RESEARCH COMMUNICATION

Long-term High-dose Proton Pump Inhibitor Administration to *Helicobacter pylori*-infected Mongolian gerbils Enhances Neuroendocrine tumor Development in the Glandular Stomach

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Abstract

Proton pump inhibitors (PPIs) are routinely used for control of upper gastrointestinal disorders, often with long-term application. However, there has been some concern about the long-term safety and the possibility of cancer induction and development of neuroendocrine tumors (NET) in the stomach. We therefore analyzed the influence of PPI use on tumor development histologically, immunohistochemically, and serologically in the glandular stomachs of Helicobacter pylori (Hp)-infected and uninfected Mongolian gerbils (MGs). 53 MGs were divided into 6 groups: Hp+25PPI, Hp+5PPI, Hp, 25PPI, 5PPI, and controls. The high-dose Hp+25PPI and 25PPI groups received the PPI (lansoprazole) at 25mg/kg/day, and the low-dose Hp+5PPI and 5PPI groups were given 5mg/kg/day. After 50 or 100 weeks, animals were sacrificed humanely, and the glandular stomach samples were evaluated histologically and phenotypically, using antibodies against chromogranin A (CgA), gastrin and gastric inhibitory polypeptide (GIP). Serum gastrin levels were also examined. NETs occurred in the Hp+25PPI, Hp+5PPI, Hp, and 25PPI groups, but there was no synergistic effect between Hp-infection and high-dose PPI administration. Serum gastrin was increased statistically by Hp infection and high-dose PPI administration, but not influenced by the low-dose. The NETs featured expression of CgA, but not gastrin or GIP. In conclusions, PPI at low dose had no influence on development of carcinomas and NETs in the Hp-infected and uninfected glandular MG stomach, suggesting clinical safety. However, PPI at high dose increased NET development and serum gastrin in the MG model.

Keywords: Neuroendocrine tumors - proton pump inhibitor - *Helicobacter pylori* - Mongolian gerbil

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Introduction

Proton pump inhibitors (PPIs) are routinely used to control upper gastrointestinal disorders such as peptic ulcers and gastro-esophageal reflux disease (GERD), often for long periods of time. However, there has been some concern about their long-term safety (Poulsen et al., 2009). Most PPI users have moderate hypergastrinemia due to the inhibition of gastric acid secretion (Lamberts et al., 1988; Klinkenberg-Knol et al., 1994), which may increase the development of neuroendocrine tumors (NETs) (Bardram et al., 1986; GillenMcColl, 2001) and carcinomas (Laine et al., 2000; Waldum et al., 2005; Kuipers, 2006) in the stomach. It has been reported that the long-term PPI use is associated with an increased incidence of atrophic gastritis (Kuipers et al., 1996), a precursor of stomach cancer (Kuipers et al., 1996; YeNyren, 2003) in patients with the *Helicobacter pylori* (Hp), but concrete conclusions have yet to be drawn.

The Mongolian gerbil (MG) model is useful for examining the link between Hp infection and human stomach disorders, as the lesions induced by Hp in this experimental animal resemble those apparent in man (Hirayama et al., 1996). The Hp-infected and chemical carcinogen-treated MG has proved very useful for the analysis of stomach carcinogenesis (Tatematsu et al., 2005). Recently, several reports have shown development of NETs in Hp-infected MGs (Kagawa et al., 2002; Cao et al., 2008). Regarding the histogenesis of cancers and NETs in the gastrointestinal tract, we have previously

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demonstrated the importance of the gastric and intestinal phenotypic expression in the human and MG model, using gastric mucous (MUC5AC, MUC6) and endocrine (gastrin), and intestinal mucous (MUC2, villin) and endocrine (gastric inhibitory polypeptide; GIP) markers (Mizoshita et al., 2003; Mizoshita et al., 2006; Cao et al., 2008; Hirata et al., 2009). However, the relations between PPI use, tumor development, and phenotypic expression have hitherto remained unclear in the Hp-infected stomach.

In the present study, we therefore analyzed the influence of PPI use on development of cancers and enterochromaffin-like lesion (ECL lesions) in the glandular stomachs of Hp-infected and uninfected MGs, evaluated histologically and phenotypically.

Materials and Methods

PPI

Lansoprazole (Takeda Pharmaceutical Co., Ltd.), mixed with CE-2 (CLEA Japan INC., Tokyo, Japan) was given as the PPI.

Animals

A total of 53 (26 Hp-infected and 27 uninfected), 13-week-old male MGs (MGS/Sea, Kyudo Co., Ltd) were housed in plastic cages on hardwood chip bedding in an air-conditioned biohazard room with 12:12 h light:dark cycle. The experimental design was approved by the Animal Care Committee of the Nagoya City University Animal Research Institute and the animals were cared for in accordance with institutional guidelines, in compliance with the instructions of the Health, Labour and Welfare Ministry concerning animal experiments.

Experimental design

The experimental design is illustrated in Figure 1. Fifty-three gerbils were divided into 6 groups. Hp+25PPI and 25PPI groups were given the PPI at 25mg/kg/day, and the Hp+5PPI and 5PPI groups at 5mg/kg/day. The animals were sacrificed humanely at 50 or 100 weeks (Figure 1).

After 24h fasting, all animals were deeply anesthetized, laparotomized, and exsanguinated from the inferior vena cava, followed by excision of their stomachs. Each glandular stomach was fixed in 10% formalin neutral buffer solution, and routinely processed for histological examination (Sasaki et al., 2007). The glandular stomach samples were serially cut into 5-mm slices in parallel with the lesser curvature and embedded in paraffin, and then sectioned and stained with hematoxylin and eosin (H&E).

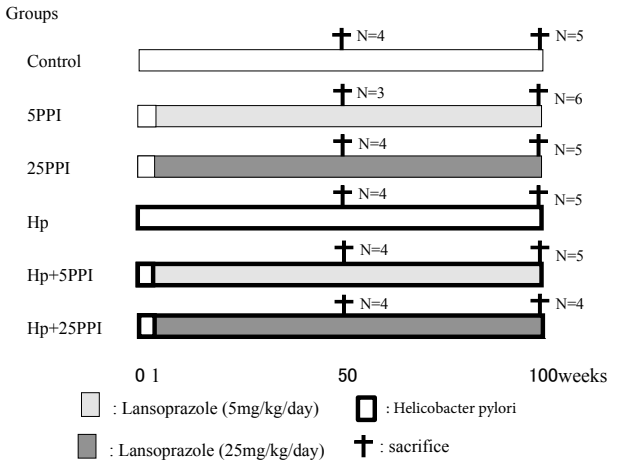

Figure 1. Experimental Design

Immunohistochemistry

Immunohistochemical staining was carried out with polyclonal antibodies against chromogranin A (CgA) (Yanaihara Institute Inc., Fujinomia, Japan), gastrin (Yanaihara Institute Inc., Fujinomia, Japan) and gastric inhibitory polypeptide (GIP) (Yanaihara Institute Inc., Fujinomia, Japan). The precise procedures were as described previously (Takenaka et al., 2006). Briefly, 4 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of ethanols. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2 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'-diaminobinzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. Two independent investigators (HT and TM) judged the histology and immunohistochemical staining.

Diagnosis of ECL lesions

The gastric ECL lesions were evaluated histologically, basically according to TNM staging classification for the NET of the foregut published by a working group of the European Neuroendocrine Tumor Society (ENETS) in 2006 (Rindi et al., 2006), but with modifications (Cao et al., 2008). The lesions were divided into hyperplasia, NET-Tis, and NET-≥T1. The NET-≥T1 lesions were tumors invading lamina propria or submucosa, while NET-Tis lesions exhibited features of in situ tumors or dysplasia. Micronodular lesions, excepting NET-Tis and NET-≥T1, were defined as hyperplasia. Areas of hyperplasia, NET-Tis, and NET-≥T1 were assessed using a micrometer; lesions per length of glandular stomach epithelium examined (mm^2/cm) were then calculated.

Phenotypic classification of ECL lesions

Gastrin is the marker of the gastric endocrine cell phenotype, whereas GIP is typical of the intestinal one (Takenaka et al., 2007). ECL lesions were classified as endocrine-gastric (e-G) type or endocrine-intestinal (e-I) type, respectively with at least one gastric or intestinal cell phenotype, and as the endocrine-gastric-and-intestinal mixed (e-GI) type when both gastric and intestinal endocrine cell markers were present. Those showing neither gastric nor intestinal phenotypic expression were grouped as endocrine-null (e-N) type, as previously described (Takenaka et al., 2007; Hirata et al., 2009).

Serum gastrin level

Serum gastrin levels were examined using a radioimmunoassay kit Gastrin-RIA KIT II inhibition (Dainabot Co., Ltd., Tokyo) after sacrifice and expressed as pg/ml values.

Statistical analyses

The unpaired t test was applied to establish the significance of differences in titers of serum gastrin levels and the areas of ECL lesions. In the case of non-normal distribution, the Mann-Whitney's U test was applied. Incidences of ECL lesions and NET-≥T1 were assessed using the Fisher's exact test. Correlation analysis was performed using the Pearson's correlation coefficient test. Differences in serum gastrin levels in MGs with and without ECL lesions were assessed using the unpaired t test. P values < 0.05 were considered to be statistically significant.

Results

Expression of CgA, gastrin, and GIP

Immunohistochemical expression of CgA is shown in Figure 2. Limited numbers of CgA-positive cells were observed at the bottoms of the normal fundic and pyloric glands in non-infected MGs (red arrows). In the pyloric glands, expression of gastrin was clearly detected, but no GIP expression was observed. In the duodenum, GIP expression was detected, but no gastrin was observed. Neither gastrin nor GIP were observed in the fundic glands (data not shown).

Histopathological findings of ECL lesions

No adenocarcinomas were observed in the MG groups at 50 and 100 weeks. Some ECL lesions invaded the submucosa, but there was no involvement of the muscularis propria or subserosa. ECL lesions were always found in fundic mucosa near the forestomach in MGs, as

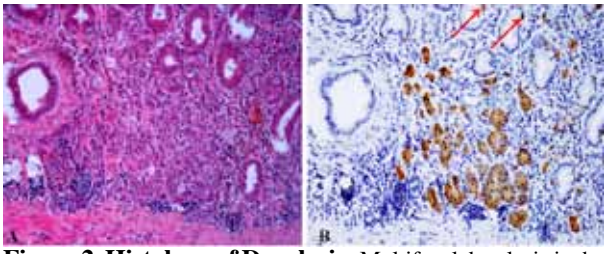

Figure 2. Histology of Dysplasia. Multifocal dysplasia in the fundic mucosa of the Hp-infected Mongolian gerbil treated with PPI (5mg/kg/day). Cytoplasmic CgA expression was detected in normal glands (red arrows) and dysplasia in the MG glandular stomach. (A: H&E staining, B: immunostaining for Cg A) (×200)

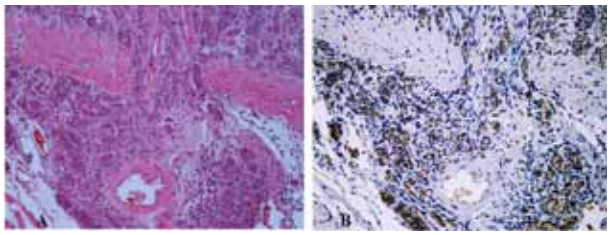

Figure 3. Histology of a Typical NET-≥T1 Lesions. NET invading the fundic submucosa through the muscularis mucosa in an Hp-infected Mongolian gerbil treated with PPI (5mg/kg/day). (A:H&E staining, B:immunostaining for CgA) (×200)

Table 1. Incidences of ECL Lesions in Mongolian Gerbils

Groups	Lansoprazole	Incidence 50 wks		Incidence 100 wks	
		NET-≥T1	NET-Tis*	NET-≥T1	NET-Tis*
Control	-	0/4[a]	0/4c	0/5[a]	0/5[c]
5PPI	5mg	0/3b	0/3d	0/6[b]	0/6[d]
25PPI	25mg	4/4[a,b]	4/4[c,d]	5/5[a,b]	5/5[c,d]
Hp	-	0/4	3/4	3/5	5/5
Hp+5PPI	5mg	0/4	1/4	2/5	2/5
Hp+25PPI	25mg	3/4	3/4	4/4	4/4

*NET-Tis + hyperplasia; [a,b,c,d]P<0.01 in Fisher's exact test

previously described (Cao et al., 2008). Angio-invasion and metastasis were also not evident, and no gerbils died of NETs in the glandular stomach. All ECL lesions were positively immunostained for CgA (Figurex 2 and 3), but neither gastrin nor GIP expression was detected in the ECL lesions, which were classified phenotypically as e-N type (data not shown).

ECL lesions in the glandular stomachs of MGs

The incidences of the ECL lesions are summarized in Table 1. At 50 weeks, NET-≥T1 lesions occurred in the groups given PPI at high dose (25PPI and HP+25PPI), but not detected in the Control, 5PPI, Hp, and Hp+5PPI groups. Regarding hyperplasia and NET-Tis, they were detected in the Hp-infected (Hp, Hp+5PPI, and Hp+25PPI) and 25PPI groups. There were the statistical differences between Control and 25PPI, as well as 5PPI and 25PPI groups, while there were no statistical differences between Hp-infected groups.

At 100 weeks, NET-≥T1 lesions occurred in the Hp-infected (Hp, Hp+25PPI, and Hp+25PPI) and 25PPI groups. Hyperplasia and NET-Tis were also detected in the Hp-infected (Hp, Hp+5PPI, and Hp+25PPI) and 25PPI groups. There were the statistical differences between Control and 25PPI and also between the 5PPI and 25PPI groups, while there were no statistical differences between Hp-infected groups.

Areas of ECL lesions in the glandular stomachs of MGs

The results for areas of ECL lesions are summarized in Figure 4. Values for ECL lesions were 0±0 (×10-2mm2/cm, average±SE), 0±0, 4.63±0.582, 0.0316±0.0140, 0.0102±0.0102 and 1.79±0.738 in Control, 5PPI, 25PPI, Hp, Hp+5PPI and Hp+25PPI at 50 weeks, respectively. At 100 weeks they were 0±0, 0±0, 11.57±3.62, 1.613±0.846, 1.380±1.006 and 16.51±6.55. The areas of ECL lesions were increased by Hp infection (P<0.05, Control vs. Hp)

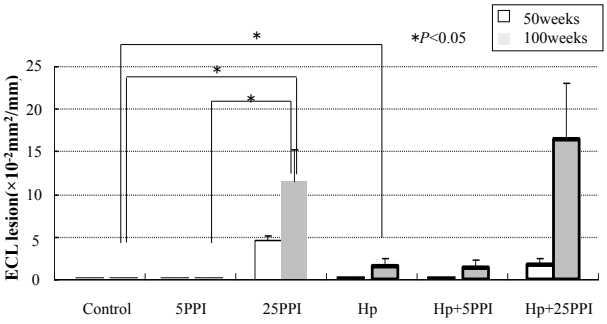

Figure 4. Areas of ECL Lesions in Each Group

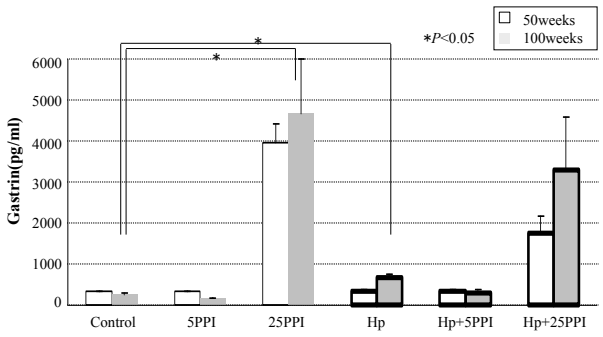

Figure 4. Serum Gastrin Levels in Each Group

and by 25PPI but not 5PPI. The areas of ECL lesions in Hp+25PPI group were greater than those in the Hp one, but without statistical significance.

Serum gastrin levels at 50 and 100 weeks

The serum gastrin levels are summarized in Figure 5. Values for the serum gastrin were 320±32 (pg/ml, average±SE), 320±26, 3950±483, 315±62, 315±56 and 1752±415 for Control, 5PPI, 25PPI, Hp, Hp+5PPI and Hp+25PPI groups at 50 weeks, respectively. At 100 weeks they were 246±65, 167±20, 4680±1324, 670±91, 277±93 and 3275±1328, respectively. The serum gastrin level was increased by Hp infection ($P<0.05$, Control vs. Hp) and by 25PPI (Fig.5). There were no significant differences between Hp-infected groups at 100 weeks.

Relationship between serum gastrin and ECL lesions

The areas of ECL lesions strongly correlated with serum gastrin levels ($P<0.01$, correlation coefficient=0.666). The serum gastrin level in the MGs with ECL lesions was 2951.9±505.3 (pg/ml, average±SE), significantly increased from the average in MGs without ECL lesions (282.8±24.7) ($P<0.01$).

Discussion

We here found that no stomach cancers developed in the Hp+PPI and PPI groups, although hypergastrinaemia is known to be induced by long-term PPI administration in the MG model. It has been reported that the long-term PPI use is associated with an increased incidence of atrophic gastritis (Kuipers et al., 1996), a precursor condition for stomach cancer (Uemura et al., 2001; YeNyren, 2003). However, long-term PPI treatment has not been documented to hasten the development of stomach cancer (Laine et al., 2000). Therefore long term PPI treatment is a safe therapy for acid peptic disorders (Kuipers, 2006), although, the development of atrophic gastritis in Hp-positive patients treated with PPIs, with the long-term concern of stomach cancer development, remains controversial (GillenMcColl, 2001). Gastric secretion may decrease during gastric cancer development in rat (Bralow et al., 1970), and long-lasting iatrogenic hypergastrinemia due to PPI might be expected to increase the occurrence of stomach cancer in the long-term (Waldum et al., 2005). Regarding the MG model, the gerbil can be easily infected with Hp, and the resultant chronic active gastritis, peptic ulcers, and intestinal metaplasia closely resemble lesions apparent in man (Hirayama et al., 1996; Tatematsu et al., 2005). The Hp-infected and chemical carcinogen-treated MG has proved very useful for the analysis of stomach carcinogenesis, providing clear evidence that Hp exerts a promoting effect on stomach carcinogenesis (Tatematsu et al., 2005). Indeed, Hp infection has been reported to induce the development of cancers in the glandular stomach of MGs (Honda et al., 1998; Watanabe et al., 1998; Hirayama et al., 1999). Thus, we consider that the finding of no cancerous lesions in the Hp-infected and uninfected stomach with PPI treatment, suggests that PPI is not a carcinogen. However, it was reported that the use of high dose (100mg/kg/day) omeprazole induced gastric adenocarcinoma (Hagiwara, 2011). This difference may be due to difference of the medicines or the doses. Further studies for the risk of carcinogenesis of PPI are needed.

In the present study, suppression of gastric acid by high-dose PPI (25PPI) induced hypergastrinaemia, and NET occurrence, although no NET-≥T1 tumor development was observed in the low-dose PPI (5PPI) groups. In rats, hypergastrinaemia is associated with an increased risk of stomach carcinoid (Havu, 1986). Gastric carcinoids also occur in patients with type-A chronic gastritis and the Zollinger-Ellison syndrome when it is associated with multiple endocrine neoplasia-1 (Borch et al., 1985; Borch et al., 1987; Solcia et al., 1990). Pronounced acid suppression has been shown to lead to elevated serum gastrin in many individuals (Lanzon-Miller et al., 1987). Whereas the relation between carcinoids and hypergastrinanemia induced by PPI has not been made clear in human, the differences between human and rodents need to be considered. Firstly, rats demonstrate a relatively greater increase in serum gastrin levels in response to inhibition of gastric acid secretion than do humans. In the present study, the serum gastrin level in the 25PPI group was approximately twentyfold the group control value. In contrast, long-term use of proton pump inhibitor therapy generally results in a two- to fourfold increase in gastrin serum levels in man (Freston, 1992). Patients with carcinoids associated with type-A chronic gastritis have marked long-standing hypergastrinanemia (>500pg/mL; normal limit <100pg/mL). Actually, in this study the MGs receiving 5PPI had a similar serum gastrin level as the control group, and no ECL lesions, including NETs, occurred. Secondly, several reports suggested that humans have a much lower density of enterochromaffin-like cells than do rats (Hakanson et al., 1976; Simonsson et al., 1988). Thus, we consider that the PPI use at clinical dose is safe, and no NET lesions would be expected to develop with standard dosing.

We also have shown that PPI at low dose (5PPI) is not associated with the occurrence of ECL lesions and serum gastrin level in the Hp-infected MG (Fig. 5). In the MG model, we and others 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; Cao et al., 2008). We also have previously reported that Hp infection induces NET development, and Hp eradication prevents its occurrence in the glandular MG stomach (Cao et al., 2008). In humans, Hp-infected individuals show hypergastrinemia, possibly due to alteration of G-cell function by specific Hp-products (McColl et al., 1997), or because of inflammation-

stimulating gastrin hypersecretion (McGowan et al., 1996). In Japan, Hp infection and hypergastrinemia are found in patients with NET without autoimmune gastritis, suggesting that Hp infection may induce corporal mucosal atrophy and hypergastrinemia that can produce a NET over time (Sato et al., 2002). 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). The Hp-induced hypergastrinemia and stomach NETs are thought to be closely linked (Bordi et al., 1991; Nilsson et al., 1993). Thus, we consider that Hp infection induces NET development, and PPI use at a clinical dose would have no influence on its occurrence in man.

After Hp infection, glands in the glandular stomach of MG start to proliferate into the submucosa, disrupting the laminal muscularis mucosa (Nozaki et al., 2002). Resultant lesions, termed heterotopic proliferative glands (HPGs) frequently develop with Hp infection in the glandular stomach of MG (Nozaki et al., 2002). HPGs often resemble differentiated adenocarcinomas, but do not appear to be malignant (Tatematsu et al., 2005). In our study, HPGs occurred in the Hp group, while no HPGs occurred in HP+5PPI and HP+25PPI groups (data not shown).

In 2006, a working group of the ENETS published a proposal for a TNM staging classification of NETs of the foregut (Rindi et al., 2006). Subsequent publication of a TNM staging classification of the midgut and hindgut NETs from the same group followed in 2007 (Rindi et al., 2007), both clearly distinguishing them from other tumors, including carcinomas. In the present study, we used the above-mentioned classification with some modifications. We have previously shown that most stomach NETs exhibit the e-G type in humans, in contrast to the e-N type predominating in MGs (Takenaka et al., 2007; Hirata et al., 2009). However, in the present study, ECL lesions had the e-N type, suggesting development from progenitor cells specializing towards the endocrine cell lineage in glandular ducts exhibiting neither gastric nor intestinal phenotypic expression.

In conclusion, PPI at low dose has no influence on development of carcinomas and NETs in the Hp-infected and uninfected glandular MG stomach, suggesting that PPI is clinically safe. However, at high dose it increases the NET development and serum gastrin level in the MG model, and clarification of whether these phenomenon are important for stomach tumorigenesis is needed.

Acknowledgments

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