Helicobacter pylori Lipopolysaccharides Upregulate Toll-Like Receptor 4 Expression and Proliferation of Gastric Epithelial Cells via the MEK1/2-ERK1/2 Mitogen-Activated Protein Kinase Pathway[▽]

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Helicobacter pylori is recognized as an etiological agent of gastroduodenal diseases. *H. pylori* produces various toxic substances, including lipopolysaccharide (LPS). However, *H. pylori* LPS exhibits extremely weakly endotoxic activity compared to the typical LPS, such as that produced by *Escherichia coli*, which acts through Toll-like receptor 4 (TLR4) to induce inflammatory molecules. The gastric epithelial cell lines MKN28 and MKN45 express TLR4 at very low levels, so they show very weak interleukin-8 (IL-8) production in response to *E. coli* LPS, but pretreatment with *H. pylori* LPS markedly enhanced IL-8 production induced by *E. coli* LPS by upregulating TLR4 via TLR2 and the MEK1/2-ERK1/2 pathway. The transcription factor NF- κ B was activated by this signal and promoted transcription of the *tlr4* gene. These MEK1/2-ERK1/2 signal-mediated activities were more potently activated by LPS carrying a weakly antigenic epitope, which is frequently found in gastric cancers, than by LPS carrying a highly antigenic epitope, which is associated with chronic gastritis. *H. pylori* LPS also augmented the proliferation rate of gastric epithelial cells via the MEK1/2-ERK1/2 pathway. *H. pylori* LPS may be a pathogenic factor causing gastric tumors by enhancing cell proliferation and inflammation via the MEK1/2-ERK1/2 mitogen-activated protein kinase cascade in gastric epithelial cells.

Helicobacter pylori is a microaerophilic, spiral gram-negative bacterium that can colonize gastric epithelial cells or gastric mucin. *H. pylori* is recognized as a causative factor of chronic gastritis, gastroduodenal ulcers, gastric cancer, and mucosa-associated lymphatic tissue lymphoma. The cell injury and inflammation caused by chronic *H. pylori* infection is believed to underlie these diseases (5). *H. pylori* produces several factors that induce inflammatory reactions. For example, vacuolating toxin (VacA), an ammonium ion produced by *H. pylori* urease, and monochloramines are cytotoxic (9, 33). The monochloramines form from an ammonium ion produced by urease and hypochlorite produced by phagocytic cells. *H. pylori* activates NF- κ B through the type IV secretion system, which consists of proteins encoded by *cag* pathogenicity island (8). *H. pylori* 60-kDa heat shock protein (HSP60) induces the production of inflammatory cytokines via the mitogen-activated protein (MAP) kinase pathway (40). The chronic inflammation induced by these substances may indirectly cause gastroduodenal diseases, including gastric cancer. In addition, direct mechanisms for carcinogenesis have been reported. For example, *H. pylori* CagA is inserted into host cells via the type IV secretion system, and it activates various host intracellular signaling pathway, which leads to the promotion of cell proliferation and motility, inhibition of apoptosis, and increased inflammatory cytokine production

(reviewed in reference 22). Another direct mechanism is that *H. pylori* induces host activation-induced cytidine deaminase, which promotes genetic mutations in tumor suppressors, such as p53 (20).

In general, gram-negative bacterial lipopolysaccharides (LPSs) are key inducers of inflammation through their role as agonists of Toll-like receptors (TLRs). However, *H. pylori* LPSs show extremely low endotoxic activity compared to typical gram-negative bacterial LPSs, such as those from *Escherichia coli* (11, 23, 25, 34). The weak endotoxic activity allows *H. pylori* to establish chronic colonization or infection, rather than causing a systemic inflammatory response, such as septic shock. The typical LPSs are recognized by the TLR4 complex expressed on host cells, which consists of TLR4, CD14, and MD2. However, there is controversy over which TLR contributes to signal transduction by *H. pylori* LPS. Some reports suggest that *H. pylori* LPS transduces signals via the TLR4 system (12, 13, 24), whereas others (15, 30), including our recent report (37), suggest that TLR2 is required for the signal transduction induced by *H. pylori* LPS. Notably, Triantafyllou et al. (32) suggested that LPS derived from some *H. pylori* strains antagonizes the TLR4 signaling activated by a typical LPS.

We have proposed that *H. pylori* LPSs are divided into three types: a smooth LPS that carries a highly antigenic epitope, a smooth LPS that carries a weakly antigenic epitope, and a rough LPS that lacks an O-polysaccharide chain (34, 39). The highly antigenic epitope and the weakly antigenic epitope are located on the O-polysaccharide chain adjacent to the core region of the LPS. Most *H. pylori*-infected individuals have high titer antibodies against the highly antigenic epitope in sera, whereas about half of infected individuals have low titers

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of antibodies against the weakly antigenic epitope (35, 39). The LPS carrying the weakly antigenic epitope is frequently found in strains obtained from patients with gastric cancer, whereas the LPS with the highly antigenic epitope is preferentially found in strains associated with chronic gastritis (34, 39), suggesting that the antigenicity of LPS is related to the pathogenicity of *H. pylori* strains. However, the relationship is still unclear. In the present study, we found that *H. pylori* LPS upregulates TLR4, thereby potentiating the effects of LPS from other organisms, such as *E. coli*. *H. pylori* LPS also increased the proliferation of gastric epithelial cells. These properties might contribute to gastric inflammation and carcinogenesis.

MATERIALS AND METHODS

Bacterial strains and preparation of LPS. *H. pylori* clinical strains were isolated from biopsy specimens obtained from patients with chronic gastritis (CG), gastric ulcer (GU), duodenal ulcer (DU), and gastric cancer (CA) (36). After fewer than three passages of laboratory cultures, these bacteria were grown in brucella broth (BBL, Cockeysville, MD) supplemented with 10% (vol/vol) horse serum at 37°C for 2 days in microaerophilic conditions by using the Campybak system (BBL). The organisms were collected by centrifugation (10,000 × g, 30 min), washed twice with deionized water, and lyophilized. Conventional LPS was prepared from lyophilized cells by the hot phenol-water method (36). LPS was further purified in several steps as previously described (37), and this highly purified LPS was used to avoid the potential influence of contaminants. The conventional LPS preparation was treated with 10 µg of DNase I (Roche Diagnostics, Tokyo, Japan)/ml, 10 µg of RNase A (Qiagen, Hilden, Germany)/ml, 2 µg of lipoprotein lipase from bovine milk (Sigma-Aldrich, St. Louis, MO)/ml, and 10 µg of lipoprotein lipase from *Pseudomonas* spp. (Sigma-Aldrich)/ml at 37°C for 12 h in 50 mM sodium phosphate buffer (pH 7.2). After the enzyme treatment, proteinase K (Wako Junyaku, Tokyo, Japan) was added at a concentration of 20 µg/ml, and the mixture was further incubated at 60°C for 4 h. The resulting material was subjected to octyl-Sepharose column chromatography (GE Healthcare Bio-Science, Piscataway, NJ), and the LPS fraction was applied to a PD-10 desalting column (GE Healthcare Bio-Science) equilibrated with 0.2% triethylamine. The material eluted at void volume was pooled, lyophilized, and used as a highly purified LPS preparation.

Cell lines. The human gastric carcinoma cell lines MKN28 and MKN45 were obtained from the Japanese Collection of Research Biosources (JCRB; Ibaraki, Japan). MKN28 and MKN45 were routinely cultured in Dulbecco modified minimum essential medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Reagents. A MEK1/2 inhibitor (PD98059), ERK1/2 inhibitor (FR180204), p38 MAP kinase inhibitor (SB202190), and JNK inhibitor (SP600125) were purchased from Calbiochem-Merck (Darmstadt, Germany). Pam₃-CSK₄ (a TLR2/TLR1 agonist) and MALP-2 (a TLR2/TLR6 agonist) were purchased from Bachem (Bubendorf, Switzerland) and Alexis (Lausen, Switzerland), respectively. *E. coli* O111:B4 LPS was purchased from Sigma-Aldrich. The LPS was further purified as described above.

ELISA. The amounts of interleukin-8 (IL-8) in culture supernatants were determined with an enzyme-linked immunosorbent assay (ELISA) development kit for human IL-8 (R&D Systems, Minneapolis, MN).

Western blotting analysis. Cells were lysed with 1% Nonidet P-40, 120 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 1 mM NaF, and 20 mM HEPES-NaOH (pH 7.5) as previously described (38). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as previously described (38). A rabbit anti-ERK polyclonal antibody (C-16), mouse anti-phospho-ERK (Tyr204) monoclonal antibody (E-4), rabbit anti-JNK polyclonal antibody (FL), and mouse anti-phospho-JNK (Thr183 and Tyr185) monoclonal antibody (G-7) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit anti-MEK1/2 polyclonal antibody and rabbit anti-phospho-MEK1/2 (Ser221) monoclonal antibody (166F8) were purchased from Cell Signaling Technology (Danvers, MA). A rabbit anti-p38 MAP kinase polyclonal antibody and mouse anti-diphosphorylated p38 MAP kinase monoclonal antibody (P38TY) were purchased from Sigma. Alkaline phosphatase-labeled goat anti-mouse and anti-rabbit immunoglobulin antibodies were purchased from BioSource International (Camarillo, CA) and used as secondary antibodies.

TABLE 1. Sequences of oligonucleotide primers used in site-directed mutagenesis

Mutant	Primer	Sequence (5'–3')
mA/E	mA/Es	CAGAGGTCAGAGTACTAATTGGG
	mA/Eas	CAATTAGTACTCTGACCTCTGCC
mPU.1_5	mPU.1_5s	GCCAACTAGCTAGTTCCTTGCTGTT
		TCTTTAG
	mPU.1_5as	CTAAAGAAACAGCAAGAACTAGC
		TAGTTGGC
mPU.1_2	mPU.1_2s	CTTCTTCTAACTAGTTCCTCTG
		TGAC
	mPU.1_2as	GTCACAGGAGAACTAGTTAGAA
		GAAG
mIRF	mIRFa	CGCTAGTACTTCCTCTCACCCCTT
	mIRFas	AAGGGTGAGAGGAAGTACT
		AGCG
mPU.1_1	mPU.1_1a	CGCTTTCACTAGTTCCTCACCCCTT
	mPU.1_1as	AAGGGTGAGAACTAGTGAA
		AGCG
mNFY	mNFYa	CAGTACTTGCTGTGGGGCGGCTC
		GAGG
	mNFYas	GCCCCACAGCAAGTACTGTATTC
		AAAGCAGTTC
mGC	mGCa	ACCAATTGCTGTTCAGCGGCTC
		GAGG
	mGCas	CTCTGTGGTTTCTGGTACCTCCT
		CTGG
mPU.1_0	mPU.1_0a	GGGCGGCTCGAACTAGAGAAGA
		CACC
	mPU.1_0as	GGTGCTTCTCTAGTTCGAGCC
		GCCC
mINR	mINRa	CTCGGTGTCCGTGTGATAGCGAG
		CCAC
	mINRas	CTATCACACGGACACCGAGCAGT
		TTCTGAGGC

Specific binding was detected by using tetrazolium bromochloroindolylphosphate-nitroblue tetrazolium as a developing substrate.

Semiquantitative RT-PCR. Total RNA was isolated from cells by using an RNeasy minikit (Qiagen). Reverse transcription-PCR (RT-PCR) was performed by using the OneStep RT-PCR kit (Qiagen). mRNA levels of TLR2, TLR4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a control, were determined as previously described (27). The quantitative nature of the RT-PCR was validated by the linearity of the determination curve at various concentrations of RNA.

Flow cytometry. Flow cytometry was performed by using a FACSCalibur (Becton Dickinson, San Jose, CA) as previously described (27, 37). Phycoerythrin-labeled mouse monoclonal antibodies against TLR2 (clone TLR2.1), TLR4 (HTA125), and CD14 (61D3), and their isotype controls, phycoerythrin-labeled mouse monoclonal immunoglobulin G1 (IgG1) and IgG2a, were purchased from eBioscience (San Diego, CA).

Luciferase reporter gene assay. Construction of the luciferase reporter plasmid containing the human TLR4 promoter was described previously (16, 26). A series of mutant reporter plasmids were generated by overlap PCR using specific primers (Table 1), together with the vector-specific primers GL2 and RV3 (Promega), and using the reporter plasmid TLR4E containing the TLR4 promoter (–385 to +190) (26) as a template. After MKN28 cells were plated in 96-well plates, the cells were transfected by using Fugene (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions with the indicated expression plasmids (0.02 µg), together with 0.0025 µg of pRL-TK (Promega) for normalization. After 24 h, cells were stimulated with *E. coli* LPS, *H. pylori* LPS, or Pam₃-CSK₄ for 6 h. Reporter activity was measured by using the dual-luciferase reporter assay system (Promega).

ELISA. Nuclear extracts were prepared by using NE-PER nuclear and cytoplasmic extraction reagents (Thermo, Rockford, IL). The DNA-binding activities of NF-Y in the nuclear extracts were determined by enzyme-linked DNA-protein interaction assay (ELISA) by using a TransAM NF-YA kit (Active Motif, Carlsbad, CA). The principle of this assay is that active NF-Y in the nuclear fraction binds to an immobilized oligonucleotide double-stranded DNA contain-

FIG. 1. Enhancement of *E. coli* LPS-induced IL-8 production by pretreatment with *H. pylori* LPS in the gastric epithelial cell lines MKN28 and MKN45. The cells were treated with *H. pylori* LPS from various strains at a concentration of 100 ng/ml for 24 h and then treated with various concentrations of *E. coli* LPS for 24 h. The IL-8 concentration in the resulting culture supernatant was determined by ELISA. *H. pylori* LPSs were derived from strains CA2 (gastric cancer, ■), CA6 (gastric cancer, □), GU2 (gastric ulcer, ▲), DU2 (duodenal ulcer, △), and CG10 (chronic gastritis, rough strain, ◆). Closed circles (●) indicate experiments without *H. pylori* LPS pretreatment. The experiments were performed in triplicate, and the data are presented as mean values.

ing the NY-Y binding motif, and the bound NF-Y is detected by an anti-NF-YA antibody.

siRNA-mediated suppression of gene expression. Small interfering RNA (siRNA) specific for human TLR2 [TLR2 siRNA(h) (sc-40256)] and a control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. Then, 0.5 μ g of siRNA was transfected into cells by using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instruction. After 48 h culture, the cells were treated with *H. pylori* LPS.

Cell proliferation assay. The cell proliferation rate was determined by the uptake of 5-ethynyl-2'-deoxyuridine (EdU) into DNA, using a Click-iT EdU microplate assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction manual. The incorporated EdU in DNA was coupled with Oregon Green-azide, and subsequently incubated with horseradish peroxidase-labeled anti-Oregon Green antibody and Amplex UltraRed. The fluorescence (expressed as relative fluorescence units [RFU]) at 490 nm excitation/585 nm emission was measured with a Labsystems Fluoroskan Ascent FL (Thermo, Waltham, MA), and expressed as the cell proliferation rate.

RESULTS

***H. pylori* LPS enhances *E. coli* LPS-induced IL-8 production in gastric cancer cell lines.** In our previous study, the gastric cancer cell lines MKN28 and MKN45 responded very weakly to *E. coli* LPS (37), presumably because these cells express low levels of TLR4. In the present study, we found that pretreatment of these cells with *H. pylori* LPS at concentrations of 10 to 100 ng/ml substantially enhanced *E. coli* LPS-induced IL-8 production in a dose-dependent manner (Fig. 1). Smooth LPS carrying the weakly antigenic epitope derived from strains isolated from gastric cancer patients and rough LPS showed more potent enhancing activity than smooth LPS carrying the highly antigenic epitope derived from strains from other gastroduodenal diseases, such as chronic gastritis and gastroduodenal ulcers.

***H. pylori* LPS upregulates TLR4.** *H. pylori* LPS upregulated TLR4 expression in a dose-dependent manner, as determined

with flow cytometry (Fig. 2A). Moreover, LPS derived from strain CA2 (weakly antigenic) was a more potent TLR4 inducer than LPS derived from strain GU2 (highly antigenic), which is consistent with enhancing the activity of responsiveness to *E. coli* LPS (Fig. 1 and 2). Significant upregulation of TLR4 was not observed after treatment with other TLR agonists, such as *E. coli* LPS, MALP-2, and Pam₃-CSK₄ (Fig. 2B). The cell surface expression levels of TLR2 and CD14 were not substantially altered by *H. pylori* LPS treatment (Fig. 2C). We examined the knockdown of TLR2 expression by specific siRNA. The siRNA for TLR2 successfully downregulated TLR2, because suppressed TLR2 expression was observed by RT-PCR and Pam₃-CSK₄-induced IL-8 production was suppressed (Fig. 3, right panels). The induction of TLR4 by *H. pylori* LPS was also suppressed by the knockdown of TLR2 (Fig. 3, left panels), indicating that *H. pylori* LPS upregulates TLR4 expression via TLR2 signaling.

Upregulation of TLR4 by *H. pylori* LPS is mediated by the MEK1/2-ERK1/2 MAP kinase pathway. We examined whether *H. pylori* LPS activates the MAP kinase pathway. MKN28 cells were treated with *H. pylori* LPS derived from strain CA2 (weakly antigenic), GU2 (highly antigenic), or *E. coli* LPS, and the lysates of these cells were analyzed by Western blotting. *H. pylori* LPS from strains CA2 and GU2 substantially upregulated phosphorylation of MEK1/2 and ERK1/2 (Fig. 4). *E. coli* LPS induced a little phosphorylation of ERK1/2 and MEK1/2, whereas it induced a higher phosphorylation of p38 than *H. pylori* LPS from strains CA2 and GU2 did. JNK was constitutively phosphorylated, and its phosphorylation tended to be upregulated by *H. pylori* LPS treatment.

We next determined the effect of MAP kinase inhibitors on TLR4 expression. An MEK1/2 inhibitor (PD98059), an ERK1/2 inhibitor (FR180204), and a JNK inhibitor (SP600125) suppressed the induction of cell surface TLR4, as determined with flow cytometry (Fig. 5A). A p38 MAP kinase inhibitor (SB202190) did not affect TLR4 expression. Similar to results of flow cytometry, RT-PCR data (Fig. 5B) indicated that the MEK1/2, ERK1/2, and JNK inhibitors, but not the p38 inhibitor, suppressed the induction of TLR4 by *H. pylori* LPS at the mRNA level.

***H. pylori* LPS augments cell proliferation of gastric cancer cell lines through the TLR2 and the MEK1/2-ERK1/2 pathway.** We also determined the effect of *H. pylori* LPS on the growth rate of MKN28 cells. *H. pylori* LPS significantly upregulated cell growth as determined by an EdU uptake assay (Fig. 6A). Enhancing activity in cell proliferation of CA2 LPS (weakly antigenic) was more potent than that of GU2 LPS (highly antigenic). Other TLR2 agonists, such as Pam₃-CSK₄ and MALP-2, also augmented cell proliferation, but with markedly weaker activity than *H. pylori* LPS. *E. coli* LPS did not significantly affect cell proliferation. The upregulation was significantly suppressed by a MEK1/2 inhibitor and ERK1/2 inhibitor (Fig. 6B), but p38 and JNK inhibitors had no significant effect on cell proliferation. Cells transfected with siRNA specific for TLR2 did not show enhancement of cell proliferation induced by *H. pylori* LPS (Fig. 6C). These results indicated that the enhancing effect of *H. pylori* LPS on cell proliferation of gastric epithelial cell lines was via TLR2, and MEK1/2-ERK1/2 MAP kinase cascade.

FIG. 2. Induction of TLR4 expression with *H. pylori* LPS in gastric epithelial cell lines. (A) Dose dependency of *H. pylori* LPS in TLR4 upregulation. MKN28 cells were treated with various concentrations of *H. pylori* LPS derived from strain CA2 or GU2 for 24 h. (B) Effect of various TLR agonists. MKN28 cells were treated with *H. pylori* LPS, *E. coli* LPS, MALP-2, or Pam₃-CSK₄ at concentrations of 100 ng/ml for 24 h. (C) Cell surface expression of TLR2, TLR4, and CD14 determined by flow cytometry. The cells were treated with *H. pylori* LPS derived from strain CA2 at a concentration of 100 ng/ml for 12 or 24 h. The resulting cells were scraped; incubated with a phycoerythrin-labeled monoclonal antibody against TLR2, TLR4, or CD14; and applied to the flow cytometer. Control experiments (negative control) were performed with an isotype-matched, phycoerythrin-labeled unrelated monoclonal antibody. The experiments were performed in triplicate, and representative data are presented.

Transcription factor NF- κ B contributes to *H. pylori* LPS-induced TLR4 expression. To elucidate the mechanism of TLR4 induction by *H. pylori* LPS, we analyzed the promoter of the human TLR4 gene by luciferase reporter gene assay. Among various promoter mutants, only the promoter with a mutated NF- κ B binding motif (mNFY) abolished induction by *H. pylori* LPS (Fig. 7). Promoters mutated in the AP-1, PU.1, and interferon regulatory factor (IRF) binding sites, which are indicated as mA/E, mPu.1-1, and mIRF, respectively, showed very low transcriptional activities in the absence of stimulation, but *H. pylori* LPS treatment induced transcription. These results suggest that NF- κ B contributes to the upregulation of TLR4 induced by *H. pylori* LPS, and AP-1, PU.1, and IRF mainly contribute to basal transcription of TLR4. The JNK inhibitor probably suppressed TLR4 expression (Fig. 5B), because AP-1, which is activated by JNK, contributes to the basal

expression of TLR4. A TLR2 antagonist, Pam₃-CSK₄, also tended to enhance promoter activities of TLR2 as similar to *H. pylori* LPS; however, the magnitude of enhancing effect was less than that of *H. pylori* LPS. The results suggested that the typical TLR2 agonist also shared the enhancing activity of TLR transcription, but the activity was not enough for the appearance of TLR4 upregulation at protein and mRNA levels.

***H. pylori* LPS activates DNA binding of NF- κ B through the MEK1/2-ERK1/2 pathway.** We assessed the presence of active NF- κ B in nuclear extracts by ELDIA. *H. pylori* LPS significantly upregulated binding of NF- κ B to a specific DNA sequence. The LPS derived from CA2 activate NF- κ B more strongly than LPS from GU2. In contrast, *E. coli* LPS increased the DNA binding of NF- κ B very little (Fig. 8A).

We next examined the effect of MAP kinase inhibitors on the activation of NF- κ B by *H. pylori* LPS. The NF- κ B DNA

FIG. 3. Upregulation of TLR4 by *H. pylori* LPS is mediated by TLR2 determined by siRNA specific for TLR2. The cells were transfected with a siRNA specific for TLR2 (siTLR2) or control siRNA (mock) and then cultured for 48 h. The cells were treated with *H. pylori* LPS at a concentration of 100 ng/ml. Total RNA was extracted from the resulting cells, and the RNA levels of TLR4 and TLR2 were determined by RT-PCR. The products were separated with 2% agarose gel electrophoresis. For control experiment, transfected cells were incubated with a TLR2 agonist, Pam₃-CSK₄, at a concentration of 100 ng/ml for 6 h. The mRNA of IL-8, TLR2, and GAPDH were determined by RT-PCR.

binding induced by *H. pylori* LPS was significantly lower in the presence of the MEK1/2 inhibitor or ERK1/2 inhibitor (Fig. 8B). In contrast, the p38 MAP kinase inhibitor and JNK inhibitor did not alter the NF- κ B DNA-binding activity. These

FIG. 4. Activation of the MAP kinase cascade by treatment with *H. pylori* LPS. MKN28 cells were treated with 100 ng of *H. pylori* LPS/ml from strain CA2 or GU2, or *E. coli* LPS for the indicated times. Treated cells were lysed, and proteins were separated with SDS-PAGE and subjected to Western blotting with anti-MEK1/2, anti-phospho-MEK1/2 (p-MEK1/2), anti-ERK1/2, anti-phospho-ERK1/2 (p-ERK1/2), anti-p38 MAPK, anti-phospho-p38 MAPK (p-p38 MAPK), anti-JNK1/2, and anti-phospho-JNK1/2 (p-JNK1/2) antibodies.

FIG. 5. Effect of MAP kinase inhibitors on *H. pylori* LPS-induced TLR4 upregulation in MKN28 cells. (A and B) MKN28 cells were treated with 100 ng of *H. pylori* LPS/ml derived from strain CA2 in the presence of one of the following MAP kinase inhibitors: PD98059 (50 μ M), FR180204 (5 μ M), SB202190 (5 μ M), or SP600125 (10 μ M). The expression levels of TLR4 were determined by flow cytometry using a phycoerythrin-labeled murine anti-TLR4 monoclonal antibody (A) and RT-PCR (B). Dashed lines indicate staining with a phycoerythrin-labeled isotype-matched murine IgG. GAPDH was examined in the RT-PCR experiment as a control.

FIG. 6. Effects of *H. pylori* LPS on cell proliferation of gastric epithelial cell line MKN28. (A) MKN28 cells were treated with *H. pylori* LPS derived from CA2 or GU2, MALP-2, Pam₃-CSK₄, or *E. coli* LPS for 18 h. (B) Effect of MAP kinase inhibitors. MKN28 cells were treated with 1 μg of *H. pylori* LPS/ml derived from strain CA2 for 18 h in the presence of one of the following MAP kinase inhibitors: PD98059 (50 μM), FR180204 (5 μM), SB202190 (5 μM), or SP600125 (10 μM). (C) Effect of downregulation of TLRs by siRNA. MKN28 cells were transfected with siRNA specific for TLR2 (siTLR2) or control siRNA (mock). After 48 h of transfection, the cells were treated with 1 or 0.1 μg of *H. pylori* LPS/ml derived from CA2 for 18 h. After treatment, cell proliferation was determined with uptake of EdU into DNA using a Click-iT EdU microplate assay kit. The resulting fluorescence (measured in RFU) was measured and is expressed as cell proliferation. The experiments were examined in triplicate, and the data are presented as means ± the standard deviations. *, $P < 0.01$.

results indicate that the MEK1/2-ERK1/2 MAP kinase pathway is important for the upregulation of TLR4 by *H. pylori* LPS through the activation of NF- κ B.

DISCUSSION

In the present study, we showed that *H. pylori* LPS has activity of upregulating TLR4 expression in gastric epithelial cell lines. Upregulation of TLR4 by *H. pylori* infection (31) and LPS (13) in gastric epithelial cell lines have been reported, and Asahi et al. (2) reported that expression levels of TLR4 were higher in the antral and corpus mucosa in biopsy specimens from *H. pylori*-infected patients than in those from *H. pylori*-negative patients. Backhed et al. reported that primary gastric antral epithelial cells derived from healthy individuals did not express TLR4; in contrast, several epithelial cell lines derived from gastric cancer examined expressed TLR4 (3). These in vitro and in vivo findings are consistent with those of the present study, and together suggest that *H. pylori* LPS contributes to the upregulation of TLR4 induced by infection. Triantafyllou et al. (32) reported that pretreatment of vascular epithelial cells and HEK293 cells expressing TLR4, MD2, and CD14 for 1 h with *H. pylori* LPS suppressed *E. coli* LPS-induced tumor necrosis factor alpha (TNF- α) production and NF- κ B activation, and these authors therefore concluded that *H. pylori* LPS acts as an antagonist of *E. coli* LPS signaling mediated by TLR4. However, the *H. pylori* LPS pretreatment in the Triantafyllou et al. (32) report was much shorter (1 h)

than that in our study (24 h). We did not observe TLR4 induction on the cell surface after a 1-h pretreatment (data not shown).

Our results indicate that *H. pylori* LPS activates the MEK1/2-ERK1/2 pathway via TLR2, which then activates NF- κ B, which in turn activates the transcription of the TLR4 gene (Fig. 9). The upregulated TLR4 enhances the production of typical LPS-induced proinflammatory cytokines, such as IL-8. NF- κ B, which is also known as CCAAT-binding factor, is a ubiquitous and evolutionally conserved transcription factor (17, 19, 21) that contributes to the transcription of numerous genes, including the cell cycle regulatory genes cyclin A2, cyclin B2, and E2F1 (10, 18). NF- κ B is composed of three subunits: NF- κ A, NF- κ B, and NF- κ C. Of these, NF- κ B activity is mainly regulated by NF- κ A (17, 19). Alabert et al. reported that transforming growth factor β (TGF- β) is an activator of NF- κ B (1). TGF- β treatment leads to translocation of NF- κ A to the nucleus. This nuclear translocation is dependent upon extracellular signal-regulated kinase (ERK) activation, but the kinetics of NF- κ A nuclear accumulation differ with cell type, probably due to differences in the basal levels of activation of the ERK and p38 signaling pathway. Consistent with this, we showed that the DNA-binding activity of NF- κ B induced by *H. pylori* LPS was dependent on the ERK1/2 pathway.

Transcriptional regulation of TLR4 is complicated and differs between human and mouse, and between myeloid cells and nonmyeloid cells (16). Our present study, which used hu-

FIG. 7. Luciferase reporter gene analysis of the TLR4 gene promoter activated by treatment with *H. pylori* LPS. (A) The results of the reporter gene assay. Luciferase reporter plasmids carrying the promoter region of TLR4 gene and its mutant series were transfected into MKN28 cells. At 24 h after transfection, the cells were treated with 100 ng of *H. pylori* strain CA2 LPS, Pam₃-CSK₄, or *E. coli* LPS/ml for 6 h. The cells were then lysed, and the luciferase activity in the lysate was measured. The experiments were performed in triplicate, and the data are presented as means $\pm$ the standard deviations. (B) Schematic of the promoter region of the human TLR4 gene. Arrows indicate transcription start sites in human nonmyeloid cells (Adapted from reference 16 with the permission of the publisher.)

man nonmyeloid cells, indicated that an AP1 box (which probably binds AP-1), a PU.1 box (which binds PU.1), and an IRF box (which binds IRF) were important for basal transcription of TLR4 (Fig. 7B). In contrast, the NF-Y binding motif was necessary for *H. pylori*-induced TLR4 transcription, but less so for basal transcription.

We found that *H. pylori* LPS not only upregulates TLR4 and contributes to inflammatory processes but also enhances cell

FIG. 8. Activation of NF-Y by treatment with *H. pylori* LPS depends on the MEK1/2-ERK1/2 MAP kinase cascade. (A) MKN28 cells were treated with *H. pylori* LPS derived from strains CA2, GU2, or *E. coli* LPS for 6 h. (B) MKN28 cells were treated with *H. pylori* LPS derived from CA2 in the presence of PD98059 (50 μ M), FR180204 (5 μ M), SB202190 (5 μ M), or SP600125 (10 μ M), which inhibit MEK1/2, ERK1/2, p38 MAP, and JNK, respectively. The nuclear fraction was extracted from the cells and applied to a microplate coated with an oligo-DNA of the NF-Y binding motif. The bound active NF-Y was detected with an anti-NF-YA antibody. The specificity of NF-Y binding was confirmed by the addition of competitive inhibitor, a soluble oligonucleotide containing the NF-Y binding motif (data not shown). The experiments were performed in triplicate, and the data are presented as means $\pm$ the standard deviations.

proliferation. On the other hand, Slomiany and Slomiany reported that activation of the MAP kinase (p38 and ERK) pathway by *H. pylori* LPS disrupts gastric mucin synthesis, increases caspase-3 activity and apoptosis, and upregulates endothelin-1 and TNF- α (28, 29). The enhancement of cell proliferation that we observed can be explained by the activated MEK1/2-ERK1/2 pathway via TLR2. The ERK pathway is mainly responsible for cell signaling of growth factor receptors (14), and inhibition of ERK1/2 results in G₀/G₁ arrest. Cyclin D1, which contributes to the G₀/G₁ transition, is upregulated by ERK1/2 signaling and downregulated by p38. Ding et al. also reported that *H. pylori* activates MAP kinase pathways, and treatment with an ERK1/2 inhibitor resulted in accumulation of cells at the G₀/G₁ stage (6). Earlier, Ding et al. had used a microarray to screen genes induced by *H. pylori* and tested the dependence of the induction on TLR2, using HEK cells with or without the transfection of TLR2 expression plasmid (7). Genes upregulated in a TLR2-dependent way during *H. pylori* infection were IL-8, growth-related oncogenes, and molecules contributing to NF- κ B pathways.

Chochi et al. found that *H. pylori* LPS enhanced cell proliferation of gastric epithelial cell lines (4). This finding was similar to our present results, but several other points contradicted our results. These authors observed an enhancement of cell proliferation not only with *H. pylori* LPS but also with *E. coli* LPS, whereas we observed little effect of *E. coli* LPS on cell

FIG. 9. Proposed mechanism for pathogenesis due to *H. pylori* LPS. *H. pylori* LPS upregulates TLR4 and augments cell proliferation via TLR2 and the MEK1/2-ERK1/2 MAP kinase pathway in gastric epithelial cells. These activities likely enhance the inflammatory response by increasing the activity of LPS from strains with high endotoxic activity, as well as tumorigenesis.

growth. Chochi et al. also showed that the enhancing effect of proliferation was effectively suppressed by the addition of an anti-TLR4 monoclonal antibody, but we have not been able to replicate this inhibitory activity with the same clone of anti-TLR4 monoclonal antibody purchased from a different manufacturer (data not shown). We have not been able to explain these contradictions, so further experiments are required.

Several studies have suggested various mechanisms for the carcinogenesis induced by *H. pylori*. In the present study, we propose a novel mechanism of inflammation and carcinogenesis induced by *H. pylori* LPS (Fig. 9). *H. pylori* LPS itself has extremely low endotoxic activity and causes a very weak inflammatory reaction compared to a typical LPS, such as that from *E. coli*. On the other hand, *H. pylori* LPS can enhance inflammatory reactions mediated by TLR4 agonists, such as bacterial LPSs that can cause sepsis, by upregulating TLR4 in cells expressing low levels of TLR4. In addition, *H. pylori* LPS augments cell proliferation. Both activities occur via activation of the MEK1/2-ERK1/2 MAP kinase pathway, starting with stimulation of TLR2 by *H. pylori* LPS. As described in the introduction, we propose that polysaccharide region of *H. pylori* LPS can be divided two types of antigenicity to humans, namely, highly antigenic type (mainly isolated from patients with chronic gastritis) and weakly antigenic type (mainly isolated from gastric cancer patients) (34, 39). We also found that *H. pylori* LPS carrying the weakly antigenic epitope activates TLR2 and the MEK1/2-ERK1/2 signaling pathway more strongly than the LPS carrying the highly antigenic epitope. This suggests that the activities of *H. pylori* LPS differ from strain to strain. The LPS carrying the weakly antigenic epitope derived from strains frequently found in gastric cancer patients may cause more inflammation and carcinogenesis than the highly antigenic type. However, the molecular basis of the difference in activity between two

antigenic types of LPS has not been discovered, and further studies are needed to clarify this issue.

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