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Effects of Aspirin on the Development of *Helicobacter pylori*-Induced Gastric Inflammation and Heterotopic Proliferative Glands in Mongolian Gerbils

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Keywords
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Abstract

Background: *Helicobacter pylori* infection is a major cause of gastritis and gastric carcinoma. Aspirin has anti-inflammatory and antineoplastic activity. The aim of the present study was to determine the effects of aspirin on *H. pylori*-induced gastritis and the development of heterotopic proliferative glands.

Methods: *H. pylori* strain SS1 was inoculated into the stomachs of Mongolian gerbils. Two weeks after inoculation, the animals were fed with the powder diets containing 0 p.p.m. (n = 10), 150 p.p.m. (n = 10), or 500 p.p.m. (n = 10) aspirin. Mongolian gerbils were killed after 36 weeks of infection. Uninfected Mongolian gerbils (n = 10) were used as controls. Histologic changes, epithelial cell proliferation and apoptosis, and prostaglandin E_2 (PGE_2) levels of gastric tissue were determined.

Results: *H. pylori* infection induced gastric inflammation. Administration of aspirin did not change *H. pylori*-induced gastritis, but alleviated *H. pylori*-induced hyperplasia and the development of heterotopic proliferative glands. Administration of aspirin accelerated *H. pylori*-associated apoptosis but decreased *H. pylori*-associated cell proliferation. In addition, the increased gastric PGE_2 levels due to *H. pylori* infection were suppressed by treatment with aspirin, especially at the dose of 500 p.p.m.

Conclusions: Aspirin alleviates *H. pylori*-induced hyperplasia and the development of heterotopic proliferative glands. Moreover, aspirin increases *H. pylori*-induced apoptosis. We demonstrated the antineoplastic activities of aspirin in *H. pylori*-related gastric carcinogenesis.

Helicobacter pylori infection is a major cause of chronic gastritis, peptic ulceration, and gastric carcinoma [1]. Studies have shown that *H. pylori* increases proliferation of gastric epithelial cells, and curing of the infection returns cell proliferation to normal levels [2–4]. The hyperplastic changes observed in *H. pylori*-induced gastritis are thought to be associated with precancerous changes [5,6]. A dynamic balance between epithelial cell proliferation and apoptosis is essential for maintaining normal gastric mucosal integrity. *H. pylori* infection induces apoptosis and proliferation of gastric epithelial cells and may lead to alterations in the balance of epithelial cell proliferation and apoptosis [7–11], which is believed to contribute to gastric ulcerogenesis or even carcinogenesis [12,13].

Previous studies have indicated that glands in the stomach of Mongolian gerbils start to proliferate into the submucosa, disrupting the laminal muscularis mucosa after *H. pylori* infection, but with minimal dysplastic change

of constituent cells, and this phenomenon was defined as the formation of "heterotopic proliferative glands (HPGs)" [14,15]. HPGs have many features that are quite different from those of well-differentiated adenocarcinomas or mucinous adenocarcinomas. These characteristics include organized polarity of their component cells, differentiation from gastric phenotype HPGs into intestinal ones with mature Paneth cells, formation of large cystic dilatations containing mucin, often with calcification, shedding of epithelial cells and necrosis at the tips of lesions, high-grade inflammation with infiltration of inflammatory cells, and organized polarity of proliferating zones [14]. Although HPGs do not appear to be malignant, they show gradual intestinalization with a shift from gastric to intestinal phenotypic expression, and thus may be considered as a risk factor for the development of gastric cancer as intestinal metaplasia is a precancerous lesion [14,16]. It has observed that eradication of *H. pylori* infection reverses HPGs and induces apoptosis and inhibits proliferation in HPGs of infected Mongolian gerbils, suggesting that these lesions are apparently reversible [14,16].

Nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, are known to inhibit production of cyclooxygenases (COX)-1 and -2 through both prostaglandin (PG)-dependent and -independent pathways [17,18]. NSAIDs are potential agents for the chemoprevention of gastric cancer, as demonstrated by a recent meta-analysis that evaluated studies on the association between use of NSAIDs and risk of gastric cancer, and concluded that NSAIDs use was associated with a decreased risk of gastric cancer in a dose-dependent manner [19]. However, whether aspirin plays a role in the regulation of *H. pylori*-induced formation of HPGs and apoptosis and cell proliferation of gastric mucosa is unknown. Therefore, the aim of the present study was to determine the effects of aspirin on the development of *H. pylori*-induced gastric inflammation and heterotopic proliferative glands.

Materials and Methods

Animals

Mongolian gerbils were purchased from Seac Yoshitomi Ltd. (Fukuoda, Japan), and were successfully bred in the Laboratory Animal Unit of Hong Kong University. Male Mongolian gerbils (5–7 weeks old, 40–50 g) were maintained on a 12-hour light/12-hour dark cycle at 22 °C in Plexiglas cages with autoclaved water, and fed autoclaved standard laboratory chow *ad libitum*. The animals were fasted for 24 hours before *H. pylori* inoculation, and drinking water was also withheld after the inoculation. From 4 hours after the inoculation, both food and water were freely available to the animals. All procedures were performed with the approval of the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.

Bacterial Inoculation

This strain, named the Sydney strain of *H. pylori* (SS1), is *CagA* and *vacA* positive, which has proven to be an excellent strain for use in the rodent colonization [20], was grown in Brucella broth (BBL, Cockeysville, MD, USA) supplemented with 10% bovine serum albumin (BSA) for 24 hours at 37 °C under microaerophilic conditions. Gerbils fasted overnight were inoculated three times by gavage with 800 µL of *H. pylori* organisms (10^8 colony-forming units (CFU)/mL) with 1 day interval. Age-matched control animals were mock-inoculated with 800 µL Brucella broth.

Aspirin Administration

Sixty Mongolian gerbils were divided into six groups. Two weeks after the first inoculation with *H. pylori*, the animals were fed the powder diets containing 0 parts per million (p.p.m.), 150 p.p.m., or 500 p.p.m. aspirin [21]. The diets containing aspirin were prepared once a week by mixing with AIN-76 A powder diet, and kept at 4 °C until use. Thirty-six weeks after the first *H. pylori* inoculation, the gerbils were sacrificed by exsanguinations during ketamine anesthesia. The blood of five gerbils of each group was collected for measurement of the serum salicylate level (an indicator of aspirin ingestion) using a commercial kit as described by Lee et al. [22]. The stomach was excised and opened longitudinally along the greater curvature.

Detection of *H. pylori* Colonization

Quantitative assessment of *H. pylori* colonization was performed as previously described [23]. Briefly, one piece of weighed stomach tissue was homogenized in 1 mL of Brucella broth by use of a hand pestle (Kontes, NJ, USA), and the homogenate was diluted 10- and 100-fold in Brucella broth. One hundred microliters of each dilution was plated on selective medium containing 10% horse blood (Hong Kong Jockey club), 100 µg/mL vancomycin, 50 µg/mL cefsulodin, 50 µg/mL trimethroprim lactate, and 50 µg/mL amphotericin B (Oxoid, Cambridge, UK). Plates were incubated at 37 °C under microaerophilic conditions produced by a gas-generating system (CampyGen, Oxoid) for 5–7 days. *H. pylori* was identified by Gram staining and by positive urease, oxidase, and catalase tests. *H. pylori* colonies were then counted to determine the number of CFU per gram of the stomach tissue.

Histologic Examination

Gastric specimens were embedded in paraffin and cut into 4-μm thick sections. The sections were stained with hematoxylin–eosin for semiquantitative histologic examination of the activity (neutrophil infiltration score) and severity (mononuclear cell infiltration score) of chronic inflammation, which were graded as follows: 0, minimal; 1, mild; 2, moderate; and 3, marked in accordance with the updated Sydney system [24]. Moreover, hyperplasia and the formation of HPGs, which were defined as described by Nozaki et al. [14], were assessed and graded as follows: 0, histologically normal (normal glands); 1, mild (hyperplastic glands in mucosa); 2, moderate (hyperplastic glands in mucosa and a few HPGs until submucosa); and 3, marked (many HPGs until subserosa) abnormality, according to the criteria described by Nozaki et al. [14]. The presence of spiral organisms was also examined in Giemsa-stained sections. Pathologists experienced in histologic examination of Mongolian gerbils who were blinded to treatment schedule read the stained slides.

Determination of Apoptosis and Cell Proliferation

Epithelial cell apoptosis was determined in situ from paraffin-embedded tissue sections using the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) technique (Apop Tag In Situ Apoptosis Detection Kit; Intergen Company, Norcross, GA, USA), in accordance with the manufacturer's protocol. Epithelial cell proliferation was determined by immunohistochemical staining for the proliferating cell nuclear antigen (PCNA). Briefly, sections were deparaffinized, placed in citrate buffer (10 mmol/L; pH 6.0), and heated in a 700-W microwave oven for 10 minutes. Endogenous peroxidase activity was quenched by use of hydrogen peroxide. After being washed in immunoassay buffer, the slides were incubated with a mouse monoclonal IgG against PCNA (DakoCytomation, Glostrup, Denmark) in a humidified chamber. Slides were incubated with biotinylated rabbit antirat IgG and peroxidase-conjugated streptavidin (DakoCytomation), developed using the DAB+ substrate-chromogen system (DakoCytomation), and counterstained with hematoxylin (Sigma Chemical CO, St. Louis, MO, USA). Pathologists experienced in histologic examination of Mongolian gerbils who were blinded to treatment schedule read the stained slides. For determination of the level of apoptosis or cell proliferation, cells were counted in 25 well-orientated gastric glands in the gastric corpus and antrum. The apoptosis index (AI) and the cell proliferation index (PI) were defined as the percentage of positively stained cells per the total number of cells counted (about 1000 cells).

Enzyme-Linked Immunosorbent Assay for Gastric PGE_2

Gastric specimens were weighed and homogenized at 4 °C in lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 100 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mg/mL glucose, 28 mmol/L indomethacin). Homogenates were vortexed and centrifuged at 13 400 *g* for 30 minutes at 4 °C. PGE_2 levels in the supernatants were measured by use of commercially available ELISA kits (Caymen Chemical Company, Ann Arbor, MI, USA), in accordance with the manufacturer's protocol. Plates were read at 410 nm by use of a microtiter plate reader.

Statistics

All numerical data are presented as means ± standard error of means. The Mann–Whitney *U*-test was used to determine the differences between groups. Statistical analyses were performed using SPSS (version 12.0 for Windows, SPSS Inc., Chicago, IL, USA). Significance was defined as a $p < .05$ (two-tailed).

Results

Quantitative Culture of *H. pylori*

All gerbils challenged with SS1 were infected. There was no significant difference in the numbers of CFUs of *H. pylori* in gastric mucosa among Mongolian gerbils with or without aspirin treatment 36 weeks after the infection (Fig. 1), and no *H. pylori* colonies were detected in uninfected groups.

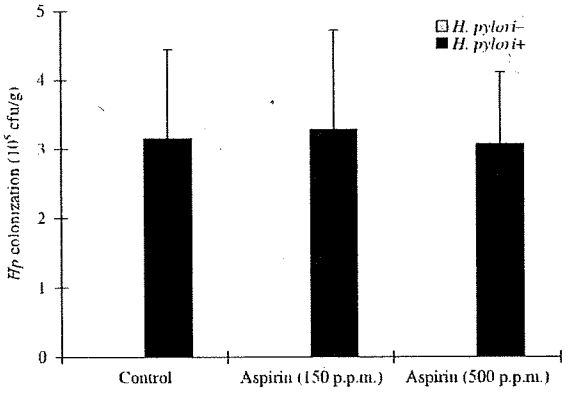

Figure 1 Levels of *H. pylori* colonization in infected Mongolian gerbils (10 per group), as assessed by counting the number of colony-forming units (CFU) per gram of stomach tissue after bacterial culture. Data are mean ± SEM.

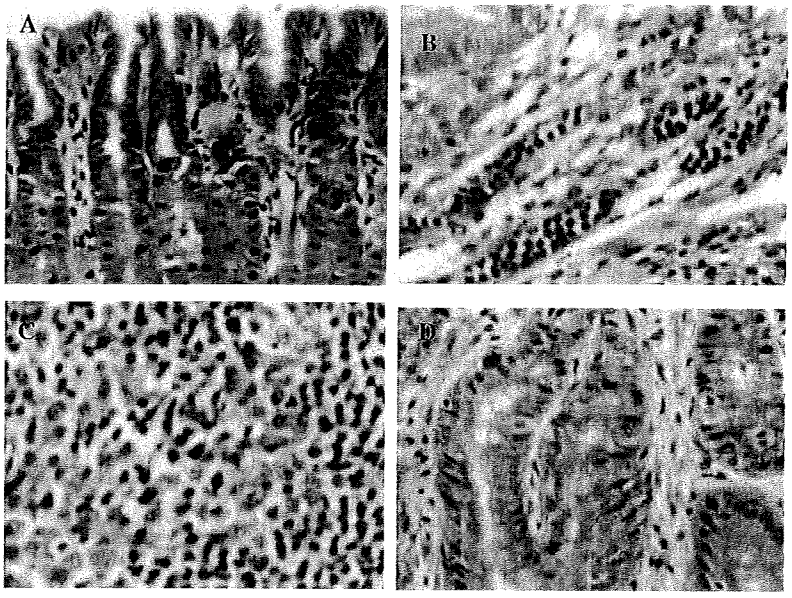

Figure 2 Representative hematoxylin–eosin-stained sections of gastric mucosa showing normal mucosa in an uninfected gerbil without aspirin treatment (*A*), moderate and marked infiltration of polymorphonuclear neutrophils and mononuclear cells in an *H. pylori*-infected gerbil without aspirin treatment (*B*), a dense lymphoid response occupying both mucosa and submucosa in an *H. pylori*-infected gerbil without aspirin treatment (*C*), and heterotopic proliferative glands with cystic dilation in an infected gerbils without aspirin treatment (*D*). Original magnification ×40.

Table 1 Serum salicylate levels (mean ± SEM) in Mongolian gerbils treated with different doses of aspirin

		Aspirin treatment	
	Control (0 p.p.m.) (n = 10)	150 p.p.m. (n = 10)	500 p.p.m. (n = 10)
Serum salicylate levels (mg/L)	0	11.40 ± 0.76[a]	16.73 ± 0.91[b]

[a] $p < .05$, compared with the control group (0 p.p.m.).
[b] $p < .05$, compared with the control (0 p.p.m.) and 150 p.p.m. aspirin groups.

Measurement of the Serum Aspirin Level

There was no significant difference in food consumption between gerbils on diet with aspirin supplementation (means, 5.9 and 5.7 g diet/day per gerbil for 150 p.p.m. and 500 p.p.m., respectively) and those without (means, 5.6 g diet/day per gerbil). Salicylate was not detected in the serum of gerbils without aspirin treatment. The serum salicylate concentrations were dose-dependently elevated in gerbils on diet with aspirin supplementation; 11.40 ± 0.76 mg/L and 16.73 ± 0.91 mg/L, in those treated with 150 p.p.m. and 500 p.p.m., respectively (Table 1).

Histopathologic Findings

No histopathologic changes were present in the stomach of uninfected gerbils without aspirin treatment (Fig. 2A, Table 2). Mild antral and corpus gastritis were observed in all except one uninfected gerbils treated with aspirin; moderate infiltration of polymorphonuclear neutrophils and mononuclear cells were found in the stomach of one gerbil treated with 500 p.p.m. aspirin. In contrast, moderate or marked active and chronic inflammation was observed in all *H. pylori*-infected gerbils without aspirin treatment (Fig. 2B, Table 2). Moderate or marked active and chronic inflammation was also observed in all except one infected gerbils with aspirin treatment; mild active and chronic gastritis was present in an infected gerbil treated with 150 p.p.m. aspirin.

Increased levels of polymorphonuclear neutrophils and mononuclear cells were found in the stomachs in the presence of *H. pylori* infection, with or without aspirin treatment. In most cases, there was a dense lymphoid response occupying both gastric mucosa and submucosa (Fig. 2C, Table 2). Hyperplasia and HPGs were found in all the stomachs of infected gerbils without aspirin treatment (Fig. 2D, Table 2). HPGs developed only in the antrum and the junctional region between the antrum and the body showing pseudopyloric metaplasia. No HPGs were observed in fundic mucosa in this experiment. The hyperplasia and HPGs were also observed in infected gerbils treated

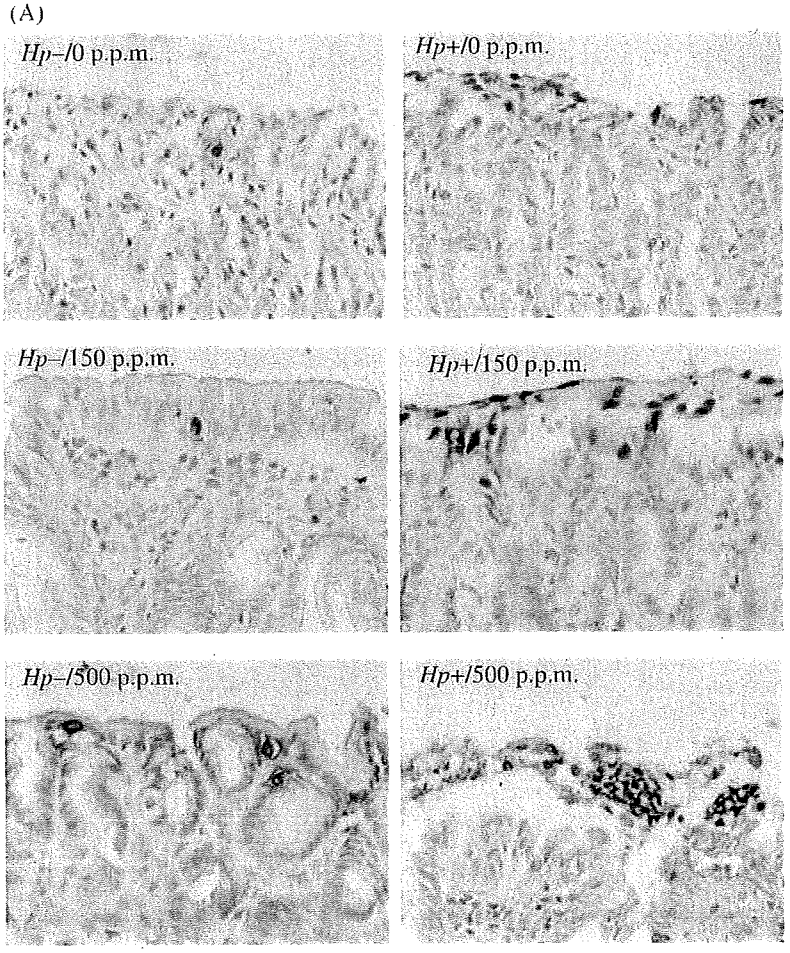

Figure 3 Representative TUNEL-stained sections of the gastric corpus (*A*: original magnification, ×400) and proliferating cell nuclear antigen-stained sections of the gastric antrumt.

Table 2 Infiltration of polymorphonuclear neutrophils and mononuclear cells and the development of hyperplasia and heterotopic proliferative glands (HPGs) in the stomachs of *H. pylori*-infected (*Hp*+) or uninfected (*Hp*–) Mongolian gerbils with or without aspirin treatment

	Aspirin treatment					
	0 p.p.m.		150 p.p.m.		500 p.p.m.	
Score of histologic changes (mean ± SEM)	*Hp*– (n = 10)	*Hp*+ (n = 10)	*Hp*– (n = 10)	*Hp*+ (n = 10)	*Hp*– (n = 10)	*Hp*+ (n = 10)
Neutrophil infiltration	0.0 ± 0.0	2.5 ± 0.2[a]	1.0 ± 0.0	2.1 ± 0.2[a]	1.0 ± 0.0	2.3 ± 0.2[a]
Mononuclear infiltration	0.0 ± 0.0	2.6 ± 0.2[a]	1.0 ± 0.0	2.8 ± 0.2[a]	1.2 ± 0.2	3.0 ± 0.0[a]
Hyperplasia and formation of HPGs	0.0 ± 0.0	2.0 ± 0.0[a]	0.0 ± 0.0	1.1 ± 0.2[ab]	0.0 ± 0.0	1.3 ± 0.2[ab]

[a] $p < .01$, compared with the corresponding uninfected gerbils.
[b] $p < .05$, compared with the infected gerbils without aspirin treatment.

with aspirin, but the grades were significantly decreased (Table 2). However, there was no significance in the grades between gerbils on 150 p.p.m. aspirin and those on 500p.p.m. aspirin (Table 2). In this study, no animals developed adenoma or adenocarcinoma.

Apoptosis and Proliferation of Gastric Epithelial Cells

Apoptosis, as indicated by TUNEL-positive cells, was observed in the surface epithelium of the gastric mucosa of

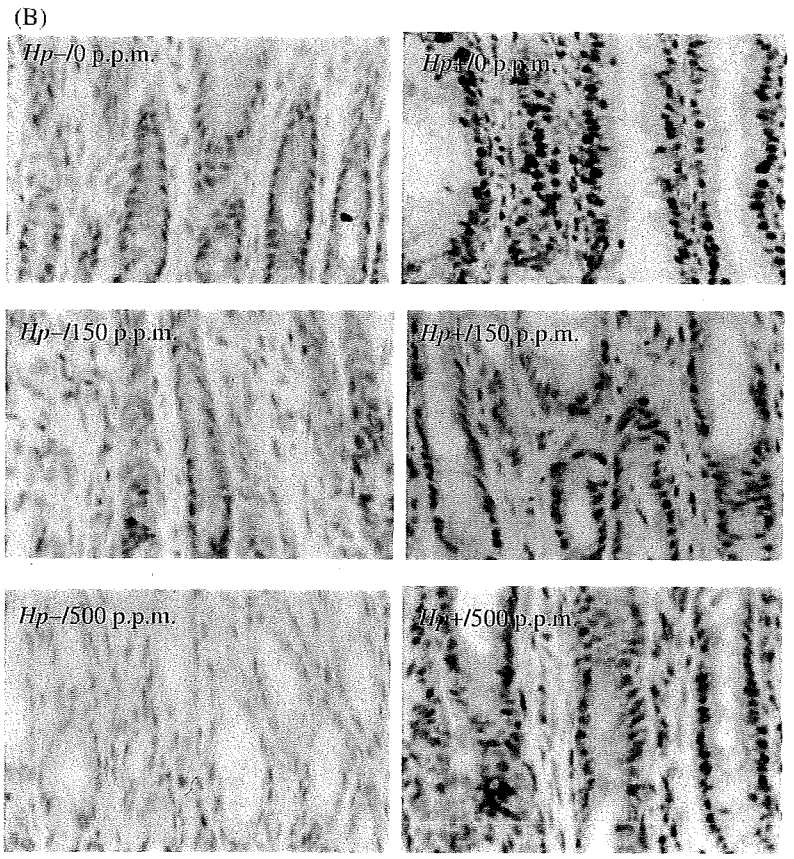

Figure 3 (*B*: original magnification, ×100) were from uninfected (*Hp–*) and *H. pylori*-infected (*Hp+*) gerbils with or without aspirin treatment.

Table 3 Apoptotic index (AI) and proliferation index (PI) in epithelial cells at gastric antrum and corpus in uninfected (*Hp–*) and *H. pylori*-infected (*Hp+*) gerbils with or without aspirin treatment 36 weeks

	Aspirin treatment					
	0 p.p.m.		150 p.p.m.		500 p.p.m.	
Score (mean ± SEM)	*Hp–* (n = 10)	*Hp+* (n = 10)	*Hp–* (n = 10)	*Hp+* (n = 10)	*Hp–* (n = 10)	*Hp+* (n = 10)
AI						
Antrum	0.15 ± 0.09	1.98 ± 0.24[a]	0.19 ± 0.09	3.65 ± 0.53[ac]	0.20 ± 0.12	3.94 ± 0.86[ac]
Corpus	0.22 ± 0.08	1.26 ± 0.21[b]	0.32 ± 0.10	1.98 ± 0.47[a]	0.34 ± 0.12	2.95 ± 0.74[bc]
PI						
Antrum	9.20 ± 2.89	36.40 ± 8.72[a]	8.56 ± 2.53	19.70 ± 7.65[bc]	6.42 ± 1.97	17.10 ± 5.81[bc]
Corpus	4.35 ± 2.78	27.60 ± 7.66[a]	3.72 ± 2.26	14.90 ± 6.14[bc]	3.18 ± 1.97	12.40 ± 5.63[bc]

[a] $p < .01$, compared with their corresponding uninfected control gerbils.
[b] $p < .05$, compared with their corresponding uninfected control gerbils.
[c] $p < .05$, compared with *H. pylori*-infected gerbils without aspirin treatment.

the infected gerbils with and without aspirin treatment (Fig. 3A). In gerbils without aspirin treatment, apoptosis in the surface epithelium of the gastric antrum and corpus mucosa was significantly increased in the presence of *H. pylori* infection, compared to that in the absence of the infection (Table 3). That is, the apoptotic index (AI) both in antrum and in corpus was 1.98 and 1.26, respectively, in the presence of *H. pylori* infection, while 0.15 and 0.22,

respectively, in the absence of the infection ($p < .01$). The AI in antrum was also significantly increased by aspirin administration in uninfected gerbils (Table 3). Moreover, aspirin administration further increased apoptosis in the antrum at a dose of 150 p.p.m. and in both antrum and corpus at a dose of 500 p.p.m. in infected gerbils ($p < .05$) (Table 3). However, there was no significant difference in AI in the antrum and corpus between gerbils treated with the two doses of aspirin (Table 3).

Immunostaining of PCNA, which reflects cell proliferation, was localized in the nuclei of epithelial cells within the proliferating compartment in the basal zone of the antrum (Fig. 3B). The cell proliferation index (PI) in both the antrum and the corpus was significantly increased by *H. pylori* infection ($p < .01$). Aspirin administration did not change PI in both antrum and corpus in infected gerbils. However, the increased level in the PI in both the antrum and the corpus of infected gerbils was significantly counteracted by aspirin administration at the doses of both 150 p.p.m. and 500 p.p.m. ($p < .05$). There was no significant difference in the effect between 150 p.p.m. and 500 p.p.m. (Table 3).

Gastric PGE_2 Level

PGE_2 was detected at very low levels in the normal gastric tissue. *H. pylori* infection significantly increased PGE_2 production of gastric tissue ($p < .01$). However, aspirin administration decreased the stimulating effect of *H. pylori* infection on gastric PGE_2 production by 8 and 67%, respectively, at the doses of 150 and 500 p.p.m. (Fig. 4).

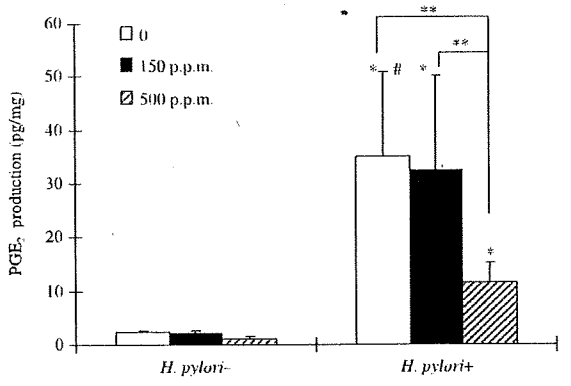

Figure 4 Expression of gastric prostaglandin E (PGE) 2 in uninfected (*Hp–*) and *H. pylori*-infected (*Hp+*) gerbils with or without aspirin treatment (n = 10 per group). Experiments were performed in triplicate. Data are mean ± SEM. *$p < .01$, compared with the corresponding uninfected gerbils. **$p < .05$, compared with *H. pylori*-infected gerbils treated with 500 p.p.m. aspirin.

Discussion

Previous studies have suggested that NSAIDs including aspirin possess bacteriostatic activity against *H. pylori* in vitro with the minimum inhibitory concentration (MIC) of 100 μg/mL [25–27]. However, the present study showed that there was no apparent difference in the bacterial density in gastric mucosa 36 weeks after inoculation of *H. pylori* between Mongolian gerbils with and without 34-week aspirin treatment, suggesting that the aspirin doses (150 and 500 p.p.m.) used in the study may not reach the MIC in the stomach and *H. pylori* colonization is not affected by the aspirin administration at these doses.

To date, many studies have investigated whether NSAIDs and *H. pylori* infection are "foes or friends" in the development of gastric mucosal damage, and majority of these studies have shown that NSAIDs enhance *H. pylori*-induced gastric mucosa inflammation and injury [28–34]. Our previous study demonstrated that genetic deficiency of the COX-1 or COX-2 isoform exacerbated the severity of *H. pylori*-induced gastritis [23], suggesting that inhibition of COX activity is at least part of the mechanism that NSAIDs exhibit the enhancing effects. However, a few studies failed to confirm the synergistic interaction between NSAIDs and *H. pylori* infection [35–38]. Kim et al. reported that indomethacin and NS-398 decreased gastric inflammation induced by *H. pylori* infection in mice [35], while Bhang et al. and Magari et al. reported that administration of selective COX-2 inhibitors, NS-398, and etodolac did not increase mucosal damage in *H. pylori*-induced gastritis [36,37]. In a clinical study conducted by Scheiman et al., rofecoxib, a COX-2 inhibitor, did not significantly affect gastritis scores in patients with *H. pylori* infection [38]. In the present study, treatment with aspirin both at 150 and at 500 p.p.m. had no significant effect on *H. pylori*-induced gastritis.

The hyperplastic changes including the formation of HPGs have been considered to increase the risk of gastric cancer. On one hand, HPGs are frequently observed in *H. pylori*-associated gastritis [14], and on the other, NSAIDs prevent the development of hyperplastic changes and subsequent gastric cancer [19,37,39,40]. However, the impact of NSAIDs on *H. pylori*-associated gastric carcinogenesis remains unclear since only a small number of studies have investigated the interaction between *H. pylori* infection and NSAID use in gastric carcinogenesis [37,39–41]. Moreover, to our knowledge, the present study is the first to investigate the effect of NSAIDs on *H. pylori*-induced HPG formation. In the present study, administration of aspirin significantly alleviated *H. pylori*-induced hyperplasia and the development of HPGs, indicating that aspirin exhibits a preventive effect on the development of *H. pylori*-induced HPG, although further studies including

clinical and pathologic studies are required to confirm our animal study.

It has been consistently demonstrated that chronic *H. pylori* infection increases apoptosis and proliferation of gastric epithelial cells and may eventually result in an imbalance between apoptosis and proliferation, which may contribute to either gastric ulceration due to excessive apoptosis or even carcinogenesis due to hyperproliferation [7–13]. Eradication of the infection normalizes apoptosis and cell proliferation [11,13]. In addition, Cao et al. observed that apoptosis of HPGs was increased while cell proliferation was reduced after eradication of *H. pylori* infection in Mongolian gerbils, and suggested that changes of cell turnover precede the reduction of HPG area and therefore are causal for reversibility, upon eradication of the infection [16]. The present animal study showed that *H. pylori* infection increased both apoptosis and proliferation of gastric cells in gerbils, which is consistent with the findings of our previous study [23] and other studies. Moreover, we observed that aspirin treatment further increased apoptosis, but partially counteracted the increased cell proliferation in *H. pylori*-infected gerbils, further indicating that aspirin prevents the *H. pylori*-induced HPG formation and subsequent the development of gastric carcinoma by affecting the balance of apoptosis and cell proliferation of gastric epithelial cells.

In the present study, *H. pylori* infection elevated the gastric mucosal production of PGE_2, but after aspirin at 150 and 500 p.p.m. was given for 34 weeks, gastric mucosa PGE_2 synthesis was reduced by 8 and 67%, respectively. This finding is in agreement with those of other studies [31,42]. It is believed that aspirin reduces the synthesis of PGs by inhibiting COX enzymes, and this is one of mechanisms by which aspirin induces apoptosis and inhibits cell proliferation of *H. pylori*-infected gastric epithelial cells, and further exhibits protective role against the development of gastric carcinoma.

In human, exposure to aspirin in *H. pylori*-infected individuals results in gastritis and occasionally peptic ulceration. There is a synergistic effect of *H. pylori* infection and use of aspirin/NSAIDs in ulcer formation. More peptic ulcers were found in *H. pylori*-positive than *H. pylori*-negative users of NSAIDs [43]. In contrast, most animals including Mongolian gerbils do not naturally host *H. pylori* [44]. Whether the difference in the duration of *H. pylori* exposure between human and Mongolian gerbils contributes to the different toxicity of aspirin in *H. pylori*-infected individuals remains to be determined.

Other mechanisms that may potentially be involved in influencing the toxic effects of aspirin in stomach may include the expression level and activity of certain gastric protective factors such as PG and nitric oxide (NO). COX-1 is the primary isoform of cyclooxygenases responsible for the production of cytoprotective PGs – PGE-2 and PGI-2 – in the human stomach. Previous studies have shown that aspirin could more significantly suppress the activity of COX-1 irrespective of the *H. pylori* infection status in human stomach. [45]. We do not know whether there is a differential expression and activity of COX-1 in the stomach of Mongolian gerbils relative to human stomach, thus, the influence of COX-1 remains to be confirmed.

In conclusion, aspirin prevents *H. pylori*-induced hyperplasia and the development of heterotopic proliferative glands although it does not affect *H. pylori*-induced gastric inflammation in Mongolian gerbils. Moreover, aspirin increases *H. pylori*-induced apoptosis, but counteracts *H. pylori*-induced cell proliferation. Aspirin may exert these antineoplastic activities, partially by inhibiting the production of gastric PGE_2.

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Synergistic upregulation of inducible nitric oxide synthase and cyclooxygenase-2 in gastric mucosa of Mongolian gerbils by a high-salt diet and *Helicobacter pylori* infection

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Summary. Aims: The intake of salt and salty food is known as a risk factor for gastric cancer. We have previously demonstrated that a high-salt diet dose-dependently enhances *Helicobacter pylori* (*H. pylori*)-associated gastritis and stomach carcinogenesis in Mongolian gerbils. In this study, we focused on the influence of excessive salt intake on the expression of inflammatory mediators involved in progression of *H. pylori*-induced chronic gastritis.

Methods and Results: A total of 45 stomach samples from Mongolian gerbils were evaluated by immunohistochemistry. The animals were infected with *H. pylori* and fed basal (0.32%) or a high-salt (10%) diet, and sacrificed after 40 weeks. Proliferative activity and expression of cyclooxygenase-2 (COX-2) in gastric mucosa were significantly increased in *H. pylori*-infected gerbils. The additional high-salt diet significantly up-regulated the expression of inducible nitric oxide synthase (iNOS) and COX-2 in *H. pylori*-infected groups ($P<0.01$ and $P<0.05$, respectively), while no significant effects were noted in non-infected animals. There was significant synergistic interaction between *H. pylori* infection and 10% NaCl diet on the expression of iNOS ($P<0.05$) and also a tendency for enhanced COX-2 expression ($P=0.0599$).

Conclusions: The present results suggest that a high-salt diet works synergistically with *H. pylori* infection to enhance iNOS and COX-2 expression in the gastric mucosa of Mongolian gerbils, and support the hypothesis that excessive salt intake may be associated with progression of *H. pylori*-induced gastritis.

Key words: Salt, Gastritis, *Helicobacter pylori*, iNOS, COX-2

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Introduction

Helicobacter pylori (H. pylori) is a major causative factor for gastric disorders and epidemiological evidence has accumulated indicating a significant relationship with gastric cancer development (Marshall and Warren, 1984; Uemura et al., 2001). In 1994, the World Health Organization/International Agency for Research on Cancer concluded that *H. pylori* is a "definite carcinogen" based on the epidemiological findings (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). Recently, the concept that inflammation is a critical component of tumor progression has received a great deal of attention (Coussens and Werb, 2002). It is now known that there is a strong association between *H. pylori*-induced chronic atrophic gastritis and development of gastric cancer (Correa, 1995). Mongolian gerbils can readily be infected with *H. pylori,* and the resultant chronic active gastritis, peptic ulcers and intestinal metaplasia resemble lesions also apparent in humans (Hirayama et al., 1996; Sugiyama et al., 1998). We have previously reported that the severity of gastritis plays an important role in *H. pylori*-associated gastric carcinogenesis in gerbils, with essential involvement of chronic inflammation and increased rates of cell proliferation (Cao et al., 2007). Thus, investigation of the progression mechanisms of gastritis and the search for crucial factors for chemoprevention of gastric cancer continues to be very important.

Environmental and host factors are also known to influence gastric carcinogenesis, and salt (sodium chloride, NaCl) and salty foods are probably of particular importance, based on evidence from a large number of case-control and other epidemiological studies (Joossens et al., 1996; Kono and Hirohata, 1996; Tsugane, 2005). In Japan, foods containing salt at concentrations up to 12% are commonly consumed, such as pickled vegetables (salt content: 1-10%) and salted fish roe or fish preserves (6-12%) (Tsugane et al., 2004), and it has been reported that restriction of salty food

intake may decrease the risk of gastric cancer (Tajima and Tominaga, 1985; Shikata et al., 2006). In addition, several studies in mice and gerbils indicate that chronic excessive salt in the diet exerts synergistic effects with *H. pylori* infection on progression of gastritis and mucosal hyperplasia, also enhancing *H. pylori* colonization (Fox et al., 1999; Gamboa-Dominguez et al., 2007). Thus, the association between *H. pylori* and NaCl appears to be important for the progression of gastritis and the associated carcinogenesis, although the detailed mechanisms remain to be resolved.

It has been reported that *H. pylori* infection induces the expression of pro-inflammatory cytokines and enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the gastric mucosa of rodents and humans (Jackson et al., 2000; Yamaoka et al., 2005; Bancel et al., 2006). In addition, a recent *in vitro* study demonstrated that NaCl could affect the production of interleukin (IL)-1ß, IL-6 and tumor necrosis factor-α induced by VacA, which is a virulence factor of *H. pylori*, in the AGS gastric cancer cell line (Sun et al., 2006). To our knowledge, however, there is limited information on the influence of long-term salt intake on *in vivo* expression of mediators of inflammation and proliferative activity. In the present study, we therefore examined whether a high-salt diet might increase epithelial proliferation and expression of iNOS and COX-2 assessed immunohistochemically in Mongolian gerbils at 40 weeks after *H. pylori* infection.

Materials and methods

Experimental design

The precise experimental design was as previously described (Kato et al., 2006). In the present study, 45 stomach samples from Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Seac Yoshitomi, Fukuoka, Japan) were examined (Fig. 1). Briefly, the gerbils were divided into 4 groups (groups A-D). Groups A and B were inoculated with $1x10^8$ colony-forming unit of *H. pylori* (ATCC43504, American Type Culture Collection, Rockville, MD, USA) intra-gastrically, while groups C and D were inoculated with sterile Brucella broth (Becton Dickinson, Cockeysville, MD, USA). *H. pylori* was prepared by the same method as described previously (Shimizu et al., 1999). From weeks 1 to 40, the animals of groups A and C received a diet including 10% sodium chloride and those in groups B and D were maintained on basal diet (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) containing 0.32% NaCl. At week 40, all gerbils were intraperitoneally injected with 5'-bromo-2'-deoxyuridine (BrdU) at a dose of 100 mg/kg, 60 minutes before sacrifice. The animals were subjected to deep anesthesia and laparotomy with excision of the stomach. The excised stomachs were fixed in 10% neutral-buffered formalin and sliced along the longitudinal axis into 4 to 8 strips of equal width, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for histological examination. The experimental design was approved by the Animal Care Committee of Aichi Cancer Center Research Institute, and the animals were cared for in accordance with the institutional guidelines.

Immunohistochemistry to assess epithelial proliferation and inflammatory enzymes

Immunohistochemical analysis of BrdU, iNOS and COX-2 was carried out as previously described (Ikeno et al., 1999; Tanaka et al., 2006). Briefly, serial sections were deparaffinized and hydrated through a graded series of ethanols, and immersed in 0.3% hydrogen peroxide/methanol solution for inhibition of endogenous peroxidase activity. For antigen retrieval, sections for iNOS and COX-2 were microwaved in 10 mM citrate buffer (pH 6.0) for 10 minutes, and sections for BrdU were incubated in 5N hydrochloric acid for 30 minutes at room temperature. The sections were then incubated with primary antibodies: a mouse monoclonal anti-BrdU antibody (clone Bu20a, diluted 1:1,000, Dako, Glostrup, Denmark), a rabbit polyclonal anti-iNOS antibody (Saito et al., 2002) (diluted 1:500, Calbiochem, San Diego, CA, USA), or a mouse monoclonal anti-COX-2 antibody (Marrogi et al., 2000; Shiotani et al., 2001) (clone 33, diluted 1:100, BD Biosciences, San Jose, CA, USA). Staining for BrdU and COX-2 was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and the Fast Red Substrate System (Dako), respectively. Sections for iNOS were incubated with biotinylated secondary antibody (swine-anti rabbit IgG, Dako), and avidin-biotinylated horseradish peroxidase complexes were visualized using 0.05% 3,3'-diaminobenzidine. All sections were counterstained with hematoxylin.

The numbers of BrdU-labeled cells in gastric mucosa were counted under a microscope, and indices were determined as the mean percentages of positive epithelial cells among totals of 90 different arbitrarily selected glands (including 60 glands in each corpus and 30 in each antrum). The degree of iNOS immuno-

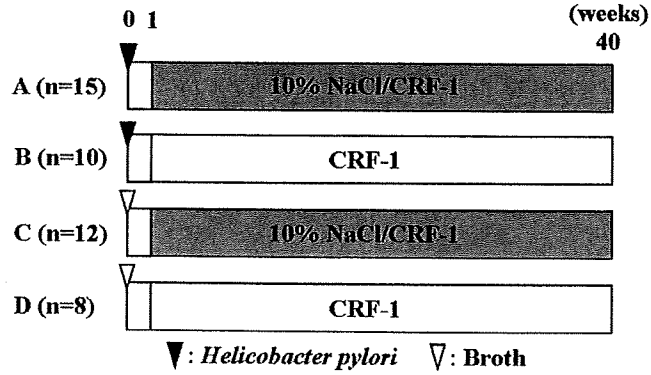

Fig. 1. Experimental design. Specific pathogen-free male, 4-month-old Mongolian gerbils were inoculated with *H. pylori* ATCC43504 strain (groups **A and B**) or Broth (groups **C and D**). Animals of groups A and C were given CRF-1 diet containing 10% NaCl from weeks 1 to 40.

positivity was expressed as the numbers of iNOS-positive cells in the total mucosal length. To quantitate the degree of COX-2 stainability, we measured the length of COX-2 positive areas per total mucosal length. The average mucosal lengths measured for evaluation of iNOS and COX-2 expression were 75.4±20.7 and 76.2±21.1 mm (means±SD), respectively.

Statistical analysis

Differences in data between groups were analyzed using the two-way factorial analysis of variance (ANOVA), followed by the Scheffe's multiple comparison procedure. P values <0.05 were considered to be statistically significant.

Results

Macroscopic and histological findings

The gastric mucosa of all gerbils in groups A and B (*H. pylori*-infected groups) was generally thickened and

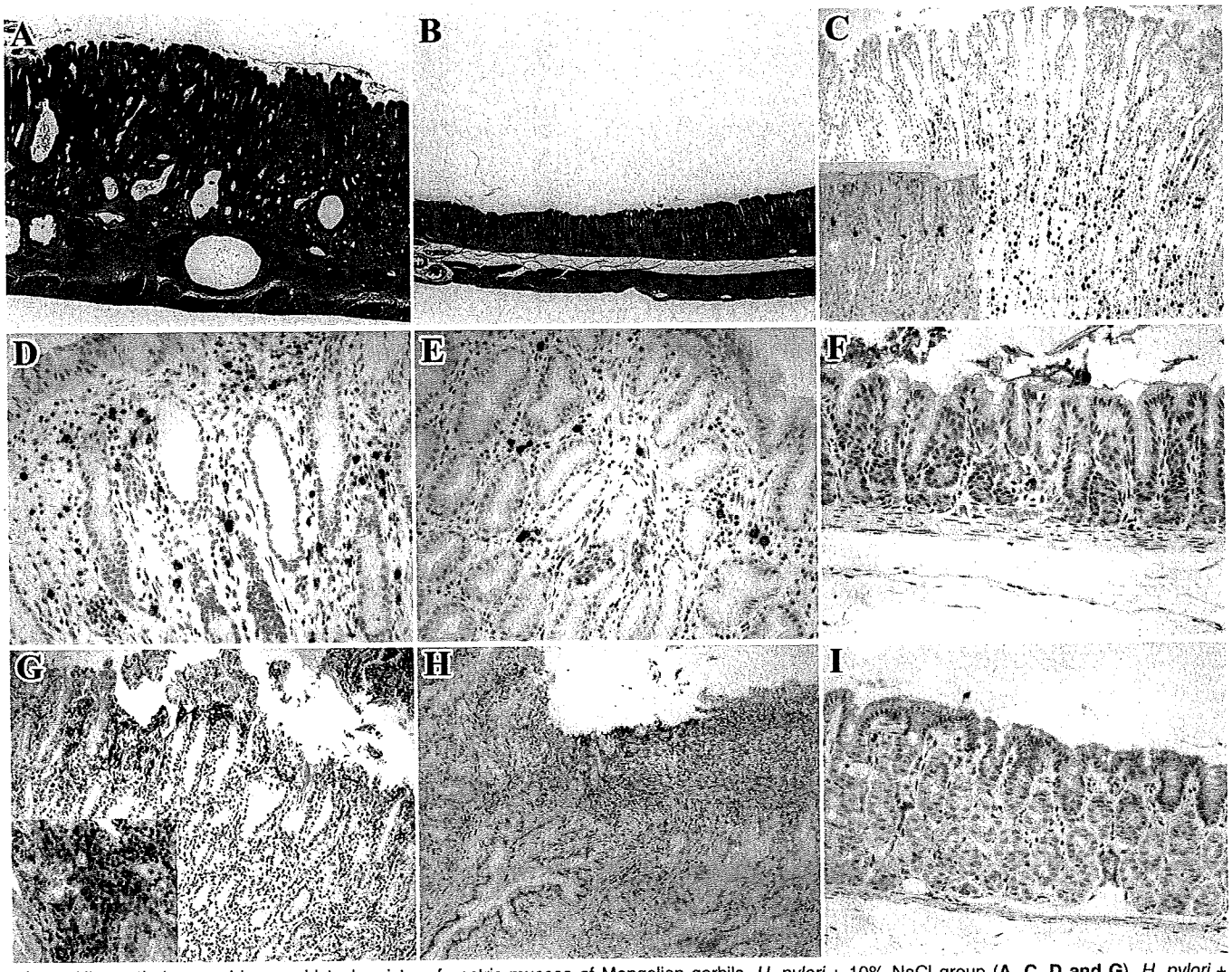

Fig. 2. Histopathology and immunohistochemistry of gastric mucosa of Mongolian gerbils. *H. pylori* + 10% NaCl group (**A, C, D and G**), *H. pylori* + basal diet group (**E and H**) and Broth + 10% NaCl group (**B, inset in C, F and I**). A and B. H&E staining. **A.** Note severe gastritis with infiltration of inflammatory cells, heterotopic proliferative glands, mucosal hyperplasia, and intestinal metaplasia at 40 weeks post-infection. x 50. **B.** No lesions were observed in gastric mucosa of non-infected and 10% NaCl diet-treated gerbils. x 50. **C.** Immunohistochemistry for BrdU. Large numbers of BrdU-positive cells are apparent in hyperplastic mucosal epithelium, while much fewer are present in the proliferative zone of a non-infected animal (inset). x 100. **D-F.** Immunohistochemistry for iNOS. **D and E.** Expression of iNOS mainly in mononuclear cells infiltrating in the lamina propria. x 200. **F.** In non-infected group, iNOS-positive cells were rarely observed in the lamina propria. x 200. **G-I.** Immunohistochemistry for COX-2. **G and H.** COX-2 is predominantly localized at the rims of areas of erosion or ulceration. Note expression localized in the cytoplasm of infiltrating mononuclear cells, fibroblasts and endothelium (**inset of G**). x 125. **I.** In the non-infected group, COX-2 staining was occasionally found in macrophages and endothelium. x 200.

edematous, occasionally with erosion and ulcers. In groups A and B, marked infiltration of neutrophils and mononuclear cells and formation of heterotopic proliferative glands were observed in the lamina propria and submucosa, occasionally with formation of lymphoid follicles. The histological examination also revealed various degrees of hyperplasia of the mucosa and intestinal metaplasia (Fig. 2A). Such macroscopic and histological lesions were not recognized in the stomachs of groups C and D (non-infected groups) (Fig. 2B). Detailed data for gastritis, including inflammation scores, have previously reported by our colleagues (Kato et al., 2006).

BrdU labeling indices for epithelial cells

BrdU-labeled epithelial cells in gastric mucosa were distributed mostly in the neck region of the hyperplastic polyps or in the proliferative zone of the non-hyperplastic mucosa (Fig. 2C). At week 40, BrdU labeling indices in *H. pylori*-infected groups were significantly greater than in non-infected groups (P<0.0001) (Fig. 3). The high-salt diet showed no significant effects of enhancement of epithelial proliferation both in *H. pylori*-infected groups and non-infected groups. There was no significant correlation between *H. pylori* infection and 10% NaCl diet in BrdU labeling indices (P=0.4785).

Immunohistochemistry of iNOS

In *H. pylori*-infected gerbils, immunostaining for iNOS was located mainly in the cytoplasm of infiltrating mononuclear cells both in the lamina propria and submucosa (Fig. 2D,E). Expression was also detected in endothelium, segmented leukocytes, and gastric epithelial cells at lower frequency. At 40 weeks, iNOS expression in group A (*H. pylori*-infected and 10% NaCl diet-treated group) was significantly higher than in

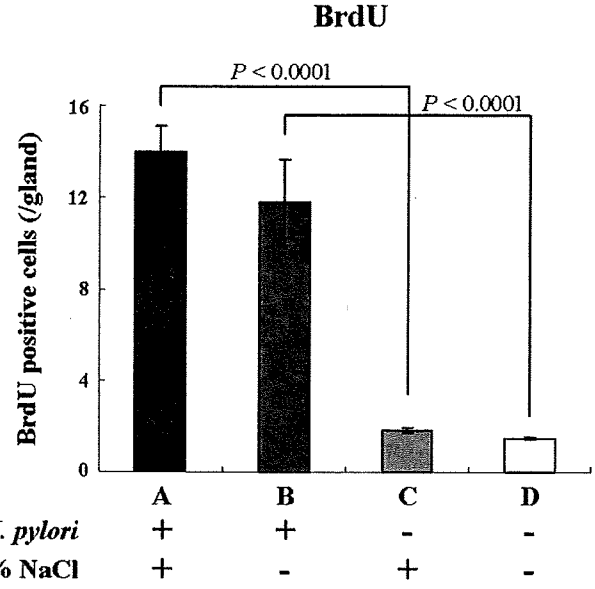

Fig. 3. Immunohistochemical analysis of epithelial cell proliferation in gastric mucosa of Mongolian gerbils by BrdU staining. Data are mean ± SE values.

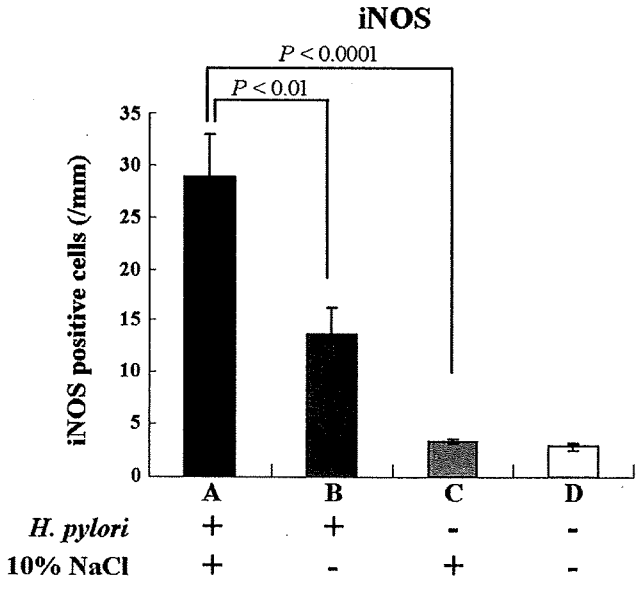

Fig. 4. Immunohistochemical analysis of iNOS expression in gastric mucosa of Mongolian gerbils. Data are mean ± SE values.

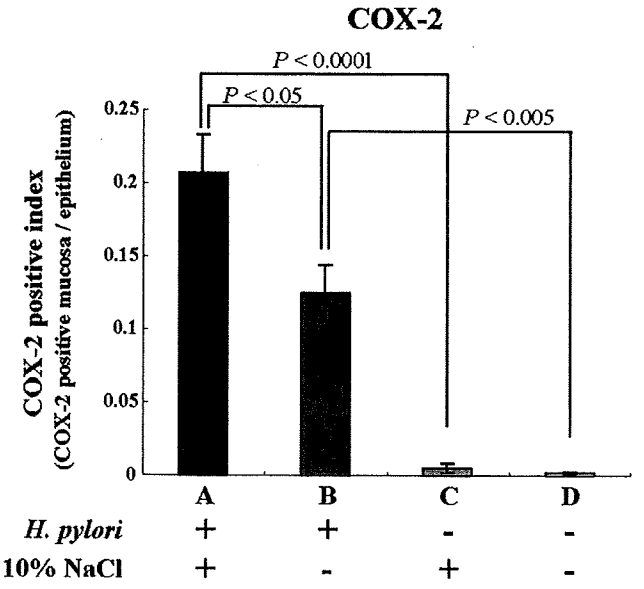

Fig. 5. Immunohistochemical analysis of COX-2 expression in gastric mucosa of Mongolian gerbils. Data are mean ± SE values.

group C (non-infected and 10% NaCl diet-treated group) ($P<0.0001$) (Fig. 4). Two-way factorial ANOVA revealed a significant interaction between *H. pylori* infection and excessive salt intake on iNOS expression ($P<0.05$). In *H. pylori*-infected groups, the numbers of iNOS-positive cells in group A (10% NaCl diet-treated) (28.8±4.12 cells/mm; means ± SE) were significantly higher than in group B (basal diet-treated) (13.7±2.63) ($P<0.01$). In non-infected groups, a high-salt diet showed no significant influence on frequency of iNOS expression (Fig. 2F).

Immunohistochemistry of COX-2

In *H. pylori*-infected groups, COX-2 protein was mainly detected in infiltrating mononuclear cells, fibroblasts, and endothelium in the lamina propria, particularly at the rims of erosion and ulcers (Fig. 2G,H), while a little COX-2 staining was observed in mononuclear cells and endothelium of non-infected gerbils (Fig. 2I). At 40 weeks, COX-2 expression in groups A and B (*H. pylori*-infected) was significantly greater than in groups C and D (non-infected) ($P<0.0001$ and $P<0.005$, respectively) (Fig. 5). Two-way factorial ANOVA showed a tendency for interaction between *H. pylori* infection and 10% NaCl diet on COX-2 expression, although this was not statistically significant ($P=0.0599$). In *H. pylori*-infected groups, the COX-2 positive index in group A (10% NaCl diet-treated) (0.21±0.03; means ± SE) was significantly higher than that in group B (basal diet-treated) (0.12±0.02) ($P<0.05$). There were no significant effects of salt on COX-2 immunoreactivity between the non-infected groups.

Discussion

It has been recognized that iNOS and COX-2 are involved in the processes of inflammation, carcinogenesis and its progression (Prescott and Fitzpatrick, 2000; Jaiswal et al., 2001). iNOS is expressed both by inflammatory cells and epithelial cells and the generated nitric oxide contributes to carcinogenesis during chronic inflammation. COX-2 is an inducible form of cyclooxygenase, which catalyzes the conversion of arachidonic acid to pro-carcinogenic eicosanoids such as prostaglandin, and is increased by various cytokines, growth factors and reactive oxygen species. A number of previous findings suggest that both iNOS and COX-2 are associated with *H. pylori*-induced gastritis in humans (Mannick et al., 1996; Jackson et al., 2000; Bhandari et al., 2005). In the present study, we showed COX-2 expression in gastric mucosa to be significantly enhanced by *H. pylori* infection in Mongolian gerbils, consistent with previous immunohistochemical studies in humans (Fu et al., 1999; Chen et al., 2006). In addition, our results demonstrated that a high-salt diet can further upregulate the expression of these two enzymes in *H. pylori*-infected gerbils. To our knowledge, this is the first report of synergistic effects of salt and *H. pylori* infection on the expression of iNOS and COX-2 in the glandular stomach of Mongolian gerbils. Rajnakova et al. (2001) reported using immunohistochemistry that iNOS and COX-2 expression may promote gastric cancer progression associated with an accumulation of p53. Furthermore, prognosis in patients expressing both iNOS and COX-2 appears to be significantly poorer than in those with single or no expression of these two genes (Chen et al., 2006). The results thus indicate a possibility that the co-expression of iNOS and COX-2 may not only promote gastric inflammation but also be a determinant factor for *H. pylori*-associated gastric carcinogenesis and prognostic outcome.

We have previously reported that excessive salt intake enhances *H. pylori*-associated gastritis and gastric cancer development in gerbils through alteration of the gastric mucus microenvironment (Kato et al., 2006). The present study showed that chronic salt administration enhances iNOS and COX-2 expression in gastric mucosa of *H. pylori*-infected gerbils. In non-infected gerbils, on the other hand, salt alone induced almost no lesions in stomach mucosa and had no promoting effects on iNOS and COX-2 expression. Furthermore, we found a significant synergistic effect between excessive salt intake and *H. pylori* infection on iNOS expression and a tendency to enhance COX-2 expression. The results suggest that increased expression of iNOS and COX-2 was induced not by a high-salt diet alone but by promoting effects of salt on *H. pylori*-activated inflammatory responses, and that excessive salt intake may be associated with the progression of *H. pylori*-induced gastritis. Further analysis is needed to clarify the interaction between co-expression of iNOS and COX-2 and progression of gastritis, because over-expression is not directly linked to functional activation.

In the present study, COX-2 immunostaining was observed in infiltrated mononuclear cells, fibroblasts and endothelium in the lamina propria, particularly at the edges of erosions and ulcers, consistent with previous reports on ulcerated gastric mucosa in humans and rodents (Mizuno et al., 1997; Takahashi et al., 1998; Jackson et al., 2000). These studies suggested that the localization of COX-2 may be associated with repair of mucosal injury. Our present results showed that excessive salt intake could significantly increase COX-2 expression in *H. pylori*-infected gerbils, without any influence on the localization. Since salt alone had no significant effects on COX-2 expression in non-infected gerbils, further up-regulation of COX-2 by salt intake in *H. pylori*-infected gerbils may be related to enhancement of *H. pylori*-induced gastritis rather than direct mucosal damage.

Several studies in rats have demonstrated that acute exposure to a highly concentrated NaCl solution may cause direct injury of the gastric surface epithelium, followed by rapid recovery with increased regenerative cellular proliferation (Charnley and Tannenbaum, 1985; Furihata et al., 1996). In the present study, on the other

hand, a 10% NaCl-containing diet had no significant effects on epithelial proliferation in gastric mucosa, independent of *H. pylori* infection. In addition, our previous study demonstrated that intermittent (once a week) administration of saturated NaCl solution for 40 weeks had no promoting effects on *H. pylori*-associated gastritis and carcinogenesis in gerbils (Kato et al., 2006). Therefore, we consider that continuous exposure to salt, rather than short-term and highly-concentrated salt intake, may be important to enhance *H. pylori*-induced gastritis, associated with increased expression of iNOS and COX-2 in the gastric mucosa. A recent study reported that osmoprotective genes promote cell survival against NaCl-induced hypertonic stress (Neuhofer et al., 2007). The osmoprotective activity might be one of the determinant factors for outcome with gastric epithelial cells exposed to various concentrations of NaCl, although detailed functions in the stomach are little understood.

In conclusion, the present study showed synergistic effects of salt with *H. pylori* infection on iNOS and COX-2 expression in the gastric mucosa of Mongolian gerbils. The results provide further support for the hypothesis that salt promotes progression of *H. pylori*-induced gastritis, and also raise the possibility that reduction of salt intake may decrease the risk of *H. pylori*-associated gastric cancer, compatible with previous epidemiological and experimental findings.

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Anti-inflammatory effects of caffeic acid phenethyl ester (CAPE), a nuclear factor-κB inhibitor, on *Helicobacter pylori*-induced gastritis in Mongolian gerbils

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Nuclear factor-κB (NF-κB) plays a major role in host inflammatory responses and carcinogenesis and as such is an important drug target for adjuvant therapy. In this study, we examined the effect of caffeic acid phenethyl ester (CAPE), an NF-κB inhibitor, on *Helicobacter pylori* (*H. pylori*)-induced NF-κB activation in cell culture and chronic gastritis in Mongolian gerbils. In AGS gastric cancer cells, CAPE significantly inhibited *H. pylori*-stimulated NF-κB activation and mRNA expression of several inflammatory factors in a dose-dependent manner, and prevented degradation of IκB-α and phosphorylation of p65 subunit. To evaluate the effects of CAPE on *H. pylori*-induced gastritis, specific pathogen-free male, 6-week-old Mongolian gerbils were intragastrically inoculated with *H. pylori*, fed diets containing CAPE (0–0.1%) and sacrificed after 12 weeks. Infiltration of neutrophils and mononuclear cells and expression of NF-κB p50 subunit and phospho-IκB-α were significantly suppressed by 0.1% CAPE treatment in the antrum of *H. pylori*-infected gerbils. Labeling indices for 5′-bromo-2′-deoxyuridine both in the antrum and corpus and lengths of isolated pyloric glands were also markedly reduced at the highest dose, suggesting a preventive effect of CAPE on epithelial proliferation. Furthermore, in the pyloric mucosa, mRNA expression of inflammatory mediators including tumor necrosis factor-α, interferon-γ, interleukin (IL)-2, IL-6, KC (IL-8 homologue), and inducible nitric oxide synthase was significantly reduced. These results suggest that CAPE has inhibitory effects on *H. pylori*-induced gastritis in Mongolian gerbils through the suppression of NF-κB activation, and may thus have potential for prevention and therapy of *H. pylori*-associated gastric disorders.
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Key words: *Helicobacter pylori*; caffeic acid phenethyl ester; chemoprevention; gastritis; Mongolian gerbils

Nuclear factor-κB (NF-κB) plays a central role in many physiological processes in the whole body such as immune responses, cell proliferation, and inflammation through promoting transcription of various cytokines, enzymes, chemokines, antiapoptotic factors and cell growth factors.[1] Because many types of cancer, including neoplasm in the stomach, are known to be associated with chronic inflammation,[2] inhibition of NF-κB activation has attracted increasing attention as a new therapeutic approach for chemoprevention of cancer development.[3,4] Several natural and synthetic compounds have been found to inhibit NF-κB activation, and to exert anti-inflammatory effects *in vitro* and *in vivo*.[5,6] Caffeic acid phenethyl ester (CAPE), one of the active components of propolis derived from honeybee hives, has been reported to be a selective inhibitor of NF-κB.[7,8] Besides that, recent study has also shown that CAPE may inhibit activator protein-1 (AP-1) activity in *Helicobacter pylori* (*H. pylori*)-stimulated gastric epithelial cells.[9] Thus, further investigation was needed, to confirm how CAPE would influence many signal transduction cascades other than NF-κB pathway. Although the mechanisms of NF-κB inhibition by CAPE are not fully understood, research has demonstrated anti-inflammatory, anticarcinogenic and immunomodulatory effects of the compound in animal models.[10–12]

H. pylori is now recognized as a major causative factor for chronic gastritis and peptic ulcer, and there is compelling evidence indicating an association between *H. pylori*-induced chronic gastritis and development of stomach cancer.[13,14] Triple therapy with a proton pump inhibitor and 2 antimicrobials, amoxicillin and clarithromycin, is usually recommended as the general therapy for *H. pylori* eradication.[15] However, considering the occurrence of antibiotic-resistance, the search for new agents for alternative therapies continues to be very important.[16] *H. pylori* infection also leads to activation of NF-κB signaling in gastric epithelial cells, and NF-κB-mediated cytokine expression is essentially involved with *H. pylori*-induced gastritis.[17–21] Thus the degree of gastritis induced by a mutant strain of *H. pylori* lacking capacity for NF-κB activation was found to be lower than that with wild type infection.[22] Inhibition of NF-κB could be a promising target for prevention and adjuvant therapy of *H. pylori*-associated gastric disorders.[3,23]

The Mongolian gerbil (*Meriones unguiculatus*) provides a useful animal model of *H. pylori*-induced chronic active gastritis, allowing investigation of morbidity-related epithelial alterations in the gastric mucosa and their development into gastric neoplasia.[24] We have previously demonstrated that some natural products in food such as a fruit-juice concentrate of Japanese apricot and nordihydroguaiaretic acid, an antioxidant to preserve food and oils, and canolol, a potent oxygen radicals scavenger contained in canola oil, have suppressive effects on *H. pylori*-induced gastric disorders in Mongolian gerbils.[25–27] The purpose of this study was to evaluate possible anti-inflammatory effects of CAPE, a naturally-occurring compound in food, in the same model.

Material and methods

Chemicals and cell culture

CAPE was purchased from Cayman Chemicals (Ann Arbor, MI) (Fig. 1). AGS cells, the human gastric cancer cell line, were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma Chemical, St. Louis, MO), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) (Invitrogen, Carlsbad, CA). Culture dishes and plates were kept in an incubator with a humidified atmosphere of 5% CO_2 at 37°C. CAPE was

Abbreviations: AI, arbitrary index; AP-1, activator protein-1; BrdU, 5′-bromo-2′-deoxyuridine; CAPE, caffeic acid phenethyl ester; CFU, colony-forming units; COX, cyclooxygenase; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; *H. pylori*, *Helicobacter pylori*; HBSS, Hanks' balanced salt solution; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor.

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