Induction of Glandular Stomach Cancers in *Helicobacter pylori*-sensitive Mongolian Gerbils Treated with *N*-Methyl-*N*-nitrosourea and *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in Drinking Water

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An animal model of stomach carcinogenesis was established using Mongolian gerbils with *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as the carcinogens. In addition, the sensitivity of these gerbils to *Helicobacter pylori* (*H. pylori*) was confirmed. One hundred and sixty specific pathogen-free male MGS/Sea animals, 7 weeks old, were treated with MNU in the drinking water (30 ppm for alternate weeks to give 10 weeks exposure, or 10 ppm or 3 ppm for 20 weeks continuous exposure), or given MNNG in the drinking water at 400 ppm or 200 ppm for 20 weeks, or orally inoculated with ATCC43504 *H. pylori* (1.7×10⁶ CFUs/animal). Adenocarcinomas in the glandular stomach were found in 2 out of 12 effective animals (2/12) treated with 30 ppm MNNG at week 20, although all were dead or moribund by week 30 due to MNU toxicity. At week 50, the incidences of gastric adenocarcinomas in groups treated with 10 ppm MNU, 3 ppm MNU, 400 ppm MNNG, and 200 ppm MNNG were 2/21 (9.5%), 1/23 (4.3%), 7/11 (63.6%), and 1/10 (10.0%). The lesions were generally well differentiated, although poorly differentiated adenocarcinoma was also found in a single gerbil in each of the 10 ppm MNU and 400 ppm MNNG groups. In control animals no tumors were found. In the infection study, the animals were killed at week 20, and *H. pylori* was detected in all cases, causing multiple erosions with marked inflammatory cell infiltration in the lamina propria and submucosa, and frequent formation of lymphoid follicles. Thus, MNU and MNNG in the drinking water induced neoplastic lesions in the glandular stomach epithelium of *H. pylori*-sensitive gerbils.

Key words: Glandular stomach cancer — *Helicobacter pylori* — Mongolian gerbil — *N*-methyl-*N*-nitrosourea — *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

*Helicobacter pylori* (*H. pylori*) has been linked to chronic atrophic gastritis, a precursor condition for gastric carcinoma.1–3) Based on epidemiological findings, *H. pylori* was defined as a “definite biological carcinogen” by WHO/IARC in 1994.4) However, there is also a report of an apparent lack of association between *H. pylori* infection and risk of gastric cancer.5) Many animals infected with human *H. pylori* have already been studied to determine the pathogenetic background,6–13) but none of the models studied mimics human *H. pylori* infection and subsequent pathology. Recently, however, a Mongolian gerbil model of human *H. pylori* infection, with the bacteria detectable throughout a 12-month study period, was described.13) In this model, gastric ulcers and intestinal metaplasia were induced, although no neoplastic lesions were found. For analysis of the role of *H. pylori* in gastric carcinogenesis, therefore, establishment of an experimental model of gastric carcinogenesis in the Mongolian gerbil is very important. In the present study, the sensitivity of this animal to *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) gastric carcinogenicity was investigated. In addition, the susceptibility of the stomach to human *H. pylori* was confirmed.

One hundred and sixty specific pathogen-free male gerbils (*Meriones unguiculatus*) (MGS/Sea) (Seac Yoshitomi, Ltd., Fukuoka), 6 weeks old, were housed in plastic cages with hard wood chips in an air-conditioned biohazard room for infection with a 12 h light-12 h dark cycle. They were given food (Oriental NMF, Oriental Yeast Co., Tokyo) and water *ad libitum*. After one week, the animals were divided into three groups for Experiments I, II and III. MNU (Sigma Chemical Co., St Louis, MO) were each dissolved in distilled water (solutions were freshly prepared three times per week) for administration in light-shielded bottles as drinking water *ad libitum*. *H. pylori* (ATCC43504, American Type Culture Collection, Rockville, MD) was inoculated on brucella agar plates (Remel, Lenexa, KS) containing 7% 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 collected with inoculating loops, dissolved in 0.5 ml of brucella broth (Remel), introduced into brucella broth supplemented with 7% heat-inactivated fetal bovine serum in tissue culture flasks with Vent caps, and incubated at 37°C under microaerobic conditions at high humidity for 24 h without shaking. The broth cultures were checked by phase contrast microscope for shape and mobility of H. pylori. Samples containing 2.1×10⁸ colony-forming units (0.8 ml) per milliliter were used as the inoculum, delivered via an oral catheter.

The animals were treated as follows (Fig. 1): Experiment I: Ninety gerbils were divided into 3 groups. They were given MNU in their drinking water at a concentration of 30 ppm for alternate weeks to give 10 weeks intermittent exposure (group 1), or at a concentration of 10 ppm (group 2) or 3 ppm (group 3) for 20 weeks continuously, and then given tap water. Experiment II: Forty gerbils were divided into 2 groups given MNNG in their drinking water at a concentration of 400 ppm (group 1) or 200 ppm (group 2) for 20 weeks, followed by tap water. Experiment III: H. pylori (1.7×10⁶ CFUs/animal) was given by intragastric intubation (i.g.) to 10 gerbils after they had been subjected to starvation for 24 h. Four hours after inoculation, they were allowed free access to water and fed again, and all animals were killed at week 20. As non-treated controls for experiments I, II and III, 20 gerbils were used. The experimental animals were initially weighed every week and then every two to three weeks after week 20. Necropsies were performed on all animals which died or were killed upon becoming moribund. In experiments I and II, ten to twelve (experiment I) and six or seven (experiment II) animals were killed at week 20 and all surviving animals were killed and autopsied at the end of the 30th experimental week (group 1 in experiment I) or at the end of the 50th experimental week (the others). Animals that survived for 20 weeks (group 1 in experiment I) or 30 weeks (others), when the first tumors appeared, were included in the effective numbers for analysis of the incidence of tumors. Survival curves of gerbils were calculated without including gerbils killed on schedule at week 20. For detection of H. pylori infection, samples of about 30 mm² of stomach mucosa from the greater curvature, containing both fundic and pyloric glands, were homogenized with one ml of brucella broth. Aliquots of 0.1 ml were inoculated on segregating agar plates for H. pylori (Eiken Chemical Co., Tokyo) and incubated at 37°C under microaerobic conditions using Anaero Pack Campyo (Mitsubishi Gas Chemical Co., Inc.) at high humidity for six days. The excised stomachs were fixed in sublimed formaldehyde and cut into about 6 strips for embedding in paraffin. Other tissues were carefully checked under the naked eye. Tumors and related lesions were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (HE), and alcian blue-PAS, as well as by the paradoxical concanavalin A (Con A) method¹⁴ and by immunohistochemistry for H. pylori (anti-H. pylori serum, Dako, Denmark). Neoplastic lesions of the glandular stomach were classified as adenomas and adenocarcinomas.¹⁵ Adenomas consisted of excessive glandular proliferation with scanty cellular atypia. Adenocarcinomas of the glandular stomach were classified into well differentiated lesions characterized by tubular structures, poorly differentiated tumors characterized by little tendency to form glandular structures with severe cellular atypia, and signet

Fig. 1. Experimental design. Animals, male Mongolian gerbils 7 weeks old; S, animals were killed. MNU in the drinking water, MNNG in the drinking water, H. pylori (1.7×10⁶ CFUs) by i.g.
ring cell carcinomas characterized by isolated tumor cells containing abundant mucin.

In experiments I and II, reductions in survival and body weight were observed in gerbils treated with MNU or MNNG in a dose-dependent manner (Figs. 2 and 3). In group 1 receiving 30 ppm of MNU in experiment I, almost all the gerbils died or became moribund from weeks 20 to 30 and the 2 remaining gerbils were therefore killed at week 30. At week 20 in the groups given MNU (30 ppm) and MNNG (400 and 200 ppm), ulcers and dysplastic regenerated epithelium were found in the glandular stomach mucosa in almost all animals. However, gerbils treated with 10 ppm or 3 ppm MNU showed minimal changes. The incidences of gastrointestinal tumors observed in each group are summarized in Tables I and II. The tumors were generally limited to the forestomach, glandular stomach and duodenum. All forestomach lesions were of squamous cell origin. Most tumors in the glandular stomach were found in the pyloric mucosa adjacent to the fundic region. At week 20, adenocarcinomas in the glandular stomach were found only in the group treated with 30 ppm MNU and no tumors were apparent in the other groups treated with carcinogens. At week 50, adenocarcinomas were found at low incidence in the 10
ppm MNU group and at high incidence in the 400 ppm MNNG group. Carcinomas induced by MNNG were mainly well differentiated adenocarcinomas (Fig. 4), though well differentiated and poorly differentiated (Fig. 5) adenocarcinomas and a signet ring cell carcinoma (Fig. 6) were induced in the glandular stomach at low incidences by MNU. Intestinal metaplasia was not evident in any stomach in experiments I and II. Sarcomas also developed in the glandular stomach, with 4 leiomyosarcomas in group 1 of experiment II (MNNG 400 ppm). In the small intestine, including the duodenum, adenocarcinomas were found at relatively high incidences in the MNU and MNNG treated groups. Adenocarcinomas originating from Brunner’s glands were included. No tumors were detected in the large intestine and control animals lacked any neoplastic lesions.

In experiment III, H. pylori was detected by culture in all gerbils previously inoculated, whereas all control animals were negative. The numbers of colonies were around $10^5$ CFUs per stomach for all inoculated gerbils.
Fig. 4. A typical well differentiated glandular stomach adenocarcinoma at week 50 in an animal of experiment II treated with 400 ppm MNNG. HE, ×200.

Fig. 5. A poorly differentiated glandular stomach adenocarcinoma at week 50 in an animal of experiment I treated with 10 ppm MNU. Alcian blue-PAS, ×200.

Fig. 6. A, A glandular stomach signet ring cell carcinoma at week 20 in an animal in experiment I treated with 30 ppm MNU. HE, ×200. B, Serial section of the same specimen as in Fig. 6A. Alcian blue-PAS, ×200.
Fig. 8. Bacteria (arrows) are evident in the gastric pits and surface mucus layer at week 20 in an animal of experiment III infected with *H. pylori*. Immunohistochemical staining of *H. pylori*, ×400.

Macroskopically, the glandular stomachs of the affected animals were edematous, with hemorrhagic spots and erosions being found. Histologically, the glandular gastric epithelium showed hyperplastic changes with variable degrees of cystic glandular dilatation and multifocal erosion. The bases of the glands had broken through the muscularis mucosae multifocally and multiple cystic glands were present in the submucosa. 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 (Fig. 7). Immunohistochemistry also demonstrated the existence of *H. pylori* in all inoculated gerbils (Fig. 8). No neoplastic or intestinal metaplastic changes were found at week 20. Moreover, there was no change in the distribution of class III mucins, so there was no apparent phenotypic alteration in the mucous membrane. In the control, there were no pathological changes of note.

The present investigation clearly demonstrated that MNU and MNNG can induce neoplasms in the glandular stomach of gerbils, with dose-dependent effects on body weight, survival time and tumor development. Intermittent administration of 30 ppm MNU for 10 one-week periods induced well and poorly differentiated adenocarcinomas and a signet ring cell carcinoma within 20 weeks. The histological types of adenocarcinomas induced by MNU...
varied considerably, as demonstrated earlier for counterpart lesions in mice. However, since toxic effects were pronounced with the high dose of MNU, lower concentrations are required to ensure longer survival. In contrast, gerbils proved resistant to MNNG, so that relatively high dosages were tolerated. The incidences of glandular stomach adenocarcinomas in the groups given 400 and 200 ppm MNNG were dose-dependent, at 7/11 and 1/10. Almost all the tumors were well differentiated, as observed in rats treated with MNU\(^{18}\) and MNNG\(^{19,20}\). In conclusion, MNU and MNNG can induce glandular stomach carcinomas in Mongolian gerbils so this animal, which is highly susceptible to \(H.\ pylori\) infection, may afford a good model for elucidation of the interaction between bacterial infection and carcinogens in the induction of stomach cancer.

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