**Helicobacter pylori** Infection Enhances N-Methyl-N-nitrosourea-induced Stomach Carcinogenesis in the Mongolian Gerbil

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**Abstract**

No previous report has demonstrated the relationship between Helicobacter pylori (HP) infection and gastric carcinogenesis in an experimental animal model. A total of 170 male Mongolian gerbils (MGs) were divided into nine groups (18 ≤ n ≤ 20 for each group). MGs of four groups were inoculated with HP before or after continuous N-methyl-N-nitrosourea (MNU) administration via the drinking water. Both intestinal-type and diffuse-type adenocarcinomas, including signet ring-cell carcinomas, were found at 40 weeks after the study commenced, but only in the HP inoculation groups with MNU exposure and not in the MNU alone or HP inoculation alone control groups. The present findings demonstrate that HP infection increases the incidence of MNU-induced adenocarcinoma of the glandular stomach in MGs.

**Introduction**

A number of epidemiological studies have indicated that there is a significant relationship between Helicobacter pylori infection and gastric carcinoma (1-4). Indeed, in 1994 the World Health Organization’s IARC designated H. pylori as a definite biological carcinogen (5). Many animals infected with human H. pylori have been studied as a way of examining the background pathogenesis, but none of the models studied thus far has been found to reproduce the features of human H. pylori infection and its subsequent pathology. For this reason, the relationship between H. pylori infection and gastric carcinoma has not been established using a well-characterized animal model. Recently, Hirayama et al. (6) reported the successful and reliable inoculation of H. pylori into the stomach of the MG. This model showed chronic active gastritis and gastric ulcer and developed intestinal metaplasia (6, 7). We recently established an MG model in which gastric carcinogenesis is induced by MNU (8). The present study was performed to explore the effect of H. pylori infection on MNU-induced gastric carcinogenesis in MGs.

**Materials and Methods**

A total of 170 specific pathogen-free, 7-week-old male MGs (Meriones unguiculatus; MGs/Sea; Seac Yoshitomi, Ltd., Fukuoka, Japan) were housed in an air-conditioned biohazard room for the purposes of infection, with a 12-h light/12-h dark cycle. They were given food (CE-2; Clea Japan Inc., Tokyo, Japan) and water ad libitum. The animals used in this study were cared for in accordance with institutional guidelines.

H. pylori (ATCC43504; American Type Culture Collection, Rockville, MD) were grown in brucella broth (Becton Dickinson, Cockeysville, MD) containing 10% v/v horse serum for 40 h at 35°C under microaerobic conditions (15% CO₂ and high humidity with shaking (150 rpm)). After each gerbil had been fasting for 24 h, 0.8 ml of a suspension of H. pylori in brucella broth containing 10⁹ CFU/ml was administered directly into the stomach via a feeding needle (4). Four h later, they were again given free access to water and food.

MNU (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water. Fresh solution was made twice per week. The solution was provided ad libitum as the drinking water in light-shielded bottles.

The animals were allocated to experiment I or II (Fig. 1). In experiment I, 75 gerbils were divided into four groups (18 ≤ n ≤ 20 for each group). One week after inoculation with H. pylori, they were given MNU in their drinking water at a concentration of 10 ppm (group A) or 3 ppm (group C) for 20 weeks continuously. Controls were given MNU at a concentration of 10 ppm (group B) or 3 ppm (group D) for 20 weeks but were not inoculated with H. pylori. In experiment II, 75 gerbils were divided into four groups (18 ≤ n ≤ 20 for each group). They were given MNU at a concentration of 30 ppm for 6 weeks of 10 (i.e., in weeks 1, 2, 3, 8, 9, and 10; group E) or 10 ppm for 10 weeks continuously (group G) and were inoculated with H. pylori 1 week after the completion of MNU administration. Control groups were given MNU according to the same protocols but without H. pylori inoculation (group F, 30 ppm for 6 weeks of 10; group H, 10 ppm for 10 weeks). In addition to the animals in experiments I and II, 20 gerbils (group I) were inoculated with H. pylori without MNU administration. All gerbils were sacrificed under ether anesthesia 40 weeks after the study commenced. Their stomachs were fixed in 20% phosphate-buffered formalin for 24 h at 4°C and then cut along the longitudinal axis into nine slices of equal thickness. Paraffin sections were prepared and stained with H&E or with alcian blue (pH 2.5)-periodic acid Schiff for the detection of mucin-containing cells.

Lesions of the glandular stomach were classified as either adenocarcinomas or other inflammatory lesions using the following histological criteria (9). Adenocarcinomas classified as well-differentiated adenocarcinomas were characterized by the presence of tubular structures with cellular atypia and infiltration at least into the muscle layer. Poorly differentiated adenocarcinomas were characterized by their having little tendency to form glandular structures with severe cellular atypia and only scant cytoplasmic mucins. Signet ring-cell carcinomas were characterized by the presence of tumor cells containing abundant mucins and depressed nuclei. Cystic glandular proliferation without cellular atypia in the submucosal layer was not classified as a form of carcinoma.

Before removal of the stomach, a blood sample was taken from the orbital plexus into a hematocrit tube. The tubes were centrifuged at 12,000 rpm for 10 min to isolate the sera. Sera were used to determine the titers of anti-H. pylori antibody by ELISA. A specific ELISA was developed to enable quantification of the serum anti-H. pylori IgG of MGs. The titer of the antibody was expressed by way of an arbitrary index; values >1.5 were accepted as indicating positive detection of H. pylori.

The statistical significance of the incidence of adenocarcinoma development was assessed by means of Fisher’s exact test. Ps < 0.05 were considered to be significant.

**Results and Discussion**

Two of the experimental animals died without developing a neoplasm, one at 26 weeks in group A and the other at 34 weeks in group C. The other animals were sacrificed at the end point of the experiments. The serum antibody (IgG) against H. pylori was positive.
HEICOBACTER PYLORI IN STOMACH CARCINOGENESIS

Table 1 Incidence of adenocarcinoma in MGs given MNU and infected with H. pylori

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of MGs</th>
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<td>B</td>
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<td>F</td>
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<td>G</td>
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<td>H</td>
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<td>I</td>
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*Group A, H. pylori inoculation followed by MNU administration (10 ppm for 20 weeks); group C, H. pylori inoculation followed by MNU administration (3 ppm for 20 weeks); group E, H. pylori inoculation following MNU administration (30 ppm for 6 weeks); group G, H. pylori inoculation following MNU administration (10 ppm for 10 weeks). Groups B, D, F, and H, in which MNU administration was conducted without H. pylori inoculation, were the control groups corresponding to groups A, C, E, and G, respectively. Group I, H. pylori inoculation alone without MNU administration.  

Well, well-differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; Signet, signet ring-cell carcinoma.*

Findings in Group I (H. pylori Inoculation Alone without MNU Administration). Macroscopically, the glandular stomach showed edema, hemorrhagic spots, and polypoid lesions, especially in the pyloric mucosa. Histologically, there were hyperplastic changes in the epithelium, cystic glandular dilation, and erosions. The bottom of the pyloric glands, as well as of the pseudopyloric glands, broke through the muscularis mucosae multifocality and formed multiple cystic glands in the submucosa. There was infiltration of lymphocytes, macrophages, and neutrophils into the lamina propria and submucosa, and lymphoid follicles had formed. Gastric ulcer and intestinal metaplasia were found in 4 and 17 gerbils, respectively. No neoplastic lesion was observed in any animal.

Findings in Experiments I and II (Groups A to H). As in group I (H. pylori inoculation alone without MNU administration), edema and elevated lesions were found in all groups infected with H. pylori. No polypoid lesions were found in the uninoculated groups (group B, 10 ppm for 20 weeks; group D, 3 ppm for 20 weeks; group F, 30 ppm for 6 weeks; and group H, 10 ppm for 10 weeks). Microscopically, gastric adenocarcinoma was found in four groups inoculated with H. pylori (group A, 10 ppm for 20 weeks; group C, 3 ppm for 20 weeks; group E, 30 ppm for 6 weeks; and group G, 10 ppm for 10 weeks); the incidence of adenocarcinoma development in these groups was 7 of 19 (36.8%), 1 of 20 (5.0%), 6 of 18 (33.3%), and 1 of 19 (5.3%), respectively (Table 1). Of these 15 adenocarcinomas, 6 were well differentiated (Fig. 2), 2 were poorly differentiated, and 7 were signet ring-cell carcinomas (Fig. 3). None of these histological types was restricted to any particular group.

One case of squamous cell carcinoma in the forestomach was found in group E, and one sarcoma was found in group A. Gastric ulcers were found only in two groups; there were four cases in group A and two in group C. Intestinal metaplasia was observed in all 39 gerbils in groups A and C, as well as 15 of 18 in group E and 10 of 19 in group G. No direct relationship was observed between carcinoma tissues and metaplastic lesions. No tumors and no intestinal metaplasia were found in control gerbils without H. pylori inoculation (groups B, D, F, and H).

In terms of the incidence of adenocarcinoma development, there was a significant difference between groups A and B (P = 0.008) and between groups E and F (P = 0.019).

The present study clearly shows that the MG model of H. pylori infection is well suited for investigating the role of H. pylori in the development of gastric disorders, including carcinogenesis. This is the first report to demonstrate the enhancement by H. pylori infection of stomach carcinogenesis.

As reported previously (6, 7) and confirmed here, H. pylori readily

Fig. 1. Experimental design. T. inoculation with H. pylori. MNU was given ad libitum in the drinking water. All gerbils were sacrificed 40 weeks after the study commenced. w. weeks.

Fig. 2. Well-differentiated adenocarcinoma in a group E animal. H&E ×40. Tubular structures with cellular atypia have infiltrated into the muscle layer and the subserosal layer.
colonizes the gastric mucosa in MGs, where it induces chronic active gastritis and gastric ulcer. Furthermore, intestinal metaplasia appears 6 months or so after inoculation. Because intestinal metaplasia has rarely been induced in experimental animals, including MGs treated with gastric carcinogens (8, 10–12). MGs inoculated with H. pylori would seem to provide an excellent model for the study of this phenomenon.

In this study, not only differentiated tubular adenocarcinoma but also signet ring-cell carcinoma was found in MGs with MNU exposure and H. pylori infection. Epidemiological studies of human gastric carcinoma have suggested that differentiated adenocarcinoma is related most closely to H. pylori infection (1–4). However, Kikuchi et al. (13) recently demonstrated a close relationship between H. pylori infection and gastric cancer. Lancet. 341: 1359–1362, 1993.


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