were obtained endoscopically from all subjects. One biopsy specimen from the corpus of the stomach and one from the antrum were cultured individually to evaluate for the presence of *H pylori* infection. Three biopsy specimens from the corpus and three from the antrum were immediately snap frozen and stored in liquid nitrogen for later use. The remaining corpus and antral specimens were fixed and stained with haematoxylin-eosin and Giemsa. Histological assessments were performed by a single observer (H Osawa) to whom no clinical data for any patient were given at the time of evaluation. *H pylori* infection was evaluated by bacterial culture and histological examination.

RNA extraction

Total RNA was isolated from biopsy specimens with Isogen (Nippon Gene, Tokyo, Japan), and 2 μ g of total RNA were reverse transcribed with random nanomers and reverse transcriptase (Toyobo, Osaka, Japan).

Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Evaluation of expression of five genes, including H⁺/K⁺-ATPase α , anion exchanger 2, M3 muscarinic receptor, intrinsic factor, and interleukin (IL)-1 β , was performed using a real time quantitative RT-PCR method with an ABI 7700 sequence detector system (PE Applied Biosystems, Foster City, California, USA). Sense primers, antisense primers, and target hybridisations used for each marker are shown in table 1. The reaction mixture was prepared according to the manufacturer's protocol using TaqMan PCR kits (PE Applied Biosystems). The probe was labelled with a reporter fluorescent dye, 6-carboxyfluorescein, at the 5' end. Thermal cycling conditions for all reactions included 50°C for two minutes and 95°C for 10 minutes, followed by 40

Figure 1 Changes in the number of parietal cells in the oxyntic mucosa before and after *Helicobacter pylori* eradication. Parietal cells were identified by immunostaining with anti-H⁺/K⁺-ATPase antibody. Numbers of parietal cells were similar before and after *H pylori* eradication (n=25; 46 (35–50) and 46 (36–56) (median (1st quartile–3rd quartile)); p=0.15 by Wilcoxon rank sum test). No significant change was observed before and after *H pylori* eradication.

cycles of 15 seconds of denaturation at 95°C and one minute of annealing and extension at 60°C. As a control, mRNA was also subjected to real time quantitative RT-PCR for measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using TaqMan GAPDH control reagents (PE Applied Biosystems). For relative quantification of expression of each marker, calibration curves were constructed using mRNA obtained from normal gastric mucosa without *H pylori* infection. Levels of each marker mRNA were normalised to GAPDH.

Counting parietal cells stained by anti-H⁺/K⁺-ATPase antibody

Parietal cells were immunostained by anti-H⁺/K⁺-ATPase antibody (1:100, developed in our laboratory) using biopsy specimens before and 12 weeks after eradication in the first consecutive 25 cases. After immunostaining slides were viewed at 200 $\times$ magnification and digitised with a digital HD microscope (VH-7000; Keyence, Tokyo, Japan). Immunoreactive cells of oxyntic glands were counted in three visual fields at 200 $\times$ magnification. Results are presented as mean number of positive cells per visual field. Parietal cells were immunostained intensely with anti-H⁺/K⁺-ATPase antibody in all specimens before and after *H pylori* cure. Thus immunohistochemical analysis did not suffer from disparities in staining. In addition, portions of patchy atrophic changes

Figure 2 Comparison of H^+/K^+ -ATPase mRNA (A), anion exchanger 2 mRNA (B), and M3 muscarinic receptor mRNA (C) expression levels in the oxyntic mucosa before and after *Helicobacter pylori* eradication. There was a 250-fold restoration of median H^+/K^+ -ATPase mRNA expression after *H. pylori* cure in comparison with levels before (4.3 (0.4–61) and 1075 (301–3127) (median (1st quartile–3rd quartile)); $p < 0.0001$ by Wilcoxon rank sum test). *H. pylori* eradication induced a slight increase in anion exchanger 2 mRNA expression levels in the oxyntic mucosa (0.23 (0.01–0.52) and 0.53 (0.31–0.83); $p < 0.0001$ by Wilcoxon rank sum test). Similarly, *H. pylori* eradication induced a slight increase in M3 muscarinic receptor mRNA expression levels in the oxyntic mucosa (0.20 (0.10–0.43) and 0.48 (0.32–0.67); $p < 0.0001$ by Wilcoxon rank sum test).

of the gastric mucosa were not selected for counting of immunoreactive cells. The numbers of H^+/K^+ -ATPase expressing cells in the gastric mucosa were also compared between subjects before and 12 weeks after *H. pylori* cure.

Statistical analyses

Number of parietal cells and levels of mRNA expression are expressed as median (first quartile to third quartile). The Wilcoxon rank sum test was used to compare respective mRNA levels before and after *H. pylori* cure. Differences at $p < 0.05$ were considered significant.

Figure 3 Comparison of interleukin 1β (IL- 1β) mRNA expression levels in the oxyntic mucosa before and after *Helicobacter pylori* cure. *H. pylori* eradication induced a 40% decrease in IL- 1β mRNA expression levels in the oxyntic mucosa (1.05 (0.70–1.90) and 0.65 (0.39–1.22) (median (1st quartile–3rd quartile)); $p = 0.005$ by Wilcoxon rank sum test).

Figure 4 Comparison of intrinsic factor mRNA expression levels in the oxyntic mucosa before and after *Helicobacter pylori* cure. *H. pylori* eradication induced a slight increase in intrinsic factor mRNA expression levels in the oxyntic mucosa (0.40 (0.20–0.80) and 0.55 (0.36–0.82) (median (1st quartile–3rd quartile)); $p = 0.009$ by Wilcoxon rank sum test).

RESULTS

H. pylori eradication did not alter the number of parietal cells in the gastric mucosa during 12 weeks of follow up

In an effort to examine the effects of *H. pylori* cure on the number of parietal cells, we first attempted to compare the number of parietal cells before and 12 weeks after treatment by counting them in 25 patients. As shown in fig 1, there was no significant difference in the number of parietal cells before and 12 weeks after treatment.

Marked restoration of H^+/K^+ -ATPase mRNA expression after *H. pylori* eradication

We next attempted to assess the effects of *H. pylori* eradication on the acid secretory machinery of parietal cells. We compared mRNA levels of H^+/K^+ -ATPase, anion exchanger 2, and M3 muscarinic receptor before and 12 weeks after *H. pylori* eradication. In addition, we also determined IL- 1β and intrinsic factor mRNA expression levels as an inflammatory reaction parameter and a control not involved in the acid secretory machinery, respectively. As shown in fig 2A, there was a 250-fold increase in median H^+/K^+ -ATPase mRNA expression levels in the gastric mucosa after *H. pylori* eradication ($n = 111$), accompanied by a 40% decrease in median IL- 1β mRNA expression levels ($n = 111$) (fig 3). These data suggest that *H. pylori* eradication restored H^+/K^+ -ATPase expression in the gastric mucosa, concomitant with improvement in gastric mucosal inflammation.

In contrast, the increase in mRNA expression of other molecules involved in acid secretion was relatively small. As

Figure 5 Representative images of biopsy specimens stained with haematoxylin-eosin used for histological assessment of gastric atrophy. The fundic mucosa before (A) and after (B) eradication in the non-severe atrophic group is shown for a subject with a pepsinogen I/II ratio of 4.7 before treatment. The fundic mucosa before (C) and after (D) eradication in the severe atrophic group is shown for a subject with a pepsinogen I/II ratio of 1.0 before treatment. The pepsinogen I/II ratio was closely correlated with gastric body atrophy grade assessed by histological examination.

shown in fig 2B and 2C, the increase in mRNA expression levels of anion exchanger 2 ($n = 111$) and M3 muscarinic receptor ($n = 111$) after *H. pylori* eradication was 2.3- and 2.5-fold, respectively, similar to intrinsic factor ($n = 111$) (fig 4). These data indicate that *H. pylori* eradication predominantly restored H⁺/K⁺-ATPase expression compared with other molecules expressed in parietal cells. As *H. pylori* eradication did not alter parietal cell numbers in the gastric mucosa, these data suggest that recovery of gastric acid secretion after *H. pylori* eradication in the gastric mucosa is due to an increase in H⁺/K⁺-ATPase expression in parietal cells.

Grade of gastric atrophy did not affect restoration levels of H⁺/K⁺-ATPase mRNA expression after *H. pylori* eradication

Based on the finding that *H. pylori* eradication restores H⁺/K⁺-ATPase expression in parietal cells, we then examined whether restoration of H⁺/K⁺-ATPase expression in parietal cells was related to the degree of gastric atrophy before *H. pylori* eradication. We thus compared gastric H⁺/K⁺-ATPase mRNA expression levels between the groups with different degrees of gastric atrophy. Pepsinogen I and pepsinogen II

differ in their location in the stomach. Both are located in chief and mucous neck cells of the oxyntic gland mucosa in the gastric corpus but only pepsinogen II is present in the gastric antrum. A pepsinogen I/II ratio <3 is considered to be a reliable marker for severe atrophic gastritis.^{11 12} We thus evaluated gastric atrophy in subjects by determining the pepsinogen I/II ratio. In addition, gastric atrophy was evaluated by histological examination. Figure 5 shows representative images of biopsy specimens stained with haematoxylin-eosin used for histological assessment of gastric atrophy. In these cases, the pepsinogen I/II ratio was closely correlated with the gastric atrophy grade assessed by histological examination. We thus classified subjects into two groups using the pepsinogen I/II ratio as a gastric atrophy parameter: severe atrophy group ($n = 66$) (pepsinogen I/II ratio <3) and non-severe atrophy group ($n = 45$) (pepsinogen I/II ratio ≥ 3). As shown in fig 6, median H⁺/K⁺-ATPase mRNA expression levels were increased from 0.9 to 960 in the severe atrophy group, and from 37 to 1273 in the non-severe atrophy group, respectively. These results indicate that H⁺/K⁺-ATPase expression in parietal cells is largely restored even in patients with high grade gastric atrophy.

Figure 6 Comparison of H^+/K^+ -ATPase mRNA expression levels in the oxyntic mucosa and serum pepsinogen levels before and after *Helicobacter pylori* cure. H^+/K^+ -ATPase mRNA expression levels rose markedly despite the serum pepsinogen I/II ratio (0.9 (0.3–24) and 960 (148–3106) (median (1st quartile–3rd quartile)) in the severe atrophy group (pepsinogen I/II ratio <3) ($p < 0.0001$); 37 (5.0–219) and 1273 (479–3343) in non-severe atrophy group (pepsinogen I/II ratio ≥ 3) ($p < 0.0001$ by Wilcoxon rank sum test)).

DISCUSSION

In the present study we have demonstrated restoration of H^+/K^+ -ATPase mRNA expression levels in the gastric mucosa at a relatively early stage after *H. pylori* eradication without alteration of parietal cell numbers. In contrast, restoration of anion exchanger 2, M3 muscarinic receptor, and intrinsic factor mRNA expression levels in the gastric mucosa after *H. pylori* eradication was much smaller than that of H^+/K^+ -ATPase. These results suggest that restoration of H^+/K^+ -ATPase expression in parietal cells plays a central role in the recovery of gastric acid secretion after *H. pylori* eradication.

It is well known that gastric acid secretion recovers after *H. pylori* eradication but the mechanism has been unclear, partly due to the controversy as to whether *H. pylori* eradication leads to improvement of gastric atrophy. Tucci *et al* indicated that there was a significant improvement in mucosal inflammation and atrophy in the corpus one year after eradication.¹³ However, several reports showed that gastric atrophy did not improve after eradication.^{14–17} Hence it has been unclear whether recovery of gastric acid secretion is a result of restoration of parietal cell numbers or functional recovery of parietal cells. Our current study simultaneously demonstrated absence of alteration of parietal cell number and restoration of H^+/K^+ -ATPase, thus providing strong evidence that recovery of gastric acid secretion after *H. pylori* eradication is caused by functional recovery of parietal cells.

The mechanism of the inhibitory effect of *H. pylori* infection on gastric acid secretion is still controversial, despite extensive studies. One hypothesis is that parietal cell function is directly affected by *H. pylori*. It has been shown in vivo that acute infection with *H. pylori* causes hypochlorhydria.¹⁸ In an in vitro experiment using human gastric adenocarcinoma (AGS) cells transfected with H^+/K^+ -ATPase α 5'-flanking sequence, *H. pylori* infection induced dose dependent inhibition of basal and histamine stimulated H^+/K^+ -ATPase α promoter activity by 80% and 66%, respectively.¹⁹ Smolka *et al* demonstrated that *H. pylori* downregulated human H^+/K^+ -ATPase α basal transcription by displacement of the AP-1 transcription factor from the homology box I region of H^+/K^+ -ATPase α 5'-flanking sequence.²⁰ In our study, restoration of gastric H^+/K^+ -ATPase after eradication of *H. pylori* strongly

suggests that inhibition of H^+/K^+ -ATPase production in gastric parietal cells during *H. pylori* infection plays a role. One possible explanation is the association with inflammatory responses induced by *H. pylori* infection. Antibacterial treatment is known to induce resolution of the acute component of *H. pylori* gastritis within a few days after treatment.²¹ IL-1 β is important in initiating and amplifying the inflammatory response against bacteria and is also a potent inhibitor of gastric acid secretion.^{22–26} Indeed, our data showed a negative association between H^+/K^+ -ATPase and IL-1 β , supporting this notion. These results also suggest that *H. pylori* derived ammonia may not be responsible for the reversible inhibition of acid secretion, as several investigators have reported.^{27, 28}

Gastric acid secretion from parietal cells is highly organised, with a stimulus-secretion coupling molecular system. In parietal cells, neurohormonal stimuli trigger parietal acid secretion through receptors present in the basolateral membranes, such as M3 muscarinic receptor.^{29–31} Then, H^+/K^+ -ATPase is transported to the apical membrane where it secretes acid into the gastric lumen. During acid secretion, anion exchanger 2 plays a role in acid loading into parietal cells by functioning in HCO_3^- efflux and Cl^- influx across the basolateral membrane.^{32, 33} Thus H^+/K^+ -ATPase, M3 muscarinic receptor, and anion exchanger 2 play crucial roles in parietal gastric acid secretion. Of these molecules, our study clearly demonstrated the extraordinary restoration of H^+/K^+ -ATPase after *H. pylori* eradication.

Consistent with our data, Furuta *et al* previously reported that both H^+/K^+ -ATPase expression and gastric acid secretion are elevated after *H. pylori* eradication.³⁴ These data suggest a relationship between the increase in H^+/K^+ -ATPase expression and recovery of gastric acid secretion. However, their data did not address the question of whether the increase in H^+/K^+ -ATPase expression was a result of an increase in parietal cell numbers or enhanced expression of H^+/K^+ -ATPase. We thus expanded their study by showing that recovery of gastric acid secretion in the gastric mucosa after eradication was not due to an increase in parietal cell numbers but to enhanced expression of H^+/K^+ -ATPase in parietal cells. In addition, we elucidated that H^+/K^+ -ATPase expression in the gastric mucosa was restored even in patients with severe gastric atrophy. We further extended knowledge of the importance of restoration of H^+/K^+ -ATPase expression in the recovery of acid secretion by comparing it with restoration of other molecules involved in parietal acid secretion. Taken together, it is reasonable to conclude that restoration of H^+/K^+ -ATPase expression plays a central role in recovery of gastric acid secretion after *H. pylori* eradication.

Furuta and colleagues³⁴ and our group observed restoration of H^+/K^+ -ATPase expression at a relatively early period: one month and three months after *H. pylori* eradication, respectively. However, recovery of gastric acid secretion has also been observed at later periods after *H. pylori* eradication. For example, Iijima *et al* reported that gastrin stimulated acid secretion was observed even seven months after *H. pylori* eradication in patients with gastric ulcer.³⁵ However, changes in parietal cell numbers and H^+/K^+ -ATPase expression during these later periods after *H. pylori* eradication have yet to be elucidated. Thus further study on parietal cell numbers and H^+/K^+ -ATPase expression at late periods after *H. pylori* eradication is warranted.

In conclusion, we have shown marked restoration of H^+/K^+ -ATPase expression in the gastric mucosa after *H. pylori* eradication without alteration of parietal cell numbers. These data provide novel insight into our understanding of the mechanism of gastric functional recovery after treatment of *H. pylori* infection.

ACKNOWLEDGEMENTS

We are indebted to Ms Kaoru Tokuda for her excellent technical assistance.

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Conflict of interest: None declared.

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