FR167653, a p38 Mitogen-Activated Protein Kinase Inhibitor, Prevents *Helicobacter pylori*-Induced Gastritis in Mongolian Gerbils

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ABSTRACT

FR167653 was discovered as a cytokine production inhibitor, but its target molecule has remained unclear. We examined the effect of FR167653 on activities of purified protein kinases. FR167653 dose dependently inhibited p38α mitogen-activated protein kinase activity without affecting the activities of other kinases. FR167653 had no effect on cyclooxygenase (COX)-1 or COX-2 activities, whereas SB203580 inhibited them. FR167653 suppressed endogenous p38 kinase activity in interleukin-1-stimulated NRK-F cells. These results indicate that FR167653 is a p38 kinase-selective inhibitor without affecting COX activity. To evaluate the role of p38 kinase in *Helicobacter pylori*-induced gastritis, we therefore examined the effect of FR167653 on *H. pylori*-induced gastritis in Mongolian gerbils. *H. pylori* infection activated p38 kinase in the gastric mucosa and caused neutrophil infiltration from 2 and 3 weeks of infection, respectively. At 4 weeks, severe mucosal inflammation with erosive injury was observed. When FR167653 was administered to *H. pylori*-infected gerbils from 2 weeks, both neutrophil infiltration and mucosal injury at 4 weeks were significantly prevented. FR167653 markedly reduced the *H. pylori*-induced increase in endogenous p38 kinase activity in the gastric mucosa, and also significantly inhibited neutrophil chemokine production. In contrast, the drug did not affect *H. pylori* colonization or acid secretion. FR167653 did not cause any pathological change in the gastric mucosa of normal animals. These results indicate that p38 kinase plays a crucial role in *H. pylori*-induced gastritis in Mongolian gerbils.

FR167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-c][1,2,4]triazin-2-yl]-2-phenylethanediole sulfate monohydrate) (Fig. 1A) was first discovered to be a potent inhibitor of interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) production in lipopolysaccharide (LPS)-stimulated human monocytes and phytohemagglutinin-M-stimulated human lymphocytes (Yamamoto et al., 1996). Takahashi et al. (1998b) and Kawano et al. (1999) also confirmed the inhibitory effect of FR167653 on cytokine production. FR167653 ameliorates endotoxin shock in rabbits and intravascular coagulation in rats (Yamamoto et al., 1996, 1997), and also ameliorates cardiac dysfunction caused by chronic infusion of LPS in rats (Gardiner et al., 1999). Furthermore, FR167653 protects lung, liver, and heart against ischemia-reperfusion injury in dogs (Kamoshita et al., 1997; Kobayashi et al., 1998; Koyano et al., 1998). However, the target molecule of FR167653 has remained unknown. We noticed that the pharmacological characteristics and chemical structure of FR167653 resemble those of pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase (MAPK)/stress-activated protein kinase (SAPK)-2, such as SB203580 and RWJ67657 (Lee et al., 1993; Cuenda et al., 1995; Wadsworth et al., 1999). p38 kinase inhibitors exhibit anti-inflammatory effects through inhibiting IL-1 and TNF-α production in a variety of models (Lee et al., 1993; Badger et al., 1996; Jackson et al., 1998; Wadsworth et al., 1999). In the present study, we examined the effect of FR167653 on various protein kinases and found that FR167653 is also a p38 kinase-selective inhibitor.

*Helicobacter pylori* is recognized as a major etiological factor of chronic gastritis and gastric/duodenal ulcers. *H. pylori* infection causes marked infiltration of neutrophils into the gastric mucosa, and expression of many cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), mitogen-activated protein kinase (MAPK), stress-activated protein kinase (SAPK), extracellular signal-regulated kinase (ERK), cyclooxygenase (COX), and myeloperoxidase (MPO). *H. pylori* infection activates mitogen-activated protein kinase (MAPK), stress-activated protein kinase (SAPK), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK), and also significantly inhibits neutrophil chemokine production. In contrast, the drug did not affect *H. pylori* colonization or acid secretion. FR167653 did not cause any pathological change in the gastric mucosa of normal animals. These results indicate that p38 kinase plays a crucial role in *H. pylori*-induced gastritis in Mongolian gerbils.

**ABBREVIATIONS:** IL, interleukin; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; COX, cyclooxygenase; MPO, myeloperoxidase; CINC, cytokine-induced neutrophil chemoattractant.
IL-1, IL-8, and TNF-α (Blaser, 1992; Noach et al., 1994; Yamaoka et al., 1995; Crabtree, 1998). It is generally suspected that cytokine-related inflammatory responses might be involved in H. pylori-induced mucosal inflammation and injury (Blaser, 1992; Ernst et al., 1997; Crabtree, 1998; Takahashi et al., 1998a). Because IL-8 is a potent neutrophil chemokine, its production is a key event in the occurrence of H. pylori-induced gastritis (Blaser, 1992; Ernst et al., 1997; Crabtree, 1998). H. pylori adhesion on gastric epithelial cells causes IL-8 gene expression and production (Crowe et al., 1995). Keates et al. (1999) have recently reported that p38 kinase mediates the H. pylori-induced IL-8 production in cultured gastric epithelial cells. To evaluate the role of p38 kinase in H. pylori gastritis in vivo, we therefore examined the effect of FR167653 on H. pylori-induced gastric pathology in Mongolian gerbils.

Experimental Procedures

Effect of FR167653 on Activities of Purified Protein Kinases. Purified protein kinases and their sources were as follows: active p38α kinase (SAPK-2α/RK), active p38γ kinase (SAPK-3/extracellular signal-regulated kinase (ERK)-6), active c-Jun N-terminal kinase (JNK)-2 (SAPK-1α/SAPK-α), and ERK-1 were from Upstate Biotechnology (Lake Placid, NY). The catalytic subunit of protein kinase A, protein kinase C, and protein kinase G were from Promega (Madison, WI). The kinase domain of epidermal growth factor receptor was from Stratagene (La Jolla, CA). Kinase assays were performed in 50 μl of reaction mixture according to each instruction manual. Each protein kinase was preincubated with FR167653, SB203580, or vehicle (control) for 3 min at 30°C in a volume of 40 μl, and then kinase reaction was initiated by adding 10 μl of substrate solution consisting of 500 μg/ml myelin basic protein (Sigma, St. Louis, MO) and 50 μM [γ-32P]ATP (1 μCi, >3000 Ci/mole)
mmol; Amersham Pharmacia, Little Chalfont, UK). The mixtures were incubated for 5 min at 30°C. The reactions were terminated by adding 10 μl of 250 mM phosphoric acid. After centrifugation at 10,000g for 10 min, the resulting supernatants were spotted onto P81 filters (Whatman, Maidstone, UK). The filters were washed with 75 mM phosphoric acid and then soaked briefly in 95% ethanol, followed by air dry. The radioactivity on the filters was measured by liquid scintillation counting (Packard, Meriden, CT). All kinase reactions linearly proceeded up to 20 min, and the activities of the kinases in the control were about 25 pmol of phosphate incorporated into the substrate per 5 min. The data are expressed as percentage of the corresponding control value.

**Effect of FR167653 on Cyclooxygenase (COX) Activities.** COX activities were assayed according to the method of Curnock et al. (1997) with a slight modification. Purified ovine COX-1 and COX-2 (Cayman, Ann Arbor, MI) were incubated in 450 μl of reaction buffer consisting of 100 mM Tris-HCl (pH 8.0) and 1 μM hematin in the presence of FR167653, SB203580, or vehicle (control) for 3 min at 37°C. Thereafter, 50 μl of 100 μM arachidonic acid was added. Ten minutes later, the reactions were terminated by adding 100 μl of 100 μM indomethacin (Sigma) and immediately chilled on ice. After centrifugation at 10,000g for 10 min, the resulting supernatants were recovered, followed by 100-fold dilution. Because prostaglandin (PG)E2 is formed enzymatically from PGG2 during the assay (Curnock et al., 1997), the amount of PGE2 in the diluted samples was determined by enzyme-immunoassay (PG2 EIA kit; Cayman). PGE2 production linearly proceeded up to at least 20 min, and the activities of COX-1 and COX-2 in the control were about 80 pmol of PGE2 per 10 min. The data are expressed as percentage of the corresponding control value.

**Effect of FR167653 on Activation and Activity of Endogenous p38 Kinase in NRK-F Cells.** NRK-F cells (rat kidney fibroblast line) were kindly supplied from Dr. Kazuhisa Nakayama (University of Tsukuba, Tsukuba, Japan) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (Life Technologies), 100 units/ml penicillin, 100 units/ml streptomycin, and 0.5 μg/ml amphotericin B at 37°C under 5% CO2 in air. NRK-F cells (1 × 105 cells/ml) were seeded on 60-mm dishes and allowed to grow to confluence. The cells were pretreated with 10 μM FR167653 or vehicle for 30 min and then were further incubated with 10 ng/ml IL-1β for 30 min. After the cells were washed with PBS, they were scraped with a rubber policeman in 0.5 ml of lysis buffer consisting of 50 mM HEPES-NaOH (pH 7.6), 300 mM NaCl, 0.5% Triton X-100, 0.2 mM EDTA, 1.5 mM MgCl2, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na3VO4, 10 mM NaF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM dithiothreitol. The lysates were vortexed, stood on ice for 10 min, and then centrifuged at 10,000g for 10 min. The resulting supernatants were used as cell extract. Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA) after nonspecific binding sites had been blocked with bovine serum albumin as a standard.

The activation of p38 kinase, JNKs, and ERKs was examined as their phosphorylated forms by means of Western blotting. After cell extracts (10 μg) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (12.5%), the separated proteins were transferred onto Hybond-P membranes (Amersham Pharmacia). The membranes were incubated with the antibody against total or phosphorylated p38 kinase, JNK, or ERK (New England Biolabs, Beverly, MA) after nonspecific binding sites had been blocked with bovine serum albumin. We confirmed that the antibodies against two forms of p38 kinase recognize p38α, but not p38γ. Immunoreactive proteins were visualized on X-ray films with Phototope-HRP Western blot detection kit (New England Biolabs).

Endogenous p38 kinase activity was evaluated as activity of MAPK-activated protein kinase (MAPKAPK)-2, which is a specific substrate for p38 kinase. MAPKAPK-2 in the cell extracts (200 μg) was immunoprecipitated by anti-MAPKAPK-2 antibody (Upstate Biotechnology) at 4°C overnight and successively by protein A-Sepharose beads (Amersham Pharmacia) for 2 h at 4°C. The immunoprecipitates were washed with the lysis buffer and then resuspended in 50 μl of kinase buffer consisting of 50 mM HEPES-NaOH (pH 7.6), 50 mM MgCl2, 20 mM β-glycerophosphate, 10 mM Na3VO4, 10 mM NaF, and 1 mM dithiothreitol. Aliquots (25 μl) were incubated with the substrate in a volume of 50 μl at 30°C for 30 min. The phosphorylation of myelin basic protein was determined as described above. Data are expressed as cpm per milligram of protein (extract).

**H. pylori Infection to Mongolian Gerbils.** The preparation and inoculation of *H. pylori* were performed as described previously (Takahashi et al., 1998a). A cagA- and cagA-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, Detroit, MI) containing 10% fetal bovine serum at 37°C overnight under a microaerophilic atmosphere and allowed to grow to a density of 2 × 108 colony-forming units (CFU)/ml. *H. pylori* (2 × 105 CFU, 1.0 ml) was orally inoculated to each animal. Normal animals received 1.0 ml of the medium alone.

**Determination of Viable H. pylori in Stomach.** Viable *H. pylori* was assayed as reported previously (Takahashi et al., 1998a). After the animals were fasted for 24 h, they were sacrificed, and their stomachs were excised. The stomachs were homogenized in 20 ml of PBS with a Polytron (Kinematica, Steinhofhalde, Switzerland). The diluted homogenates were applied onto Brucella agar (Life Technologies) plates containing 10% horse blood (Nippon Bio-Test, Tokyo, Japan), 2.5 μg/ml amphotericin B, 9 μg/ml vancomycin, 0.32 μg/ml polymyxin B, 5 μg/ml trimethoprim, and 50 μg/ml 2,3,5-triphenyl-tetrazolium chloride. The plates were incubated at 37°C under a microaerophilic atmosphere for 7 days. The number of colonies was counted, and viable *H. pylori* was expressed as CFU per stomach.

**Determination of Myeloperoxidase (MPO) Activity.** MPO activity was determined as we previously reported (Takahashi et al., 1998a). Gastric specimen (about 100 mg) was cut off from the fundus near the antrum and then homogenized in 1 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma) with a Polytron, followed by freeze-thawing. The homogenates were centrifuged at 1600g for 10 min. After an aliquot (5 μl) of the supernatants had been mixed with 145 μl of phosphate buffer containing 0.167 mg/ml o-dianisidine dihydrochloride (Sigma) and 0.0005% H2O2, the change in the rate of absorbance at 450 nm was measured with a microplate reader (Thermo Max; Molecular Devices, Sunnyvale, CA). MPO activity was expressed as the degradation of H2O2 (μmol) per minute per gram of tissue. Horseradish peroxidase (Sigma) was used as a standard.

**Determination of Activation and Activity of p38 Kinase in Gastric Mucosa.** Gastric specimen (about 100 mg) was cut off from the fundus near the antrum and then homogenized in 1 ml of the lysis buffer. After the homogenates were centrifuged at 10,000g for 10 min, p38 kinase and MAPKAPK-2 in the resulting supernatants (500 μg) were immunoprecipitated as described above. The immunoprecipitates were resuspended in 100 μl of the kinase buffer. Aliquots (20 μl) were subjected to Western blot analysis of p38 kinase activation. Intensities of p38 kinase bands (total and phosphorylated forms) on the X-ray films were determined as arbitrary values by densitometric analysis, and then the levels of activated p38 kinase were expressed as the ratio to total p38 kinase. For MAPKAPK-2 assay, aliquots (25 μl) were incubated with the substrate in a volume of 50 μl for 60 min at 30°C. Data for MAPKAPK-2 activity are expressed as cpm per milligram of protein.

**Evaluation of H. pylori-Induced Gastritis.** Gastric pathology was blindly evaluated. Normal and *H. pylori*-infected animals were sacrificed, and their stomachs were excised. The stomachs were incised along the greater curvature and spread out with pins on a corkboard. Mucosal erosions (mm2) were examined under a dissecting microscope (magnification, 10×). Thereafter, four gastric specimens were cut off from the fundus near the antrum and fixed in 4% paraformaldehyde in PBS. Frozen sections (12 μm in thickness) were...
prepared, and neutrophil-specific MPO activity-dependent staining was carried out (Fujita et al., 1998). In brief, the sections were incubated in 50 mM Tris-HCl (pH 7.6) containing 0.2 mg/ml 3′,3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) in the presence of 0.005% H2O2 at room temperature. After washed with PBS, the sections were successively stained with hematoxylin. Black-stained cells in the mucosa were apparently identified as neutrophils because morphological features such as polymorphonuclear were confirmed under a light microscope at high magnification, and the cells were not stained in the absence of 3′,3′-diaminobenzidine or H2O2. Mucosal inflammation was graded 0–3 for each specimen under a light microscope (magnification, 25×), and the net score per animal was used. 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.

**Determination of Gastric Acid Secretion.** Gastric acid secretion was determined by the pylorus ligation method. After the animals were fasted for 24 h, under ether anesthesia, the pylorus was ligated 1 h after the final administration of FR167653. Two hours later, the animals were sacrificed, and their stomachs were excised. The gastric contents were collected and analyzed as to volume and acidity. Acidity was determined by titration of the contents against 10 mM NaOH to pH 7.0. Total acid output was calculated as volume \( \times \) acidity, and expressed as \( \mu \text{Eq} \) per 2 h.

**Determination of Neutrophil Chemokine Production.** The production of cytokine-induced neutrophil chemotractant (CINC/KC; IL-8 family chemokine in rodents) was assayed according to the method of Noach et al. (1994). Gastric specimen (about 80 mg) was cut off from the fundus near the antrum. After being washed with PBS, the tissues were incubated in 1 ml of DMEM supplemented with 2.5% fetal bovine serum and the above-mentioned antibiotics at 37°C for 20 h under 5% CO2 in air. Thereafter, the tissues were homogenized in the same culture medium in the presence of 0.1 mM phenylmethylsulfonyl fluoride and 1 \( \mu \text{g/ml} \) leupeptin. The homogenates were centrifuged at 10,000 g for 20 min. The amount of CINC/KC in the resulting supernatants was determined by enzyme-linked immunosorbent assay (CINC/KC EIA kit; Immuno Biological Laboratories, Fujioka, Japan). CINC/KC production was expressed as picograms of CINC/KC per milligram of tissue per 20 h.

**Materials.** FR167653 and recombinant human IL-1β were kindly provided by Fujisawa Pharmaceutical Company (Osaka, Japan) and Otsuka Pharmaceutical Company (Tokushima, Japan), respectively. In vitro studies, FR167653, SB203580 (Calbiochem, La Jolla, CA), and indomethacin were dissolved in dimethyl sulfoxide, followed by dialution with the buffer to the desired concentrations. The final concentration of dimethyl sulfoxide was less than 0.5%, at which concentration cell viability and kinase activities were unaffected. IL-1β was dissolved in DMEM.

Male Mongolian gerbils (6 week old, 40–50 g) were from Nihon SLC (Hamamatsu, Japan). The animals were maintained in an isolated clean room with regulated temperature (20–22°C) and humidity (approximately 55%) with a 12/12-h light/dark cycle. The animals were fasted for 24 h before \( \text{H. pylori} \) 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.

For animal study, FR167653 was dissolved in saline and orally administered at 30 mg/kg twice daily. FR167653 was administered for 2 weeks from 2 weeks of \( \text{H. pylori} \) infection. The animals were given the drug in a volume of 10 ml/kg body weight. Control animals received saline alone. The animals were sacrificed 1 h after the final administration, except for determination of acid secretion, and then the indicated assays were performed.

**Statistical Analysis.** Data are presented as means±S.E. Statistical differences in the dose-response studies were evaluated by Dunnett's multiple comparison test. Student's \( t \) test or Mann-Whitney \( U \) test was used for the comparison between two groups. \( P \) value of <0.05 was regarded as significant. EC50 values were calculated by the Litchfield-Wilcoxon method.

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**Results**

**Pharmacological Property of FR167653.** We examined whether FR167653 affects the activities of various protein kinases (Table 1). FR167653 at 10 \( \mu \text{M} \) potently inhibited p38α kinase activity. However, the compound had no effect on the activities of p38γ, ERK-1, JNK-2, protein kinase A, protein kinase C, protein kinase G, or epidermal growth factor receptor kinase.

The inhibitory effect of FR167653 on p38α kinase activity was compared with that of SB203580, which is a well known p38 kinase inhibitor (Fig. 1B). FR167653 inhibited p38α kinase activity in a dose-dependent manner, and a significant inhibition was observed from 0.1 \( \mu \text{M} \). SB203580 also dose dependently and significantly inhibited p38α kinase activity. EC50 values of FR167653 and SB203580 were 0.71 and 0.56 \( \mu \text{M} \), respectively.

Börsch-Haubold et al. (1998) reported that SB203580 directly inhibits both COX-1 and COX-2 activities. We therefore examined the effect of FR167653 on the activities of COX-1 and COX-2 (Fig. 1C). FR167653 even at 10 \( \mu \text{M} \) failed to reduce COX-1 and COX-2 activities. In contrast, SB203580 dose dependently suppressed both COX-1 and COX-2 activities. SB203580 was significantly effective at more than 3 \( \mu \text{M} \), and the EC50 value against COX-2 was 5.1 \( \mu \text{M} \).

We next examined the effect of FR167653 on endogenous p38 kinase in cultured cells. After NRK-F cells were stimulated with 10 ng/ml IL-1β in the presence or absence of 10 \( \mu \text{M} \) FR167653, activation (phosphorylation) of p38 kinase was examined (Fig. 2A). IL-1β induced phosphorylation of p38 kinase, whereas FR167653 had no effect on p38 activation caused by IL-1β. Endogenous p38 kinase activity was evaluated that FR167653 potently inhibited the increased MAPKAPK-2 activity caused by IL-1β, indicating that FR167653 serves as a p38 kinase inhibitor in the cells. Although JNKs and ERKs were also activated by IL-1β, their activation was not affected by FR167653 (data not shown).

**p38 Activation in the Gastric Mucosa by \( \text{H. pylori} \) Infection.** We have previously reported that \( \text{H. pylori} \) is colonized for at least 10 months in the gastric mucosa of all gerbils given the bacteria (Takahashi et al., 1998a; Kato et al., 1999). As shown in Fig. 3A, the number of viable \( \text{H. pylori} \) in the stomach reached a plateau level from 2 weeks after the inoculation. In addition, severe gastritis with mucosal ero-

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**TABLE 1**

Effect of FR167653 on the activities of various protein kinases

<table>
<thead>
<tr>
<th>Activity</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38α</td>
<td>94.4±2.9*</td>
</tr>
<tr>
<td>p38γ</td>
<td>93.7±4.5</td>
</tr>
<tr>
<td>ERK-1</td>
<td>109.6±6.8</td>
</tr>
<tr>
<td>JNK-2</td>
<td>102.9±12.2</td>
</tr>
<tr>
<td>Protein kinase A</td>
<td>101.0±5.6</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>90.5±8.8</td>
</tr>
<tr>
<td>Protein kinase G</td>
<td>79.6±14.4</td>
</tr>
<tr>
<td>EGFR kinase</td>
<td>107.2±11.6</td>
</tr>
</tbody>
</table>

* Significantly different from the control.
sions and gastric ulcers are generated in the fundus near the antrum from 4 and 20 weeks of the infection, respectively. Thus, the region is highly sensitive to *H. pylori*, and the following parameters except for *H. pylori* viability were measured in the region. MPO activity in the gastric mucosa (a neutrophil-specific enzyme, an indicator of mucosal inflammation) is also shown in Fig. 3A. MPO activity was negligible in normal mucosa. MPO activity was not elevated at 2 weeks of *H. pylori* infection, but there was significant increase in MPO activity from 3 weeks. At 4 weeks, the increased MPO activity was about 40-fold higher than that in normal mucosa. We actually confirmed that a large number of neutrophils were infiltrated in the gastric mucosa with *H. pylori* infection for 4 weeks, as described in Fig. 4.

We examined whether p38 kinase in the gastric mucosa is activated (phosphorylated) by *H. pylori* infection (Fig. 3B).

**On Western blot analysis, phosphorylated form of p38 kinase could be detected in normal mucosa, but its intensity was faint. *H. pylori* infection for 1 week slightly promoted the phosphorylation of p38 kinase compared with normal mucosa. Furthermore, its phosphorylation was more profoundly enhanced as the term of *H. pylori* infection became longer. It was evident that p38 phosphorylation was induced by *H. pylori* infection for more than 2 weeks.**

**Prevention of *H. pylori*-Induced Gastritis by FR167653.** To further clarify the role of p38 kinase in the pathogenesis of *H. pylori*-induced gastritis, FR167653 at 30 mg/kg was administered twice daily to *H. pylori*-infected gerbils from 2 weeks of the infection. After 2 weeks treatment with FR167653, we evaluated gastric pathology. Gross observation revealed that *H. pylori* infection induced mucosal erosions in all control animals, and the lesion area was 2.5 ± 0.2 mm² (*n* = 8). FR167653 significantly prevented the formation of hemorrhagic lesions caused by *H. pylori* infection, the area being 1.5 ± 0.3 mm².

On histological analysis, *H. pylori* infection caused marked infiltration of neutrophils in the gastric mucosa of the control
animals (Fig. 4A). Epithelium damage was also generated. In contrast, in the FR167653-treated animals, both neutrophil infiltration and epithelium damage were obviously attenuated. As shown in Fig. 4B, FR167653 significantly inhibited the *H. pylori*-induced neutrophil infiltration in the gastric mucosa, compared with that in the controls.

The number of viable *H. pylori* in the stomach and gastric acid secretion after treatment with FR167653 were also determined. The number of *H. pylori* colonized in the mucosa was $6.01 \pm 0.63 \times 10^5$ and $6.24 \pm 0.51 \times 10^5$ CFU in the control and FR167653-treated animals, respectively ($n = 8$). Acid secretion in normal animals was $22.1 \pm 2.5 \mu$Eq/2 h. *H. pylori* infection for 4 weeks failed to affect acid secretion in the absence ($17.8 \pm 1.7 \mu$Eq/2 h) and presence ($18.8 \pm 2.5 \mu$Eq/2 h) of FR167653. There was no difference of *H. pylori* infection or acid secretion between the control and FR167653-treated groups. When FR167653 at 30 mg/kg was similarly administered to normal animals for 2 weeks, the drug did not cause any pathological changes in the gastric mucosa.

**Inhibition by FR167653 of *H. pylori*-Induced Increases in Endogenous p38 Kinase Activity and Neutrophil Chemokine Production.** We examined the effect of FR167653 on the *H. pylori*-induced p38 activation in the gastric mucosa (Fig. 5A). *H. pylori* infection promoted the phosphorylation of p38 kinase in the gastric mucosa of the control animals. The *H. pylori*-induced increase in p38 phosphorylation was observed in FR167653-treated animals, but the level of its activation was lower in FR167653-treated animals than in the controls. Densitometric analysis revealed that the levels of activated p38 kinase were 0.1, 1.2, and 0.7 in normal, *H. pylori*-infected (control) and FR167653-treated *H. pylori*-infected animals, respectively.

In addition, the effect of FR167653 on p38 kinase activity in the gastric mucosa was also evaluated as MAPKAPK-2 activity (Fig. 5B). *H. pylori* infection significantly increased MAPKAPK-2 activity by 4.8-fold. FR167653 markedly inhibited the *H. pylori*-increased MAPKAPK-2 activity, and the inhibition was 77.9%, indicating that p38 kinase in the mucosa was suppressed by FR167653. It was evident that the inhibitory effect of FR167653 on p38 kinase activity was
stronger than that on p38 activation in the *H. pylori*-infected gastric mucosa.

We next examined the effect of FR167653 on the production of CINC/KC (a predominant neutrophil chemokine in rodents) in the gastric mucosa (Fig. 6). CINC/KC production in normal mucosa was negligible (0.14 ± 0.06 pg/mg/20 h). In contrast, at 4 weeks of *H. pylori* infection, CINC/KC production was remarkably increased by 9.8-fold. Treatment with FR167653 caused a significant decrease in the *H. pylori*-promoted CINC/KC production in the mucosa. The inhibition by FR167653 was 77.8%.

**Discussion**

In the present study, we showed that FR167653 is a p38 kinase-selective inhibitor both in vitro and in vivo. FR167653 was discovered as an inhibitor of IL-1 and TNF-α production (Yamamoto et al., 1996). The inhibitory effect of FR167653 on p38 kinase activity accounts for suppression of LPS-induced production of IL-1 and TNF-α. Furthermore, FR167653 inhibited p38α kinase activity, but not p38γ kinase activity. These results are consistent with the previous findings that SB203580 and RWJ67657 inhibit the α- and β-isoforms of p38 kinase, but not the γ- and δ-isoforms (Kumar et al., 1997; Wadsworth et al., 1999). Gum et al. (1998) demonstrated that both the α- and β-isoforms, but not the γ- and δ-isoforms, possess three amino acid residues near the hinge of ATP binding pocket, which are sensitive to pyridinyl imidazole inhibitors of p38 kinase. These results suggest that FR167653 is able to inhibit p38β as well as p38α. FR167653 is as potent as SB203580 for inhibition of p38α kinase activity, but differs from SB203580 in respect of COX inhibition. SB203580 significantly inhibited both COX-1 and COX-2 activities, as reported by Börsch-Haubold et al. (1998), whereas FR167653 did not affect the COX activities. Because COX inhibition causes gastrointestinal side effects (Eberhart and DuBois, 1995; Mitchell and Warner, 1999), anti-inflammatory drugs without inhibiting COX activities have been explored. FR167653 may be useful as a new anti-inflammatory drug without gastrointestinal side effects. In fact, it has been reported that FR167653 is effective on several diseases (Yamamoto et al., 1996, 1997; Kamoshita et al., 1997; Kobayashi et al., 1998; Koyano et al., 1998; Gardiner et al., 1999).

FR167653 inhibits endogenous p38 kinase activity without affecting its activation, as observed in p38 kinase inhibitors such as SB203580 (Cuenda et al., 1995). This is due to the binding property of these p38 kinase inhibitors, i.e., this class of compounds competes with ATP at the ATP-binding site of p38 kinase, but does not bind to its phosphorylation sites (Frantz et al., 1998; Gum et al., 1998).

The present results indicate that *H. pylori* infection induces neutrophil infiltration from 3 weeks of the infection and causes gastritis with mucosal erosive lesions at 4 weeks in Mongolian gerbils. Furthermore, we found that p38 kinase in the gastric mucosa is activated by *H. pylori* infection. This is the first report concerning the *H. pylori*-induced p38 activation in vivo, although Keates et al. (1999) reported that exposure of gastric epithelial AGS cells to *H. pylori* results in the activation of p38 kinase. Interestingly, p38 kinase was activated from 2 weeks of *H. pylori* infection, followed by the occurrence of mucosal inflammation and injury. FR167653 markedly inhibited the *H. pylori*-activated p38 kinase activity and prevented both neutrophil infiltration and mucosal injury. These results demonstrate that p38 kinase plays a crucial role in generation of the *H. pylori*-induced gastritis in Mongolian gerbils.

In *H. pylori* infection, both bacterial and host factors are believed to contribute to gastric mucosal injury. Regarding host factors, it is suspected that inflammatory responses may be involved in *H. pylori*-induced gastritis (Ernst et al., 1997; Crabtree, 1998; Takahashi et al., 1998a). Blaser (1992) speculated that mucosal inflammation disrupts gastric epithelial functions and therefore might be deleterious to the mucosa. It is generally accepted that IL-8, a potent neutrophil chemokine, is a key factor for gastric mucosal inflammation in *H. pylori*-infected humans. In the patients with *H. pylori*-positive gastritis, IL-8 level in the gastric mucosa is significantly higher than in ones with *H. pylori*-negative gastritis (Crabtree et al., 1994; Noach et al., 1994; Yamaoka et al., 1995). Similarly, our previous study revealed that neutrophil chemotactic activity is increased (Takahashi et al., 1998a), and, in the present study, we confirmed that CINC/KC production is significantly promoted in the gastric mucosa of *H. pylori*-infected gerbils. Neutrophil infiltration was associated with the increases in neutrophil chemotactic activity and CINC/KC production. CINC/KC is considered to play an important role in neutrophil infiltration in rodents, because CINC/KC belongs to IL-8 chemokine family, and rodent counterparts of IL-8 have not been identified (Watanabe et al., 1993; Utsunomiya et al., 1996). These results indicate the pathological relevance of CINC/KC in *H. pylori*-induced gastritis in gerbils. FR167653 reduced the increased CINC/KC production in the *H. pylori*-infected gastric mucosa. The inhibitory effect of FR167653 on the chemokine production accounts for a decrease in neutrophil infiltration. In addition, neutrophil migration in response to chemotactic factors may be inhibited by FR167653. Several studies showed that SB203580 inhibits neutrophil chemotaxis caused by formylpeptide, platelet-activating factor, and transforming growth factor-β (Nick et al., 1997; Hannigan et al., 1998; Zu

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**Fig. 6.** Effect of FR167653 on CINC/KC production in the gastric mucosa of *H. pylori*-infected gerbils. FR167653 at 30 mg/kg or vehicle (control) was administered from 2 weeks of *H. pylori* infection. At 4 weeks, gastric tissues were isolated, and then CINC/KC production for 20 h by the tissues was determined by enzyme-linked immunosorbent assay. Data are presented as means ± S.E. (n = 8). * and #, significantly different from the normal and control, respectively.
We found that p38 kinase is involved in CINC/KC production in the gastric mucosa of H. pylori-infected gerbils. Keates et al. (1999) reported that SB203580 inhibits H. pylori-increased IL-8 production in gastric epithelial AGS cells. Immunohistological studies demonstrate that gastric epithelial cells serve as a predominant source of IL-8 in H. pylori positive patients (Crabtree et al., 1994). These results suggest that H. pylori infection activates p38 kinase in gastric epithelial cells and induces CINC/KC production in gerbils. However, further investigation is needed to clarify the cellular localization of CINC/KC and activated p38 kinase.

The inhibition by FR167653 of production of other inflammatory cytokines such as IL-1 and TNF-α might also contribute to prevention of H. pylori-induced gastritis. It is known that up-regulation of IL-1 and TNF-α gene expression is mediated by p38 kinase in various models, and p38 kinase inhibitors, including FR167653, suppress the gene expression and production of these cytokines (Lee et al., 1993, 1994; Badger et al., 1996; Jackson et al., 1998; Takahashi et al., 1998b; Kawano et al., 1999; Wadsworth et al., 1999). IL-1 and TNF-α are potent activators of p38 kinase cascade, and there has been accumulating evidence that multiple responses to these cytokines are mediated by p38 kinase (Lee et al., 1993; Cuenda et al., 1995; Karnitz and Abraham, 1995). IL-1 and TNF-α stimulate IL-8 and CINC/KC production in gastric epithelial cells (Yasumoto et al., 1992) (our unpublished data). In both the direct effect of H. pylori adhesion and the stimulatory effects of IL-1 and TNF-α toward gastric epithelial cells, p38 kinase might play an important role in CINC/KC production in H. pylori-infected gastric mucosa in gerbils.

On Western blot analysis, the level of activated p38 kinase (phosphorylated form) by H. pylori infection in the gastric mucosa of FR167653-treated animals was lower than that in the controls. However, FR167653 did not affect the activation of p38 kinase in the IL-1β-stimulated NRK-F cells. In addition, FR167653 failed to prevent the H. pylori-induced p38 activation in gastric epithelial MKN28 cells (our unpublished data). Because it is known that p38 kinase in neutrophils is activated by chemotactic factors (Nick et al., 1997; Hannigan et al., 1998; Ziu et al., 1998), the infiltrated neutrophils as well as epithelial cells in H. pylori-infected mucosa might contain activated p38 kinase. In the gastric mucosa of the FR167653-treated gerbils, the decrease in p38 activation might be due to a reduction of neutrophil infiltration.

It is also found that p38 kinase is not involved in H. pylori colonization in the gastric mucosa or gastric acid secretion. These results support the pathological relevance of inflammatory reactions in H. pylori-induced gastritis.

In conclusion, FR167653 is a p38 kinase-selective inhibitor without affecting COX activities, and prevents the H. pylori-induced gastric mucosal inflammation and erosive injury in Mongolian gerbils. The present results indicate that p38 kinase plays a crucial role in H. pylori-induced gastritis.

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References


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