Marked Enhancement by Fish Meal of Helicobacter pylori-induced Gastritis in Mongolian Gerbils

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In a search for dietary factors influencing Helicobacter pylori-induced gastritis, the effects of fish meal in the diet were examined in Mongolian gerbils. When a conventional diet containing 10% fish meal was given to Mongolian gerbils for 4 weeks after inoculation of H. pylori, edematous thickening with severe neutrophil and mononuclear cell infiltration in both the mucosa and submucosa was observed in the glandular stomach of 19 out of the 20 animals, and hemorrhagic spots were evident in 11 cases. These gastric lesions were enhanced by a 20% fish meal supplement, and edema and hemorrhage in the gastric mucosa were observed in 19 and 17 out of 20 animals, respectively. Although almost the same levels of viable bacteria were detected independent of the diet, edema and hemorrhage were seen in only 2 and 1 of 20 gerbils fed a diet containing 10% casein, instead of 10% fish meal, respectively. Neither edema nor hemorrhage was observed in 10% beef diet animals. These results suggest that fish meal contains factors which greatly enhance H. pylori-induced gastritis in Mongolian gerbils. Since the incidences of gastritis and gastric cancer are very high throughout the world, it is very important to identify these gastritis-enhancing factors.

Key words: H. pylori—Mongolian gerbils—Gastritis—Fish meal

Gastric cancer continues to be one of the most common malignancies in the world. Its pathogenesis is known to be associated with a high intake of salted foods and an insufficient intake of fresh fruits and vegetables.1 In addition, several epidemiological studies have suggested a link to infection with Helicobacter pylori.1–4 Recently, Hirayama et al. reported Mongolian gerbils to be easily colonized with H. pylori, with associated development of chronic gastritis, gastric ulcers and intestinal metaplasia after prolonged infection.5

Using this animal model, the effects of H. pylori infection on gastric carcinogenesis have been studied. Infection with H. pylori in N-methyl-N-nitrosourea (MNU) or N-methyl-N’-nitro-N-nitrosoguanidine (MNNG)-treated Mongolian gerbils was found to enhance significantly the development of both well-differentiated and undifferentiated adenocarcinomas in the stomach.6–9 These enhancing effects were diminished by eradication of H. pylori.10 Furthermore, two papers have documented that long-term colonization (15–18 months) by H. pylori alone may induce well-differentiated gastric cancers at incidences of around 40% in Mongolian gerbils.7,11 However, the data are somewhat equivocal, since Tatematsu et al. pointed out that glandular stomach epithelium having multifocal cystic glandular proliferation without any cellular atypia, and penetrating into the muscularis mucosae, could possibly be misidentified as well-differentiated adenocarcinoma of the stomach.12 Consistent with that suggestion, Hirayama et al. reported the development of carcinoids at a relatively high incidence, whereas poorly differentiated adenocarcinoma was found in only 1 out of 56 gerbils after almost 2 years of H. pylori infection.13

It is suggested that development of H. pylori-induced gastritis and ulceration are affected by H. pylori strain diversity, as well as host and environmental factors, including diet.14,15 During the search for dietary factors affecting gastritis associated with H. pylori infection, we incidentally noticed that the conventional diet containing fish meal showed an enhancing activity for H. pylori-induced gastritis. Therefore, in the present study, we examined the effects of fish meal on H. pylori-induced gastritis in Mongolian gerbils.

The ingredients for the conventional diet, used for animal experiments in Japan, were purchased from Nippon Formula Feed Manufacturing Co., Ltd. (Tokyo), and blended in our laboratory. The concentration of protein in the standard diet is 25.0%, with white fish meal made from whole codfish and soybean meal as the main protein sources. The amount of fish meal in the diet is usually

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10%, and therefore the control diet was named 10% fish meal diet in the present study. Fish meal was additionally supplemented to give a 20% fish meal diet. For comparison, fish meal was replaced by casein and beef to make a 10% casein diet and 10% beef diet, respectively. The compositions of all diets are given in Table I.

Specific pathogen-free male Mongolian gerbils (Meriones unguiculatus, MGS/Sea; Seac Yoshitomi, Ltd., Fukuoka), 6 weeks old, were housed in polycarbonate cages with hard wood chip bedding in an air-conditioned biohazard room with a 12 h light/dark cycle. All animals had free access to food and water.

H. pylori (ATCC 43504) was obtained from the American Type Culture Collection (Rockville, MD) and grown in 300-ml baffled flasks containing 100 ml of brucella broth (BBL, Cockeysville, MD) supplemented with 10% heat-inactivated horse serum (Nacalai Tesque, Kyoto). The flasks were incubated at 37°C for 24 h under microaerophilic conditions with agitation on a rotary shaker at 125 rpm. The broth culture (0.5 ml) of H. pylori (1.0×10⁹ CFU/ml) was inoculated orally, using a feeding needle after the gerbils had been fasted for 24 h. Animals of the non-infected control group were dosed with broth culture media alone.

All animals were sacrificed under ether anesthesia 4 weeks after H. pylori inoculation. Their stomachs were excised and opened, the contents were gently removed, and then macroscopic observation and counts of hemorrhagic spots were performed. A half of each stomach was used for histological examination after fixation in Carnoy’s fixative (30% chloroform, 60% ethanol, 10% acetic acid) for 1–2 h at room temperature. The other halves were homogenized with 0.3 ml of phosphate-buffered saline (pH 7.6), further diluted and aliquots (0.1 ml) were inoculated onto Skirrow’s agar plates with sterile horse blood (7%, v/v) in blood agar base no. 2 (Oxoid, Ltd., Basingstoke, England), containing a Campylobacter selective supplement (Oxoid, Ltd.), and incubated at 37°C for 7 days under microaerophilic conditions. Bacterial colonies

### Table I. Compositions of the Diets Used in the Present Study

| Ingredient           | 10% fish meal diet (%) | 20% fish meal diet (%) | 10% casein diet (%) | 10% beef diet (%) |
|----------------------|------------------------|------------------------|---------------------|-------------------|
| Water                | 7.3                    | 7.2                    | 8.2                 | 9.5               |
| Protein (Crude)      | 25.0                   | 29.2                   | 28.0                | 25.6              |
| Fat (Crude)          | 4.7                    | 4.7                    | 3.6                 | 5.1               |
| Fiber (Crude)        | 4.0                    | 3.6                    | 3.0                 | 3.2               |
| Ash (Crude)          | 6.0                    | 7.7                    | 3.8                 | 4.8               |
| Nitrogen free extract| 53.0                   | 47.6                   | 53.4                | 51.8              |
| Sodium chloride⁴     | 0.9                    | 1.0                    | 0.7                 | 0.7               |

Analysis was performed at Tokyo Kenbikyoin Foundation (Tokyo) referring to Annotation of Official Methods of Feed Analysis in Japan.

⁴ Sodium was measured by flame-spectrochemical analysis.

### Table II. H. pylori Colonization and Macroscopic Lesions in the Glandular Stomachs of Mongolian Gerbils

| Group | Diet       | No. of animals | H. pylori inoculation | No. of colonies of H. pylori⁴ (log CFUs/stomach) | No. of animals (%) with Edema | Hemorrhage | No. of hemorrhagic spots/animal⁴ |
|-------|------------|----------------|-----------------------|-----------------------------------------------|------------------------------|------------|-------------------------------|
| 1     | 10% fish meal | 20  +          | 5.7±0.4               | 19/20 (95)                                   | 11/20 (55)                   | 4.7±5.5    |
| 2     | 20% fish meal | 20  +          | 5.4±0.4               | 19/20 (95)                                   | 17/20 (85)                   | 13.3±8.0⁶  |
| 3     | 10% casein   | 20  +          | 4.8±0.5               | 2/20 (10)³                                  | 1/20 (5)³                    | 0.2±0.7⁶   |
| 4     | 10% beef     | 20  +          | 5.0±0.5               | 0/20 (0)³                                  | 0/20 (0)³                    | 0³         |
| 5     | 10% fish meal | 5   −           |                       | 0/5 (0)                                     | 0/5 (0)                       | 0           |
| 6     | 20% fish meal | 5   −           |                       | 0/5 (0)                                     | 0/5 (0)                       | 0           |
| 7     | 10% casein   | 5   −           |                       | 0/5 (0)                                     | 0/5 (0)                       | 0           |
| 8     | 10% beef     | 5   −           |                       | 0/5 (0)                                     | 0/5 (0)                       | 0           |

All animals were sacrificed at week 4, then H. pylori colonization and the lesions in the glandular stomach were analyzed.

⁴ Mean±SD.

³ Significantly different from group 1 by Fisher’s exact test (P<0.01).

⁶ Significantly different from group 1 by Mann-Whitney’s U-test (P<0.001).
Fig. 1. Macroscopic views of glandular stomach of Mongolian gerbils inoculated with or without *H. pylori*. A. 10% fish meal diet with *H. pylori* infection (group 1); B. 20% fish meal diet with *H. pylori* infection (group 2); C. 10% casein diet with *H. pylori* infection (group 3); D. 10% fish meal diet without *H. pylori* infection (group 5).

Fig. 2. Microscopic views of glandular stomach of Mongolian gerbils inoculated with or without *H. pylori*. Sections were stained with hematoxylin and eosin for histological examination. Original magnification ×8 and ×80 are shown in the upper and lower parts, respectively. Group numbers corresponding to A–D are the same as those in Fig. 1.
were identified by the rapid urease test and Gram staining for morphology, and counted.

Table II shows experimental groups for the present study and animal numbers. Body weight gains and diet intake of the gerbils were similar in all groups during the experimental period. *H. pylori* was detected by culture in all gerbils of groups 1–4, but was absent in animals of groups 5–8, not inoculated with bacteria. Data for numbers of colonies obtained from samples of stomach in group 1–4 animals are given in Table II. In all gerbils inoculated with the broth culture of *H. pylori*, around 10⁵ viable bacteria were counted, without any significant intergroup differences.

Four weeks after the *H. pylori* inoculation, edematous thickening was observed in the gastric mucosa, especially in the pylorus, of 19 of the 20 animals in the 10% fish meal group (group 1), and in 19 of the 20 animals in the 20% fish meal group (group 2). Hemorrhagic spots were evident in 11 of 20 animals in group 1, and 17 of 20 in group 2. In contrast, edema and hemorrhage were observed in only 2 and 1 of 20 animals, respectively, in the 10% casein group (group 3), and neither were observed in 10% beef diet animals (group 4). The average numbers of hemorrhagic spots were 4.7 for group 1, and 13.3 for group 2, and 0.2 in group 3 (Table II). No gastritis was observed in the 10% or 20% fish meal, 10% casein and 10% beef diet animals without *H. pylori* inoculation (groups 5–8). In addition, no fish meal-counteracting effect of casein on *H. pylori*-induced gastritis was observed when a combined diet containing 10% casein plus 10% fish meal diet was given to Mongolian gerbils. Therefore, *H. pylori*-induced gastric lesions were enhanced dose-dependently by the 10% and 20% fish meal diets.

Figs. 1 and 2 show macroscopic and histological findings, respectively. Histological examination revealed gastritis in Mongolian gerbils inoculated with *H. pylori*, but not in non-infected controls. In the gerbils fed 10% and 20% fish meal diets after inoculation of bacteria, moderate to severe gastritis featuring mononuclear cell and neutrophil infiltration into the submucosal and lamina propria layers, and hyperplastic and cystic changes of glandular epithelium in the pyloric region were observed in a dose-dependent manner (Fig. 2, A and B). Such gastric changes were not evident in the fundic region. Only mild hyperplastic gastritis was seen in Mongolian gerbils inoculated with *H. pylori* and fed the 10% casein diet (Fig. 2C). Similar mild hyperplastic changes were observed in the gerbils fed 10% beef diet after inoculation of *H. pylori*. The lack of gastritis in Mongolian gerbils fed 10% fish meal diet without *H. pylori* inoculation was also noteworthy (Fig. 2D).

The present study showed that fish meal in the diet markedly enhances *H. pylori*-induced gastritis in Mongolian gerbils. Infected animals maintained on a fish meal-free diet had few grossly visible gastric lesions, even though almost the same numbers of *H. pylori* were cultured from the gastric mucosa at necropsy as in the fish meal diet cases. No gastric lesions were detected in the 10% and 20% fish meal groups without *H. pylori* inoculation. On the basis of these observations, synergistic effects between fish meal components and *H. pylori* colonization appear to be responsible for severe gastritis in the stomachs of Mongolian gerbils. Several epidemiological studies have suggested that salted, dried and broiled fish may be risk factors for gastric carcinogenesis. The main reason for the association of fish intake with gastric carcinogenesis has been thought to be its high salt content, in addition to its impact as a source of precursors for N-nitroso compounds. The concentration of sodium chloride was 0.9% for 10% fish meal diet, 1.0% for 20% fish meal diet and 0.7% for both 10% casein and beef diets. These doses of sodium chloride in the diet could not significantly affect the induction of gastritis in Mongolian gerbils. Thus, the possibility that salt in the diet influenced the gastric mucosal injury induced by *H. pylori* can be ruled out. Fish oil is reported to suppress ethanol- and aspirin-induced gastritis, but enhances ethanol-induced liver injury in animals. Thus, it should be studied how fish oil influences *H. pylori*-induced gastritis in Mongolian gerbils.

Since the incidences of gastritis and gastric cancer incidence are high throughout the world, including Japan, it is very important to study whether fish other than the codfish used in the present study might induce gastritis in *H. pylori*-infected Mongolian gerbils, and what component in the fish meal is involved.

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