Activation of IkB Kinase B and NF-κB Is Essential for Helicobacter pylori-Induced Chronic Gastritis in Mongolian Gerbils

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The Mongolian gerbil model of Helicobacter pylori infection resembles human gastritis. In this study, we investigated the role of NF-κB activation in H. pylori-infected gerbils. Activated macrophages were significantly increased in H. pylori-infected gastric mucosa and were identified as being active cells with potent activation of NF-κB, which plays an important part in producing proinflammatory cytokines. Macrophage depletion by the administration of clodronate resulted in milder inflammation in gerbils infected with H. pylori. In macrophages, the inhibition of IkB kinase B (IKKB), which is a critical kinase for NF-κB activation, resulted in lower proinflammatory cytokine expression caused by heat-killed H. pylori cells. Furthermore, treatment with IKKB inhibitor resulted in milder inflammation in gerbils with H. pylori gastritis. Collectively, our data suggest that H. pylori-mediated gastric inflammation critically depends on the efficient recruitment and activation of macrophages, with sufficient NF-κB activation.

Helicobacter pylori, a gram-negative pathogen, induces chronic inflammation in the stomach (21). H. pylori infection is known to be causally related to chronic active gastritis and peptic ulcers. Epidemiological studies have also demonstrated its association with gastric malignant diseases, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (6, 19, 26). H. pylori was defined as a definite carcinogen at the World Health Organization/International Agency for Research on Cancer meeting in 1994 (15). The association between H. pylori infection and gastric adenocarcinoma was demonstrated in a recent prospective study (25).

In the H. pylori-infected gastric mucosa, numerous inflammatory cells, such as neutrophils, macrophages, and even lymphocytes, are infiltrated (4). Among these, macrophages are thought to be critical cell types for the innate immune response against H. pylori and to express abundant proinflammatory cytokines. However, the function of macrophages in H. pylori-associated gastritis has not been determined in vivo.

NF-κB transcription factors are critical regulators of genes involved in inflammation, innate immunity, and suppression of apoptosis (7). In resting cells, NF-κB is retained in the cytoplasm by IkB inhibitors, which are rapidly degraded in response to stimuli such as tumor necrosis factor alpha (TNF-α) and bacterial lipopolysaccharide (LPS), resulting in NF-κB nuclear entry (5). This process requires the phosphorylation of IkBs by the IkB kinase (IKK) complex, which is composed of three subunits: IKKa, IKKB, and IKKγ. IKKB is critical for IkB degradation and the activation of NF-κB in response to proinflammatory stimuli and pathogen-associated molecular patterns. NF-κB dimers can translocate to the nucleus, in which they modulate the transcription of genes encoding cytokines, chemokines, and antiapoptotic factors (5, 7). IKKB deficiency results in the absence of innate immunity, as well as a deficiency of p65, an NF-κB subunit (8, 9). Thus, selective inhibition of the IKK complex has been raised as a promising target to block aberrant NF-κB activity in inflammatory diseases such as H. pylori infection (12).

To determine whether the macrophages and the NF-κB activation in gastric mucosa infected with H. pylori are involved in gastric inflammation, we used the Mongolian gerbil model, which shows inflammation similar to that in the human stomach. Surprisingly, macrophage depletion markedly reduced H. pylori-induced gastric inflammation. In addition, the inhibition of IKKB by its inhibitor also resulted in a marked decrease in gastric inflammation, suggesting that NF-κB signaling in the gastric epithelium, a critical regulator of its inflammation, has important implications for understanding the pathogenetic mechanisms of gastric inflammation and carcinogenesis.

MATERIALS AND METHODS

Bacterial strains and culture. The clinical isolate H. pylori TN2 (a type I H. pylori) and its cogE mutant were used in this study (16). Bacteria were maintained under microaerophilic conditions in brucella broth culture medium (Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% fetal bovine serum (Gibco Laboratories, Grand Island, NY). Heat-killed H. pylori cells were prepared from cultured H. pylori cells (10⁷ CFU/ml). The number of the infecting bacteria in the stomach was measured as previously described (16).

Cell lines. Mouse macrophage cell line J774A.1 was maintained in RPMI medium containing 10% fetal bovine serum and 50 µg/ml kanamycin.

Reagents. Human TNF-α was purchased from R&D Systems (Minneapolis, MN). Escherichia coli LPS was purchased from Sigma (St. Louis, MO). The polyclonal anti-phospho-IkBα (Ser32), anti-phospho-IκBα N-terminal peptide kinase (JNK) (Thr183/Tyr185), and anti-phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA). Anti-IκBα, anti-α-tubulin, and anti-β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Clodronate liposomes were a gift from Roche GmbH (Mannheim, Germany).

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We initially investigated gastric inflammation in Mongolian gerbils inoculated with *H. pylori*. Twelve weeks after inoculation, *H. pylori* was detected in the stomach by immunohistochemistry. On September 29, 2017 by guest http://iai.asm.org/ Downloaded from

**RESULTS**

*Helicobacter pylori* induces chronic inflammation and macrophage accumulation in the stomach of Mongolian gerbils. We initially investigated gastric inflammation in Mongolian gerbils inoculated with *H. pylori*. Twelve weeks after inoculation, severe gastritis characterized by neutrophil and mononuclear cell infiltration could be seen in the stomach. In contrast, inflammation was not observed in the stomach of uninfected gerbils (Fig. 1A). Infiltration of mature macrophages, one of the major cell types in the human gastric epithelium infected with *H. pylori*, was assessed by immunohistochemistry with monoclonal antibody against F4/80, a specific marker for tissue macrophages. Macrophage infiltration in gerbils infected with *H. pylori* was markedly elevated compared to that in uninfected gerbils, which showed only a few macrophages (Fig. 1B).

**Improvement of chronic gastritis in Mongolian gerbils after depletion of macrophages.** Clodronate liposomes were used to specifically deplete macrophages. A single injection of clodronate into mice was reported to induce depletion of macrophages within 2 days (27). Mongolian gerbils were given clodronate liposomes, and the degree of macrophage depletion was assessed by immunostaining with F4/80, a specific marker for resident tissue macrophages, 2 days after administration. In Mongolian gerbils treated with clodronate, two- to fivefold reductions in F4/80-positive cells were observed in the colon, liver, and spleen (Fig. 1C).

To investigate the role of macrophages in chronic gastritis, we used a long-term *H. pylori* infection model. Mongolian gerbils were inoculated with *H. pylori* cells, and 12 weeks later, PBS or clodronate liposomes were injected intravenously three times a week (Fig. 1D). After 1 week, *H. pylori* cells were recovered from all animals in the challenged groups, and bacterial density was not different between the groups. The inflammation level in the stomach was evaluated by H&E staining or F4/80 immunostaining. Marked infiltration of macrophages was observed in gerbils treated with PBS liposomes. In contrast, gerbils injected with clodronate liposomes showed not only elimination of F4/80-positive macrophages, but also a decrease in infiltration with other inflammatory cells, such as neutrophils (Fig. 1A, B, E, and F). These results indicate that macrophage accumulation leads to full expression of gastric inflammation.

**Macrophages are an important source of NF-κB-regulated proinflammatory cytokines in *H. pylori*-infected gastric mucosa.** To investigate the response to *H. pylori* in macrophages, J774A.1 cells were cultured with heat-killed *H. pylori* cells for 24 h, and cytokine production was analyzed by ELISA. Inflammatory cytokines, such as IL-6 and TNF-α, in macrophage cultures were significantly increased by heat-killed *H. pylori* cells (Fig. 2A and B). We also analyzed intracellular signaling pathways that regulate proinflammatory cytokine expression. IkBα was phosphorylated and degraded in J774A.1 cells cultured with heat-killed *H. pylori* cells (Fig. 2C). In addition, we investigated the intracellular signaling status in vivo, we performed immunohistochemistry for phospho-IkBα in the stomach of infected gerbils. The number of phospho-IkBα-positive cells, which were likely F4/80-positive macrophages, increased markedly in gerbils infected with *H. pylori* (Fig. 2D).

**Improvement of gastritis by inhibition of IKK in gerbil stomach.** To investigate the relationship between gastric inflammation induced by *H. pylori* and NF-κB activation, we used IMD-0354 to inhibit IKKβ, a critical kinase for NF-κB activation (18, 23). In J774A.1 cells, IkBα degradation mediated by heat-killed *H. pylori* cells was strongly suppressed by treatment with IMD-0354 (Fig. 3A). Furthermore, the levels of production of IL-6 and TNF-α by heat-killed *H. pylori* cells and *E. coli* LPS were significantly suppressed, in a dose-dependent manner, by treatment with IMD-0354 (Fig. 3B and C). We also found that IL-6 and TNF-α production by live *H. pylori* cells...
Fig. 1. Effect of clodronate treatment on *Helicobacter pylori*-induced gastritis. (A) Mongolian gerbils were inoculated with *H. pylori*, and after 12 weeks, PBS or clodronate liposomes (4 ml/kg body weight) were injected intravenously. After 7 days, the gerbils were killed; their stomachs were removed, fixed, and stained with H&E (middle and right panels). Left panel shows uninfected control. Original magnification, ×100. (B) Gerbils were treated as described above. Paraffin-embedded stomach sections were prepared after PBS or clodronate injection and were immunostained for F4/80 (middle and right panel). Left panel shows stomach section of uninfected control. Original magnification, ×100. (C) Clodronate or PBS liposomes were injected intravenously into Mongolian gerbils. After 48 h, systemic inflammation was induced by intraperitoneal injection of LPS (5 mg/kg body weight). Ten hours later, the gerbils were killed; F4/80 expression in the colon, liver, and spleen was determined by immunohistochemistry. (D) Schematic representation of the experimental protocol for in vivo depletion of macrophages by clodronate. Gerbils were inoculated with *H. pylori*, and after 12 weeks (w), PBS or clodronate liposomes (4 ml/kg body weight) were injected intravenously. After 7 days, the gerbils were killed and their stomachs analyzed. (E) Numbers of F4/80-positive cells were compared for gerbils treated with PBS or clodronate as described above. (F) Inflammatory cell infiltration was evaluated by the updated Sydney system. The results shown in panels E and F are the means ± SEM. **, *P* < 0.05 by Student’s *t* test.
was significantly suppressed by the treatment with IMD-0354. In contrast, NF-κB activation by live *H. pylori* cells was not significantly suppressed in gastric epithelial cells (data not shown). We investigated the role of NF-κB activation in the inflammatory response to *H. pylori* infection in Mongolian gerbils (Fig. 4A). Infiltration of neutrophils and mononuclear cells decreased markedly in the animals injected with IKKβ inhibitor (Fig. 4B and C). *H. pylori* cells were recovered from all

FIG. 2. Effect of *Helicobacter pylori* on NF-κB signaling pathway in macrophages. (A) J774A.1 cells were cultured with heat-killed *H. pylori* cells for 24 h. IL-6 production levels were analyzed by ELISA. (B) Cells were treated as described above, and TNF-α production was analyzed by ELISA. The results shown in panels A and B are the means ± SEM. **, *P* < 0.05 by Student’s *t* test. (C) J774A.1 cells were incubated with heat-killed *H. pylori* cells. Cell lysates were prepared at the indicated times, and the levels of phospho-IκBα, IκBα, phospho-JNK, JNK, phospho-p38, p38, phospho-ERK, and ERK were measured by Western blotting. (D) Mongolian gerbils were inoculated with *H. pylori*, and their stomachs were removed, fixed, and sectioned 12 weeks later. Serial sections were immunostained with phospho-IκBα (left and middle panels) and F4/80 (right panel). Original magnification, ×100.
animals in the challenged groups, and bacterial density was not different between the groups. Infiltration of F4/80- or phospho-IκBα-positive cells was also significantly lower in the gerbils injected with IKKβ inhibitor (Fig. 4B, D, and E). These results indicate that NF-κB activation is a critical regulator for H. pylori-mediated gastritis.

DISCUSSION

Helicobacter pylori infection and macrophage recruitment. In this study, we showed that H. pylori induced chronic inflammation and macrophage infiltration in gerbils, and that treatment with clodronate for depletion of macrophages caused milder inflammation. The infiltration of neutrophils and lymphocytes was also significantly suppressed in gerbils with macrophage depletion. H. pylori may make contact with epithelial cells and simultaneously stimulate tissue-localized macrophages by soluble factors to produce proinflammatory cytokines, which accumulate macrophages or neutrophils to the locus of inflammation. It has been reported that H. pylori infection induces monocyte chemoattractant protein 1 gene transcription by activating NF-κB via the NF-κB-inducing kinase-IKK signaling complex in gastric epithelial cells (14). This might be one of the reasons why the inhibition of IKK/NF-κB reduced macrophage accumulation induced by H. pylori. In addition, soluble factors, such as LPS, stimulate tissue-localized macrophages to produce proinflammatory cytokines and promote more macrophage accumulation. It is suggested that not only gastric epithelial cells, but also activated macrophages, lead to the accumulation of more macrophages and characterize H. pylori gastritis.

Macrophage accumulation and carcinogenesis. One common tumor-promoting mechanism may involve inflammation (20). It is estimated that inflammation plays a role in the etiology of 15% of human cancers, mostly acting as a tumor promoter (2). However, precise details of the mechanism are still unknown. Tumor-associated macrophages are involved in tumor progression and metastasis via the release of matrix metalloproteinase, TNF-α, vascular endothelial growth factor, and other molecules. Recently, it was reported that decreasing the number of tumor-associated macrophages in the tumor stroma effectively suppresses tumor growth and metastasis by altering the tumor microenvironment (10). In an H. pylori-related cancer model, H. pylori increased the incidence of carcinogen-induced adenocarcinoma in the glandular stomach, suggesting that H. pylori infection may have tumor-promoting activity (22, 24). It is suggested that macrophage accumulation controlled by NF-κB activation at inflammatory sites has an important role in the induction of chronic inflammation, cell proliferation, and tumor promotion in chronically inflamed mucosa.

FIG. 3. Effect of IKK inhibitor on Helicobacter pylori-mediated NF-κB activation and cytokine expression. (A) J774A.1 cells were treated with IKK inhibitor. After 1 h, cells were incubated with or without heat-killed H. pylori for 15 min. Cell lysates were prepared, and the degradation of IκBα was evaluated by Western blotting. Tubulin was used as a control. (B) J774A.1 cells were pretreated with or without IKK inhibitor for 1 h, and cells were incubated with or without live H. pylori cells, heat-killed H. pylori cells, or LPS (25 ng/ml). After 24 h, supernatants were collected, and IL-6 production was analyzed by ELISA. (C) Cells were treated as described above, and TNF-α production was analyzed by ELISA. The results shown in panels B and C are the means ± SEM. **, P < 0.05 by Student’s t test.
NF-κB activation and gastric inflammation. In this study, we showed that most of the cells with phosphorylated 1kβ were F4/80-positive macrophages. This observation suggests that NF-κB was mainly and strongly activated in macrophages and may play an important role in the inflammatory response. However, we also detected phosphorylated 1kβ in the epithelial cells in other areas.

We showed that the cag pathogenicity island (cagPAI) is
responsible for various gastric lesions induced in the gerbil model (Fig. 4C) (16). It has also been suggested that cagPAI plays an important role in the pathogenesis of gastric diseases related to H. pylori infection in humans. In in vitro studies, signal transduction pathways, such as NF-κB or mitogen-activated protein kinase, are activated by cagPAI-positive, but not -negative, strains in gastric epithelial cells (11, 13). In contrast, several studies have reported that cagPAI does not contribute to the activation of NF-κB in monocytes or lymphocytes (11, 17). These in vivo and in vitro results suggest that H. pylori triggers gastric inflammation through gastric epithelial cells in a cagPAI-dependent manner, following amplification of inflammation due to inflammatory cells such as cagPAI-independent macrophages.

NF-κB transcription factors are critical regulators of genes involved in inflammation, innate immunity, and suppression of apoptosis (7). In this study, we used an inhibitor of IKKβ, a critical kinase for NF-κB activation, and found that the inhibitor was effective for regression of the inflammatory response to H. pylori infection. Although numerous reports suggest the importance of NF-κB activation by H. pylori in vitro, this is, in fact, the first report to show that NF-κB activation is important for H. pylori-mediated gastritis in vivo. The same concentration of inhibitor used in macrophages was not effective for the inhibition of H. pylori-mediated NF-κB activity in gastric epithelial cells (data not shown). We assume that the concentration or delivery of the inhibitor we used in the gastric epithelial cells was not sufficient for inhibition. As the inhibitor may also act mainly on mononuclear cells in vivo, our results indicate that NF-κB-mediated inflammatory cytokine expression from mononuclear cells, such as macrophages, might be the main avenue of H. pylori-mediated gastritis. This result is consistent with the result of macrophage depletion shown in this study.

In conclusion, depletion of macrophages and inhibition of IKKβ, which is mainly activated in macrophages, resulted in a marked decrease in gastric inflammation. These results suggest that NF-κB signaling, a critical regulator of inflammation in the gastric epithelium, has important implications for understanding the mechanisms involved in the pathogenesis of gastric inflammation and cancer.

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REFERENCES