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Nonsteroidal anti-inflammatory drug-induced acute gastric injury in *Helicobacter pylori* gastritis in Mongolian gerbils

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Abstract

We examined the acute ulcerogenic effects of indomethacin and *N*-(2,cyclohexyloxy-4-nitrophenyl)methane sulfonamide (NS-398) on the gastric mucosa in *Helicobacter pylori*-infected Mongolian gerbils. *H. pylori* infection for 4 and 12 weeks caused moderate and severe gastritis, respectively, with cyclooxygenase-2 expression and an increase in prostaglandin E_2 production. In normal animals, gastric injury was caused by indomethacin, but not by NS-398. At 4 weeks infection, gastric lesions were synergistically aggravated by indomethacin, and NS-398 at high doses. However, at 12 weeks, the synergistic effects of indomethacin and NS-398 with *H. pylori* were not observed. Indomethacin and NS-398 at high doses inhibited prostaglandin E_2 production in both normal and the infected mucosa. NS-398 at low dose reduced only the *H. pylori*-increased prostaglandin production. These results suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) synergistically aggravate gastric lesions in moderate *H. pylori* gastritis, but not in severe gastritis. Cyclooxygenase-2 inhibition only might not induce acute gastric injury in *H. pylori* gastritis.

Keywords: *Helicobacter pylori*; NSAID; Prostaglandin; Cyclooxygenase; Gastric ulceration

1. Introduction

Helicobacter pylori and nonsteroidal anti-inflammatory drugs (NSAIDs) are major risk factors in the pathogenesis of gastric injury and ulcers in humans. Both the American and European consensus have recommended eradication of *H. pylori* in NSAID users, but the synergistic interaction between *H. pylori* and NSAIDs remains controversial. Some groups reported that NSAID-induced gastric injury and ulcers are markedly aggravated by *H. pylori* infection (Chan et al., 1997; Santucci et al., 1995b; Taha et al., 1992, 1999). The other groups have shown that there is no convincing evidence for synergy between the ulcerogenic effects of *H. pylori* and NSAIDs (Goggin et al., 1993; Graham et al., 1991; Hawkey et al., 1998; Lipscomb et al., 1996; Yeomans et al., 1998). This discrepancy is not clear, but one of the reasons is likely to be that the definitive studies have not been performed (Veldhuyzen van Zanten, 1997), i.e., there were differences of lifestyle, type and dosage of NSAIDs, duration of NSAID administration, the strain of *H. pylori*, eradication therapy or the severity of *H. pylori*-associated gastric diseases. Moreover, the diversity of human races may also be related to the different results. To evaluate the synergistic interaction between *H. pylori* and NSAIDs under the condition where the above parameters can be uniformly arranged, we used a Mongolian gerbil model of *H. pylori* infection. The gerbil model is quite useful because it exhibits pathological features that mimic those of human patients with *H. pylori* infection. Gastritis, gastric ulcers and cancers are all generated in *H. pylori*-infected gerbils, whereas only gastritis is observed in the mice and rats with *H. pylori* infection (Hirayama et al., 1996; Ikeno et al., 1999; Keto et al., 1999; Takahashi et al., 1998a,b; Watanabe et al., 1998).

In addition, recent studies revealed that cyclooxygenase-2 is induced in the gastric mucosa of *H. pylori*-infected patients (McCarthy et al., 1999; Sawaoka et al., 1998; Fu et al., 1999). Several cyclooxygenase-2-selective inhibitors are used as new NSAIDs without ulcerogenic

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effects. However, cyclooxygenase-2-selective NSAIDs as well as conventional NSAIDs also impair the healing of pre-existing gastric ulcers in mice and rats (Mizuno et al., 1997; Schmassmann et al., 1998; Shigeta et al., 1998). It is an important clinical issue whether or not cyclooxygenase-2-selective NSAIDs affect *H. pylori*-induced gastric diseases, but their ulcerogenic effects on the *H. pylori*-infected gastric mucosa remain undefined.

In the present study, we examined the acute ulcerogenic effects of indomethacin (a conventional NSAID) and *N*-(2,cyclohexyloxy-4-nitrophenyl)methane sulfonamide (NS-398; a cyclooxygenase-2-selective NSAID) on *H. pylori*-infected gastric mucosa of Mongolian gerbils.

2. Materials and methods

2.1. Animals

Male Mongolian gerbils (6 weeks old, 40–50 g) were obtained from Nihon SLC (Hamamatsu, Japan). The animals were kept in an isolated clean room with regulated temperature (20–22°C) and humidity (approximately 55%) with12/12-h light/dark cycle. The animals were fasted for 24 h before *H. pylori* inoculation, and drinking water was also withheld after the inoculation. From 4 h after the inoculation, both food and water were freely available to the animals. In the case of NSAID-induced gastric injury, the animals were deprived of food for 24 h and of water for 6 h before administration of drugs.

2.2. H. pylori preparation and inoculation to Mongolian gerbils

The preparation and inoculation of *H. pylori* were performed as described previously (Takahashi et al., 1998a). A *cagA*- and *vacA*-positive standard strain of *H. pylori* (NCTC11637; American Type Culture Collection, Rockville, MD) was used. The bacteria were incubated in brain–heart infusion broth (Difco Laboratories, Detroit, MI) containing 10% fetal bovine serum (GIBCO, Gaithersburg, MD) at 37°C overnight under a microaerophilic atmosphere and allowed to grow to a density of approximately 2.0×10^8 colony-forming units/ml. *H. pylori* (2.0×10^8 colony-forming units, 1.0 ml) was orally inoculated to each animal. Normal animals received 1.0 ml of the medium alone and used 4 weeks later.

2.3. Evaluation of H. pylori-induced gastritis

Four gastric specimens were taken from the fundus of normal and *H. pylori*-infected animals and fixed in 10% formalin. Thereafter, 4 μm paraffin sections were prepared and stained with hematoxylin and eosin. Histological features of mucosal inflammation and epithelial damage were graded 0–3 for each specimen, and the median score was used. The investigator determining gastric pathology was unaware of the treatment given to the animals. According to the Sydney system (Price, 1991), neutrophil infiltration into the mucosa was evaluated as follows: 0, none; 1, mild; 2, moderate; and 3, severe. As described by Atherton et al. (1997), epithelial damage was graded as follows: 0, no exfoliation; 1, exfoliation of $< 30\%$ of epithelium; 2, exfoliation of 30–70% of epithelium; and 3, exfoliation of $> 70\%$ of epithelium.

2.4. Western blot analysis of cyclooxygenase-1 and cyclooxygenase-2 proteins

Expression of cyclooxygenase-1 and cyclooxygenase-2 proteins was examined by Western blotting. Gastric specimens were taken from normal and *H. pylori*-infected animals. Cyclooxygenase proteins were partially purified according to the method of Gierse et al. (1995). In brief, the specimens were homogenized in 25 mM Tris–HCl (pH 8.0 buffer containing 250 mM sucrose, followed by centrifugation at $10{,}000 \times g$ for 20 min. The pellet was resuspended in 25 mM Tris–HCl (pH 8.0) buffer containing 1% 3-[(3-cholamidopropyl)]dimethylammonio]-1-propanesulfonate (CHAPS), and the mixture was gently stirred for 2 h at 4°C. The supernatant was recovered after centrifugation at $30{,}000 \times g$ for 30 min and applied onto a DEAE-Sepharose CL-4B column (Amersham Pharmacia, Buckinghamshire, UK) which had been equilibrated with 25 mM Tris–HCl (pH 8.0) buffer containing 0.5% CHAPS, 1 mM phenylmethylsulfonyl fluoride and 0.2 mM EDTA. After the column was washed with the same buffer supplemented with 50 mM NaCl, elution was carried out with 200 mM NaCl. After aliquots (20 μg) of the eluted proteins had been subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (10%), the separated proteins were electrophoretically transferred onto Hybond-P membranes (Amersham Pharmacia). The membranes were incubated with the antibodies (Cayman, Ann Arbor, MI) against cyclooxygenase-1 or cyclooxygenase-2 protein after nonspecific binding sites had been blocked with non-fat milk. Cyclooxygenase proteins were detected on X-ray films (Fuji Film, Tokyo, Japan) with an enhanced chemiluminescence kit (Immuno Star, Wako, Osaka, Japan).

2.5. Evaluation of NSAID-induced gastric mucosal injury

Six hours after NSAID administration, the animals were sacrificed, and then their stomachs were excised. The stomachs were opened along the greater curvature and spread out with pins on a cork board. The area (mm^2) of mucosal erosive lesions was measured blindly under a dissecting microscope with a squared grid (X10; Olympus, Tokyo, Japan).

2.6. Determination of prostaglandin E_2 production

Gastric specimens were taken from normal and *H. pylori*-infected animals. After washing with phosphate-buffered saline, the tissues were incubated in 1 ml of Dulbecco's modified Eagle's medium supplemented with 2.5% fetal bovine serum at 37°C for 1 h under 5% CO_2 in air. The amount of prostaglandin E_2 in the medium was determined by enzyme immunoassay (prostaglandin E_2 EIA kit; Cayman). Prostaglandin E_2 production was expressed as picograms prostaglandin E_2 per milligrams tissue per hour.

2.7. Drugs

Indomethacin (Sigma) and NS-398 (kindly synthesized by Nippon Chemiphar, Tokyo, Japan) were finely dispersed in a trace of Tween 80 (about 50 μl) and then suspended by adding saline to the desired concentrations. The drugs were administered subcutaneously in a volume of 20 ml/kg body weight. Control animals received the vehicle alone.

2.8. Statistical analysis

Data are presented as means ± S.E. from six to eight animals per group. Statistical differences in the dose–response studies were evaluated by Dunnett's multiple comparison test. Student's *t*-test was used for the comparison between two groups. Mann–Whitney *U*-test was also applied to the comparison of scores. P value of < 0.05 was regarded as significant.

3. Results

3.1. Cyclooxygenase-2 expression in the gastric mucosa of *H. pylori*-infected gerbils

In our model of *H. pylori* infection, *H. pylori* is detected for at least 10 months in the gastric mucosa of all gerbils given the bacteria (Keto et al., 1999; Takahashi et al., 1998a). The number of viable *H. pylori* in the stomach reached a plateau level at around 1×10^5 colony-forming units/stomach from 2 weeks after the inoculation. Fig. 1 shows *H. pylori*-induced gastric pathology in Mongolian gerbils. At 4 weeks of *H. pylori* infection, both neutrophil infiltration into the mucosa and superficial mucosal damage were found to be moderate. Such pathological changes became more severe after 12 weeks infection. These results indicate that moderate and severe gastritis occurs at 4 and 12 weeks of *H. pylori* infection, respectively.

We examined the expression of cyclooxygenase-1 and cyclooxygenase-2 proteins in the gastric mucosa by means of Western blot analysis (Fig. 2A). Cyclooxygenase-1 protein was observed both in normal and the *H. pylori*-infected mucosa. The expression of cyclooxygenase-1 protein was kept in the same level during the infection. Cyclooxygenase-2 protein was not detected in normal mucosa, but *H. pylori* infection for more than 4 weeks caused the expression of cyclooxygenase-2 protein. The cyclooxygenase-2 level at 12 weeks was higher than that at 4 weeks. In addition, prostaglandin E_2 production in the mucosa was elevated by *H. pylori* infection for more than 4 weeks, compared with normal level, and was associated with cyclooxygenase-2 expression (Fig. 2B).

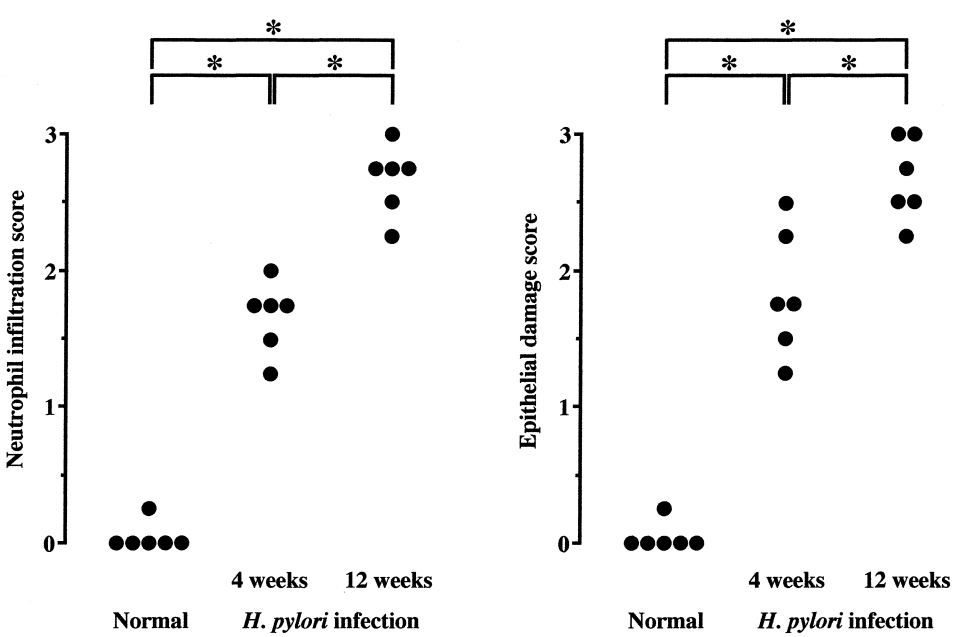

Fig. 1. *H. pylori*-induced gastric pathology in Mongolian gerbils. Neutrophil infiltration into the gastric mucosa and epithelial damage were evaluated in the gerbils with *H. pylori* infection for 4 and 12 weeks. Data are presented as means ± S.E. ($n = 6$). * Significant differences between the indicated groups.

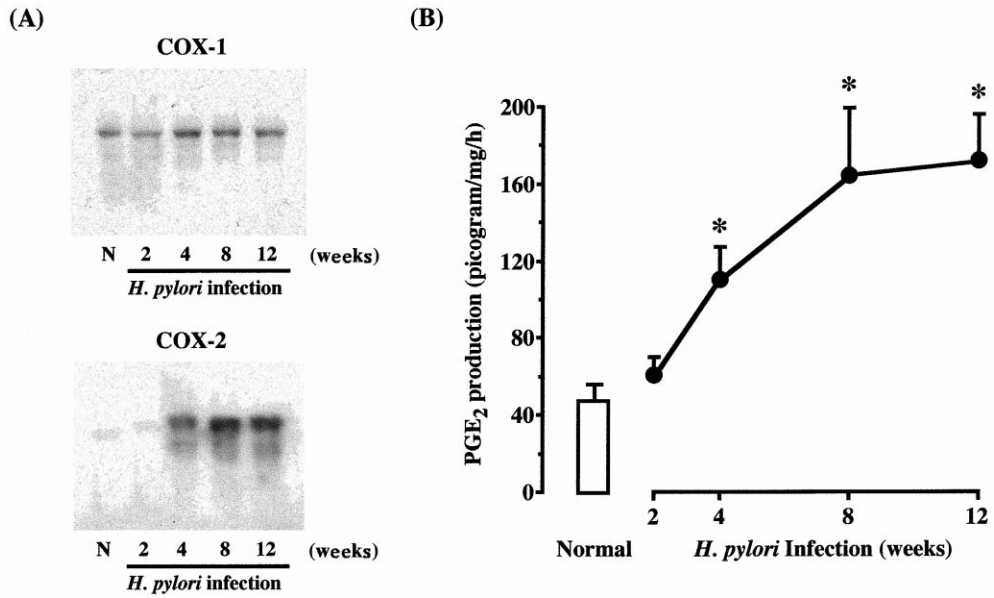

Fig. 2. Cyclooxygenase protein expression and prostaglandin E_2 production in the gastric mucosa of *H. pylori*-infected gerbils. (A) Expression of cyclooxygenase-1 and cyclooxygenase-2 proteins was examined by Western blot analysis. N represents normal mucosa. (B) Prostaglandin E_2 production in normal and *H. pylori*-infected mucosa was determined. Data are presented as means ± S.E. ($n = 5$). * Significant differences from normal mucosa.

3.2. Acute gastric injury caused by indomethacin and NS-398 in H. pylori-infected gerbils

We examined the acute ulcerogenic effects of indomethacin and NS-398 on the gastric mucosa of *H. pylori*-infected gerbils. In normal animals, indomethacin at 10 mg/kg did not cause any gastric injury, while higher doses of indomethacin induced erosive lesions in a dose-dependent manner (Fig. 3). In contrast, NS-398 had a negligible gastric side-effect even at 50 mg/kg. The lesion area caused by 50 mg/kg indomethacin and 50 mg/kg NS-398 was 7.4 ± 1.3 and 0.5 ± 0.3 mm^2, respectively.

H. pylori infection for 4 weeks caused small gastric lesions to appear, the area being 1.3 ± 0.5 mm^2. Over the

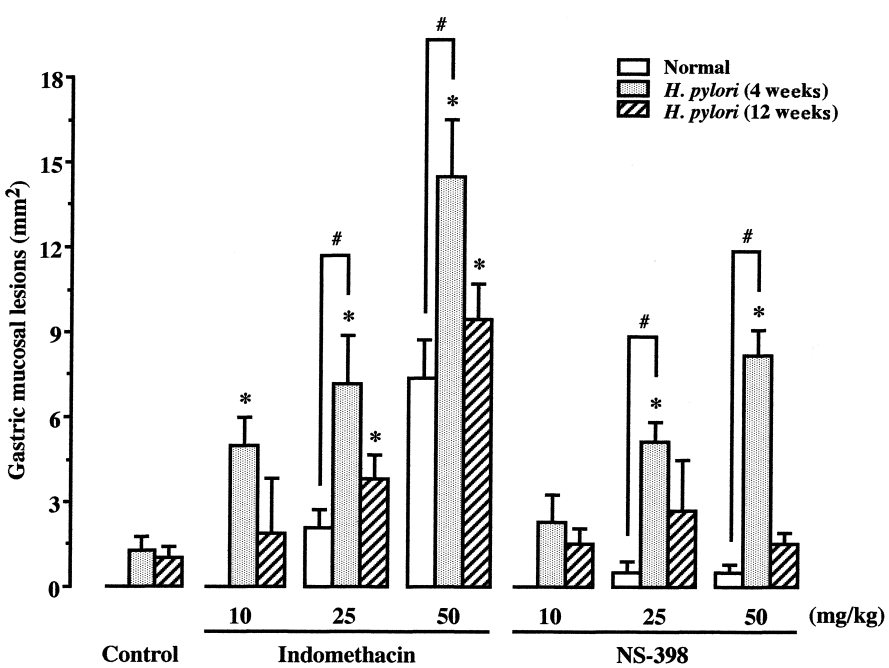

Fig. 3. NSAID-induced gastric injury in normal and *H. pylori*-infected gerbils. Indomethacin and NS-398 were subcutaneously administered to normal gerbils, and the gerbils with *H. pylori* infection for 4 and 12 weeks, and the area of gastric lesions was determined 6 h later. Data are presented as means ± S.E. ($n = 8$). *, #Significant differences from the corresponding control and normal animals, respectively.

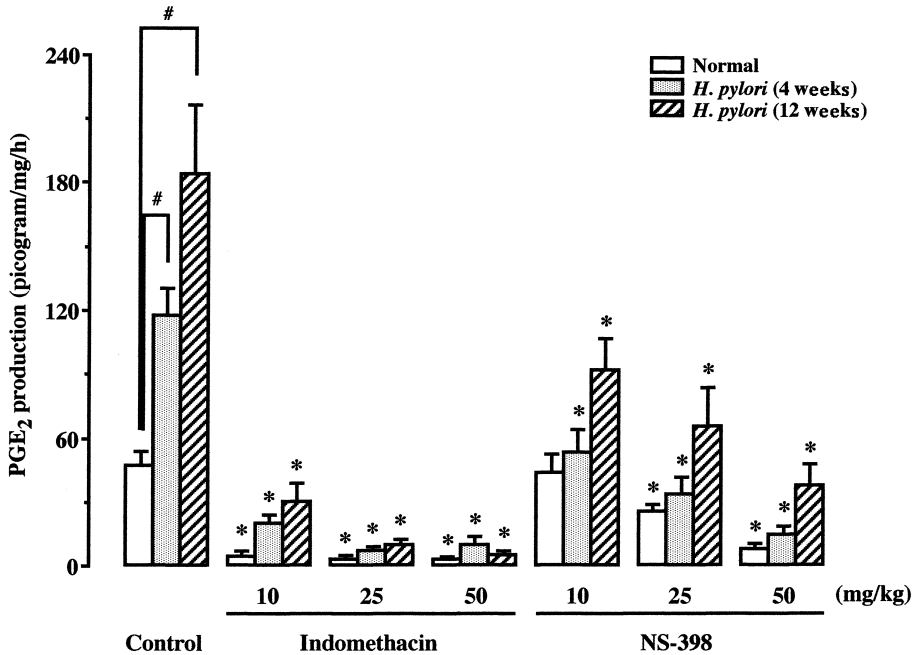

Fig. 4. Effect of NSAIDs on prostaglandin E_2 production in the gastric mucosa of *H. pylori*-infected gerbils. Indomethacin and NS-398 were subcutaneously administered to normal gerbils, and the gerbils with *H. pylori* infection for 4 and 12 weeks and prostaglandin E_2 production in the gastric mucosa was determined 6 h later. Data are presented as means ± S.E. ($n = 8$). *, #Significant differences from the corresponding control and normal animals, respectively.

range that was tested, indomethacin dose-dependently and significantly aggravated the *H. pylori*-induced gastric injury, compared with the control. It should be noted that the ulcerogenic effect of indomethacin was quite stronger in the gerbils with *H. pylori* infection for 4 weeks, compared with normal gerbils. The lesion area was 14.5 ± 2.0 mm^2 at 50 mg/kg indomethacin. NS-398 at 10 mg/kg only slightly worsened the *H. pylori*-induced gastric injury. At higher doses, the drug significantly potentiated the injury, although the effect of NS-398 was weaker than that of indomethacin. The lesion area was 8.2 ± 0.9 mm^2 at 50 mg/kg NS-398.

Similarly, there were small erosions in the gerbil stomach with *H. pylori* infection for 12 weeks, the area being 1.0 ± 0.4 mm^2. Gastric lesions were increased by indomethacin in a dose-dependent manner, but the synergistic interaction between the ulcerogenic effects of indomethacin and *H. pylori* infection was not observed. In contrast, NS-398 did not affect the *H. pylori*-induced gastric injury. The lesion area caused by 50 mg/kg indomethacin and 50 mg/kg NS-398 was 9.4 ± 1.2 and 1.5 ± 0.4 mm^2, respectively.

3.3. Effects of H. pylori infection and NSAIDs on mucosal prostaglandin E_2 production

We examined prostaglandin E_2 production in gastric tissues of the animals given indomethacin or NS-398 (Fig. 4). Mucosal prostaglandin E_2 production was significantly higher in *H. pylori*-infected gerbils than in normal ones. Prostaglandin E_2 production in normal and the *H. pylori*-infected (4 and 12 weeks infection) mucosa amounted to 46.6 ± 6.1, 117.4 ± 12.3 and 183.6 ± 32.3 picograms/mg/h, respectively. Indomethacin even at 10 mg/kg potentially inhibited prostaglandin E_2 production in both normal and the *H. pylori*-infected gerbils. At 25 and 50 mg/kg, it produces nearly complete inhibition of prostaglandin E_2 production. NS-398 at 10 mg/kg did not affect prostaglandin E_2 production in normal animals, but significantly reduced the *H. pylori*-increased prostaglandin E_2 production. The drug at 25 and 50 mg/kg dose-dependently and significantly inhibited both the normal and the *H. pylori*-increased prostaglandin E_2 production.

4. Discussion

In the present study, *H. pylori* infection for more than 4 weeks caused cyclooxygenase-2 expression in the gastric mucosa of Mongolian gerbils. In contrast, cyclooxygenase-1 protein was constitutively present in both normal and *H. pylori*-infected mucosa, and its level remained constant during *H. pylori* infection. Recent studies with human gastric specimens also revealed that cyclooxygenase-2 expression is absent in normal mucosa, but profoundly induced in *H. pylori*-positive gastritis (McCarthy et al., 1999; Sawaoka et al., 1998; Sidong et al., 1999). Prostaglandin E_2 production was elevated with cyclooxygenase-2 protein expression, and the increased production was markedly inhibited by NS-398 at 10 mg/kg, at the

dose of which prostaglandin E_2 production in normal mucosa was unaffected. These results indicate that cyclooxygenase-2 significantly contributes to the elevation of prostaglandin E_2 production in the gastric mucosa with *H. pylori* infection. Romano et al. (1998) reported that adhesion of *H. pylori* on cultured gastric cancer MKN28 cells results in cyclooxygenase-2 mRNA expression. However, it is unclear whether the direct effect of *H. pylori* on gastric cells is crucial for cyclooxygenase-2 expression in vivo, because cyclooxygenase-2 protein was not expressed even at 2 weeks of *H. pylori* infection in our model. Cyclooxygenase-2 expression may result from *H. pylori*-associated mucosal inflammation, because 4 weeks of *H. pylori* were required for gastritis and clycooxygenase-2 expression. It is also known that *H. pylori* infection causes the expression of cytokines such as interleukin-1 and tumor necrosis factor-α which serve as potent cyclooxygenase-2 inducers (Takahashi et al., 1998b; Yamaoka et al., 1995). Further investigation is needed to clarify the relationship between *H. pylori* infection and cyclooxygenase-2 expression in vivo.

In response to *H. pylori* infection, mucosal defense mechanism might be activated. Since prostaglandins are important defensive factors in the gastric mucosa (Eberhart and DuBois, 1995; Robert et al., 1979), cyclooxygenase-2 induction is reasonable for protection of the mucosa against *H. pylori*. Prostaglandin E_2 increases blood flow and secretion of mucus and bicarbonate, inhibits acid secretion, and directly protects gastric cells against toxic stimuli (Eberhart and DuBois, 1995). We have reported that *H. pylori* infection for 2 and 4 weeks causes an increase in mucus synthesis in the gastric mucosa of gerbils, and that both NS-398 and indomethacin suppress the increased mucus synthesis (Takahashi et al., 1997).

We found that the NSAID-induced acute gastric injury appears more facilely and severely in moderate *H. pylori* gastritis than in both normal mucosa and severe *H. pylori* gastritis. In clinical studies, it remains controversial whether conventional NSAID-induced gastric injury is aggravated by *H. pylori* infection. Several groups reported that incidence of gastric injury and ulcers by NSAIDs is significantly higher in *H. pylori*-positive patients than in *H. pylori*-negative ones (Chan et al., 1997; Santucci et al., 1995b; Taha et al., 1992, 1999). In contrast, there has been no convincing evidence for the synergistic interaction between the ulcerogenic effects of *H. pylori* and NSAIDs (Goggin et al., 1993; Graham et al., 1991; Hawkey et al., 1998; Lipscomb et al., 1996; Yeomans et al., 1998). Based on our results in this study, the discrepancy between the above clinical reports may be due to the severity of gastritis or the term of *H. pylori* infection, i.e., NSAIDs and *H. pylori* synergistically induce gastric injury in moderate gastritis, whereas there is no interaction between them in severe gastritis.

NS-398 is a cyclooxygenase-2-selective inhibitor and exhibits an anti-inflammatory action without an ulcerogenic effect in normal animals (Futaki et al., 1993, 1994). Cyclooxygenase-2-selective NSAIDs such as nimesulide and celecoxib are clinically used as new anti-inflammatory drugs, and the incidence of their gastric side-effects is considerably lower than those of conventional NSAIDs. Likewise, NS-398 even at higher doses did not induce the formation of gastric lesions in normal gerbils. NS-398 at 10 mg/kg, at the dose of which only cyclooxygenase-2 activity was inhibited, failed to aggravate the *H. pylori*-induced gastric lesions. This indicates that the inhibition of cyclooxygenase-2 activity only might not aggravate gastric injury in *H. pylori*-infected mucosa. However, gastric lesions in moderate *H. pylori* gastritis were worsened by 25 and 50 mg/kg NS-398, at the dose of which it inhibited both cyclooxygenase-1 and cyclooxygenase-2 activities. Wallace et al. (1998) reported that, to achieve desirable anti-inflammatory effects, cyclooxygenase-2-selective NSAIDs are needed to be given at high doses in which both cyclooxygenase-1 and cyclooxygenase-2 activities are inhibited.

Several mechanisms concerning the acute ulcerogenic effects of NSAIDs on the gastric mucosa have been proposed (Appleyard et al., 1996; Santucci et al., 1995a; Takeuchi et al., 1986; Wallace et al., 1990), but are not fully understood. It is evident that acute gastric injury is not caused or worsened solely by cyclooxygenase inhibition. Takeuchi et al. (1986) reported that indomethacin-induced gastric injury is not correlated with mucosal prostaglandin E_2 and 6-keto-prostaglandin F_{1a} level in rats. Using cyclooxygenase-1 gene knock-out mice, Langenbach et al. (1995) clearly revealed that gastric injury is not generated by prostaglandin deficiency, while indomethacin causes mucosal lesions. In this study, the inhibition of mucosal prostaglandin E_2 production is not correlated with gastric lesions in both the cases of normal and *H. pylori*-infected animals, although the ulcerogenic effects of NSAIDs were observed with cyclooxygenase-1 inhibition. The inhibition of cyclooxygenase-1-dependent prostaglandin E_2 production in the mucosa may trigger the induction of NSAID injury, but other factors such as tumor necrosis factor-α may be important for the acute ulcerogenic effects of NSAIDs in *H. pylori*-infected as well as normal animals (Appleyard et al., 1996; Santucci et al., 1995a). It still remains unclear how *H. pylori* infection causes gastric mucosal erosions. It is highly suspected that cytokine-induced inflammatory responses might be involved in the pathogenesis of *H. pylori*-induced gastric injury (Blaser, 1992; Crabtree, 1998; Ernst et al., 1997; Yokota et al., 1999). *H. pylori*-induced epithelial damage was associated with the severity of mucosal inflammation, but there was no correlation between NSAID-induced mucosal lesion area and the severity of pre-existing mucosal inflammation. It is suggested that there may be a key factor, which causes microvascular damage in the formation of *H. pylori*-induced gastric erosions. Gastric lesion formation caused by NSAIDs and/or *H. pylori*, therefore,

may depend on the production and interaction of the unknown causal factors. Further investigation will be needed to clarify the ulcerogenic effects of NSAIDs and *H. pylori*.

In conclusion, the present result suggest that NSAIDs synergistically aggravate gastric lesions in moderate *H. pylori* gastritis, but there is no synergistic interaction between NSAIDs and *H. pylori* in severe gastritis. In addition, cyclooxygenase-2 inhibition only might not induce acute gastric injury even in *H. pylori* gastritis.

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