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Synergistic Promoting Effects of *Helicobacter pylori* Infection and High-salt Diet on Gastric Carcinogenesis in Mongolian Gerbils

Koji Nozaki,[1,3] Nobuyuki Shimizu,[3] Ken-ichi Inada,[1] Tetsuya Tsukamoto,[1] Manami Inoue,[2] Toshiko Kumagai,[4] Atsushi Sugiyama,[5] Tsutomu Mizoshita,[1] Michio Kaminishi[3] and Masae Tatematsu[1,6]

[1]Divisions of Oncological Pathology and [2]Epidemiology & Prevention, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681, [3]Department of Gastrointestinal Surgery, Postgraduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, [4]Central Clinical Laboratories, Shinshu University Hospital and [5]First Department of Surgery, Shinshu University, 3-1-1 Asahi, Matsumoto 390-8621

***Helicobacter pylori* (*Hp*) infection and high-salt diet administration are both considered to be important factors in gastric carcinogenesis in man. To investigate the interaction of these two factors on gastric carcinogenesis, an experimental study of the carcinogenesis model was performed. Mongolian gerbils were treated with 20 ppm of *N*-methyl-*N*-nitrosourea (MNU) in their drinking water for alternate weeks for a total of 5 weeks' exposure (groups 1, 2, 3 and 4) or were maintained as controls (groups 5, 6, 7 and 8). At week 11, the animals were inoculated with *Hp* (groups 1, 2, 5 and 6) or the vehicle alone (groups 3, 4, 7 and 8), and after week 12, animals were fed a 10% high salt diet (groups 1, 3, 5 and 7) or the control diet (groups 2, 4, 6 and 8). At week 50, the incidence of adenocarcinomas in group 1 (32.1%, 6 well-differentiated, 2 poorly-differentiated adenocarcinomas, and one signet-ring cell carcinoma) was significantly higher than in groups 3 (0%) ($P<0.005$) and 4 (0%) ($P<0.01$). The incidence of adenocarcinomas in group 2 (11.8%, one well-differentiated adenocarcinoma, and one signet-ring cell carcinoma) was also higher than in groups 3 and 4. A high-salt diet enhanced the effects of *Hp* infection on gastric carcinogenesis, and these two factors acted synergistically to promote the development of stomach cancers. Moreover, *Hp* infection promoted gastric carcinomas more than the high-salt diet.**

Key words: *Helicobacter pylori* — Gastric cancer — NaCl — Mongolian gerbil — *N*-methyl-*N*-nitrosourea

Helicobacter pylori (*Hp*) infection is well accepted to be a very important factor for gastric carcinogenesis in the human stomach, and strong epidemiological evidence has accumulated indicating a significant relationship with the development of chronic gastritis,[1,2] peptic ulcers,[3] intestinal metaplasia,[4] and adenocarcinoma[5–8] or lymphoma.[9] In 1994, the World Health Organization/International Agency for Research on Cancer concluded that *Hp* is a 'definite carcinogen' based on the epidemiological findings.[10] However, the pathogenic role of *Hp* in gastric carcinogenesis remains unclear. Infection with the bacteria almost always results in chronic antral gastritis, but only a small proportion of patients develop stomach cancers. Some *Hp* strains may be more 'carcinogenic' than others, but host factors may also be important.[11–16]

Mongolian gerbils can be easily infected with *Hp*, and the resultant chronic active gastritis, peptic ulcers, and intestinal metaplasia resemble lesions apparent in man.[17] Thus, they can be an ideal experimental animal for detailed analysis of the role of *Hp* in gastric disorders. We have established a gastric carcinogenesis model using Mongolian gerbils,[18,19] and demonstrated that all histological types of gastric cancer development are enhanced by *Hp* infection in gerbils treated with the chemical carcinogens *N*-methyl-*N*-nitrosourea (MNU)[20] or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).[21] Eradication of the bacteria resulted in curtailment of the enhancing effects.[22]

A high-salt diet is also considered to be an important enhancing factor in gastric carcinogenesis.[23] We have studied enhancing effects of a high-salt diet on gastric carcinogenesis in rats.[24] However, the interaction of the two factors, *Hp* infection and/or high-salt diet, on gastric carcinogenesis remains unclear. So the present experimental study was conducted to explore their interactive influences on gastric carcinogenesis.

MATERIALS AND METHODS

MNU (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water at a concentration of 20 ppm and freshly prepared three times per week or administered in drinking water in light-shielded bottles *ad libitum*. *Hp* strain ATCC 43504 (*cagA*$^+$, *vacA*$^+$) (American Type Cul-

[6] To whom correspondence should be addressed.
E-mail: mtatemat@aichi-cc.jp

ture Collection, Rockville, MD) was inoculated on *Brucella* agar plates (Becton Dickinson Co., Cockeysville, MD) containing 7% (v/v) heat-inactivated fetal bovine serum and incubated at 37°C under microaerobic conditions using Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Inc., Tokyo) at high humidity. Two days later, the bacteria grown on the plates were introduced into *Brucella* broth (Becton Dickinson Co.) supplemented with 7% (v/v) heat-inactivated fetal bovine serum and incubated under the same conditions for 24 h. The broth cultures of *Hp* were checked under a phase contrast microscope for bacterial shape and mobility. Samples containing 1.0×10^8 colony-forming units were used as the inoculum, delivered intra-gastrically via an oral catheter to the animals fasted for 24 h.

Specific pathogen-free, male, 7-week-old Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Seac Yoshitomi, Ltd., Fukuoka) were housed in steel cages on hardwood chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. They were given food (Oriental CRF-1: containing 0.32 g sodium in 100 g, or high salt-CRF-1: containing 10% w/w sodium chloride, Oriental Yeast Co., Ltd., Tokyo) irradiated with 30 kGy γ-rays and autoclaved distilled water *ad libitum*. The experimental design described below was approved by the Animal Care Committee of the Aichi Cancer Center Research Institute.

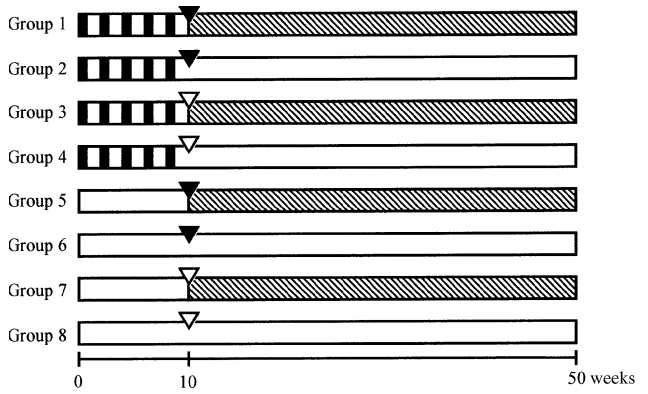

Fig. 1. Experimental design. Mongolian gerbils in groups 1–4 were given MNU in their drinking water at the concentration of 20 ppm for alternate weeks for a total of 5 weeks, exposure. Animals in groups 5–8 received no MNU as controls. On completion of the regimen, all were given autoclaved distilled water. One week thereafter, animals in groups 1, 2, 5, and 6 were inoculated with *Hp* while those in groups 3, 4, 7, and 8 received only *Brucella* broth without *Hp*. After week 12, Animals in groups 1, 3, 5, and 7 were given high-salt (10%, w/w) diet, while their counterparts in groups 2, 4, 6, and 8 received a control diet. ▮▮ MNU administration (20 ppm, alternate weeks), ▼ inoculation of *Hp*, ▽ inoculation of *Brucella* broth, ▨ high-salt diet (NaCl 10%, w/w), ▭ control diet.

Gerbils were divided into eight groups. Animals in groups 1–4 received MNU in their drinking water at the concentration of 20 ppm for alternate weeks for a total of 5 weeks' exposure. Animals in groups 5–8 received no MNU initiation. On completion of this stage, all were given autoclaved, distilled water for 1 week, and then animals were inoculated with *Hp* in groups 1, 2, 5, and 6. Animals in groups 3, 4, 7, and 8 received *Brucella* broth without *Hp*. After week 12, animals in groups 1, 3, 5, and 7 were given a high-salt (10%, w/w) diet, while animals in groups 2, 4, 6, and 8 received the control diet (Fig. 1). The animals were weighed every week. At week 50, after 24-h fasting, all animals were subjected to deep anesthesia, laparotomized, and exsanguinated from the inferior vena cava, with excision of their stomachs. Tissues were processed for microbiological and histopathological examinations.

Anti-*Hp* antibodies were measured as described earlier.[21,22] Briefly, blood samples containing a small amount of EDTA were centrifuged at 8000 rpm for 5 min to isolate sera, which were then stored at −80°C until measured by ELISA with anti-*Hp* IgG antibody (GAP-IgG; Biomerica, Newport Beach, CA). Titers were expressed using an arbitrary index (AI). Serum gastrin levels were examined using a gastrin radioimmunoassay kit Gastrin-RIA KIT II (Dainabot Co., Ltd., Tokyo) and expressed as pg/ml values.

The excised stomachs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2), Bouin's solution, or 95% ethanol containing 1% acetic acid and embedded in paraffin. Tissues were sectioned at 5 μm for staining with hematoxylin and eosin (H&E), alcian blue-periodic acid-Schiff (AB-PAS) and by immunohistochemistry for *Hp* (anti-*Hp* serum, DAKO A/S, Glostrup, Denmark). Histopathological findings of active chronic gastritis were characterized by severe infiltration of inflammatory cells, ulceration, hyperplasia, submucosal proliferation evidenced by invagination of glands into the submucosal layer, and intestinal metaplasia. Heterotopic proliferative glands[25] without cellular atypia were distinguished from carcinomas. Carcinomas of the glandular stomach of this animal species[18–22] were classified into well-differentiated lesions characterized by tubular structures with cellular atypia, poorly-differentiated tumors with severe cellular atypia and little tendency to form glandular structures, and signet-ring cell carcinomas featuring isolated tumor cells containing abundant mucin.

The unpaired *t* test was applied to establish the significance of differences in body weight distributions, titers of anti-*Hp* antibodies, and serum gastrin levels. Incidences of adenocarcinomas were assessed by the Fisher's exact test. P values <0.05 were considered to be statistically significant.

RESULTS

Total intake of MNU per animal and the body weights at experimental week 50 are shown in Table I. There were no significant differences in MNU intake among groups 1–4, and there were no significant differences in body weight among groups 1–8.

In all *Hp*-infected animals, active chronic gastritis was evident. Immunohistochemical staining for *Hp* allowed the observation of bacteria in *Hp*-infected animals. The glandular stomachs were edematous with hemorrhagic spots and erosions apparent macroscopically, and the fundic glands were atrophic with loss of parietal cells. Pyloric mucosa adjacent to the fundic region showed inflammation with hyperplasia and variable degrees of multifocal cystic glandular dilatation and erosion. The bases of the glands penetrated the muscularis mucosa multifocally, which is tantamount to heterotopic submucosal proliferation (Fig. 2A). There was marked infiltration, predominantly of lymphocytes and some macrophages, as well as neutrophils, in the lamina propria and submucosa, with frequent formation of lymphoid follicles. Submucosal proliferations and intestinal metaplasia were noted in all *Hp*-infected gastric mucosa of gerbils. Intestinal metaplasia with intestinal absorptive cells with striated cell borders and goblet cells with mucin stained blue with AB-PAS were characteristically observed in *Hp*-infected cases. On the other hand, high-salt diet administration, without *Hp* infection, did not induce active chronic gastritis, submucosal proliferations, or intestinal metaplasia. Because of the strong inflammation in *Hp*-infected gastric mucosa, no obvious histological difference was detected between animals in groups 1 and 2, and 3 and 4, with or without high-salt administration, after *Hp* infection.

Tumors were mostly found in the lesser curvature of the pyloric mucosa adjacent to the fundic region, and comprised well-differentiated (Fig. 2, B and C) and poorly-differentiated adenocarcinomas as well as signet-ring cell carcinomas (Fig. 2, D and E). Vessel invasion and lymphatic invasion of carcinomas were also found (Fig. 2F). The incidence of adenocarcinomas in group 1 was 32.1%, including not only well-differentiated, but also poorly-differentiated adenocarcinomas and a signet-ring cell carcinoma (Table I). Adenocarcinomas noted in group 2 (11.8%) included one well-differentiated, one poorly-differentiated adenocarcinoma and a partly signet-ring cell carcinoma. No carcinoma was found in group 3.

The incidence of adenocarcinomas in group 1 (9/28=32.1%) was significantly higher than in groups 3 (0/27=0%) and 4 (0/20=0%) ($P<0.005$). Groups 2 or 3 (11.8% and 0%) and group 4 (0%) showed no significant difference. The incidence of carcinomas in group 2 was higher than in group 3. The incidences of carcinomas are summarized in Table I.

Data on titers of anti-*Hp* antibodies are summarized in Table I. Values for *Hp*-inoculated animals in groups 1, 2 and 5, 6 were significantly higher than in groups 3, 4 ($P<0.0001$), and 7, 8. Values for animals in group 5,

Table I. Incidence of Carcinoma

Group	Treatment	*n*	Carcinoma				Anti *Hp* titer (AI) [SD]	Serum gastrin (pg/ml) [SD]	MNU intake (ml) [SD]	BW at 50 wk (g) [SD]
			Incidence (%) [95%CI]	Well-diff.	Poorly-diff.	Signet-ring cell type				
1	MNU 20 ppm +*Hp*+NaCl	28	32.1[*, **, †] [15.8–52.4]	6	2	1	337.51[a, b, c] [152.93]	499.33[a, b, c] [195.83]	379.47 [35.33]	93.03 [16.67]
2	MNU 20 ppm +*Hp*	17	11.8[‡] [1.5–36.4]	1	0	1	254.71[a, b] [125.40]	381.98[a, b] [197.70]	352.67 [28.91]	98.79 [11.80]
3	MNU 20 ppm +NaCl	27	0.0[‡] [0.0–10.5]	0	0	0	1.74[d] [0.93]	177.18[d] [54.45]	348.80 [16.94]	92.49 [11.58]
4	MNU 20 ppm	20	0 [0.0–13.9]	0	0	0	2.13 [1.32]	186.78 [37.07]	377.95 [28.61]	106.81 [9.38]
5	*Hp*+NaCl	11	0	0	0	0	283.88[e, f, g] [149.77]	554.45[e, h, i] [287.25]	0	87.74 [16.03]
6	*Hp*	6	0	0	0	0	120.95[f, g] [51.63]	273.93[h, i] [52.90]	0	108.95 [6.30]
7	NaCl	4	0	0	0	0	1.50[j] [0.32]	189.60[j] [6.00]	0	101.60 [9.00]
8	Control	4	0	0	0	0	2.10 [1.25]	162.97 [24.51]	0	107.55 [6.38]

∗ $P<0.005$ vs. group 3, ∗∗ $P<0.01$ vs. group 4, † NS vs. group 2, ‡ NS vs. group 4. Fisher's exact test.
a) $P<0.0001$ vs. group 3, b) $P<0.0001$ vs. group 4, c) NS vs. group 2, d) NS vs. group 4, e) $P<0.05$ vs. group 6, f) $P<0.005$ vs. group 7, g) $P<0.01$ vs. group 8, h) $P<0.05$ vs. group 7, i) $P<0.05$ vs. group 8, j) NS vs. group 8. Unpaired *t* test.

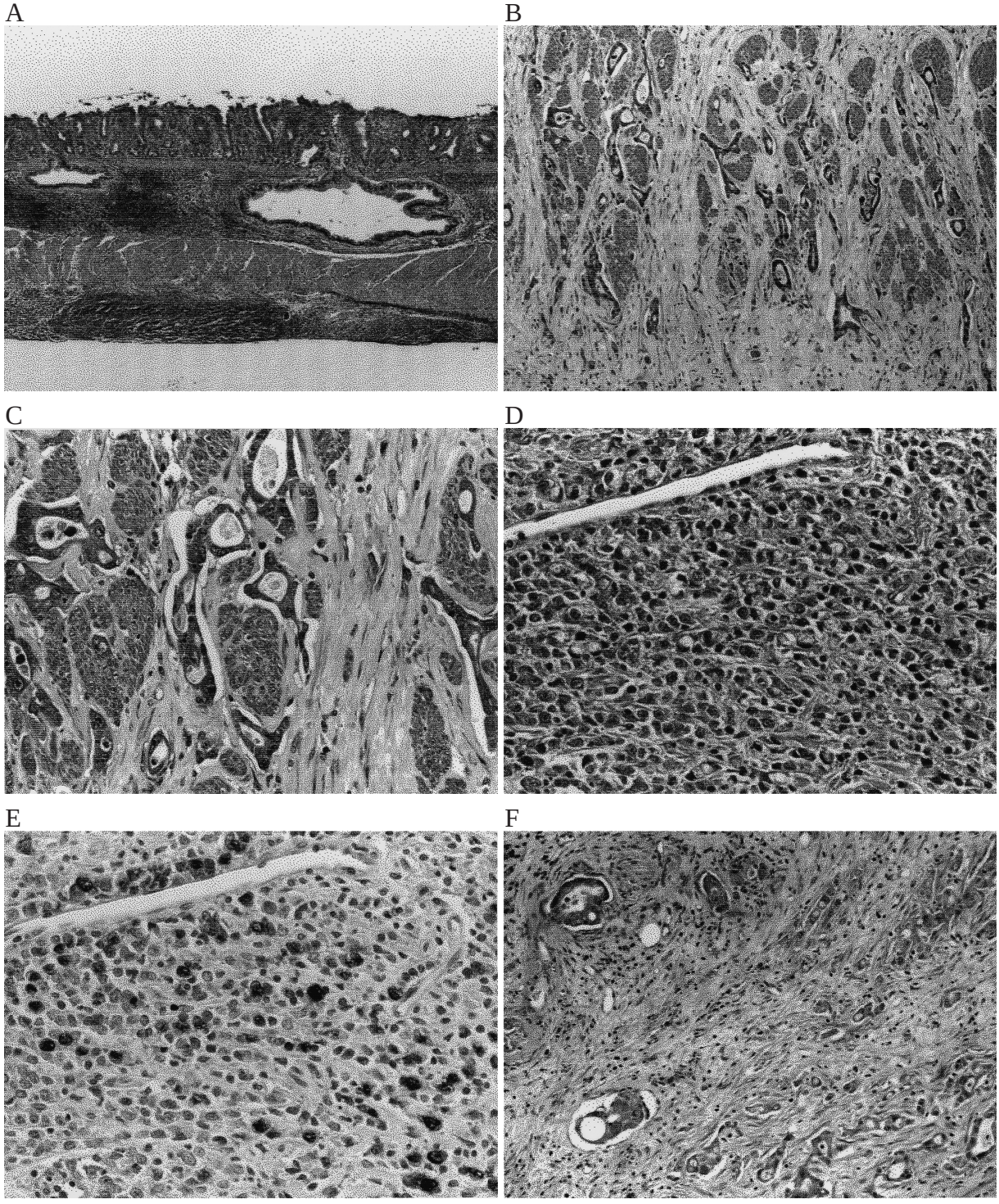

Fig. 2. A. Multiple heterotopic proliferative glands, proliferating into submucosa. Infiltration of inflammatory cells in the lamina propria and submucosa with formation of lymphoid follicles is also seen (H&E, ×10). B. Well- to moderately-differentiated adenocarcinoma, penetrating the muscle layer (H&E, ×25). C. Higher magnification of B. Well- to moderately-differentiated adenocarcinoma with marked cellular atypia (H&E, ×80). D. Signet-ring cell carcinomas with poorly-differentiated carcinomas (H&E, ×80). E. Signet-ring cell carcinomas containing mucin (AB-PAS, ×80). F. Vessel invasion and lymphatic invasion of carcinomas (H&E, ×40).

infected with *Hp* and given a high-salt diet, were significantly higher than in group 6, infected with *Hp* and given a control diet ($P<0.05$). Data on serum gastrin levels are also summarized in Table I. Values in *Hp*-infected animals of groups 1, 2, and 5, 6 were significantly higher than in groups 3, 4 ($P<0.0001$), and 7, 8. Values for animals in group 5 were significantly higher than in group 6 ($P<0.05$).

DISCUSSION

The incidence of adenocarcinoma was high in animals both infected with *Hp* and given a high-salt diet after administration of MNU. While administration of a low concentration (20 ppm) of MNU did not induce gastric carcinogenesis, in line with previous results,[20] subsequent exposure to both *Hp* infection and a high-salt diet resulted in marked development of tumors. The incidence of carcinomas in group 1 (32.1%), with 20 ppm of MNU, infection with *Hp* and high-salt diet, was almost the same as that of animals administered 30 ppm of MNU and infected with *Hp* (33.3%) in our previous study.[20] The present study demonstrated that these two factors act synergistically to enhance development of stomach cancers. Furthermore, the incidence of adenocarcinoma in animals infected with *Hp* after administration of MNU was higher than in those given a high-salt diet thereafter. The 10% (w/w) sodium chloride concentration of the diet used in this experiment is considered to be almost the maximum concentration consistent with keeping the experimental animals alive. So the results imply that *Hp* infection alone might be a stronger factor than an extremely high-salt diet administration in promoting gastric carcinogenesis.

Hp can infect the stomachs of gerbils persistently. The organism preferentially colonizes and forms micro-colonies within the mucous gel layer of surface mucous cell type mucins in the human stomach.[26] Mucins from gland mucous cells may disturb the movement of *Hp* within the mucous gel layer.[27] The gastric mucosa contains several kinds of acid mucopolysaccharides, and the presence of NaCl decreases the viscosity of the gastric mucus, reduces the protective mucous barrier in a similar manner to surface-active agents, and allows direct contact of carcinogens with the gastric mucosa.[24] Fox *et al.* demonstrated that a high-salt diet enhances *Hp* colonization in C57BL/6 mice.[28] *Hp* infection has in fact been shown to exacerbate stomach mucosal damage due to MNU or a salty diet in mice.[29]

Other mechanisms of high-salt action in gastric carcinogenesis have been considered to be increased cell replication leading to gastric epithelial proliferation, and increased incidence of endogenous mutations. A greater increase in replicative DNA synthesis was observed with a high concentration of NaCl, suggesting that a high-salt diet is responsible for the initial tissue damage and the resulting temporary cell proliferation during gastric tumor promotion.[30]

Humoral immunity with T helper-2 response was also considered to be dominant with regard to neoplasia.[21] It has been proposed that chronic inflammation enhances cell proliferation,[31] which may promote carcinogenesis by increasing the turnover of initiated cells. Serum anti-*Hp* IgG levels differed in gerbils with a different status of gastric lesions.[32] In the present study, the titers of anti-*Hp* antibodies were relatively higher in animals on a high-salt diet. This implies that the high-salt diet affects humoral responses after *Hp* infection, and in some way contributes to the carcinogenesis-enhancing effect. However, there were no statistically significant differences between groups 1 and 2 in anti-*Hp* titers ($P=0.0670$), whereas groups 5 and 6 showed a significant difference ($P<0.05$). A possible explanation for these results is changes in the immune status and immunosuppression during carcinogen administration.[33] Similarly, there was a significant difference in serum gastrin levels between groups 5 and 6 ($P<0.05$), whereas groups 1 and 2 exhibited no statistically significant difference ($P=0.0587$). The serum gastrin levels were relatively higher in animals on a high-salt diet after *Hp* infection. MNU administration might also have exerted some influence on gastrin production. Higher serum gastrin levels after *Hp* infection might be caused by a direct effect of IL-1β on gastrin release from G cells, or gastrin release corresponding to a decrease in intragastric acidity.[34] Long-term *Hp* infection resulted in decreased parietal cells in the glandular stomach of gerbils,[25] and the alteration of the acidity following the loss of parietal cells might also affect gastrin production. In the present study, the presence of *Hp* infection greatly influenced both the serum anti-*Hp* IgG levels and serum gastrin levels.

A cross-sectional study conducted in humans revealed a significant association between the prevalence of *Hp* and frequent intake of salty food in Japan.[35] In Western countries, the declining incidence of stomach cancer has been attributed to improved food hygiene and increasingly available refrigeration facilities, and not to decreasing salt concentrations in the diet.[36] However, especially in Japan, the high prevalence of *Hp* infection and dietary habits of consuming high salt, particularly in soy sauce, pickles and other foods might greatly affect gastric carcinogenesis. First-generation immigrants from prefectures in Japan with a population at high risk of developing gastric cancer still had a high risk in Hawaii, whereas their second-generation offspring did not,[37] implying that the gastric cancer was closely linked with the salty foods consumed.

Hp, a causative factor in gastric disorders, has been closely linked to an increased risk of the development of gastric adenocarcinoma. However, it is also well known that most persons infected with *Hp* will not develop stomach cancer, and several populations have been identified

with low rates of gastric cancer despite high infection prevalence.[38] Host factors may also have an important role in gastric carcinogenesis. Gerbils appear ideal for investigating the role of *Hp* in carcinogenesis, because of their susceptibility and response to *Hp* infection, mimicking the human case. In this study, we observed signet-ring cell carcinomas, as well as poorly- and well-differentiated adenocarcinomas. This variety of induced stomach cancers, as well as the similarity to the human response to *Hp* infection, suggests the advantages of this model for research purposes. Sequential examinations using this model should allow detailed assessment of the risk of *Hp* infection in terms of the mechanisms of gastric neoplasia.

The highest incidence of adenocarcinomas was induced by the combined use of a high-salt diet, carcinogen exposure and *Hp* infection. These findings demonstrate that these factors interact to produce gastric cancers and have serious implications for cancer prevention.

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