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ABSTRACT

CCL20 attracts immature dendritic cells and memory T cells, and plays a role on mucosal surfaces in inflammation. However, whether *H. pylori* infection induces CCL20 in human gastric epithelial cells remains to be determined. The aim of this study was to analyze the molecular mechanism of *H. pylori*-induced CCL20 expression. Expression of CCL20 mRNA was assessed by reverse transcription-PCR. Five normal and five *H. pylori*-infected gastric tissue samples were stained immunohistochemically for CCL20. Luciferase assay was used to monitor activation of the CCL20 gene promoter, and electrophoretic mobility shift assay was used to explore the binding of transcription factors to this promoter. CCL20 expression in epithelial cells of *H. pylori*-positive tissues was higher than in *H. pylori*-negative tissues. *H. pylori* induced CCL20 expression in gastric epithelial cell lines, and such induction was dependent on an intact *cag* pathogenicity island. Activation of the CCL20 promoter by *H. pylori* occurred through the action of NF-κB. Transfection of dominant negative mutants of IκB kinase and NF-κB-inducing kinase inhibited *H. pylori*-mediated activation of CCL20. Treatment with inhibitor of Hsp90 suppressed *H. pylori*-induced CCL20 mRNA due to deactivation of NF-κB. Collectively, these results suggest that *H. pylori* activates NF-κB through an intracellular signaling pathway that involved IκB kinase and NF-κB-inducing kinase, leading to CCL20 gene transcription, and that Hsp90 is a crucial regulator of *H. pylori*-induced CCL20 expression, presumably contributing to immune response in *H. pylori*.

INTRODUCTION

Helicobacter pylori plays an important role in the pathogenesis of human gastric disease. In 10-20% of infected individuals, the *H. pylori*-induced chronic gastric inflammation progresses to peptic ulcer, gastric cancer, or gastric mucosa-associated lymphoid tissue lymphoma (22, 23, 50). Bacterial, environmental, and host genetic factors may affect the progress and outcome of gastric disease. One such factor that is responsible for severe disease is the virulence of individual *H. pylori* strains. Several virulence factors have been described and include the presence of a vacuolating cytotoxin (*vacA*) and *cag* pathogenicity island (PAI) (12, 41, 43). *H. pylori* strains that carry *cag* PAI genes, called type I strains, are highly prevalent in patients with peptic ulcer and gastric cancer (4, 10, 13).

There is abundant evidence that T lymphocytes play a pivotal role in the pathogenesis of *H. pylori*-induced chronic gastric inflammation (46). This pathological state is considered a Th1-mediated process characterized by increased of gamma interferon, which is implicated in perpetuating the inflammatory changes that lead to disease (14, 32). Several trafficking mechanisms are involved in the selective recruitment of T lymphocytes in the mucosa, such as selectins, the immunoglobulin superfamily, $\alpha_4\beta_7$, $\alpha_4\beta_1$, and $\alpha_E\beta_7$ integrins, and chemokines (29).

The elucidation of a T lymphocyte response is currently believed to require interaction with professional antigen-presenting cells, (e.g., dendritic cells [DCs]) (3). Because the normal gastric mucosa has no mucosa-associated lymphoid system, very little is known about the role of DCs in the mucosal immune system of the stomach.

However, several groups have shown that human DCs are activated and secrete cytokines when cultured in the presence of *H. pylori* (20, 33). A recent study demonstrated also the recruitment of DCs to the gastric mucosa after *H. pylori* infection in mice (30).

Chemokines acting through seven transmembrane-spanning G-protein-coupled receptors are believed to be critical in the migration of lymphocytes and DCs out of blood vessels into peripheral tissue and secondary lymphoid organs (2, 37). In the multistep model of immune cell recruitment, various adhesion molecules, including selectins, mediate transient (rolling) interactions with endothelial cells whereas chemokines have been shown to induce firm arrest via integrins in vitro (9) and in vivo (8, 17). CCL20 (also known as macrophage inflammatory protein 3α [47], liver and activation-regulated chemokine [21], and Exodus [24]) is one of a small number of chemokines that have been demonstrated to induce arrest of lymphocytes under flow conditions (9, 17). The receptor for CCL20, namely CCR6, is restricted to $CD45RO^+$ memory T cells (34). Furthermore, CCL20 has recently been identified as the chemokine that recruits immature $CD11b^+$ myeloid DCs to mucosal surfaces, allowing for their antigen uptake and further migration toward secondary lymphoid organs (15, 16, 27). Because CCR6 mediates DCs localization, lymphocyte homeostasis, and immune responses in mucosal tissue (11), and CCL20 preferentially attract memory T cells (34), CCL20 may play a role in gastric inflammation. This capacity of CCL20 to recruit immature DCs (capable of *H. pylori* antigen uptake and thus potentially promoting further T cell activation) and T cells with homing

properties for the gastric mucosa prompted us to investigate the expression of CCL20 in human *H. pylori*-infected gastritis.

The present study was designed to evaluate the effect of *H. pylori* on the expression of CCL20 in gastric epithelial cells both in vitro and in vivo and to study the role of *H. pylori* virulence factors in any such effect. We detected the expression of CCL20 in human gastric mucosa of patients infected with *H. pylori*. We found that *H. pylori* activated CCL20 gene expression in gastric epithelial cells. We also showed that the NF-κB element is essential for *H. pylori*–induced activation of CCL20 gene expression. This was related to the expression of *cag* PAI responsible for cytokine/chemokine production.

The 90-kDa heat shock protein, Hsp90, is a major molecular chaperone and appears to have particular significance to cellular regulatory processes. Recent studies revealed that the majority of client proteins of Hsp90 are protein kinases or transcription factors that play important roles in cellular carcinogenesis (45). Several groups also documented that Hsp90 plays a critical role in the inflammatory response and its inhibitor resulted in a reduced immune response as indicated by a decrease of proinflammatory mediator production (7, 59). Furthermore, a recent study indicated that IκB kinase α (IKKα) and IKKβ are clients of Hsp90 (6). Finally, we demonstrated that Hsp90 acted as a crucial regulator in *H. pylori*-induced CCL20 expression. We postulate that CCL20 plays a role in the development of gastric inflammation associated with *H. pylori*.

MATERIALS AND METHODS

Antibodies and reagents. Goat polyclonal antibody to CCL20 was purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies to IκBα and NF-κB subunits p50, p65, c-Rel, p52, and RelB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies to Hsp90 and actin were purchased from BD Transduction Laboratories (San Jose, CA) and NeoMarkers (Fremont CA), respectively. Mouse monoclonal antibody to phospho-IκBα (Ser-32 and Ser-36) and rabbit polyclonal antibodies to IKKα and IKKβ were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibody to IKKγ was purchased from Sigma-Aldrich (St Louis, MO).
N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) and Bay 11-7082 were purchased from Sigma-Aldrich and Calbiochem (La Jolla, CA), respectively.
17-allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from Alomone Labs (Jerusalem, Israel).

Bacterial strains. *H. pylori* (ATCC 49503; American Type Culture Collection, Rockville, MD) was used in most experiments described in this study. Other clinical strains (OHPC0001, OHPC0002, and OHPC0003), isolated from patients with chronic gastritis, were kind gifts from T. Kitahora (Ohkura Hospital, Tokyo, Japan). An isogenic *H. pylori* mutant lacking the *cag* PAI (51) was also studied together with its parental wild-type strain (26695). The presence of *cag* PAI and *vacA* in these strains was determined previously by PCR using specific sets of primers (1, 53). *H. pylori*

strains were plated on blood agar plate at 37°C for 3 days under microaerophilic conditions. Using cotton swabs, bacteria harvested from the plates were suspended in 200 ml of brain heart infusion broth containing 10% fetal bovine serum (FBS) and then liquid cultured at 37°C for 2 days in a controlled microaerophilic environment. Bacteria were harvested from the broth culture by centrifugation and then resuspended at the indicated concentrations in antibiotic-free medium. Cultured bacteria reached a density of 3×10^9 CFU/ml. All procedures were performed with the approval of the appropriate institutional biosafety review committees and in compliance with their guidelines for biohazards.

Cell culture. Human gastric epithelial cells (MKN45 and MKN28) were maintained in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. On the day of the experiment, cells were refed with fresh serum- and antibiotic-free medium, and cocultured with *H. pylori* to a final concentration of 10^7 CFU/ml for the indicated time intervals.

Tissue samples. Five histopathologically normal stomach biopsy specimens and stomach biopsy specimens from five patients with *H. pylori* gastritis were studied for reverse transcription (RT)-PCR analysis and histopathologically examined for CCL20. The presence of *H. pylori* infection was determined by culture, serology (anti-*H. pylori* IgG antibody), rapid urease test, and visualization through histology with Giemsa staining. *H. pylori* gastritis showed polymorphonuclear neutrophil infiltration in the gastric epithelium, in conjunction with the presence of bacterial forms,

consistent with *H. pylori* infection. All samples were obtained after receiving informed consent from the subjects.

RT-PCR. Total cellular RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 1 μg total cellular RNA using an RNA PCR kit (Takara Bio Inc., Otsu, Japan) with random primers. Thereafter, cDNA was amplified for 30 and 28 cycles for CCL20 and β-actin, respectively. Specific primers used were as follows: CCL20 forward 5'-ATGTGCTGTACCAAGAGTTTGC-3', reverse 5'-CCAATTCCATTCCAGAAAAGCC-3'; β-actin forward 5'-GTGGGGCGCCCCAGGCACCA-3', reverse 5'-CTCCTTAATGTCACGCACGATTTC-3'. Product sizes were 320 bp for CCL20 and 548 bp for β-actin. Thermocycling conditions for the targets were as follows: 94°C for 60 s for CCL20 and 30 s for β-actin, 60°C for 60 s for CCL20 and 30 s for β-actin, and 72°C for 60 s for CCL20 and 90 s for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Plasmids. The dominant negative mutants of IκBα, IκBαΔN, and IκBβ, IκBβΔN (kindly provided by D. W. Ballard, Vanderbilt University School of Medicine, Nashville, TN) are deletion mutants of IκBα and IκBβ lacking the NH_2-terminal 36 amino acids and 23 amino acids, respectively (5, 38). The dominant negative mutants of IKKα, IKKα (K44M), IKKβ, IKKβ (K44A), and IKKγ, IKKγ (1-305) as well as the dominant negative mutant of NF-κB-inducing kinase (NIK), NIK

(KK429/430AA), have been described previously (19, 25). Reporter plasmid κB-LUC is a luciferase expression plasmid controlled by 5 tandem repeats of the NF-κB-binding sequences of the interleukin-2 (IL-2) receptor (IL-2R) α chain gene. The CCL20 promoter pGL2 luciferase reporter plasmid containing the wild-type sequence (-874/+58) or the NF-κB site mutant was used to map *H. pylori*-responsive regions (26).

Transfection and luciferase assay. MKN45 cells (2×10^6) were transfected with 1 μg of appropriate reporter and 5 μg of effector plasmids using Lipofectamine (Invitrogen). After 24 h, *H. pylori* was added and incubated for 6 h. The ratio of bacteria to cells was 20:1. Preliminary studies with MKN45 cells using varying numbers of *H. pylori* indicated that higher concentrations of bacteria (final concentrations, 500 organisms/cell) induced the death of epithelial cells, as determined by morphological analysis. The cells were washed in phosphate-buffered saline (PBS) and lysed in reporter lysis buffer (Promega, Madison, WI). Lysates were assayed for reporter gene activity with the dual luciferase assay system (Promega). Luciferase activities were normalized relative to the *Renilla* luciferase activity from phRL-TK.

Preparation of nuclear extracts and EMSA. NF-κB binding activity to NF-κB element was examined by electrophoretic mobility shift assay (EMSA) as described previously (40). To examine the specificity of the NF-κB element probe, we preincubated unlabeled competitor oligonucleotides with the nuclear extracts for 15

min before incubation with probe. The probe or competitors used were prepared by annealing the sense and antisense synthetic oligonucleotides as follows: NF-κB element of CCL20 gene, 5'-gatcGATCAATGGGGAAAACCCCATGTG-3'; NF-κB element of the IL-2R α chain gene, 5'-gatcCGGCAGGGGAATCTCCCTCTC-3'; and AP-1 element of the IL-8 gene, 5'-gatcGTGATGACTCAGGTT-3'. The oligonucleotide 5'-gatcTGTCGAATGCAAATCACTAGAA-3', containing the consensus sequence of the octamer binding motif was used to identify specific binding of the transcription factor Oct-1. The underlined sequences represent the NF-κB, AP-1, or Oct-1 binding site. To identify NF-κB proteins in the DNA protein complex shown by EMSA, we used antibodies specific for various NF-κB family proteins, including p50, p65, c-Rel, p52, and RelB to elicit a supershift DNA protein complex formation. These antibodies were incubated with the nuclear extracts for 45 min at room temperature before incubation with radiolabeled probe.

Western blot analysis. Cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol, and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing sequentially with the specific antibodies. The bands were visualized with the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

CCL20 measurement. The CCL20 content in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). MKN45 and MKN28 cells were cultured in RPMI 1640 supplemented with 10% FBS in 24-well plates. Subconfluent monolayers of cells were cocultured with *H. pylori* for 24 h. The supernatants were then collected after centrifugation to remove bacteria and stored at –80°C until assayed for CCL20 by ELISA. The concentration of CCL20 was determined using a standard curve obtained with recombinant CCL20.

Immunohistochemical analysis. Serial sections were deparaffinized in xylene and dehydrated through a graded ethanol series. For better detection, sections were pretreated with ready-to-use proteinase K (Dako, Inc., Carpinteria, CA) for 20 min at 37°C. This procedure increases the number of antigenic sites available for binding by the antibody. In the next step, the tissues were placed in 3% hydrogen peroxide and absolute methanol for 5 min to reduce endogenous peroxidase activity, followed by washing in PBS. The tissue sections were incubated with goat anti-human CCL20 polyclonal antibody (diluted 1:250) or a control mouse IgG for 3 h at 37°C. After washing with PBS, the sections were covered with EnVision plus (Dako, Santa Barbara, CA) for 40 min at 37°C and washed in PBS. Antigenic sites bound by the antibody were identified by reacting these sections with a mixture of 0.05% 3,3’-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl buffer and 0.01% hydrogen peroxide for 7 min. Sections were then counterstained with methyl green for 10 min, hydrated in ethanol, cleaned in xylene, and mounted.

RESULTS

High expression levels of CCL20 in gastric mucosa of patients with *H. pylori* gastritis. RT-PCR showed the presence of CCL20 transcripts in the specimens of all the 5 patients with *H. pylori* gastritis (Fig. 1A). Analysis of *H. pylori*-negative control specimens showed very low to undetectable levels of CCL20 mRNA.

Immunohistochemical studies. We also investigated the presence of CCL20 protein in *H. pylori*-positive gastric diseases and determined its cellular source. For this purpose, we immunostained *H. pylori*-positive gastritis tissues (n = 2). CCL20 staining was exclusively detected in the mucosal epithelial cells of specimens of patients with *H. pylori*-positive gastritis (Fig. 1D and E). In contrast, CCL20 staining was faint in the normal mucosa (Fig. 1B and C).

***H. pylori* increases steady-state CCL20 mRNA levels in gastric epithelial cells.** Using RT-PCR, we next examined whether coculture of two gastric epithelial cell lines, MKN45 and MKN28, with *H. pylori* leads to the induction of CCL20 mRNA. Coculture with *H. pylori* significantly enhanced steady-state levels of CCL20 mRNA in both cell lines (Fig. 2A). CCL20 transcript levels clearly increased 1 h after addition of *H. pylori* to the MKN45 and MKN28 cells, and reached peak levels at 6 h, but were weaker at 24 h after cocultivation (Fig. 2A). Supernatants derived from *H. pylori* cultures failed to induce CCL20 mRNA expression in MKN45 cells (Fig. 2B). Moreover, neither heat-killed bacteria nor live bacteria, separated by a permeable

membrane, induced CCL20 mRNA expression in MKN45 cells (data not shown). These results suggest that interaction with live *H. pylori* itself, rather than products secreted by these bacteria, upregulates the steady-state levels of CCL20 mRNA. In the next step, we examined whether CCL20 was secreted into the culture media of MKN45 and MKN28 cells cocultured with *H. pylori*. ELISA indicated that CCL20 was secreted into the media of MKN45 and MKN28 cells cocultured with *H. pylori* over a 24-h period and that its concentration was dependent on the density of *H. pylori* (Fig. 3).

***H. pylori*-induced CCL20 mRNA expression is strain-dependent.** Because recent studies indicated that expression of multiple genes in the *cag* PAI is necessary for cytokine production by gastric epithelial cells in vitro (10, 52), we examined the ability of various *H. pylori* strains, possessing or lacking the *cag* PAI, to induce CCL20 mRNA expression. Infection with *H. pylori* strains ATCC 49503, OHPC0001, and OHPC0003, which contain the entire *cag* PAI (53), led to increased CCL20 mRNA levels in MKN45 cells (Fig. 2B). On the other hand, strain OHPC0002, which lacks the *cag* PAI (53), failed to induce CCL20 mRNA expression (Fig. 2B). The ability of *H. pylori* to induce CCL20 mRNA expression was independent of the *vacA* locus, because all bacteria isolates used in this experiment had this gene (Fig. 2B) (1). Furthermore, strain OHPC0001 induced CCL20 mRNA expression despite the absence of vacuolating cytotoxic activity (1). To determine whether the observed difference in CCL20 mRNA expression was specific to the *cag* PAI, we next examined a wild-type *cag* PAI-positive *H. pylori* strain (26695) and an isogenic *cag*

PAI mutant (Δ*cag* PAI). As expected, stimulation with the wild-type strain 26695 induced CCL20 mRNA expression in MKN45 and MKN28 cells, while the isogenic mutant that lacked the expression of *cag* PAI did not (Fig. 2C). These results suggest that the *H. pylori cag* PAI plays an important role in the induction of CCL20 mRNA expression.

***H. pylori* regulates CCL20 gene transcription.** In the next series of experiments, we investigated whether *H. pylori*-mediated upregulation of CCL20 gene expression could directly enhance the activity of its promoter. MKN45 cells were transiently transfected with a reporter gene construct containing the –874 to +58 segment of the CCL20 upstream regulatory sequences. Coculture of *H. pylori* caused a dose-dependent increase in the activity of this CCL20-driven reporter construct (Fig. 4A). We further investigated the involvement of *cag* PAI in the induction of CCL20 promoter activity in MKN45 cells. The wild-type strain 26695 increased CCL20-driven reporter gene activity, but activation of this reporter was not observed by the isogenic mutant Δ*cag* PAI (Fig. 4B). Therefore, *cag* PAI appears to be required for activation of the CCL20 promoter.

NF-κB is essential for activation of the CCL20 promoter by *H. pylori* infection. The NF-κB signaling pathway is activated in epithelial cells infected with *cag* PAI-positive *H. pylori*, but not in those infected with *cag* PAI-negative strains of *H. pylori* (18, 35, 49). Indeed, the wild-type strain 26695 increased transcription of the NF-κB-dependent reporter gene (κB-LUC), while the isogenic *cag* PAI mutant did not

(Fig. 4B). To test the relative contribution of NF-κB binding site to *H. pylori*-mediated activation of CCL20, the plasmid with point mutations in this site of the CCL20 promoter was transfected (Fig. 5). Mutation of the NF-κB binding site (CCL20/κBM) reduced *H. pylori*-mediated activation of this reporter construct. Therefore, the NF-κB binding site contributes to activation of the CCL20 promoter induced by *H. pylori* infection.

***H. pylori* infection of gastric epithelial cells induces binding of NF-κB family proteins to the NF-κB element of the CCL20 promoter.** Because the mutational analysis of the CCL20 promoter indicated that *H. pylori* infection activated transcription through the NF-κB site, it was important to identify the nuclear factors that bind to this site. The NF-κB sequence derived from the CCL20 promoter was used as a probe in electrophoretic mobility shift assay (EMSA). MKN45 and MKN28 cells were infected with *H. pylori*, at different time intervals after challenge, nuclear protein extracts were prepared and analyzed for NF-κB DNA-binding activity. As shown in Figure 6A, a complex was induced in MKN45 and MKN28 cells within 10 min after infection with *H. pylori*. This binding activity was reduced by the addition of either cold probe or the typical NF-κB sequence derived from the IL-2Rα enhancer but not by an oligonucleotide containing the AP-1 binding site (Fig. 6B, lanes 2 to 4). Next, we characterized the *H. pylori*-induced complexes identified by the CCL20 NF-κB probe. These complexes were supershifted by the addition of anti-p50 or anti-p65 antibody (Fig. 6B, lanes 5 to 9), suggesting that *H. pylori*-induced CCL20 NF-κB binding activity is composed of p50 and p65. Based on these results, *H. pylori*

infection seems to induce CCL20 gene expression at least in part through the induced binding of p50 and p65 to the NF-κB site in the CCL20 promoter region.

As described above, *cag* PAI-positive strains induced significantly more CCL20 mRNA than *cag* PAI-negative *H. pylori* strains. Next, we determined whether *cag* PAI-positive *H. pylori* strains better induced NF-κB. Markedly increased NF-κB DNA-binding activity was induced by the wild-type strain 26695 compared with the isogenic *cag* PAI mutant (Fig. 6C). These results indicate that the better activation of NF-κB binding by *cag* PAI-positive strains is the underlying mechanism of the observed activation of the CCL20 promoter by these bacterial strains. Considered together, these results indicate that *H. pylori* infection induces CCL20 gene expression at least in part through the induced binding of p50 and p65 NF-κB family members to the NF-κB element of the CCL20 promoter and this effect is dependent on *cag* PAI products.

NF-κB signal is essential for induction of CCL20 expression by *H. pylori*. We next examined whether *H. pylori*-mediated upregulation of CCL20 gene expression involves signal transduction components in NF-κB activation. The activation of NF-κB requires phosphorylation of two conserved serine residues of IκBα (Ser-32 and Ser-36) and IκBβ (Ser-19 and Ser-23) within their NH_2-terminal domain (31). Phosphorylation leads to ubiquitination and the 26S proteasome-mediated degradation of IκBs, thereby releasing NF-κB from the complex to translocate to the nucleus and activate genes. The signal is eventually terminated through cytoplasmic resequestration of NF-κB, which depends on IκBα synthesis, a process requiring

NF-κB transcriptional activity (31). *H. pylori* infection induced the phosphorylated IκBα. Kinetic analysis of *H. pylori*-induced degradation of IκBα in MKN45 cells revealed gradual replacement of IκBα levels (Fig. 7A). The high-molecular-weight complex, IKK complex, which is composed of the two catalytic subunits (IKKα and IKKβ) and the regulatory subunit (IKKγ) phosphorylates IκBs (31). Previous studies indicated that members of the mitogen-activated protein kinase kinase kinase kinase family mediate physiologic activation of IKK (57). These kinases include NIK (54). Dominant interfering mutants of IκBα, IκBβ, and IKKγ, and kinase-deficient mutants of IKKα, IKKβ, and NIK were tested for their ability to inhibit *H. pylori*-mediated activation of the CCL20-driven reporter gene. Expression of these various inhibitory mutants abolished *H. pylori*-induced CCL20 expression (Fig. 7B). These data show that signaling components involved in the activation of NF-κB are necessary for *H. pylori* activation of the CCL20 promoter.

Because activation of the CCL20 promoter by *H. pylori* infection required activation of NF-κB, we blocked NF-κB activation with Bay 11-7082, an inhibitor of IκBα phosphorylation (44), or LLnL, a proteasome inhibitor (28). The proteasome inhibitor is known to inhibit the activation of NF-κB by blocking the degradation of the IκBα protein. These studies served to demonstrate a link between NF-κB activation and upregulation of CCL20 expression in *H. pylori*-infected cells. As shown in Figure 7C, Bay 11-7082 or LLnL markedly inhibited *H. pylori*-induced expression of CCL20 mRNA. Bay 11-7082 or LLnL inhibited *H. pylori*-induced NF-κB DNA-binding (Fig. 7D).

Inhibition of Hsp90 reduces CCL20 expression induced by *H. pylori*. Hsp90 plays a critical role in the inflammatory response and a requirement of Hsp90 for activation of NF-κB has been suggested (6, 7, 59). As a possible mechanistic link between *H. pylori* infection and inflammation, we hypothesized the involvement of Hsp90. To test this hypothesis, we investigated the effect of *H. pylori* infection on Hsp90 and evaluated the effect of Hsp90 inhibitor, 17-AAG, on *H. pylori*-induced CCL20 expression. MKN45 cells constitutively expressed Hsp90 protein, but *H. pylori* infection did not affect its expression (Fig. 8A). Furthermore, pretreatment with 17-AAG completely inhibited *H. pylori*-induced CCL20 expression (Fig. 8B). These findings suggest possible involvement of Hsp90 in *H. pylori*-induced CCL20 signaling.

Next, we tested the direct influence of 17-AAG on *H. pylori*-induced transcriptional activity of NF-κB using EMSA. Pretreatment with 17-AAG decreased the retardation of gel mobility through the inhibition of DNA binding activity of NF-κB complex, indicating repression of transcriptional activity of NF-κB (Fig. 8C). Of note, no differences in binding to the octamer motif on DNA were noted in the absence or presence of 17-AAG. Next, we examined the effect of 17-AAG on expression of IKKα and IKKβ since IKKα and IKKβ are known clients of Hsp90 (6). Treatment of MKN45 cells with 17-AAG in the absence of *H. pylori* reduced the amounts of IKKα and IKKβ proteins but not those of IKKγ and Hsp90 (Fig. 8D).

Several Hsp90 client proteins are degraded by the proteasome following Hsp90 inhibition (36). To examine whether proteasomal degradation was responsible for decreased levels of client proteins after 17-AAG treatment, MKN45 cells were

cultured in a medium containing 17-AAG and proteasomal inhibitor LLnL. 17-AAG-mediated degradation of IKKα and IKKβ proteins was partially blocked by LLnL (Fig. 8D). In contrast, IKKγ and Hsp90 were not destabilized by 17-AAG, and LLnL did not change the protein level of IKKγ and Hsp90. The reversal of 17-AAG-induced degradation of IKKα and IKKβ proteins by LLnL suggests that these proteins are subject to ubiquitin-dependent turnover.

DISCUSSION

A recent study reported that *H. pylori* infection upregulated CCL20 expression in gastric epithelial cells and induced an influx of myeloid DCs in the lamina propria of the gastric mucosa in neonatally thymectomized mice (42). However, CCL20 expression in human *H. pylori*-induced gastritis remains unclear. We demonstrated here the upregulation of CCL20 mRNA expression in *H. pylori*-infected gastritis compared with normal controls. Immunohistochemical analysis showed that CCL20 protein expression is exclusively localized in the mucosal epithelium. After the completion of this work, Wu et al. reported that there was significantly increased CCR6 expression in $CD3^+$ T cells infiltrating the gastric mucosa, and its ligand CCL20 was selectively expressed in inflamed gastric tissues from *H. pylori*-infected subjects (55). In the present study, we examined the hypothesis that *H. pylori* infection of gastric epithelial cells modulates the host cell CCL20 expression levels. Coculture with *H. pylori* significantly enhanced steady-state levels of CCL20 mRNA in gastric epithelial cells. The results showed that the NF-κB binding site present in

the CCL20 promoter is required for CCL20 induction by *H. pylori*. However, mutagenesis of the NF-κB binding site did not reduce *H. pylori*-induced CCL20 promoter activation to baseline levels, suggesting that additional pathways are involved in stimulating CCL20 expression. The CCL20 promoter region contains a putative TATA and CAAT box, and possible binding sites for various transcription factors other than NF-κB, like AP-1, AP-2, CAAT/enhancer-binding protein, Sp1, and the epithelium-specific Ets nuclear factor ESE-1 (48). The NF-κB binding site and to a lesser degree the AP-1 binding site are involved in *H. pylori*-induced IL-8 gene activation (1). Therefore, the AP-1 binding site may also be involved in *H-pylori*-induced CCL20 gene activation.

Because neither supernatants of *H. pylori* cultures nor *H. pylori* separated by a permeable membrane induced CCL20 expression, components of the *H. pylori* bacterium most likely trigger the induction of CCL20 in gastric cells. That a *cag* PAI-negative strain of *H. pylori* could not induce CCL20 expression suggests the involvement of the *cag* PAI gene products in the induction of CCL20 gene expression. In this study, we analyzed the capacities of different *H. pylori* strains to induce CCL20 and identified the signaling components NIK and IKKs as likely participants in *H. pylori*-mediated NF-κB activation. Compared with the *cag* PAI-negative *H. pylori* strain, the more virulent *cag* PAI-positive strains potently induced CCL20 promoter activity and NF-κB binding activity. This was consistent with our observations that increased induction of CCL20 in MKN45 cells was associated with *cag* PAI-positive strains. These results show that the *cag* PAI-positive *H. pylori*-induced CCL20 expression is dependent on prior activation of NF-κB p50 and

p65 subunits. The *cagE*, *cagG*, *cagP*, or surrounding genes in the *cag* PAI has a function related to adhesion to epithelial cells (39, 58). However, the isogenic *cag* PAI mutant adhered a little less than the wild-type strain (26695) to MKN45 cells, suggesting that the less induction of CCL20 expression by the *cag* PAI mutant is not due to the reduced adherence to epithelial cells.

The mammalian signaling pathway(s) triggered by *H. pylori* remains largely unknown. In this study, we identified the cellular kinases NIK and IKKs as participants in NF-κB-dependent CCL20 induction by *H. pylori* in gastric epithelial cells. In addition, we documented the effect of Hsp90 inhibitor, 17-AAG, on *H. pylori*-induced CCL20 expression and identified its molecular mechanism. 17-AAG inhibited CCL20 mRNA expression in *H. pylori*-infected gastric epithelial cells. This finding may be due to inactivation of NF-κB signaling induced by *H. pylori* infection. Hsp90 is a regulator of NF-κB signaling through its general involvement in IKK activation (6). 17-AAG decreased IKK complex proteins, IKKα and IKKβ. The loss of IKK reduced NF-κB DNA-binding, resulting in reduction of *H. pylori*-induced CCL20 mRNA expression. In agreement with our results, previous studies have shown that the blockage of Hsp90 inhibits *H. pylori*-induced IL-8 production through the inactivation of NF-κB (56).

In conclusion, we provided evidence of upregulated CCL20 expression in *H. pylori*-infected human gastric epithelial cells. Because CCL20 is important in the migration of myeloid DCs into the lamina propria of the gastric mucosa (42), modification of the CCL20/CCR6 might be a potentially useful strategy in the pharmacological management of *H. pylori*-induced gastritis.

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FIGURE LEGENDS

FIG. 1. Expression of CCL20 in *H. pylori*-infected gastric mucosa. (A) RT-PCR analysis of CCL20 in human gastric tissues. Lanes 1 to 5, normal mucosa, lanes 6 to 10, *H. pylori*-positive gastritis. β-actin expression served as control. Representative example of three similar experiments. (B to E) Immunohistochemical detection of CCL20 in tissues of patients with *H. pylori*-positive gastritis. The serial sections of gastric biopsy specimens were stained with goat polyclonal antibody to CCL20. Sections were counterstained with methyl green. B and C are representative examples of normal mucosa. D and E are representative examples of *H. pylori*-positive gastritis. Note the positive staining for CCL20 in the epithelial cells of *H. pylori*-positive gastritis. (Original magnification 400×)

FIG. 2. *H. pylori*-induced CCL20 mRNA expression in gastric epithelial cells. (A) Dynamics of *H. pylori*-induced CCL20 mRNA expression. Total RNA was extracted from MKN45 and MKN28 cells infected with *H. pylori* (ATCC 49503) for the indicated time intervals and used for RT-PCR. The bacterium to cell ratio was 20:1. (B) *cag* PAI-positive and *cag* PAI-lacking *H. pylori* strains differ in their ability to induce CCL20 expression. (C) The *cag* PAI of *H. pylori* is required for induction of CCL20 expression in MNK45 cells. Total RNA was extracted from the cells infected with *H. pylori* for 6 h and used for RT-PCR. β-actin expression served as control. Representative examples of three similar experiments in each section.

FIG. 3. Increased secretion of CCL20 into the supernatants of MKN45 and MKN28 cultures in response to *H. pylori* infection at 24 h. Cells were infected with varying densities of *H. pylori* (ATCC 49503). CCL20 concentrations in the supernatants were determined by ELISA. Data are mean ± SD of three experiments.

FIG. 4. *H. pylori* infection activates the CCL20 promoter in gastric epithelial cells. (A) *H. pylori* infection increased CCL20 promoter activity in a dose-dependent fashion. Either pGL2-CCL20 or pGL2 (promoter-less luciferase vector) was transfected into MKN45 cells, and the cells were subsequently infected with *H. pylori* (ATCC 49503) for 6 h. The activities are expressed relative to that of cells transfected with pGL2-CCL20 without further treatment, which was defined as 1. (B) The *cag* PAI is required for induction of CCL20 promoter activity. pGL2-CCL20, pGL2, or κB-LUC was transfected into MKN45 cells, and the cells were subsequently infected with the wild-type strain 26695 (WT) or the isogenic mutant Δ*cag* PAI (Δ*cag*) for 6 h. The bacterium to cell ratio was 20:1. The activities are expressed relative to that of cells transfected with pGL2-CCL20 without further treatment, which was defined as 1. Data are mean ± SD values of three independent experiments.

FIG. 5. *H. pylori* activates the CCL20 promoter through NF-κB binding site. (A) Schematic representation of the CCL20 reporter constructs containing the wild-type (pGL2-CCL20) and mutant (pGL2-CCL20/κBM) NF-κB site; underlined letters indicate substituted bp. (B) The NF-κB element in the CCL20 promoter is critical for the *H. pylori*-induced activity. Either pGL2-CCL20 or pGL2-CCL20/κBM was

transfected into MKN45 cells, and the cells were subsequently infected with *H. pylori* (ATCC 49503) for 6 h. The bacterium to cell ratio was 20:1. The activities are expressed relative to that of cells transfected with pGL2-CCL20 without further treatment, which was defined as 1. Data are mean ± SD values of three independent experiments.

FIG. 6. *H. pylori* infection induced NF-κB binding activity. (A) Time course of NF-κB activation in MKN45 and MKN28 cells infected with *H. pylori*, evaluated by EMSA. Nuclear extracts from cells infected with *H. pylori* (ATCC 49503) for the indicated time periods were mixed with NF-κB ^{32}P-labeled probe. The bacterium to cell ratio was 20:1. (B) Sequence specificity of NF-κB binding activity and characterization of NF-κB proteins that bound to the NF-κB binding site of the CCL20 gene. Competition assays were performed with nuclear extracts from MKN45 and MKN28 cells infected with *H. pylori* (ATCC 49503) for 1 h. Where indicated, 100-fold excess amounts of each specific competitor oligonucleotide were added to the reaction mixture with labeled probe NF-κB (lanes 2 to 4). Supershift assay of NF-κB DNA-binding complexes in the same nuclear extracts was also performed. Where indicated, appropriate antibodies were added to the reaction mixture before the addition of ^{32}P-labeled probe (lanes 5 to 9). Arrows show the specific complexes, while arrowheads point to the DNA-binding complexes supershifted by antibodies. (C) *cag* PAI products of *H. pylori* are required for induction of NF-κB binding activity in MKN45 cells. Nuclear extracts from MKN45 cells cocultured with variable

densities of the wild-type strain 26695 or the isogenic mutant Δ*cag* PAI were analyzed for NF-κB. Representative examples of three similar experiments in each section.

FIG. 7. NF-κB signal is essential for activation of CCL20 expression by *H. pylori*. (A) *H. pylori* infection leads to IκBα phosphorylation and degradation. MKN45 cells were infected with *H. pylori* (ATCC 49503), for the indicated time periods. The bacterium to cell ratio was 20:1. The cells were then lysed and analyzed by immunoblot with phospho-specific IκBα, IκBα, and actin antibodies. Representative examples of three similar experiments. (B) Functional effects of IκBα, IκBβ, and IKKγ dominant interfering mutants and kinase-deficient IKKα, IKKβ, and NIK mutants on *H. pylori*-induced activation of the CCL20 promoter. MKN45 cells were transfected with pGL2-CCL20 and the indicated mutant plasmids or empty vector (pCMV4), and then infected with *H. pylori* (ATCC 49503) for 6 h. Open bar: luciferase activity of the pGL2-CCL20 and pCMV4 without *H. pylori* infection. All values were first calculated as a fold induction relative to the basal level measured in uninfected cells. Data are mean ± SD values of three independent experiments. (C) Bay 11-7082 and LLnL inhibit CCL20 mRNA expression induced by *H. pylori*. MKN45 cells were pretreated with Bay 11-7082 (20 μM) and LLnL (20 μM) for 1 h prior to *H. pylori* infection. They were subsequently infected with *H. pylori* for 6 h. CCL20 mRNA expression on harvested cells was analyzed by RT-PCR. Representative examples of three similar experiments. (D) Bay 11-7082 and LLnL inhibit *H. pylori*-induced NF-κB DNA-binding. MKN45 cells were pretreated with Bay 11-7082 (20 μM) and LLnL (20 μM) for 1 h prior to *H. pylori* infection. They

were subsequently infected with *H. pylori* for 1 h. Nuclear extracts from harvested cells were analyzed for NF-κB. Representative examples of three similar experiments.

FIG. 8. Inhibitory effects of 17-AAG on *H. pylori*-induced CCL20 expression. (A) *H. pylori* infection does not affect Hsp90 expression. MKN45 cells were infected with *H. pylori* (ATCC 49503), for the indicated time periods. The bacterium to cell ratio was 20:1. The cells were then lysed and analyzed by immunoblot with Hsp90 and actin antibodies. (B) MKN45 cells were incubated with 1 μM 17-AAG for 16 h prior to infection with variable densities of *H. pylori* for 6 h. RT-PCR was performed to check the changes of CCL20 mRNA expression after 17-AAG treatment in *H. pylori*-infected MKN45 cells. (C) Attenuation of *H. pylori*-induced NF-κB DNA-binding by 17-AAG treatment. MKN45 cells were treated with (+) or without (-) 17-AAG for 16 h prior to infection with *H. pylori* for 1 h. The nuclear extracts were isolated from MKN45 cells infected with *H. pylori* and analyzed for NF-κB. (D) Hsp90 protects IKKα and IKKβ from proteasomal degradation. MKN45 cells were either pretreated with the proteasomal inhibitor LLnL (20 μM) for 1 h, followed or not by the addition of 17-AAG (1 μM) for 16 h, or were treated with 17-AAG for 16 h or left untreated in the absence of *H. pylori* as indicated. Samples were analyzed for each protein by western blotting. Representative examples of three similar experiments in each section.