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Advances in Brief

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 ($18 \leq n \leq 20$ 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.[2] 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 unguiculatu*; 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% CO_2) 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 (1×10^9 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 ($18 \leq n \leq 20$ 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 ($18 \leq n \leq 20$ 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 titer 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. *P*s < 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

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[2] The abbreviations used are: MG, Mongolian gerbil; MNU, *N*-methyl-*N*-nitrosourea.

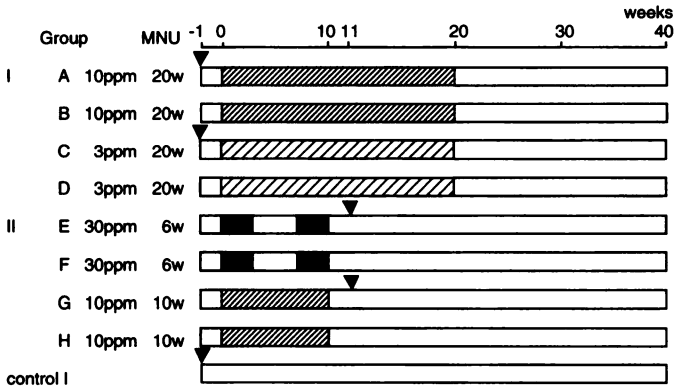

Fig. 1. Experimental design. ▼, inoculation with *H. pylori*. MNU was given *ad libitum* in the drinking water. All gerbils were sacrificed 40 weeks after the study commenced. *w*, weeks.

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

Group[a]	No. of MGs	Histological type of cancer[b]			Total
		Well	Poor	Signet	
I					
A	19	1	1	5	7 (36.8%)[c]
B	18				0
C	20	1			1 (5.0%)
D	18				0
II					
E	18	4	1	1	6 (33.3%)[d]
F	18				0
G	19			1	1 (5.3%)
H	20				0
I	20				0

[a] 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.

[b] Well, well-differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; Signet, signet ring-cell carcinoma.

[c] $P < 0.01$ compared with group B.

[d] $P < 0.05$ compared with group F (Fisher's exact test).

(arbitrary index >1.5) for all gerbils of the inoculated groups (groups A, C, E, G, and I) and was negative for all animals of groups B, D, F, and H.

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

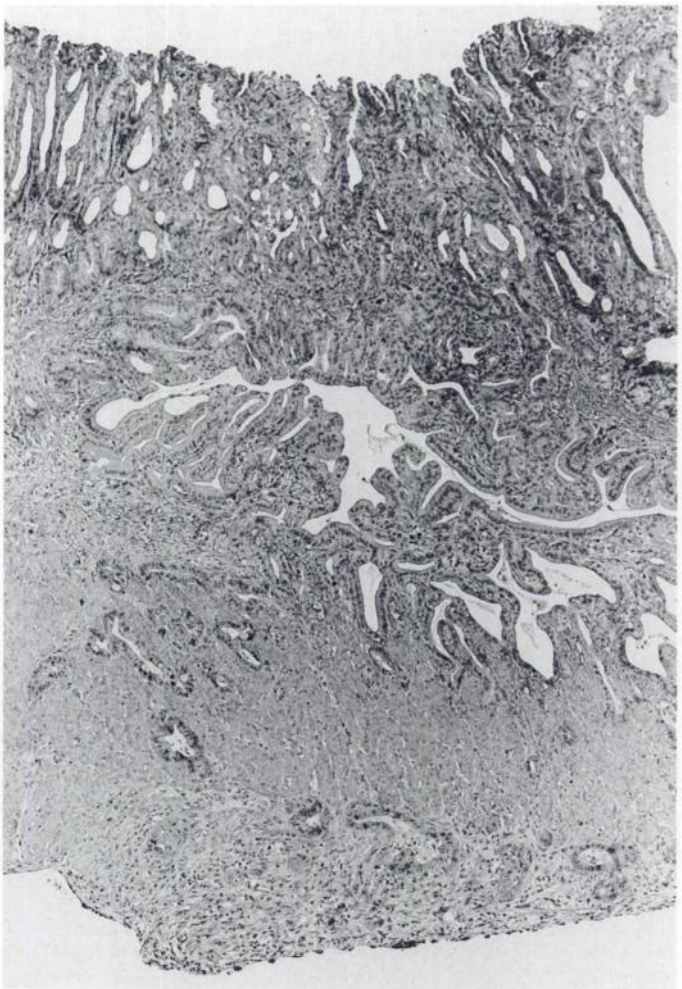

Fig. 2. Well-differentiated adenocarcinoma in a group E animal. H&E ×40. Tubular structures with cellular atypia have infiltrated into the muscle layer and the subserosal layer.

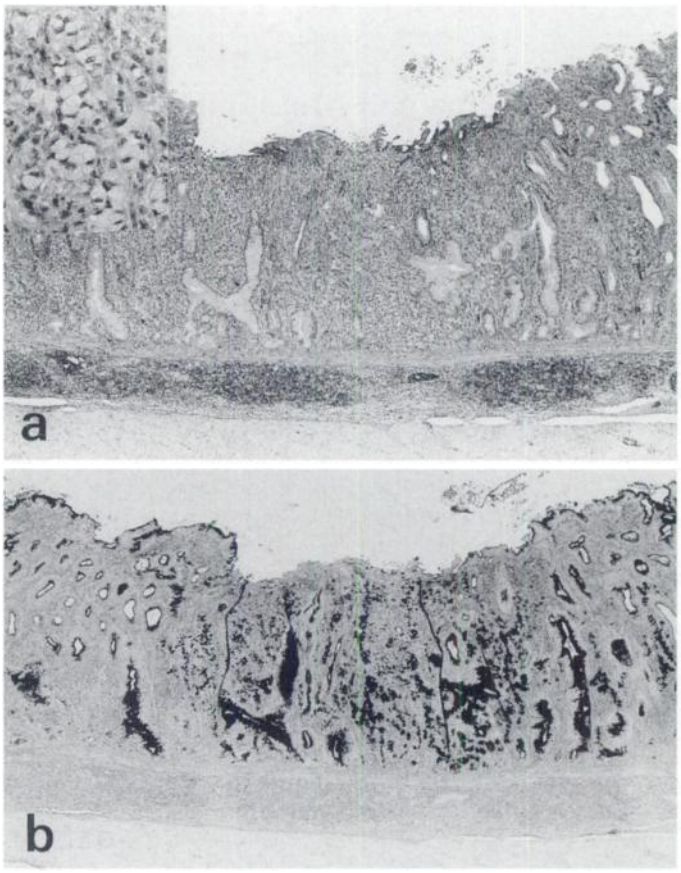

Fig. 3. Signet ring-cell carcinoma in a group A animal. *a*, H&E ×25; *inset*, H&E ×125. *b*, adjacent serial section of the same specimen as in *a*. Alcian blue-periodic acid Schiff ×25. Signet ring cells have infiltrated widely into the mucosal layer.

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 diffuse-type carcinoma in a younger generation of patients. Our data were obtained in relatively young MGs, up to 48 weeks of age, at a stage at which mucosal atrophy was not evident. Further study of more aged MGs will be needed to clarify the relationship between mucosal atrophy and carcinogenesis, because differentiated adenocarcinoma (*i.e.*, intestinal-type carcinoma) occurs mainly in atrophic gastric mucosa.

Although MNU has been reported to induce a low incidence of glandular stomach cancer in MGs at 50 weeks (8), no carcinoma was found in the MNU-treated groups without *H. pylori* inoculation at 40 weeks in experiments I and II in the present study. The data from experiment II in which the animals were infected after completion of MNU treatment fit perfectly the initiation-promotion theory (14) and clearly indicate a promoter effect of *H. pylori* infection on gastric carcinogenesis. On the other hand, the animals in experiment I were exposed almost simultaneously to MNU and *H. pylori* infection, which has been reported to induce a marked increase in proliferating cells in the inflammed mucosa (15). Because the presence or induction of cell proliferation is a major requirement for the carcinogenic activity of many chemicals (16, 17). *H. pylori* might also have a co-initiator effect when present with MNU.

Factors such as the ammonia produced by *H. pylori* urease (18), decreased ascorbic acid secretion from the mucosa (19), and oxygen radicals originating from neutrophils (20) have been reported to be related to the induction of carcinogenesis by *H. pylori*. Because all of these factors are present in gastric mucosa showing active inflammation, the precise mechanism by which carcinogenesis is induced in the inflammed mucosa remains to be clarified. Nevertheless, we should perhaps consider eradicating *H. pylori* in symptom-free carriers to reduce the risk of gastric carcinoma.

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