Enhanced Expression of Inducible Nitric Oxide Synthase and Nitrotyrosine in Gastric Mucosa of Gastric Cancer Patients

Toyoko Goto, Ken Haruma, Yasuhiko Kitadai, Masanori Ito, Masaharu Yoshihara, Koji Sumii, Norihiko Hayakawa, and Goro Kajiyama
First Department of Internal Medicine [T. G., K. H., Y. K., M. I., M. Y., K. S., G. K.], Hiroshima University School of Medicine, and Department of Epidemiology [N. H.], Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, 734-8551, Japan

ABSTRACT
Recent studies (K. Komoto et al., Am. J. Gastroenterol., 93: 1271–1276, 1998) have shown that Helicobacter pylori infection is associated with gastric cancer. However, the mechanism of H. pylori in carcinogenesis has not been clarified. H. pylori infection leads to a sustained production of reactive nitrogen species that may contribute to cause DNA damage. In this study, we examined the expression of inducible nitric oxide synthase (iNOS) and nitrotyrosine in gastric mucosa. The expression of iNOS and nitrotyrosine was examined by immunohistochemistry in 93 patients who initially underwent gastric biopsies between 1975 and 1992. Thirty-four individuals were later found to have gastric cancer at least 2 years after the initial biopsies (group A). The other 59 subjects have shown no evidence of gastric cancer during long-term follow-up. Fifty-one of these patients were positive for H. pylori (group B), and eight were negative for H. pylori (group C). The expression of iNOS and nitrotyrosine in the gastric mucosa was significantly higher in H. pylori-positive groups A and B than in H. pylori-negative group C. Among the H. pylori-positive patients, the expression of iNOS and nitrotyrosine was significantly higher in group A than in group B. These results suggest that high production of iNOS and nitrotyrosine in the gastric mucosa infected with H. pylori may contribute to the carcinogenesis of gastric cancer.

INTRODUCTION
Gastric cancer is one of the most common malignancies in the world and is the leading cause of death in Japan (1).

Epidemiological studies have indicated that infection with Helicobacter pylori is considered a risk factor for gastric cancer (2), and the WHO IARC has classified this bacterium as a definite biological carcinogen in 1994 (3). However, the mechanisms linking H. pylori infection and gastric carcinogenesis remain unclear.

It has been proposed that reactive oxygen and nitrogen species may play a role in human carcinogenesis. The reactive nitrogen species are derived from the synthesis of NO, stimulated by iNOS (4) in a variety of cell types including activated macrophages and neutrophils. Increased iNOS activity has been observed in patients with chronic gastritis and gastric cancer (4, 5). Furthermore, recent studies have revealed that H. pylori infection leads to the formation of nitrotyrosine, which may contribute to DNA damage and apoptosis in gastric mucosa (4).

We hypothesized that H. pylori-positive subjects with high levels of reactive nitrogen species in gastric mucosa may be a high-risk group for gastric cancer. To clarify this hypothesis, we used immunohistochemical analysis to detect iNOS and nitrotyrosine in the initial gastric biopsy specimens of subjects who had been followed-up over long-term periods before developing gastric cancer.

MATERIALS AND METHODS

Study Population. From 1975 to 1992 at Hiroshima University Hospital, 7359 subjects underwent routine gastric biopsies to evaluate the grade of gastritis. During endoscopic examination, two biopsy specimens were obtained from the lesser curvature of the antrum and two from the anterior and posterior walls of the corpus. Of the total group, 4652 had no malignant lesion at the first endoscopy, and gastric cancer was detected in 83 of the subjects at various intervals after the first gastric examination. These cases were documented in the Hiroshima Tissue Registry. Malignant cases of patients residing in the city are identified through the Tissue Registry and added to the Tumor Registry file. Information placed in the Tumor Registry includes name, address, sex, date of birth, tumor site, and morphology, methods of diagnosis, and date of diagnosis. Cases identified from various hospitals and sources are collated, and data are stored in computerized files (6). In the present study, 16 cancer patients were excluded because the gastric adenocarcinoma was diagnosed less than 2 years after they underwent endoscopy. Another 33 patients were excluded because the biopsy specimens of their first endoscopic examinations could not be obtained. Therefore, we examined 34 subjects who developed gastric cancer more than 2 years after the initial biopsy (group A). All of them tested positive for H. pylori infection. The mean follow-up of group A was 18.3 years (range 13–21 years).

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2 To whom requests for reprints should be addressed, at the First Department of Internal Medicine, Hiroshima University School of Medicine, 1–2–3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan. Phone: 81-82-257-5192; Fax: 81-82-257-5194.

3 The abbreviations used are: iNOS, inducible NO synthase; NO, nitric oxide; IL, interleukin; TNF, tumor necrosis factor.
years). For control subjects, we randomly chose 59 individuals who were matched with group A in relation to age, sex, and year of initial visit and who had no macroscopic abnormalities. Fifty-one of these subjects (group B) had H. pylori infection (mean follow-up, 18.5 years; range, 13–21 years). The remaining eight (group C) were negative for H. pylori (mean follow-up, 17.4 years; range, 16–21 years). Table 1 presents the characteristics of the three groups.

**Histological Assessment.** Biopsy specimens were placed in 10% formalin, embedded in paraffin, and cut in sequential 4-μm sections. Subsequently, one section was stained with H&E and another with Giemsa. Each slide was assessed for gastric mucosal atrophy, gastritis, and H. pylori infection. In accordance with the Sydney System, the degree of mucosal atrophy, inflammation, activity (neutrophil infiltration), intestinal metaplasia, and the H. pylori colonization was classified into four grades as follows: 0, none; 1, mild; 2, moderate; 3, severe. H. pylori-positive determination was made when H. pylori was found in at least one specimen stained with Giemsa (7–10).

**Immunohistochemistry.** Immunohistochemical analysis was performed according to the method described by Mannick et al. (4) with minor modification. Fixed tissues were deparaffinized and rehydrated. Pepsin (Biomedica, Foster City, CA.) was applied to a section for 10 min at room temperature. Endogenous peroxidase was quenched using 3% H2O2 in methanol for 10 min, followed by rinsing with PBS. Nonspecific binding was blocked with PBS containing 1.5% normal goat serum for 20 min. The sections were rinsed with Tris buffer and incubated with rabbit polyclonal IgG for human iNOS (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit polyclonal IgG for nitrotyrosine (1:250) (Upstate Biotechnology, Lake Placid, NY) overnight in a humidity chamber at 4°C. Antirabbit biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) was incubated for 30 min and then rinsed three times with PBS. The sections were developed with the diaminobenzidine (DAB) chromagen (MERCK, Germany) and then counterstained with Mayer’s hematoxylin, dehydrated, and mounted.

**Grading for iNOS and Nitrotyrosine.** iNOS and nitrotyrosine slides were randomly read by two independent investigators (T. G., Y. K.) blinded to patient history with less than 10% differences between the two observers. iNOS measurements consisted of a four-level scale (0 to 3) of positive staining (0, no cells are stained; 1, a few cells are stained; 2, a moderate amount are stained; 3, almost all of the cells are stained). Nitrotyrosine scores were determined using a four-level scale (0 to 3) based on the density of positive cell staining in the surface and neck epithelium and stroma observed in three consecutive high-power fields.

**Statistics.** Results are expressed as the mean (M) ± SE. An analysis of unpaired observations between groups was performed using an unpaired t test for normally distributed data. The significance level was set at P = 0.05.

**RESULTS**

**Endoscopic Diagnosis and the Prevalence of H. pylori Infection.** Endoscopic examination and four-specimen biopsy were performed in the 34 patients of group A more than 2 years before the diagnosis of gastric cancer. Thirty-one (91.2%) of them originally revealed no localized lesion in the stomach, and the other 3 had benign diseases (gastric ulcer, duodenal ulcer, and gastric polyp). Every subject in group A was infected with H. pylori (Table 1). Localized gastric disease has not been detected in any of the subjects in groups B and C in the long-term follow-up. There were no differences in age and sex among these groups.

**Histological Score and H. pylori Colonization.** Next, we compared the degree of atrophy, intestinal metaplasia, inflammation, and neutrophil activity between the groups. As shown in Table 2, groups A and B had a significantly high degree of inflammation and atrophy and H. pylori colonization in the corpus and antrum compared with that found in group C (P < 0.01). The degree of intestinal metaplasia and activity in the corpus and antrum was significantly higher in group A and group B than in group C (P < 0.05). There was no significant difference in the gastric score and degree of H. pylori colonization between group A and group B.

**Immunohistochemistry.** Using immunohistochemistry, we detected positive staining for iNOS and nitrotyrosine in gastric biopsies from patients in all of the groups. Representative pictures of immunohistochemistry for iNOS and nitrotyrosine in group A are shown in Fig. 1. iNOS immunoreactivity

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**Table 1** Characteristics of the patients in the three groups

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>34</td>
<td>51</td>
<td>8</td>
</tr>
<tr>
<td>Age (range) at first visit</td>
<td>56.5 (30–77)</td>
<td>55.9 (34–72)</td>
<td>56.4 (36–72)</td>
</tr>
<tr>
<td>Men/Women</td>
<td>18/16</td>
<td>25/26</td>
<td>6/2</td>
</tr>
<tr>
<td>H. pylori infection</td>
<td>positive</td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>Endoscopic Dx</td>
<td>Normal</td>
<td>31</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Gastritis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ga</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Du</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Gastric polyp</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2** Comparison of scorea of gastritis and H. pylori colonization of the three groups

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrophy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antrum</td>
<td>2.35 ± 0.15b</td>
<td>1.96 ± 0.13b</td>
<td>0.38 ± 0.38</td>
</tr>
<tr>
<td>Corpus</td>
<td>1.91 ± 0.19b</td>
<td>1.70 ± 0.15b</td>
<td>0.25 ± 0.25</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antrum</td>
<td>1.17 ± 0.19c</td>
<td>1.21 ± 0.17c</td>
<td>0.13 ± 0.13</td>
</tr>
<tr>
<td>Corpus</td>
<td>0.66 ± 0.15c</td>
<td>0.53 ± 0.13c</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antrum</td>
<td>1.86 ± 0.14c</td>
<td>1.65 ± 0.13c</td>
<td>0.25 ± 0.25</td>
</tr>
<tr>
<td>Corpus</td>
<td>1.73 ± 0.17c</td>
<td>1.47 ± 0.12c</td>
<td>0.25 ± 0.25</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antrum</td>
<td>1.14 ± 0.18c</td>
<td>0.73 ± 0.15c</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Corpus</td>
<td>1.21 ± 0.20c</td>
<td>0.96 ± 0.14c</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>H. pylori</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antrum</td>
<td>1.44 ± 0.13b</td>
<td>1.82 ± 0.10b</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Corpus</td>
<td>1.58 ± 0.13b</td>
<td>1.73 ± 0.11b</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

* Mean ± SE.

b P < 0.01, compared with Group C.

P < 0.05, compared with Group C.
was predominantly localized in inflammatory cells including polymorphonuclear leukocytes and mononuclear cells; nitrotyrosine staining was observed in epithelial cells, inflammatory cells, and components of the extracellular matrix. The iNOS and nitrotyrosine scores in group A were significantly higher than those in group B or group C. These differences were observed in both the antrum and corpus. Comparison of iNOS and nitrotyrosine expression between groups is shown in Table 3 and in Figs. 2 and 3. There is significant association between the grade of iNOS and the grade of nitrotyrosine in both antrum and corpus by contingency table analysis ($P < 0.01$).

### DISCUSSION

*Helicobacter pylori* infection leads to type B chronic gastritis, which slowly develops into atrophic gastritis and subsequently into intestinal metaplasia in a significant number of *H. pylori*-infected subjects (11, 12). A model proposed for the etiology of stomach cancer contains a sequence of events progressing from inflammation to atrophy, to metaplasia, to dysplasia, to carcinoma *in situ*, and finally to the possibility of gastric carcinoma (13–16). Although *H. pylori* has been reported to be associated with an increased risk of gastric carcinoma, most persons infected with *H. pylori* will never have gastric carcinoma. Therefore, other factors that increase the risk of gastric carcinoma among those infected with *H. pylori* may be assumed to exist.

It has been previously reported (17–22) that NO plays an important role in carcinogenesis as a mediator of carcinogenic nitrosamine formation, DNA damage, and tissue injury associ-
In the present study, we demonstrated that the expression of nitrotyrosine in both the antrum and corpus of gastric mucosa. The grade of nitrotyrosine staining in group A (cancer group) was greater than that in group B (H. pylori-positive control group) or group C (H. pylori-negative control group); **, P < 0.01. The grade of iNOS staining in group B was greater than that in group C; *, P < 0.05.

Fig. 3 The expression of nitrotyrosine in both the antrum and corpus of gastric mucosa. The grade of nitrotyrosine staining in group A (cancer group) was greater than that in group B (H. pylori-positive control group) or group C (H. pylori-negative control group); **, P < 0.01. The grade of iNOS staining in group B was greater than that in group C; *, P < 0.05.

Recent studies (23–25) have revealed that H. pylori infection in humans is associated with the enhanced expression of iNOS by tissue neutrophils and mononuclear cells.

In the present study, we demonstrated that the expression level of iNOS and nitrotyrosine in the gastric mucosa was significantly higher in patients with H. pylori infection (groups A and B) than in those without it (group C). These results correspond well with those reported by Mannick et al. (4), who concluded that H. pylori infection is accompanied by the formation of endogenous reactive nitrogen intermediates, which may contribute to DNA damage and apoptosis. More interestingly, when we limited the analysis to H. pylori-positive patients, significant differences in the staining of iNOS and nitrotyrosine were found between group A (cancer group) and group B (H. pylori-positive control group). The histological scores for inflammation, activity, atrophy, intestinal metaplasia, and H. pylori colonization in group A were similar to those in group B.

It is not clear why the grade of iNOS and nitrotyrosine in group A was higher than that in group B. Blaser et al. (26) reported that infection with H. pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. H. pylori strains with cagA gene induce inflammatory cytokines—such as IL-1, IL-6, IL-8, and TNF—at high levels as compared with cagA-negative strains (26). Because IL-1, TNF, and IFN influence iNOS production by activating macrophages and neutrophils (27, 28), H. pylori strains may be associated with differences in the grade of iNOS and nitrotyrosine. In addition, host immunoresponses may be different individually.

In conclusion, high production of reactive nitrogen species in the gastric mucosa may be one of the factors that contribute to gastric carcinogenesis. Patients with high scores for iNOS and nitrotyrosine should be carefully followed-up on a long-term basis.

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