ABSTRACT

Background. Recently, the acquisition by Helicobacter pylori of resistance to antibiotics has become a serious problem. Therefore, nonantibiotic substances are required to diminish H. pylori-induced gastric lesions. In the present study, the effects of Cladosiphon fucoidan were examined in terms of H. pylori attachment to porcine gastric mucin in vitro and Helicobacter pylori-induced gastritis in vivo.

Methods. The inhibitory effect of Cladosiphon fucoidan and other polysaccharides on H. pylori attachment to porcine gastric mucin was assayed in vitro with mucin-coated microtiter plates. The effect of Cladosiphon fucoidan on H. pylori-induced gastritis was examined in vivo using Mongolian gerbils. H. pylori-inoculated gerbils were given fucoidan in drinking water. Six weeks after H. pylori-inoculation, gerbils were sacrificed for macroscopic and microscopic examination of gastric lesions and counting of viable H. pylori in the gastric mucosa.

Results. Cladosiphon fucoidan inhibited the H. pylori attachment to porcine gastric mucin at pH 2.0 and 4.0. Two other sulfated polysaccharides, Fucus fucoidan and dextran sulfate sodium, also inhibited the attachment but only at pH 2.0. Inhibitory effects of these three sulfated polysaccharides were not observed at pH 7.2 and nonsulfated polysaccharides, such as mannan and dextran, exerted no influence at any pH. In the in vivo experiment, the H. pylori-induced gastritis and the prevalence of H. pylori-infected animals were markedly reduced by fucoidan in a dose-dependent manner, at doses of 0.05 and 0.5% in the drinking water.

Conclusion. Cladosiphon fucoidan may deserve particular attention as a safe agent that can prevent H. pylori infection and reduce the risk of associated gastric cancer.

Keywords. Helicobacter pylori, fucoidan, Mongolian gerbil, prevention.

Preventive Effects of Cladosiphon Fucoidan Against Helicobacter pylori Infection in Mongolian gerbils

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involve alteration of the bacterial surface. *Cladosiphon* fucoidan has an α1-3 fucopyranosyl backbone, as is the case with *Fucus vesiculosus* fucoidan; the two compounds differ in their contents of sulfate and branched sugars [11,12]. About half of the fucose residues in *Cladosiphon* fucoidan are sulfated at the C-4 position. In contrast, almost all the fucose residues in *Fucus* fucoidan are sulfated at the same position. Approximately 15% of fucose residues in the main chain of *Cladosiphon* fucoidan have α1, 4-linked fucose branches, while 25% of those of *Fucus* fucoidan have α1,2-linked glucuronic acid. In a previous paper, we described that *Fucus* fucoidan induces an inflammatory response to rat macrophages and polymorphonuclear cells [13]. Other sulfated polysaccharides, such as dextran sulfate sodium and carrageenan, are also reported to be inflammatory agents [14] causing colitis in animal models [15–17]. In contrast, *Cladosiphon* fucoidan showed no inflammatory response in vitro [13] and therefore might have advantages as a nutritional agent in terms of application for *H. pylori*-derived diseases.

In the present study, we therefore compared the inhibitory effects of *Cladosiphon* fucoidan and other polysaccharides on *H. pylori* attachment with porcine gastric mucin in vitro as well as *H. pylori*-induced gastritis in vivo using Mongolian gerbils.

**Materials and Methods**

**Materials**

*Cladosiphon* fucoidan was prepared as described previously [18]. In brief, the wet seaweed was suspended in a 0.01-M hydrochloric acid and then heated at 95°C for 10 minutes. Then the extract was centrifuged for 60 minutes at 10 000 g at 20°C. The supernatant was neutralized to pH 6.0 with a diluted sodium hydroxide and then dialyzed against distilled water. The dialylyzate was lyophilized and used as *Cladosiphon* fucoidan. This fucoidan is stable at room temperature under dry conditions. For in vivo administration, fucoidan was dissolved in distilled water every 2 days. *Fucus* fucoidan, dextran sulfate sodium, dextran and mannan were purchased from Sigma (St. Louis, MO, USA). Dextran sulfate sodium with a molecular mass of 40,000 was obtained from ICN Biomedicals (Aurora, OH, USA). Porcine gastric mucin was purchased from Seikagaku Co. (Tokyo, Japan).

**Bacteria**

*H. pylori* ATCC 43504 (American Type Culture Collection, Manassas, VA, USA) was grown in Brucella broth (Becton Dickinson Co., Cockeysville, MD, USA) supplemented with 10% heat-inactivated horse serum (Nacalai Tesque, Kyoto, Japan) for 24 hours at 37°C under microaerobic conditions (5% O₂, 10% CO₂ and 85% N₂), as previously described [19]. Broth cultures (2.0 × 10⁸ CFU/ml) were centrifuged to collect the bacteria. After washing twice with saline, the *H. pylori* were re-suspended in saline and stored at −80°C until the in vitro experiment. For in vivo use, broth culture was diluted 100-fold with Brucella broth and orally inoculated by gavage into animals.

**Inhibition Assay of the H. pylori Attachment to Gastric Mucin**

The attachment of *H. pylori* to porcine gastric mucin was assayed according to the method described previously [20]. A sample of 100 µl of porcine gastric mucin (400 µg/ml) was added to each well of microtiter plates and incubated at 37°C. After 3 hours, the plates were washed and phosphate-buffered saline (PBS), supplemented with 0.05% Tween 20. The bacterial suspension was then mixed with *H. pylori* suspension (10⁸/ml) with polysaccharide solution of pH 7.2, 4.0 and 2.0 was also prepared by a similar method used for bacterial suspension at concentrations of 2–2000 µg/ml. The bacterial suspension was then mixed with an equal volume of one of the five polysaccharides solutions: *Cladosiphon* fucoidan, *Fucus* fucoidan; dextran sulfate sodium, dextran and mannan. The pH of the suspension was stable even by adding polysaccharide. A sample of 100 µl of *H. pylori* suspension (10⁸/ml) with polysaccharide thus obtained was added to each well of microtiter plates. After incubation for 2 hours at 37°C, the plates were washed and fixed with 4% paraformaldehyde for 30 minutes at 4°C. The plates were then incubated with monoclonal antibodies to *H. pylori* (Sanbio, Uden, Netherlands) followed by horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Pharmacia, Uppsala, Sweden). After a final washing, the plates were developed with the
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ABTS peroxidase substrate system (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA). Each determination was made in triplicate.

**Effect of Fucoidan on \textit{H. pylori}-Induced Gastritis in Mongolian Gerbils**

Specific pathogen-free male Mongolian gerbils (Seac Yoshitomi, Ltd, Fukuoka, Japan), 6 weeks old, were housed in an air-conditioned biohazard room with a 12-hour light-dark cycle. They were fed a normal diet (CE-2; Clea Japan Inc., Tokyo, Japan) and water ad libitum until the start of the experiment. At week 7, each animal was fasted for 24 hours and then \textit{H. pylori} was orally inoculated by gavage (0.5 ml, $1.0 \times 10^6$ CFU). The control animals without infection were given sterilized broth alone. After inoculation, each animal was kept without food for 4 hours and then given a normal powder diet (CE-2 powder). Drinking water was provided ad libitum throughout the experiment.

The animals in the control group were given distilled water throughout the experimental period. Experimental groups received water supplemented with fucoidan (0.05 or 0.5%) from 3 days before \textit{H. pylori} inoculation until the end of the experimental period. Others were given fucoidan-supplemented water (0.05 or 0.5%) from 2 weeks after inoculation until the end of the experiment. Body weight, diet intake and drink intake were measured once, twice and three times a week, respectively, and animals were monitored daily for their general health.

At 6 weeks after the inoculation of \textit{H. pylori}, all animals were sacrificed and their stomachs resected under ether euthanasia. Each was opened along the greater curvature and washed with saline. Then macroscopic gastric lesions (edema and hemorrhage) were recorded, followed by measurement of the wet weight of the whole stomach, including forestomach and glandular stomach. Half of the glandular mucosa was scraped for detection of colonizing \textit{H. pylori}, and the residual part was formalin-fixed and embedded in paraffin for histological observation. Colonies were also resected from vehicle- or polysaccharide-treated animals and opened longitudinally, fixed with formalin and embedded in paraffin, for histological examination. Pathological diagnosis of gastritis was made according to the criteria described previously [19]. Liver, spleen and kidney tissues were histologically reviewed when lesions were apparent on macroscopic observation.

**Detection of \textit{H. pylori} Colonization in the Gastric Mucosa**

To detect \textit{H. pylori} colonization, scraped mucosa samples were homogenized with 0.3 ml of PBS. An aliquot (100 µl) of serially diluted homogenate was inoculated onto segregating agar plates for \textit{H. pylori} (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) and incubated at 37°C under microaerobic conditions. After 5 days, the colonies were counted to determine the level of \textit{H. pylori} colonization of each stomach.

**Statistical Analysis**

The significance of differences in quantitative data for gastric lesions and \textit{H. pylori} infection was analyzed by the Fisher’s exact test. Other data were examined using the Steel test. Significance was determined as $p < .05$.

**Results**

**Inhibition of \textit{H. pylori} attachment to gastric mucin**

The \textit{H. pylori} attachment to porcine gastric mucin in the presence of the five polysaccharides tested is shown in Fig. 1. At pH 2.0 and 4.0, \textit{Cladosiphon} fucoidan inhibited \textit{H. pylori} attachment to mucin in a dose dependent manner, in the range from 10 µg/ml to 1 mg/ml (Fig. 1A). The inhibition was approximately 60% at a concentration of 100 µg/ml, and almost complete loss of attachment was noted at a concentration of 1 mg/ml. Inhibitory activity of the other fucoidan, \textit{Fucus} fucoidan, and dextran sulfate sodium were only seen under pH 2.0 (Fig. 1B,C). Under neutral conditions, however, the effects of the above three sulfated polysaccharides were weak and the inhibition was less than 25% even at a concentration of 1 mg/ml (Fig. 1A–C). Non-sulfated polysaccharides, dextran and mannan, showed no inhibitory influence under either acidic or neutral conditions at doses of 10 µg/ml to 1 mg/ml, as shown in Fig. 1(D,E).

**Prevention of \textit{H. pylori} infection in Mongolian gerbils**

The results for effects of \textit{Cladosiphon} fucoidan on \textit{H. pylori}-induced gastritis in Mongolian gerbils are shown in Table 1. With \textit{H. pylori}}
After inoculation, all gerbils developed severe gastritis with edema and hemorrhage in the glandular stomach of the *H. pylori*-inoculated control group given the vehicle alone. Microscopic erosion with infiltration of many polymorphonuclear leukocytes and lymphocytes was also observed in almost all animals of this group. Such gastric changes were evident in the pyloric region, but not in the fundic region. The microscopic score for gastritis of the vehicle group was 5–7 (6.5 ± 0.7), as shown in Fig. 2. The average stomach weight of control gerbils infected with *H. pylori* was approximately 2-fold that for animals without *H. pylori* inoculation (Fig. 3). When *Cladosiphon* fucoidan was administered from 3 days before *H. pylori* inoculation until the end of the examination at week 6, the *H. pylori*-induced gastritis was strongly suppressed in a dose-dependent manner (Table 1). At a dose of 0.05%, the number of gerbils with edema was reduced to 40%. The hemorrhagic spots were observed in only half of the animals with edema, while all animals were with hemorrhagic spots in the *H. pylori*-inoculated control group given the vehicle alone. *H. pylori* colonization was detected only in gerbils with gastric edema. The microscopic score of the animals with edema was 5–7, while that of those without edema was 0 (Fig. 2). At a dose of 0.5%, *H. pylori* colonization ratio was reduced to 20%. Gastric edema was observed in only one animal

### Table 1  Effect of fucoidan on gastritis development in *H. pylori*-inoculated Mongolian gerbils

<table>
<thead>
<tr>
<th>Fucoidan treatment</th>
<th>No. of animals with</th>
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<tr>
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<td>Edema</td>
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<td>With <em>H. pylori</em> inoculation</td>
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<td>Control</td>
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<tr>
<td>From 3 days before <em>H. pylori</em> inoculation</td>
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<tr>
<td>0.05%</td>
<td>4/10*</td>
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<tr>
<td>0.5%</td>
<td>1/10*</td>
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<tr>
<td>From 2 weeks after <em>H. pylori</em> inoculation</td>
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</tr>
<tr>
<td>0.05%</td>
<td>7/10</td>
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<tr>
<td>0.5%</td>
<td>7/10</td>
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<tr>
<td>Without <em>H. pylori</em> inoculation</td>
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<tr>
<td>Control</td>
<td>0/5</td>
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<td>0.5%</td>
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Control groups were given vehicle alone.
*The total score varies from 0 to 7, according to the criteria reported previously [19]. Data are expressed as mean ± SD.

"Significantly different (p < .05) from the *H. pylori*-inoculated control value (Fisher’s exact test)."

### Figure 1  Inhibitory effects of five polysaccharides on *H. pylori* attachment to porcine gastric mucin. *H. pylori* suspensions (107 bacteria/ml) supplemented with *Cladosiphon* fucoidan (A), *Fucus* fucoidan (B), dextran sulfate sodium (C), dextran (D) or mannan (E) were incubated in mucin-coated plates at pH 7.2 (open circle), 4.0 (solid circle), and 2.0 (solid triangle) for 2 hours. Each value represents the mean of three determinations (± SD).

### Figure 2  Scattered plots of microscopic score for gastritis developed in Mongolian gerbils. The solid circle represents the score for vehicle-treated gerbils with *H. pylori*-inoculation. Fucoidan treatment was performed starting from 3 days before *H. pylori* inoculation until the end of the experiment (hatched circle) or from 2 weeks after *H. pylori* inoculation until the end of the experiment (meshed circle) at doses of 0.05% and 0.5%. Bars represent the mean of each group. *p < .05, **p < .01 vs. the *H. pylori*-inoculated vehicle group (Steel test).
with *H. pylori* colonization. All animals in this treatment group were without hemorrhagic spots. The microscopic score of the animals with edema was 2–5, while that of those without edema was 0–1 (Fig. 2). The average stomach weights of *Cladosiphon* fucoidan-treated animals were also reduced in a dose dependent manner (Fig. 3).

In the experiment with *Cladosiphon* fucoidan given 2 weeks after the *H. pylori* inoculation, the number of animals with gastric edema and hemorrhage was decreased to 70% and 60%, respectively, by the 0.05% and 0.5% fucoidan treatment. However, there was no dose dependence or statistical significance. The stomach weights of gerbils given 0.05% and 0.5% fucoidan were reduced compared with those of controls (Fig. 3). In both of the groups with 0.05 and 0.5% fucoidan, 30% of gerbils were without *H. pylori* infection, whose microscopic score was 0. In the remaining 70%, the level of gastritis, scored 6–7 by microscopic observation, was similar to that in control gerbils without fucoidan treatment (Fig. 2).

Throughout the experiment, *Cladosiphon* fucoidan administration did not affect food intake and water intake of the animals. The body weights of the animals were also not affected by *Cladosiphon* fucoidan administration. No changes in the stomach and colon were observed with 0.5% fucoidan administration in the animals without *H. pylori* inoculation.

**Discussion**

In the present study, inhibition of *H. pylori* attachment to porcine gastric mucin could be clearly demonstrated with *Cladosiphon* fucoidan under acidic conditions by in vitro assay. Two other sulfated polysaccharides, *Fucus* fucoidan and dextran sulfate sodium, also inhibited *H. pylori* attachment to mucin only under acidic conditions. On the other hand, the nonsulfated polysaccharides, dextran and mannan, did not inhibit *H. pylori* attachment under either acidic or neutral conditions. Thus sulfated polysaccharides may generally possess a potential to inhibit *H. pylori* attachment to the gastric mucin under acidic conditions, and may prevent *H. pylori* infection in vivo. The mechanism with fucoidan is considered to involve polysaccharide attachment to the bacterial surface so that it acts competitively against mucin. We are now investigating which portion of sulfated polysaccharide molecules are required for inhibition of *H. pylori* attachment to gastric mucosa.

For the present in vivo studies of *H. pylori* infection, the Mongolian gerbil model [21] was selected. In this and other infection models, such as that in the hairless mouse [22], an excess number of *H. pylori* (10^8–10^9 CFU per animal) is generally inoculated to obtain a high infection rate. However, human beings with a normal lifestyle are never exposed to such numbers of the bacteria. To approach the actual situation of *H. pylori* infection in man, we investigated the influence of a dose of inoculated *H. pylori* in the glandular stomach of Mongolian gerbils by inoculating 10^5–10^8 CFU per animal. The prevalence of infection with 10^6 CFU per animal was more than 80%, this being suitable for animal experiments to detect substances influencing *H. pylori*-induced gastric lesions in Mongolian gerbils. To investigate preventive effects of *Cladosiphon* fucoidan, samples were dissolved in drinking water and given from 3 days before *H. pylori* inoculation until the end of the experiment (hatched circle) or from 2 weeks after *H. pylori* inoculation until the end of the experiment (meshed circle) at doses of 0.05% and 0.5%. Bars represent the mean of each group. *p < .05, **p < .01 vs. the *H. pylori*-inoculated vehicle group (Steel test).
colonization or gastritis occurrence in Mongolian gerbils. It has been reported that viable \textit{H. pylori} in gastric mucosa reaches a plateau and microscopic gastritis is first observed 2 weeks after inoculation of bacteria [21]. Consequently, our data suggest that the potential of \textit{Cladosiphon} fucoidan to eradicate \textit{H. pylori} would be weak.

Dextran sulfate sodium has been reported to be effective against \textit{H. pylori} infection in the mouse infection model [23]. However, these animals showed slight colon inflammation. In our preliminary study, Mongolian gerbils given 0.5% of dextran sulfate sodium showed colitis with infiltration of inflammatory cells into the lamina propria. Furthermore, this polysaccharide is reported to induce intestinal tumors in rats [24]. \textit{Fucus} fucoidan is also demonstrated to induce activation of inflammatory cells in vitro [13]. While dextran sulfate sodium and \textit{Fucus} fucoidan showed a potential to inhibit \textit{H. pylori} attachment to porcine gastric mucin in vitro, these polysaccharides are unsuitable as agents to be taken daily, due to their inflammatory or carcinogenic actions. In contrast, animals given \textit{Cladosiphon} fucoidan in the present study showed no colorectal lesions or changes observed in other organs. Thus \textit{Cladosiphon} fucoidan deserves particular attention as a safe sulfated polysaccharide that can prevent \textit{H. pylori} infection.

Fucoidan is a component of a large number of algae. Among the different species, \textit{Cladosiphon okamuranus} TOKIDA, frequently consumed as an edible foodstuff in Japan, contains particularly large amounts. However, even with ingestion of this seaweed, the amount of fucoidan released by our digestive processes in the stomach may be very small, because the conditions are too mild for release of this polysaccharide from algal extra cellular matrix. Thus pre-extracted fucoidans might be a useful dietary factor for anti-\textit{H. pylori} infection, leading to reduction in the risk of gastric cancer. The epidemiological data of the relation between fucoidan intake and \textit{H. pylori} infection are not available. Therefore, it is very important to investigate the effect of fucoidan on \textit{H. pylori} infection in humans.

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