

RESEARCH COMMUNICATION

Development of Carcinoid Tumors of the Glandular Stomach and Effects of Eradication in *Helicobacter Pylori*-Infected Mongolian Gerbils

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Abstract

The relation between *Helicobacter pylori* (Hp) eradication and prevention of stomach carcinoid development has hitherto remained unclear. We therefore examined this problem using an Hp-infected and Hp-eradicated Mongolian gerbil (MG) model. Enterochromaffin-like (ECL) lesions (hyperplasia/dysplasia and carcinoid) were histopathologically evaluated in the glandular stomachs of Hp-infected and Hp-eradicated MGs. In addition, serum gastrin levels were analyzed. Hp infection induced significant increase in the development of ECL lesions in the glandular stomach, as well as serum gastrin levels as compared with non-infected MGs, while Hp eradication was associated with significant alleviation. The development of ECL lesions in the glandular stomach strongly correlated with titers of anti-Hp antibodies and serum gastrin levels in MGs. In conclusion, Hp infection induces carcinoid development, and Hp eradication prevents its occurrence in the glandular MG stomach, this being strongly linked with reduction in serum gastrin levels.

Key Words: Stomach carcinoid - Mongolian gerbil - *Helicobacter pylori*

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Introduction

Several seroepidemiological studies have indicated that *Helicobacter pylori* (Hp) infection is closely related to chronic gastritis, peptic ulcers, atrophic gastritis, intestinal metaplasia, and stomach adenocarcinoma (Asaka et al., 1997). Hp-infected Mongolian gerbils (MG) have been found to be a useful animal model (Hirayama et al., 1996; Hirayama et al., 1999) whose reproducibility has been confirmed by many researchers (Ikeno et al., 1999; Takahashi et al., 1998). In the model, proliferation and neoplasia of stomach enterochromaffin-like (ECL) cells also occur in the glandular stomach. Although pathogenic roles of Hp in development of ECL neoplasms (carcinoid tumors) have been documented in both men (Solcia et al., 1995) and MGs (Kagawa et al., 2002; Nozaki et al., 2003), the relationships among chronic gastritis, gastrin levels, and pathological features of carcinoids have hitherto remained unclear. Recently, the effects of Hp treatment on prevention of stomach cancer development in patients with chronic gastritis have become a topic of discussion (Tatematsu et al., 2005). However, whether Hp eradication can prevent stomach carcinoid

development also has remained unclear.

In present study, we therefore evaluated stomach carcinoids histologically in the glandular stomachs of Hp-infected and Hp-eradicated MGs. In addition, serum gastrin levels were analyzed.

Materials and Methods

Animals and Hp challenge and eradication

Specific pathogen-free male MGs (*Meriones unguiculatus*; MGS/Sea) were purchased from Seac Yoshitomi, Ltd. (Fukuoka, Japan) at the age of 7 weeks, and housed in steel cages on hardwood chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. Gerbils were given food (Oriental CRF-1; Oriental Yeast Co., Tokyo) and water ad libitum. All experiments and procedures carried out on the animals were approved by the Animal Care Committee of Aichi Cancer Center Research Institute. Hp (ATCC 43504, American Type Culture Collection, Rockville, MD, USA) was used for this study. The bacteria were revived from freezer stocks, grown for 72 h, and harvested in Brucella broth. Samples (0.8 ml) containing about 1.0×10^8 colony-

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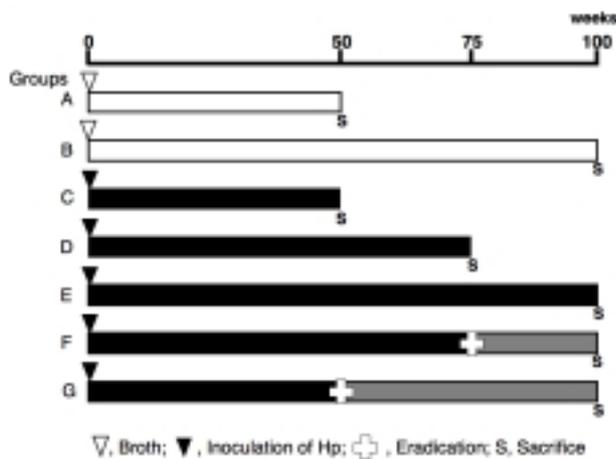


Figure 1. Experimental Design

forming units per milliliter were used as the inoculum for intragastric delivery via an oral catheter after the animals had been deprived of food for 24h, as described previously. Uninfected gerbils underwent sham inoculation using the same sterile *Brucella* broth without Hp. For eradication of Hp, a "triple therapy" was employed. The drugs, lansoprazole (Takeda Chemical Industries, Ltd.), amoxicillin (Kyowa Hakko Kogyo Co., Ltd.), and clarithromycin (Taisho Pharmaceutical Co., Ltd., Tokyo) were suspended in 0.5% w/w carboxymethyl cellulose sodium salt solution and administered intragastrically twice a day for 2 days at doses of 10, 3, and 30 mg/kg body weight, respectively. (Nozaki et al., 2002; Shimizu et al., 2000)

Experimental protocol

In this experiment, 109 gerbils were divided into 7 groups (A-G) (Fig. 1 and Table 1). Animals in groups A and B underwent sham inoculation using *Brucella* broth without Hp and were sacrificed after 50 and 100 weeks as controls. Hp was inoculated into groups C-G. Groups C, D, and E were sacrificed at 50, 75, and 100 weeks after infection of Hp, respectively. Groups F and G received eradication of Hp with "triple therapy" after 75 and 50 weeks, respectively. At the end of experiments, after 24h fasting, animals were deeply anesthetized, laparotomized, and exsanguinated from the inferior vena cava, after which each stomach was excised, fixed in 95% ethanol plus 1% acetic acid and embedded in paraffin. Tissues were sectioned at 4 μ m for staining with hematoxylin and eosin (H&E) and immunohistochemistry for chromogranin A.

Immunohistochemistry (IHC)

Immunohistochemical staining was carried out with a polyclonal antibody against chromogranin A (Dako, Glostrup, Denmark). The precise procedures for immunohistochemical techniques were as described previously (Takenaka et al., 2006). Briefly, 4 μ m-thick consecutive sections were deparaffinized and hydrated through a graded series of ethanols. After inhibition of endogenous peroxidase activity by immersion in 3% H₂O₂ methanol solution, sections were incubated with the primary antibody, washed thoroughly in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibody followed by the avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H₂O₂ and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. Two independent investigators (L.C. and T.T.) judged the histology and immunohistochemical staining of chromogranin A.

Diagnosis of ECL hyperplasia, dysplasia, and neoplasia

Histopathological classification of gastric endocrine cell lesions was into hyperplasia, dysplasia, and neoplasia (carcinoid) categories, basically according to Solcia et al (Solcia et al., 1988) but with modifications as follows. As the morphological distinction between micronodular or adenomatoid hyperplasia and dysplasia was difficult and became arbitrary, in this study, we combined them as one category. Areas of hyperplasia/dysplasia and carcinoids were assessed using a micrometer; lesions per length of glandular stomach epithelium examined (mm²/cm) were then calculated.

Serum anti-Hp IgG antibody and gastrin levels

Blood samples containing a small amount of ethylenediaminetetraacetic acid (EDTA) were centrifuged at 8,000 rpm for 5 min to isolate plasma, which was then stored at -80°C until measurement by ELISA with anti-Hp IgG antibodies (GAP-IgG; Biomerica, Newport Beach, CA). Titers were measured and expressed using an arbitrary index (A.I.). Serum gastrin levels were examined using a gastrin radioimmunoassay kit Gastrin-RIA KIT II (Dainabot Co., Ltd., Tokyo) and expressed as pg/ml values.

Table 1. Anti-Hp Antibody Titer, Serum Gastrin, and Incidence of ECL Lesions in Mongolian Gerbils

Groups	Hp infection	Eradication at	Sacrificed at	Effective no.	IgG (A.I.) ^{a,c}	Gastrin (pg/ml) ^{a,c}	Hyperplasia/Dysplasia (%)	Neoplasia (%) ^c
A	-		50 w	9	2.9 \pm 2.5	181.2 \pm 185.9	0/ 9 (0.0)	0/9 (0.0)
B	-		100 w	4	2.7 \pm 1.7	188.9 \pm 85.8	0/ 4 (0.0)	0/4 (0.0)
C	+		50 w	18	126.8 \pm 135.2 ^a	401.3 \pm 131.2 ^a	5/18 (27.7) ^a	3/18 (17.7) ^{a,d}
D	+		75 w	16	173.4 \pm 68.9	506.3 \pm 148.7	5/16 (31.2)	5/16 (31.2) ^d
E	+		100 w	24	168.8 \pm 89.0 ^b	652.9 \pm 198.9 ^b	14/24 (58.3) ^b	15/24 (62.5) ^{b,d}
F	+	75 w	100 w	16	143.3 \pm 77.8	306.1 \pm 224.8	4/16 (25.0)	6/16 (37.5)
G	+	50 w	100 w	13	37.1 \pm 31.4 ^c	127.2 \pm 47.3 ^c	2/13 (15.4) ^c	3/13 (23.1) ^c

^aMean \pm SD, ^a*P*<0.01 vs. group A, ^b*P*<0.01 vs. group B, ^c*P*<0.05 vs. group E, ^d*P*<0.01 among groups C, D, and E in trend analysis
^eCorrelations: *P*<0.01 between anti-Hp IgG and serum gastrin; *P*<0.001 between serum gastrin and incidence of carcinoids

Statistical analyses

The unpaired t test was applied to establish the significance of differences in titers of anti-Hp antibodies and serum gastrin levels. Incidences of hyperplasia/dysplasia and carcinoids were assessed using the Fisher's exact test. The Mann-Whitney's U test was applied to establish the significance of differences in areas of hyperplasia/dysplasia and carcinoids. Correlation analysis was performed using the ANOVA Bonferroni test. P values < 0.05 were considered to be statistically significant.

Results

Histopathological findings

Limited numbers of chromogranin A-positive cells were observed at the bases of the normal fundic glands in non-infected MGs (control groups, A and B).

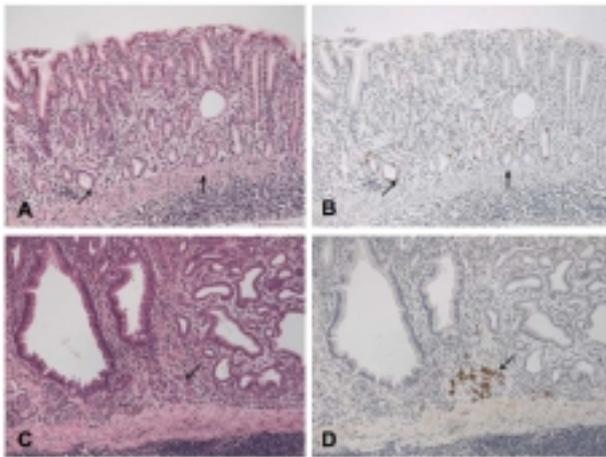


Figure 2. Histology of Simple Hyperplasia of Gastric Endocrine Cells. (A and B) A few scattered chromogranin A-positive cells are apparent in the middle-deep part of the fundic mucosa of a Mongolian gerbil with Hp infection at 50 weeks. (C and D) Micronodular hyperplasia at 50 weeks. A and C, H&E staining, B and D, chromogranin A immunohistochemistry. Original magnification, 100x

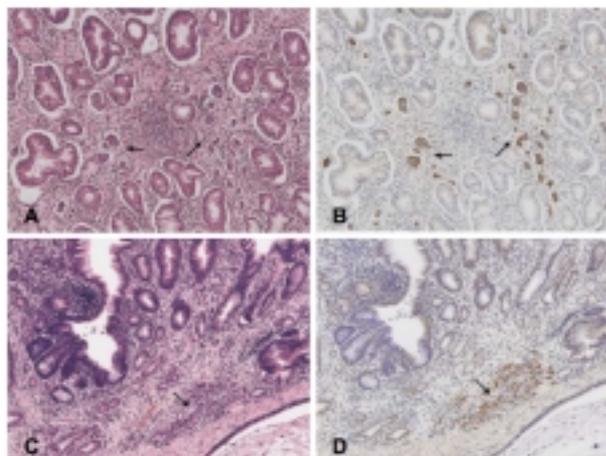


Figure 3. Histology of Dysplasia. Multifocal dysplasia (A, B) and nodular dysplasia (C, D) in fundic mucosa of a Mongolian gerbil at 75 weeks after inoculation of Hp. A and C, H&E, B and D, chromogranin A. Original magnification, 100x.

At 50 weeks after Hp infection (Group C), precursor and neoplastic lesions of chromogranin A-positive cells, such as hyperplasia, dysplasia, and carcinoids were apparent. Hyperplasia appeared as focal or scattered collections of ECL in the basal half of the mucosa and sporadically along the neck regions of the gland. In some cases, this hyperplasia became a continuum from microfocal hyperplasia to mere coalescing bands or nodular scattering of chromogranin A-positive cells that eventually merged into dysplasia or tumor-like proliferation.

Carcinoids were always found in fundic mucosa near the forestomach in MGs and localized in the lamina propria mucosa and submucosa. The histological appearance varied from microfocal and multifocal coalescing foci through anastomosing cords of cells to solid nodular, sometimes also with pseudo-acinar patterns. The proliferating cells showed a monomorphic pattern with regular cells containing uniform rounded nuclei and fairly abundant, pale, fine-granular cytoplasm. There was no cellular or nuclear pleomorphism, and the mitotic activity was very low. No invasion of the muscular wall or any spread beyond the body of the stomach occurred. Angioinvasion or metastasis was also not evident. No gerbils died of stomach carcinoids. All the hyperplastic/dysplastic and neoplastic lesions were positively immunostained for chromogranin A (Figures 2, 3, and 4).

Incidences of hyperplasia/dysplasia and carcinoids in Hp-infected and Hp-eradicated MG glandular stomachs

The incidences of the hyperplasia/dysplasia and carcinoid are summarized in Table 1. No hyperplasia/dysplasia and neoplastic lesions composed of chromogranin A-positive cells were observed in the 2 control groups, A and B. The incidences of hyperplasia/dysplasia and carcinoids demonstrated a significant increase from C, through D, to E Hp-infected groups (Table 1, groups A vs. C; B vs. E). Inversely, those demonstrated a significant decrease from E, through F, to

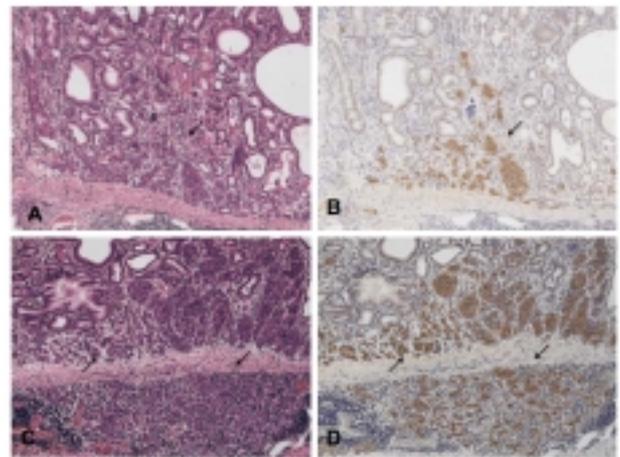


Figure 4. Histopathological Appearance of Typical Gastric Carcinoid Tumors. (A and B) Intramucosal lesion in the fundic region of a Mongolian gerbil at 100 weeks after Hp inoculation. (C and D) The tumor shows extensive involvement of the submucosa. A and C, H&E, B and D, chromogranin A. Original magnification, 100x

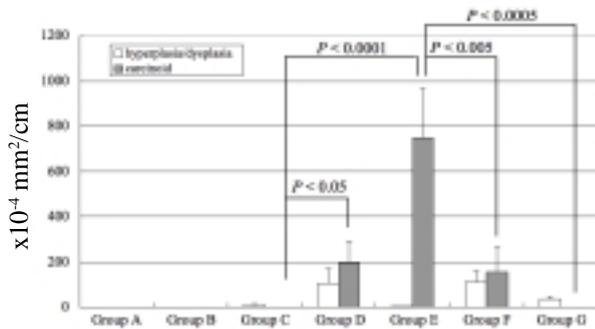


Figure 5. Areas of Gastric Endocrine Cell Hyperplasia/Dysplasia and Carcinoids in Mongolian Gerbils

G Hp-eradicated groups (Table 1, groups E vs. G). Areas of hyperplasia/dysplasia and neoplasia in Hp-infected and Hp-eradicated MG glandular stomach

The results for areas of ECL lesions are summarized in Figure 5. Values for hyperplasia/dysplasia were 8.0 ± 3.2 ($\times 10^{-3}$ mm²/cm, average \pm SE), 103 ± 67 , 2.2 ± 1.0 , 110 ± 49 , and 34.6 ± 12.9 in C, D, E, F, and G groups, respectively. For carcinoids, the figures were 0.58 ± 0.37 , 195 ± 99 , 748 ± 221 , 152 ± 117 , and 0 ± 0.00 . The areas of carcinoids demonstrated a significant increase from C, through D, to E groups in Hp-infected groups ($P < 0.05$, C vs. D; $P < 0.0001$, C vs. E). Inversely, the areas of carcinoids demonstrated a significant decrease from E, through F to G Hp-eradicated groups ($P < 0.005$, E vs. F; $P < 0.0005$, E vs. G).

Titers of anti-Hp antibodies and serum gastrin levels in Hp-infected and Hp-eradicated MGs

Titers of anti-Hp antibodies and serum gastrin levels are summarized in Table 1. Hp infection induced the significant increase of titers of anti-Hp antibodies and serum gastrin levels compared with the non-infected MGs (groups A and B) ($P < 0.01$, A vs. C; $P < 0.01$, B vs. E, respectively). Hp eradication resulted in significant decrease of titers of anti-Hp antibodies and serum gastrin levels compared with the Hp-infected MGs ($P < 0.01$, E vs. G).

Relationships among titers of anti-Hp antibodies, serum gastrin levels, and incidences of ECL lesions

Hp infection caused a gradual increase of titers of anti-Hp antibodies, serum gastrin levels and incidences of the hyperplasia/dysplasia and carcinoid compared with non-infected gerbils (groups A and B). Inversely, Hp eradication was associated with a gradual decrease of titers of anti-Hp antibodies, serum gastrin levels and incidences of the hyperplasia/dysplasia and carcinoid compared with Hp-infected gerbils (group G). The incidences of the hyperplasia/dysplasia and carcinoid were strongly correlated with serum gastrin levels, and the increase and decrease of these factors were linked with Hp infection and eradication ($P < 0.01$) (Table 1).

Discussion

Our present data provide clear evidence that Hp eradication prevents the development of carcinoids in the

MG glandular stomach. Hirayama et al (2002) also showed the frequency of carcinoids in eradication therapy groups to be remarkably reduced compared with values for control and vehicle groups. Regarding the development of stomach cancer in the MG model, we also have demonstrated that eradication of infection results in curtailment of enhancing effects on neoplasia, particularly in early stages of associated inflammation (Cao et al., 2002; Nozaki et al., 2003), similar to the our present data for MG glandular stomach carcinoids. Eradication of Hp induces apoptosis and suppresses proliferation in heterotopic proliferative glands of infected MGs (Cao et al., 2004). Taking into account the previous reports and our present data, we consider that Hp eradication is useful for the prevention of not only stomach cancer but also carcinoid development.

In animal models, *mastomys* or rats treated with H2 receptor antagonists exhibit hypergastrinemia and ECL tumors (Betton et al., 1988). However, the relation between Hp infection and carcinoid occurrence could not be analyzed in these animal models. In the MG model, several studies have shown that long-term Hp colonization produces hyperplasia of gastrin-producing antral G-cells and carcinoid tumors (Hirayama et al., 1999; Kagawa et al., 2002). In humans, Hp-infected individuals show hypergastrinemia, possibly due to alteration of G-cell function by Hp-produced specific products (McCull et al., 1997), or because of inflammation-stimulating gastrin hypersecretion (McGowan et al., 1996). It has been reported that Hp is an important factor in the progression of fundic gastritis and the development of ECL cell hyperplasia during long-term treatment with lansoprazole (Eissele et al., 1997). Thus, Hp-induced hypergastrinemia and stomach carcinoids are thought to be closely linked (Bordi et al., 1991; Nilsson et al., 1993). In the present study, Hp infection induced significant increase of serum gastrin levels compared with the non-infected MGs, while Hp eradication reversed this process, strongly associated with reduced development of ECL lesions (Table 1). Several studies have shown that Hp eradication results in a decrease in serum gastrin concentrations in humans (Graham et al., 1993; Oderda et al., 1989). In addition, Diamaline et al (1993) suggested that the production of chromogranin A, an endocrine cell differentiation marker, in ECL cells of the rat stomach was part of the functional response of these cells to circulating gastrin. We also have pointed out a correlation between chromogranin A and gastrin in Hp-infected MGs (Takenaka et al., 2006).

Gastric and intestinal phenotypic expression is important for the histogenesis of stomach tumors (Takenaka et al., 2007) and the phenotype of each tumor cell can be clearly classified on the basis of gastrointestinal exocrine and endocrine cell markers. We have previously demonstrated that Hp infection may trigger intestinalization of both stomach cancer and non-neoplastic mucosa (Mizoshita et al., 2006). However, most stomach cancers retain a gastric exocrine phenotype in the MG glandular stomach, suggesting that the origin of stomach cancer is from progenitor cells specializing towards exocrine differentiation not of intestinal metaplasia, but rather of gastric glands (Mizoshita et al.,

2006; Tatematsu et al., 2005). In addition, we have shown that the endocrine cell phenotype is in line with that of the exocrine counterpart in the glands of Hp-infected MG stomachs, supporting the concept that development of intestinal metaplasia is due to abnormal differentiation of a stem cell (Takenaka et al., 2006; Tsukamoto et al., 2006). Regarding the phenotypic expression of human stomach carcinoids, most cases exhibit a gastric endocrine cell phenotype, and have no exocrine counterpart, suggesting an origin from progenitor cells specializing towards endocrine differentiation of gastric glands (Takenaka et al., 2007). We therefore consider that analyses of phenotypic expression in the Hp-infected and Hp-eradicated MG model are important for elucidating the histogenesis of stomach carcinoids.

In the present study, the carcinoids in the MG glandular stomach were small, multicentric, and localized in lamina propria mucosa and submucosa of the fundic mucosa. No angiogenesis and metastasis were shown, suggesting low-grade malignancy. These findings are similar to those for stomach carcinoids accompanying type-A chronic atrophic gastritis, whose malignant potential is very low (Bordi et al., 1991).

In conclusion, Hp infection induces carcinoid development and Hp eradication prevents its occurrence in the MG glandular stomach, this being strongly linked with changes in serum gastrin levels.

Acknowledgments

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