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Essential Role of MD-2 in TLR4-Dependent Signaling during Helicobacter pylori-Associated Gastritis

Shunji Ishihara,1* Mohammad Azharul Karim Rumi,* Yasunori Kadowaki,* Cesar Francisco Ortega-Cava,* Takaumi Yuki,* Nagisa Yoshino,* Youichi Miyaoka,* Hideaki Kazumori,* Norihisa Ishimura,* Yuji Amano,† and Yoshikazu Kinoshita*

TLR4, a member of pattern recognition receptors, is the main receptor of LPS. MD-2 physically associates with TLR4 on the cell surface and confers LPS responsiveness. Helicobacter pylori LPS is one of the major virulence factors for induction of gastritis. We demonstrated in this study the role of MD-2 in TLR4-dependent signaling in H. pylori-associated gastritis. Gastric biopsy samples collected from patients with and without H. pylori infection and four gastric cancer cell lines were used for this study. TLR-4 and MD-2 expression in biopsy specimens and the cell lines was examined by using RT-PCR. Localization of TLR-4 in histological sections was evaluated by immunohistochemistry. For in vitro functional assays, we established stable transfectants of AGS cells expressing TLR4 and MD-2. Cellular distribution of TLR4 was examined by flow cytometry. NF-κB expression in biopsy specimens and the cell lines was examined by using RT-PCR. Localization of TLR-4 in histological sections was evaluated by immunohistochemistry. For in vitro functional assays, we established stable transfectants of AGS cells expressing TLR4 and MD-2. Cellular distribution of TLR4 was examined by flow cytometry. NF-κB activation and activation of IL-8 and MD-2 promoters were assessed by reporter gene assay. H. pylori infection up-regulated the TLR4 and MD-2 expression in gastric mucosa. TLR4 staining was observed predominantly in epithelial cells, located in both the cytoplasm and at the apical surface. MD-2 transfection in AGS cells markedly increased cell surface expression of TLR4 and augmented the activation of NF-κB and IL-8 promoter upon stimulation with H. pylori LPS. Live H. pylori also stimulated transcriptional activation of MD-2. This study revealed that MD-2 expression is elevated in gastric epithelial cells during H. pylori infection, suggesting that the TLR4/MD-2 system is a potent receptor complex involved in the response to H. pylori LPS in the stomach. The Journal of Immunology, 2004, 173: 1406–1416.

Helicobacter pylori infection is now accepted as a crucial event in the development of peptic ulcer disease and atrophic gastritis, and it is implicated in the development of gastric carcinoma (1–5). Host innate and inflammatory responses to this organism have been well documented in H. pylori-infected individuals (6–10). LPS, a component of the outer membrane of Gram-negative bacteria, is a potent activator of cells of the immune and inflammatory systems. It has been shown that H. pylori LPS is one of the virulence factors involved in the induction of gastritis. H. pylori LPS-dependent activation of monocytes and gastric epithelial cells leads to the production of several proinflammatory cytokines (11–13), oxygen radicals (14, 15), and inducible NO (16). However, little detail is known about the recognition system for H. pylori LPS and its related intracellular signaling in gastric mucosal cells.

Host innate immunity is triggered by pattern recognition receptors that sense pathogen-associated molecular patterns, including LPS, mannans, peptidoglycans, and bacterial DNA (17–19). An important class of pattern recognition receptors is the TLR family (TLRs), the members of which are type I transmembrane proteins with extracellular domains composed largely of leucine-rich repeats and intracellular signaling domains with homology to the IL-1R (20, 21). The intracellular signaling pathways through Toll/IL-1R domains result in recruitment of the cytoplasmic adaptive molecule myeloid differentiation factor 88, with subsequent activation of a signaling cascade leading to NF-κB (22). TLR4 is a member of the TLR family and the main receptor of LPS (23–25). TLR4 alone, however, cannot sense the presence of LPS on the cell surface. MD-2, a small, secreted protein, is required for TLR4-mediated recognition of LPS (26). MD-2 binds to the extracellular domain of TLR4 and can enhance the sensitivity and functional responses to LPS through interaction with TLR4 (27–33). In addition, it has been suggested that MD-2 is essential for functional cellular distribution of TLR4 (34). Recently, we demonstrated that gastric and intestinal epithelial cells in mice constitutively express TLR4, and that it is up-regulated during gut inflammation (35). Other studies have also shown that TLR-dependent signaling in epithelial cells may play an important role in the development of certain intestinal inflammatory diseases (36, 37). These findings are helpful for considering innate gut immune systems because the intestinal epithelial cell monolayer is the first line of defense that is exposed to pathogens in the luminal environment. It has been reported that TLR4 regulates the response of gastric epithelial cells to H. pylori infection and that H. pylori LPS-induced transcription of several genes depends on the activation of NF-κB (38). Although MD-2 is a key molecule in TLR4-mediated immune recognition, the role of MD-2 in H. pylori-associated gastritis is not clear. In this study, we investigated the in vivo expression of both TLR4 and MD-2 in the human gastric mucosa, with and without H. pylori infection. Furthermore, we established stable transfectants of human gastric epithelial cell lines expressing TLR4 and MD-2, and evaluated the effects of MD-2 on the TLR4 function in gastric epithelial cells in relation to H. pylori infection.
Materials and Methods

Patients and biopsy specimens
Ten patients with *H. pylori*-induced chronic gastritis (six males, four females; age range, 34–43 years; mean age, 36.3 years) and 10 patients with *H. pylori*-negative nonulcer dyspepsia (five males, five females; age range, 27–38 years; mean age, 38.3 years) were studied. *H. pylori* infection was detected by the C13-urea breath test. None of the subjects had received any antibiotics, proton pump inhibitors, H2-antagonists, nonsteroidal anti-inflammatory drugs, or corticosteroids during the 2 mo before this study. From each patient, multiple endoscopic biopsy specimens were taken from the gastric antrum, and these were immediately snap frozen in liquid nitrogen stored at −80°C until the assays. Before this study, written informed consent was obtained from each patient. The study protocol was prepared according to the Helsinki Declaration.

Immunohistochemistry
The anti-TLR4 mAb HTA125 was a gift from K. Miyake (Tokyo University, Tokyo, Japan). Five-micrometer cryostat tissue sections were mounted on poly(l-lysine)-coated slides and stored at −20°C. The slides were air dried and fixed in 100% (v/v) acetone at −4°C for 10 min. The samples were incubated with TLR4 mAb or isotype control IgG (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. The sections were washed with PBS, then incubated with goat anti-mouse IgG (Sigma-Aldrich) for 30 min. Bound Ab was detected using the avidin-biotin peroxidase method (ABC kit; Vector Laboratories, Burlingame, CA). Subsequently, peroxidase activity was detected by incubating the samples with diaminobenzidine hydrochloride for 10 min at room temperature, then staining with hematoxylin. The sections were dehydrated in a graded alcohol series, then cleared in xylene and mounted.

Laser capture microdissection
To investigate the effect of *H. pylori* infection on MD-2 expression in gastric epithelial cells, laser capture microdissection (LCM)3 coupled with RT-PCR was performed. Fresh frozen biopsy tissues were cut into 5-μm sections in a cryostat and mounted on uncoated glass slides. The slides were fixed immediately in 70% (v/v) ethanol for 30 s and washed in diethylpyrocatechol-treated water for 30 s, after which the sections were stained rapidly with hematoxylin for 30 s, dehydrated through an ethanol gradient, counterstained with eosin Y for 1 min, dehydrated through an ethanol gradient, and finally cleared in xylene. After the sections were air dried, they were laser microdissected using an LCM system LM200 (Olympus, Tokyo, Japan), as we have described previously (39, 40). In brief, slides with sectioned tissue were placed on the stage of a microscope and an area was selected. The chosen sections were covered with LCM transfer film. Under direct microscopic observation, specific portions of the tissue section, focused to the appropriate size of the desired target, were pulsed directly above the targeted cells with brief laser pulses, using a laser beam 7.5 μm in diameter and a laser power of 60 mW. The samples captured from 3000 shots on one transfer film cap were immersed in RNA extraction solution. To exclude the possibility that infiltrating cells within the gastric epithelial preparation might have been responsible for detection of MD-2 mRNA expression, RT-PCRs for the detection of cytokeratin-20 (an epithelial marker), CD3 (a T cell marker), and CD11b (an Ag-presenting cell marker) mRNA in LCM samples were also performed.

Cell lines and culture
Five human cell lines, AGS, MKN-7, MKN-28, MKN-45, and THP-1, were used in this study. AGS and THP-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA), and MKN-7, MKN-28, and MKN-45 from Riken Cell Bank (Tsukuba, Japan). AGS cells were grown in Ham’s F12 (ICN Biomedicals, Aurora, OH), and the others in RPMI 1640 (ICN Biomedicals), supplemented with 10% (v/v) heat-inactivated FBS (ICN Biomedicals) under an atmosphere of 95% (v/v) air-5% CO2 at 37°C in a humidified incubator.

RNA extraction and RT-PCR
Total RNA from each sample was extracted by a single-step guanidinium thiocyanate-phenol-chloroform method (Isogen; Nippon Gene, Tokyo, Japan). After extraction of the RNA, reverse transcription (RT) was performed using a first-strand synthesis kit 5′-TGGAGATTTACCAAGAACGAC-3′ and 5′-TTCTCTCCTAAGTTGTTCC-3′ for CD11b (325 bp), 5′-CAAGAGAGGCGACGCAACG-3′ and 5′-TTCCCTCAACTGGTTCC-3′ for CD3 (386 bp), 5′-AAGTCAAGGACGAGGAC-3′ and 5′-ACCT AGTTTTCTCGACTTGAG-3′ for CD4 (325 bp), 5′-TGGAGATTTACCAAGAACGAC-3′ and 5′-TTCTCTCCTAAGTTGTTCC-3′ for CD11b (325 bp), 5′-CAAGAGAGGCGACGCAACG-3′ and 5′-TTCCCTCAACTGGTTCC-3′ for CD3 (386 bp), 5′-AAGT CAAGGACGAGGAC-3′ and 5′-ACCT AGTTTTCTCGACTTGAG-3′ for CD4 (325 bp). RT coupled with real-time PCR
Quantitative evaluation of TLR4 and MD2 mRNA expression in *H. pylori*-positive or -negative gastric biopsy samples was performed by RT, followed by real-time PCR. TLR-4 cDNA was amplified by using the primers 5′-CTCAACCAAGAACCTGGACCT-3′ and 5′-GAGGTGTTGCTT AGGCTGAT-3′, and MD2 cDNA was amplified by the primers 5′-GAATCTCTTCACTTTCTGAAGAC-3′ and 5′-TGGAATG TGGGGATT-3′. The primers were also designed on different exons. Real-time PCR was performed on ABI PRISM 7700 Sequence Detection System using the SYBR Green PCR Master Mix and following the manufacturer’s instructions (Applied Biosystems), except for the annealing temperature, which was 56°C for 20 s in our experiments. Relative quantitiy of TLR4 or MD2 mRNA expression, normalized with GAPDH expression, was estimated by the Q-Gene analysis (Biotechnique Softwares, Westborough, MA).

Expression plasmids and stable transfectants
The sequences of human TLR4 and MD-2 were obtained from the National Center for Biotechnology Information database (TLR4, accession U88880; MD-2, accession AB018549). The cDNAs encoding full-length TLR4 and MD-2 were amplified by PCR using the normal 5′-AGCTTCTGAGCT CGGAGGCTCCAGC-3′, 5′-GTTGGACCCAGGCTGGCAAAAGATG-3′ (for TLR4), and 5′-GAGCTCTGAGATTTCGATGCTGC-3′ and 5′-CTAATTGAATTTAGGGTGTTAGAGA-3′ (for MD-2). The amplified DNA were cloned into eukaryotic TA cloning pCR3.1 expression vectors (Invitrogen, Carlsbad, CA). The plasmid clones were confirmed by the construct. AGS cells were transfected with each expression construct, and a bulk empty vector was used as a negative control, using DOTAP liposomal transfection reagent (Roche, Mannheim, Germany). The selection of bulk populations of cells was performed in the presence of the neomycin analog G418 (1 mg/ml; Calbiochem, San Diego, CA), and clonal cell lines were obtained by limiting dilution. To evaluate the effect of TLR4 and MD-2 coexpression on *H. pylori* LPS responsiveness, transient transfection of TLR4 was performed in stable transfectant of MD-2. These cells were used in several experiments with both TLR4 and MD-2.

Flow cytometry
Surface and permeabilized cell staining for TLR4 was evaluated. Cell permeabilization was performed using the IntraPre permeabilization reagent (Immunotech, Marseille, France). AGS, THP-1, stable transfectants, and control mdm-383, with or without cell permeabilization, were incubated with TLR4 mAb or isotype control IgG (Sigma-Aldrich) for 30 min, washed with staining buffer, then incubated with goat anti-mouse IgG FITC (Sigma-Aldrich) for 30 min. The cells were then washed three times with PBS, and analyzed by FACSscan (BD Biosciences, San Jose, CA); 10,000 cells were counted for each condition.

Vector construction for reporter gene assays
Three reporter vectors were constructed for the luciferase assay. pNF-κB-Luc contained five copies of κB element 5′-TGGGACTTTCGCCG-3′.

3 Abbreviations used in this paper: LCM, laser capture microdissection; RT, reverse transcription.
cloned upstream to the minimal TATA promoter (Stratagene Cloning Systems, La Jolla, CA). pGL3-IL-8-Luc, which includes the sequence, was constructed by PCR cloning using the primers 5′-TTCCTCAGTTGGTGTTGGAGAAAG-3′ and 5′-TTCCTCGAGCTTGTGTGCTCTGCTGTCTCT-3′. For the construction of pGL3-MD-2-Luc, accession NT_008209 was used to identify the 1-kb (1013-bp) sequence upstream of the start site, as reported previously (41). Amplification was preformed by PCR using the primers 5′-GCTTTACAAATGCAAAGAGGATCAG-3′ and 5′-CATGGCCTGTTAGGAATCTGGT-3′. The cloned promoter sequences (IL-8 promoter and MD-2 promoter) were confirmed by sequencing of the plasmid clones. As an internal control for the dual luciferase assay, pRL-TATA-Renilla-Luc was also constructed; this expressed Renilla luciferase under a minimal TATA promoter.

Luciferase assay
The effect of H. pylori LPS on cellular NF-κB activity and transcriptional activation of IL-8 was evaluated. H. pylori LPS (ATCC43594) was kindly provided by Otuska Pharmaceutical (Tokushima, Japan). AGS, MKN-45, stable transfectants, and cotransfectant were cultured in 24-well plates (5 × 10^4 cells/well) and transfected with 0.5 μg of pNL-κB-Luc or pGL3-IL-8-Luc and 0.02 μg of pRL-TATA-Renilla-Luc per well. The cells were then stimulated with various concentrations of H. pylori LPS or vehicle for 8 h, and cell lysates were used for the measurement of luciferase activity using the PicaGene dual luciferase kit (Toyoini, Tokyo, Japan). A recent study suggests that TLR2 may mediate H. pylori LPS signaling in gastric epithelial cells (42). Therefore, to examine the specificity of TLR4 in stable transfectant of MD-2, the effect of a neutralizing Ab against TLR4 was investigated. The cells were preincubated with the TLR4 mAb (HTA125, 10 μg/ml) or a control mAb (10 μg/ml) for 30 min, and then stimulated with H. pylori LPS (1 ng/ml) for 8 h. For plasmid DNA preparation and transfection procedures, we used all endotoxin-free reagents and kits to avoid the nonspecific activation of NF-κB and IL-8 promoter. Further studies of live H. pylori- and LPS-dependent transcriptional activation of MD-2 in AGS cells were performed in vitro. H. pylori strain 43504 was obtained from ATCC, and another strain, TN583 (a gift from Takeda Chemical Industries, Osaka, Japan), was isolated clinically from a patient with dyspepsia. The Cag and Vac statuses were positive in both strains. The effects of Escherichia coli LPS (Sigma-Aldrich) and Salmonella LPS (Sigma-Aldrich) on MD-2 promoter activity in AGS cells were also evaluated. Transient transfection was conducted in 24-well plates (5 × 10^4 cells/well) using 0.5 μg of pGL3-MD-2-Luc and 0.02 μg of pRL-TATA-Renilla-Luc per well. After 6 h of stimulation with live H. pylori (ATCC43504 or TN583, 5 × 10^6 and bacilli/well, respectively) or LPS (0.1 ng/ml, 1.0 ng/ml), luciferase activity was measured, as described above. The data are expressed as the n-fold increase in luciferase activity of live H. pylori- or LPS-stimulated samples over that of nonstimulated samples.

The effects of live H. pylori on TLR4 and MD-2 expression in AGS cells
AGS cells were cultured in six-well plates (2 × 10^5 cells/well) for 24 h; the cells were then cocultured with H. pylori (ATCC43504 or TN583, 2 × 10^7 and bacilli/well, respectively). At 0, 3, 6, and 12 h after coculture, total RNA was extracted from cultured AGS cells for RT-PCR. To detect cell surface expression of TLR4 by flow cytometry, the cells were collected at 24 h after coculture.

FIGURE 1. RT-PCR analysis of TLR4 and MD-2 expression in H. pylori-positive (A) and -negative samples (B). Gastric biopsies were taken from 10 patients with H. pylori-positive chronic gastritis and 10 patients with H. pylori-negative nonulcer dyspepsia. TLR4 was expressed constitutively in all subjects. MD-2 expression was observed clearly in the H. pylori-positive subjects, but expression was weak and seen in only 3 of 10 of H. pylori-negative subjects.
Statistical analysis
All data are expressed as mean ± SEM. The values were compared by Student’s t test, using Stat-View 4.0 software (Abacus Concepts, Berkeley, CA). A p-value of <0.05 was considered significant.

Results
TLR4 and MD-2 mRNA expression in gastric biopsies
TLR4 and MD-2 mRNA expression in biopsy specimens from the gastric antrum were examined using RT-PCR. TLR4 mRNA was constitutively expressed in all subjects, and greater amplification of TLR4 mRNA was observed in H. pylori-infected individuals (Fig. 1A). MD-2 mRNA expression was more common among H. pylori-infected subjects (10 of 10) than among H. pylori-uninfected subjects (3 of 10), and the extent of amplification was also stronger in H. pylori-infected subjects (Fig. 1B). In real-time RT-PCR, TLR4 mRNA expression was detectable in all gastric biopsy samples with or without H. pylori infection, and the mean values for normalized TLR4 mRNA in H. pylori-infected subjects were higher than that in H. pylori-negative subjects (H. pylori positive, 2.45 ± 0.84 E-2, n = 10 vs H. pylori negative, 2.04 ± 0.24 E-2, n = 10). MD-2 expression could not be detected in 5 of 10 H. pylori-negative samples, although it was detected in all of the H. pylori-positive samples. In H. pylori-negative samples with MD-2 expression, the mean value for MD-2 mRNA level was much lower than that in H. pylori-positive samples (H. pylori positive, 2.31 ± 0.61 E-2, n = 10 vs H. pylori negative, 1.16 ± 0.46 E-2, n = 5). In both TLR4 and MD-2 mRNA expression, the differences were not statistically significant, because the sample size was very small. These results suggest that H. pylori infection can accelerate gene expression of both TLR4 and MD-2, especially that of MD-2.

TLR4 expression in gastric biopsies
Immunohistochemical analysis for TLR4 was performed on frozen sections of antral biopsy specimens obtained from H. pylori-infected and -uninfected individuals. In H. pylori-uninfected subjects, TLR4 was expressed mainly in lamina propria mononuclear cells (Fig. 2, D and F, and little was observed in epithelial cells (Fig. 2E). In contrast, the expression of TLR4 in H. pylori-infected subjects could be detected clearly in both epithelial cells and infiltrating mononuclear cells (Fig. 2, A and B). The staining of TLR4 in epithelial cells was located in both the cytoplasm and at the apical surface (Fig. 2C). Isotype mAb did not show significant staining in either H. pylori-infected or -uninfected histological sections (data not shown).

MD-2 mRNA expression in gastric epithelial cells
Because TLR4 expression typically was increased in H. pylori-infected gastric epithelial cells, we investigated whether H. pylori infection can also stimulate MD-2 mRNA expression in gastric epithelial cells by LCM coupled with RT-PCR, as we have reported previously. Frozen histological sections, with and without H. pylori infection, were used for LCM, and gastric epithelial cells were captured carefully. The gastric antral mucosa after removal of the epithelial cells (Fig. 3A) and LCM-captured epithelial cells (Fig. 3B) are shown in Fig. 3. The results of RT-PCR clearly showed significant expression of MD-2 mRNA in H. pylori-infected epithelial cells, but not in H. pylori-uninfected cells (Fig. 3C). Cytokeratin-20 mRNA was detected in all LCM samples with or without H. pylori infection. However, CD3 and CD11b mRNA were detected only in the H. pylori-positive whole tissue samples, but not in LCM samples (Fig. 3C). These findings suggested that MD-2 mRNA expression is specifically up-regulated in the gastric epithelium of H. pylori-infected patients.

Cellular distribution of TLR4 in AGS, THP1, and transfectants
Recently, it has been suggested that cell surface expression of TLR4 is essential for the recognition of LPS. However, little is known about the cellular distribution of TLR4 in intestinal epithelial cells in relation to its function. THP-1 cells markedly expressed cell surface TLR4. However, cell surface expression of TLR4 in AGS and MKN-45 cells was weak (Fig. 4C). The results of cell surface expression of TLR4 in these cells clearly reflected...
H. pylori LPS-stimulated NF-κB activation in these cells (Fig. 4B). Next, using AGS cells, we established stable transfectants that expressed TLR4 and MD-2, and we evaluated both cell surface and cytoplasmic expression of TLR4 in these transfectants. The results of flow cytometry are shown in Fig. 5. No significant cell surface expression of TLR4 was found in the vector-only transfectant, but the level increased after permeabilization. Even in the TLR4 transfectants, cell surface expression of TLR4 was not high, although the level of cytoplasmic TLR4 protein was markedly higher after permeabilization. In contrast, transfection of MD-2 resulted in significantly higher cell surface expression of TLR4 without additional transfection of TLR4. Similar results were also obtained with the cotransfectant of TLR4 and MD-2. These results suggest that MD-2 regulates the cellular distribution of TLR4 and that the constitutive expression of MD-2 may be insufficient for the functional activation of TLR4 in AGS cells.

Effects of H. pylori LPS on NF-κB activation and IL-8 promoter activity in transfectants

NF-κB activation in stable transfectants and cotransfectant after stimulation with H. pylori LPS was evaluated using the reporter gene assay. H. pylori LPS significantly increased NF-κB activation in TLR4 or MD-2 stable transfectant and cotransfectant, but not in the vector transfectant. In particular, NF-κB activation in the MD-2 transfectant was stimulated markedly by H. pylori LPS as well as in the cotransfectant (Fig. 6A). The neutralizing Ab against TLR4 decreased ~60% of the H. pylori LPS-stimulated NF-κB activation in stable transfectant of MD-2, suggesting that such NF-κB activation up-regulated by MD-2 overexpression is mainly mediated through TLR4 signaling in AGS cells. Next, we investigated the effect of H. pylori LPS on IL-8 promoter activity in transfectants, because IL-8 is the major proinflammatory cytokine...
FIGURE 4. A, The results of RT-PCR for the expression of TLR4 and MD-2 in AGS, MKN-7, MKN-28, MKN-45, and THP-1 cells. All cells used in this study expressed both TLR4 and MD-2.

B, The results of NF-κB activity in AGS, MKN-45, and THP-1 cells after the stimulation with *H. pylori* LPS. Cells were cultured in 24-well plates (5 × 10⁴ cells/well) and transfected with 0.5 μg of pNF-κB-Luc and 0.02 μg of pRL-TATA-Renilla-Luc per well. The cells were stimulated with 0.1 and 1.0 ng of *H. pylori* LPS or vehicle for 8 h, and cell lysates were used for the measurement of dual luciferase activity. Data are expressed as the n-fold increase in luciferase activity of *H. pylori* LPS. Error bars indicate the SEM of the values obtained from three independent experiments performed in triplicates. Differences in luciferase activity were analyzed by Student’s *t* test. *p* < 0.05.

C, The results of surface and permeabilized cell staining for TLR4 by flow cytometry. AGS, MKN-45, and THP-1 cells, with or without cell permeabilization, were stained with TLR4 mAb (HTA125) or isotype control IgG. The significance of surface and permeabilized cell staining of TLR4 (gray histograms) was evaluated by comparing with the staining of control IgG.
produced in *H. pylori*-infected gastric mucosa in relation to infiltration of polymorphonuclear leukocytes. Transcriptional activation of IL-8 promoter was augmented significantly by *H. pylori* LPS in stable transfectant of MD-2 and in cotransfectant of TLR4 and MD-2 (Fig. 6B).

The effects of live *H. pylori* on TLR4 and MD-2 expression in AGS cells

Effects of live *H. pylori* on TLR4 and MD-2 expression were assessed by RT-PCR and flow cytometry. Two different strains of *H. pylori*, ATCC43504 and TN583, were used for this study. Both strains of *H. pylori* significantly induced expression of MD-2 mRNA in AGS cells, acting in a time-dependent manner (Fig. 7A). In addition, significant cell surface expression of TLR4 was also found after 24-h stimulation with both strains of *H. pylori* (Fig. 7A).

Effect of live *H. pylori* and LPS on MD-2 promoter activity in AGS cells

As suggested by the above data, MD-2 is thought to regulate TLR4-dependent signaling in gastric epithelial cells associated with *H. pylori* infection. Therefore, we also examined transcriptional activation of MD-2 in AGS cells cultured with *H. pylori*.

The construction of the luciferase vector for the reporter gene assay for MD-2 promoter is shown in Fig. 7B. After the 6-h stimulation with *H. pylori*, both strains had significantly higher transcriptional activation of MD-2 in AGS cells (Fig. 7C). Although the effects of three kinds of LPS purified from *H. pylori*, *E. coli*, and *Salmonella* were investigated, they did not influence MD-2 promoter activation in AGS cells.

Discussion

We have demonstrated that *H. pylori* infection up-regulates the host’s innate immunity through activation of the TLR4-MD-2 system in the stomach. Several published studies have addressed the expression of TLR4 in relation to *H. pylori* infection, but none has evaluated the role of MD-2. Because MD-2 is essential for LPS responses, we focused the present study on MD-2 expression in gastric epithelial cells and investigated its function in TLR4-dependent signaling in *H. pylori*-associated gastritis.

*H. pylori* LPS has not been considered to play a major role in the pathogenesis of gastritis because its biological activity is lower than that of the LPS of pathogens such as *E. coli* and *Salmonella* spp (45, 46). In contrast, several studies have demonstrated significant roles of *H. pylori* LPS in the induction of gastritis. Teshima et al. (14, 15) have shown that *H. pylori* LPS stimulates...
NF-κB activation markedly in cultured guinea pig gastric mucosal cells and leads to abundant release of superoxide anion from the cells. In addition, the severity of atrophic gastritis induced by long-term infection of *Haemobartonella felis* in CH3/He mice is significantly higher than that in LPS-nonresponder CH3/HeJ mice (47), which have an inactivating point mutation in *Tlr4* (48). More recently, Karhukorpi et al. (49) reported elevated serum levels of a soluble form of the CD14 molecule in *Haemobartonella pylori*-infected individuals. Soluble CD14 binds to LPS and facilitates its signaling, and this study has shown that CD14 promoter polymorphism is associated with the disease outcome. All of these findings suggest that *H. pylori* LPS may be a potent mediator in modulating *H. pylori* infection-induced gastritis.

Various studies have suggested that surface epithelial cells have an essential, front-line, defensive role in the mucosal immune system in the gastrointestinal tract. TLR4 expression has been reported to be minimal in the intestinal epithelial cells of healthy individuals, but significantly high in patients with inflammatory bowel disease (36, 37, 50). We have found recently that intestinal epithelial cells constitutively express TLR4, and that this expression is increased by dextran sodium sulfate-induced gut inflammation (35). Previously, Kawahara et al. (51) have shown that guinea pig gastric epithelial cells express TLR4, which regulates epithelial cell responses to *H. pylori* LPS in vitro. To clarify in vivo expression of TLR4 in the human stomach, in the present study we used biopsy specimens obtained from *H. pylori*-infected

**Figure 6.** A. The results of NF-κB activity in TLR4 or MD-2 stable transfectant and cotransfectant after stimulation with *H. pylori* LPS. Cells were cultured in 24-well plates (5 × 10⁴ cells/well) and transfected with 0.5 μg of pNF-κB-Luc and 0.02 μg of pRL-TATA-Renilla-Luc per well. The cells were stimulated with 0.1 and 1.0 ng of *H. pylori* LPS or vehicle for 8 h, and cell lysates were used for the measurement of luciferase activity. B. The results of IL-8 promoter activity in TLR4 or MD-2 stable transfectant and cotransfectant after stimulation with *H. pylori* LPS. The procedure for the reporter gene assay is described in Materials and Methods. Data are expressed as the n-fold increase in luciferase activity of *H. pylori* LPS. Error bars indicate the SEM of the values obtained from three independent experiments performed in triplicates. Differences in luciferase activity were analyzed by Student’s t test. *, p < 0.05; **, p < 0.01.
FIGURE 7. A. The effects of live H. pylori on MD-2 mRNA expression and cell surface expression of TLR4 in AGS cells (a, ATCC43504; b, TN583). The cells were cultured in six-well plates (2 x 10^5 cells/well) for 24 h; the cells were then cocultured with H. pylori (2 x 10^7 bacilli/well). At 0, 3, 6, and 12 h after coculture, total RNA was extracted from cultured AGS cells for RT-PCR. For flow cytometry, the cells were collected at 24 h after coculture and stained with TLR4 mAb (HTA125). Gray histograms showed significant cell surface expression of TLR4 in H. pylori-stimulated cells. B, Vector construction of MD-2 promoter for reporter gene assay. pGL3-MD-2-Luc, which induces a 1-kb (1013-bp) sequence upstream of the start site, was constructed using PCR. C, The results of MD-2 promoter activity in AGS cells after stimulation of live H. pylori (ATCC43504 or TN583) and LPS. Cells were cultured in 24-well plates (5 x 10^4 cells/well) and transfected with 0.5 µg of pGL3-MD-2-Luc and 0.02 µg of pRL-TATA-Renilla-Luc per well. The cells were stimulated with live H. pylori and LPS (0.1 or 1.0 ng/ml) for 6 h; cell lysates were used for the measurement of luciferase activity. Data are expressed as the n-fold increase in luciferase activity with H. pylori LPS. Error bars indicate the SEM of the values obtained from three independent experiments performed in triplicates. Differences in luciferase activity were analyzed by Student’s t test. **, p < 0.01.
and -uninfected individuals, and observed that \textit{H. pylori} infection accelerated TLR4 expression predominantly in the epithelial cells, although some epithelial cells seem to stain well for TLR4, while others do not. Because \textit{H. pylori} colonization is not homogeneous on gastric epithelial monolayers, the different density of \textit{H. pylori} organisms may influence such TLR4 expression pattern in gastric epithelial cells. Gastric epithelial cells have been reported to be a main source of IL-8 production in \textit{H. pylori}-infected mucosa (52–54). In addition, previous immunohistochemical studies have revealed significant nuclear staining of p65, which indicates activation of NF-κB, in gastric epithelial cells during \textit{H. pylori} infection (55, 56). These findings support our hypothesis that epithelial cells may play an essential role in the innate immune system through the activation of TLR4 in \textit{H. pylori}-associated gastritis.

Physical association of MD-2 with TLR4 on the cell surface has been demonstrated to confer LPS responsiveness (27–33). Several in vitro studies have shown LPS hyporesponsiveness in cells expressing TLR alone or TLR4 with mutant MD-2 (57). Although MD-2 expression is absolutely necessary for the recognition of LPS, no reports of in vivo expression of MD-2 and its association with the outcome of intestinal inflammatory disease have been published. In the present study, we were able to detect significant expression of MD-2 mRNA in \textit{H. pylori}-infected gastric mucosa, and LCM coupled with RT-PCR clearly showed that \textit{H. pylori} infection can activate MD-2 expression in epithelial cells. In addition, we also observed significant transcriptional activation of MD-2 in AGS cells cultured with \textit{H. pylori} by reporter gene assay. Because \textit{H. pylori} LPS as well as \textit{E. coli} and \textit{Salmonella} LPS did not influence MD-2 promoter activation in AGS cells, \textit{H. pylori} infection itself may be responsible for up-regulation of the MD-2 promoter activation and increased MD-2 expression, which ultimately leads to cell surface expression of functional TLR4. The Cag and Vac statuses are positive in both \textit{H. pylori} strains used in our study, and these bacterial virulence factors may also be involved in MD-2 promoter activation in AGS cells. Furthermore, \textit{H. pylori} infection can induce the production of several proinflammatory cytokines in gastric epithelial cells in vivo and in vitro, which may eventually stimulate MD-2 expression via an autocrine or paracrine manner. Abreu et al. (41) reported that MD-2 was expressed weakly in lamina propria cells, but not epithelial cells in uninvolved areas of intestine, and that IFN-γ, a major Th-1 cytokine, can stimulate MD-2 promoter activity in colon cancer cell lines. Because \textit{H. pylori} infection also predominantly induces a host Th1 immune response at the inflammatory site (58–61), these findings suggest that \textit{H. pylori} infection-dependent inflammation may up-regulate MD-2 expression in gastric epithelial cells and lead to stimulation of the TLR4-associated innate immune systems in the stomach.

Recently, Nagai et al. (34) reported an interesting study about the role of MD-2 in LPS responsiveness and cellular distribution of TLR4. Their immunohistochemical analysis of embryonic fibroblasts isolated from MD-2 null mice showed that TLR4 cannot reach the plasma membrane and resided predominantly in the Golgi apparatus in the cells. These findings suggest that MD-2 can activate TLR4 functionally in the cells by translocating TLR4 to the cell membrane. In our study, we used a gastric epithelial cell line and established stable transfectants that expressed TLR4 and MD-2. We found that MD-2 overexpression dramatically up-regulated the cell surface expression of TLR4 without additional TLR4 transfection. Significant increases in the activation of NF-κB and IL-8 promoter activity were also observed in MD-2 transfectants after stimulation with \textit{H. pylori} LPS. Interestingly, our immunohistochemical study showed significant TLR4 staining in the cytoplasm and at the apical surface of epithelial cells in \textit{H. pylori}-infected subjects. These in vivo results suggest that \textit{H. pylori} infection accelerates TLR4 expression in gastric epithelial cells and functionally activates TLR4 in the cells through increased expression of MD-2. Several studies using colon cancer cell lines have shown that overexpression of both TLR4 and MD-2 is essential for LPS responsiveness in the cells (30). In contrast, \textit{H. pylori} LPS has been reported to stimulate primary cultured gastric epithelial cells without transfection of TLR4 and MD-2 (14, 15, 38, 51), although AGS cells needed overexpression of MD-2 to recognize \textit{H. pylori} LPS. In our study, additional effects of TLR4 cotransfection on MD-2-transfected AGS cells were relatively low, showing that \textit{H. pylori} LPS responsiveness was mainly dependent on the increased expression of MD-2 in AGS cells. These findings suggest that LPS responsiveness in gastrointestinal epithelial cells varies and may depend on the cellular distribution of TLR4. Thus, the precise function of the TLR4/MD-2 system in intestinal epithelial cells in response to LPS has not been described fully. Further investigations about the molecular mechanism of TLR4 and MD-2 will be necessary for a complete understanding of the immune system of the gut. In summary, we investigated the role of TLR4-dependent signaling in \textit{H. pylori}-associated gastritis. The present study showed for the first time that MD-2 expression is significantly elevated in gastric epithelial cells during \textit{H. pylori} infection, which suggests that the TLR4/MD-2 system may be a potent receptor complex involved in the response to \textit{H. pylori} LPS in the stomach. Our findings provide evidence of a novel link between \textit{H. pylori} and gastric mucosal immunity that may help to explain the complexity and diversity of the disease associated with \textit{H. pylori} infection.

References

TLR-4/MD-2 SIGNALING IN H. pylori-DEPENDENT GASTRITIS


