Eradication Diminishes Enhancing Effects of Helicobacter pylori Infection on Glandular Stomach Carcinogenesis in Mongolian Gerbils

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Abstract

To investigate the nature of the link between Helicobacter pylori (Hp) infection and stomach carcinogenesis, a study of the glandular stomach of Mongolian gerbils (MGs) was performed. MGs were treated with N-methyl-N-nitrosourea (MNU), followed by inoculation with Hp (groups 1 and 2) or without Hp (group 3), or infected with Hp (groups 4 and 5) or inoculation without Hp (group 6) followed by MNU administration. At week 21, the animals in groups 2 and 5 underwent an eradication procedure. At week 50, the incidences of adenocarcinomas in group 1 (15 of 23) and group 4 (9 of 26) were significantly higher than in group 3 (1 of 15) and group 6 (1 of 18), respectively. Moreover, those in group 2 (5 of 24) and group 5 (2 of 22) were lower than in groups 1 and 4, respectively. This study shows that Hp eradication may be useful as a prevention approach against stomach cancer.

Introduction

From epidemiological findings, there is little a room for doubt that Hp infection has “positive correlation” with stomach cancer development (1–4). To demonstrate a “causal link” between Hp infection and stomach carcinogenesis, as well as for intervention studies, it is essential to establish an experimental animal model. We have demonstrated that Hp infection enhances glandular stomach carcinogenesis using MGs. All histological types of stomach cancer are increased on treatment with chemical carcinogens, mimicking human cases (5, 6). The present study was performed to explore the influence of eradication measures on stomach carcinogenesis induced by MNU administration and Hp infection in MGs.

Materials and Methods

MNU (Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water at concentrations of 30 or 10 ppm and freshly prepared three times per week. The solutions were given as drinking water in light-shielded bottles ad libitum. Hp (ATCC 43504, Rockville, MD) was inoculated on Brucella agar plates (Becton Dickinson and Company, 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, Japan) at high humidity. Two days later, the bacteria grown on the plates were introduced into Brucella broth (Becton Dickinson and Company) supplemented with 7% v/v heat-inactivated fetal bovine serum and incubated under the same conditions for 24 h. Samples containing about 1.0 × 10^7 colony-forming units (0.8 ml) per milliliter were used as the inoculum, delivered i.g. using an oral catheter after fasting for 24 h. For eradication of Hp, a “triple therapy” was performed. The drugs lansoprazole, amoxicillin, and clarithromycin were suspended in 0.5% w/w carboxymethyl cellulose sodium salt solution and administered i.g. twice a day for two days at doses of 10, 3, and 30 mg/kg body weight, respectively.

A total of 158 specific pathogen-free male, 7-week-old MGs (Meriones unguiculatus; MGS/Sea, Seac; Yoshitomi, Ltd., Fukuoka, Japan), were housed in steel cages on hardwood chip bedding in an air-conditioned biohazard room (for infection) with a 12-h light/12-h dark cycle. They were given food (Oriental MF; Oriental Yeast Co., Tokyo, Japan) irradiated with 30 kgy gamma 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. The animals were treated as follows. In experiment I, 79 gerbils were divided into three groups. They were given MNU in their drinking water at the concentration of 30 ppm for alternate weeks for a total of 5 weeks exposure. On completion of this regimen, they were given autoclaved distilled water; 1 week thereafter, Hp were inoculated (groups 1 and 2, n = 32 and 27, respectively). Group 3 (n = 20) received Brucella broth without Hp. At week 30, five animals in group 1 were killed to check for bacterial infection, and at week 21, all animals in group 2 underwent treatment for eradication. In experiment II, 79 gerbils were divided into three groups. One week after inoculation of Hp (groups 4 and 5, n = 32 and 27, respectively) or the Brucella broth vehicle alone (group 6; n = 20), they were given MNU in their drinking water at the concentration of 10 ppm for 20 weeks continuously. At week 30, five animals in group 4 were killed to check for bacterial infection, and at week 21, all animals in group 5 underwent Hp eradication treatment (Fig. 1). At the 50th experimental week, after 24 h fasting, all animals were subjected to deep ether anesthesia, laparotomized, and exsanguinated from the inferior vena cava, with excision of their stomachs. Tissues were processed for microbiological and histopathological examinations.

For detection of Hp infection, ~30-mm² samples of stomach mucosa from the greater curvature, containing both fundic and pyloric glands, were homogenized with 1 ml of Brucella broth and used for culture of Hp. Aliquots of 100 μl were then inoculated on segregating agar plates for Hp (Eiken Chemical Co., Tokyo, Japan) and incubated at 37°C under microaerobic conditions for 6 days. Anti-Hp antibodies were measured as described earlier (5). 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. Using the Mg sera thus obtained, anti-Hp IgG antibody (GAP-IgG; Biomerica, Newport Beach, CA) was measured by ELISA. The antibody titers were expressed using an A.I. The excised stomachs were fixed in 4% paraformaldehyde in PBS (pH 7.2) or 95% ethanol containing 1% acetic acid, cut into ~16 strips, processed by standard methods, and embedded in paraffin. Tissues were sectioned at 5 μm for staining with H&E, alcinian blue-periodic acid-Schiff stain and by immuno-histochimistry for Hp (anti-Hp serum; DAKO, Copenhagen, Denmark). Adenocarcinomas of the glandular stomach were classified into well-differentiated lesions, characterized by tubular structures with cellular atypia, poorly differentiated tumors, characterized by little tendency to form glandular struc-
tures with severe cellular atypia, and signet ring cell carcinomas, characterized by isolated tumor cells containing abundant amounts of mucin (7).

The two-tailed $t$ test or the Mann-Whitney $U$ test was applied to establish the significance of differences in body weight distributions and titers of anti-Hp antibodies. Survival curves were calculated by the Kaplan-Meier method, and the differences were evaluated using the log-rank test. The adenocarcinoma incidences were assessed by the Fisher’s exact probability method. P $< 0.05$ were considered to be statistically significant.

**Results**

Survival rates for each group were $>75\%$, with no differences among groups by the log-rank test. The body weights in groups 1–6 at experimental week 50 were $71.9 \pm 3.5, 83.4 \pm 3.0, 85.3 \pm 3.6, 92.3 \pm 4.2, 96.7 \pm 2.8$, and $103.1 \pm 3.6$ g (average $\pm$ SE), respectively, with the value for group 1 being significantly lower than those for groups 2 and 3 ($P < 0.05$). Hp were detected by culture in all MGs in groups 1 and 4 at week 30. Hp were also detected in all MGs in groups 1 and 4 at week 50. In group 5, 2 of 24 animals were Hp positive by culture at week 50, and these were excluded from later analyses. Other animals in group 5 and all animals in groups 2, 3, and 6 were negative for culture. Titers of anti-Hp antibodies for animals in groups 1, 2, and 3 were $136.1 \pm 23.0, 114.2 \pm 2.3$, and $23.6 \pm 0.6$ (A.I.; average $\pm$ SE), respectively. There are significant differences between each pair ($P < 0.001$). Values for groups 4, 5, and 6 were $466.7 \pm 65.1, 100.6 \pm 32.4$, and $2.5 \pm 0.4$ (A.I.), respectively, again with significant intergroup differences ($P < 0.001$).

Histologically, in all MGs in groups 1 and 4, the glandular stomach epithelium showed hyperplastic changes with variable degrees of multifocal cystic dilation and erosion. 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. Intestinal metaplasia was also noted in 23 among 24 gerbils in group 1 and in 25 among 26 animals in group 4. Intestinal metaplasia was not observed in any but the 2 animals that failed to be eradicated for Hp in group 2 and was lacking in groups 3, 5, and 6.

**Table 1** Induction of glandular stomach adenocarcinomas with MNU, Hp infection, and Hp eradication

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment $^a$</th>
<th>Effective no. of mice</th>
<th>No. of tumor-bearing mice (%) $^b$</th>
<th>No. of cancer $^c$</th>
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<tr>
<td>1$^d$</td>
<td>MNU→Hp</td>
<td>23</td>
<td>15 (65.2)</td>
<td>11 2 6</td>
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<tr>
<td>2$^e$</td>
<td>MNU→Hp→Er</td>
<td>24</td>
<td>5 (20.8)$^d$</td>
<td>5 0 0</td>
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<td>3$^f$</td>
<td>MNU→Br</td>
<td>15</td>
<td>1 (6.7)$^d$</td>
<td>0 0 1</td>
</tr>
<tr>
<td>4</td>
<td>Hp→MNU</td>
<td>26</td>
<td>9 (34.6)</td>
<td>9 0 0</td>
</tr>
<tr>
<td>5</td>
<td>Hp→MNU→Er</td>
<td>22</td>
<td>2 (9.1)$^d$</td>
<td>2 0 0</td>
</tr>
<tr>
<td>6$^g$</td>
<td>Br→MNU</td>
<td>18</td>
<td>1 (5.6)$^d$</td>
<td>1 0 0</td>
</tr>
</tbody>
</table>

$^a$ Hp, Hp (i.g.); Br, Brucella broth (i.g.); Er, eradication.

$^b$ Well, well-differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; Sig, signet ring cell carcinoma.

$^c$ Four animals in group 1 demonstrated double cancers.

$^d$ $P < 0.01$ versus group 1 by Fisher’s exact test.

$^e$ $P < 0.001$ versus group 1 by Fisher’s exact test.

$^f$ $P < 0.05$ versus group 4 by Fisher’s exact test.

$^g$ $P < 0.05$ versus group 4 by Fisher’s exact test.
5, and 6. The incidences of adenocarcinomas of the glandular stomach are summarized in Table 1. Tumors were mostly found in the antral mucosa adjacent to the oxyntic region and comprised well-differentiated adenocarcinomas (Fig. 2), as well as signet ring cell carcinomas (Fig. 3c). The incidences of adenocarcinomas in group 1 was significantly higher than in group 2 (P < 0.01) and group 3 (P < 0.001). The incidence in group 4 was also significantly higher than those in groups 5 and 6 (P < 0.05).

Discussion

Our present study provides direct evidence that Hp eradication may be useful as a prevention approach. This is the first report of such eradication leading to prevention, to our knowledge. Recently, many clinicians have been prescribing Hp eradication for medical care of patients not only suffering from peptic ulcers (8) and MALT lymphomas (9) but also dyspepsia (10). However, after complete clearance of the bacteria, reflux esophagitis often occurs (11), and this side effect is thought to be an important risk factor for esophageal adenocarcinoma development (12). Therefore, establishment of criteria for Hp eradication is now a top priority.

The most important factors that may influence stomach carcinogenesis are strain diversities of Hp and host reactions. Concerning Hp strains, investigators have studied Cag A and Vac A intensively. Tomb et al. (13) elucidated the complete Hp genome sequence in 1997, and a full understanding of the pathogenicity of Hp itself should be generated in the near future.

Concerning host factors, we have emphasized the stomach mucosal condition and serological response of the host (6). One interesting point in the MG model is the persistent Hp infection in the stomach, not found in mice and rats. A decrease in the rate of Hp infection with aging and with stomach mucosal damage in MGs and increase in Hp infection related to stomach mucosal damage in mice (14) imply important roles for mucosal conditions of the stomach with regard to Hp infection. T helper 1 cellular immune responses also contribute to Helicobacter-associated gastritis in mice and humans (15), and D’Elios et al. (16) showed that Hp-specific T helper 1 effectors may play a role in generation of peptic ulcers in humans. In our previous study, the titers of anti-Hp antibodies in tumor-bearing animals were found to be higher than in those which were tumor free treated in the same manner (6). This implies that the T helper 2 humoral response may play an important role in stomach carcinogenesis.

For application of Hp eradication to human, timing will presumably be of essential significance. In our previous study, we eradicated Hp in the period of active chronic gastritis but before intestinal metaplasia or dysplasia occurred. The early phase might be expected to be of essential significance. In our present study, we eradicated Hp in the near future.

Using the present carcinogenesis model and adding eradication at various periods, sequential histopathological and molecular biological examinations should allow detailed assessment of the underlying mechanisms.

Acknowledgments

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References


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