ORIGINAL

Helicobacter pylori lipopolysaccharide from type I, but not type II strains, stimulates apoptosis of cultured gastric mucosal cells

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Abstract: The *cag* pathogenicity island (*cag* PAI) genes are a major determinant of virulence of *Helicobacter pylori (Hp)*. Lipopolysaccharide (LPS) purified from the *cag* PAI-positive (type I) strains induced apoptosis of primary cultures of guinea pig gastric mucosal cells. Lipid A catalyzed this apoptosis. These cells expressed the Toll-like receptor 4 (TLR4) mRNA and its protein, and type I *Hp* LPS phosphorylated transforming growth factor β-activated kinase 1 (TAK1) and TAK1-binding protein 1 (TAB1) in association with up-regulation of the TLR4 expressions, suggesting that type I *Hp* LPS evoked distinct TLR4 signaling. In contrast, *Hp* LPS from type II strains with complete or partial deletion of the *cag* PAI genes did not phosphorylate TAK1 and TAB1 and failed to induce apoptosis. Accelerated apoptosis of gastric epithelial cells is one of the important events relevant to chronic, atrophic gastritis caused by *Hp* infection. The difference in proapoptotic action of LPS between the type I and II strains may support an important role of the *cag* PAI genes in the pathogenesis of gastric lesions caused by *Hp* infection. J. Med. Invest. 48 : 166-174, 2001

Keywords **:** *apoptosis, Helicobacter pylori , cag PAI gene, LPS, gastric mucosal cells*

INTRODUCTION

Accelerated apoptosis of gastric epithelial cells is one of the important events relevant to chronic, atrophic gastritis caused by *Helicobacter pylori* (*Hp*) infection (1, 2). Increased expression of suicide ligands and their receptors, such as tumor necrosis factor-α (TNF-α) (3) and the Fas receptor (4), has been suggested to participate in this apoptosis. However, it is still unclear whether distinct bacterial factors are involved in this apoptosis.

Hp strains are grouped into two families, type I and type II. Patients with the gastric lesions are most often infected with type I strains that are characterized by the presence of the cytotoxin-associated gene A (*cagA*) and the vacuolating cytotoxin gene A (*vacA*) (5). Type I strains have an insertion of approximately 40 kb of foreign DNA, named the *cag* pathogenicity island (PAI), and the *Hp cag* PAI contains 31 genes, including *cagA* (6). These genes are now recognized as transmissible DNA that encodes virulence factors and maps in the chromosome of pathogenic organisms. Among the *cag* PAI genes, *cagE* (*picB*), *cagG*, *cagH* , *cagI* , *cagL* , and *cagM* are involved in the activation of nuclear factor (NF)-κB and stimulation of interleukin (IL)-8 secretion from gastric epithelial cells (7, 8). Six of the *cag* PAI genes code for the core subunits of the type IV export machinery that can transfer CagA protein into host epithelial cells, and translocated CagA has been shown to be tyrosine

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phosphorylated by host cells (9-12).

Compared to these virulent factors, *Hp* lipopolysaccharide (LPS) is generally accepted as a low-toxic substance, compared with those of *Salmonella enterica* or *Escherichia coli* (*E. coli*) (13, 14). However, *Hp* LPS was been shown to stimulate histamine release and DNA synthesis in rat enterochromaffin-like cells more effectively than *E. coli* LPS (15). We also demonstrated that *Hp* LPS could enhance production of superoxide anion in primary cultures of guinea pig gastric mucosal cells (16, 17), suggesting that *Hp* LPS has an ability to trigger innate immune responses including activation of NF-κB.

We report here that LPS from type I *Hp*, but not type II strains, acts as a potent inducer of apoptosis of cultured cells of gastric mucosa from guinea pigs. The present findings suggest that the difference in apoptosis-inducing action between the type I and type II may explain, at least in part, an important role of the *cag* PAI genes in the pathogenesis of *Hp* infection.

MATERIALS AND METHODS

Preparation and culture of gastric mucosal cells under LPS-free conditions

Gastric mucosal cells were isolated aseptically from guinea pig fundic glands (17). In this study, all reagents used for culture were free from detectable amounts of LPS by the *Limulus* amebocyte lysate assay (Endospecy ; Seikagaku Kogyo Co., Tokyo, Japan). The isolated cells were cultured for 2 days in RPMI 1640 (GIBCO, Grand Island, NY), containing 50 μg/ml gentamicin, 100U/ml penicillin G, and 10% LPS-free fetal bovine serum (FBS ; ICN Biomedicals, Aurora, OH). This complete medium contained < 0.01 EU/ml of LPS. After culturing for 2 days, the growing cells were composed of pit cells (about 90%), pre-pit cells (about 5%), parietal cells (4-5%), mucous neck cells (less than 1%), and fibroblasts (less than 1%) (17).

Isolation and culture of clinical Hp strains

The present experiments were approved by the ethics committees of the Medical Faculty of Hokkaido University. All procedures involving animals were approved by the Animal Care and Use Committee of the University of Tokushima. Clinical isolates of *Hp* were established from gastric biopsy specimens and were cultured for up to 5 days on *Hp*-selective agar plates (Eiken Chemical Co., Tokyo, Japan) under microaerophilic conditions (12% CO_2-5% O_2-83% N_2). The organisms were identified as *Hp* by Gram-staining, colony morphology, and positive-oxidase, -catalase, and-urease reactions. A single colony on the agar plate was picked up and cultured in brucella broth (GIBCO) supplemented with 5% FBS and 10 μg/ml vancomycin.

Determination of genotypes of clinical isolates

Bacterial genomic DNA was extracted, and PCR was performed using the following primer sets : *vacA*, 5'-ATGGAAATACAACAAACACA-3' and 5'-CTCCAGAACCCACACGATT-3' or 5'-TACAAACC TTATTGATTGATAGCC-3' and 5'-AAGCTTGATT GATCACTCC-3' ; *cagA*, 5'-GGGGATCCATGACT AACGAAACC-3' and 5'-GGCTTAAGTGATGGGA CACCCAA-3' ; *cagE*, 5'-GCTAGTTATAGAGCAA GAGGTTCAA-3'and 5'-TAGTTGTTAGTAAGGAT CACCCCAT-3' ; and *cagG*, 5'-CCCTAATATCGGT GGTAAAAA-3'and 5'-CTATTTGCTTGGTGTCTT ATC-3'. The sequences of these primers corresponded to the *cag* PAI genes of *Hp* NCTC 11638. PCR was performed under the following conditions : 35 cycles of 1 min at 92°C, 1 min at 52°C, and 1 min at 72°C.

For Southern blot analysis, 10 μg of genomic DNA of *Hp* was digested with HaeIII, HindIII, or EcoRI (New England Biolabs., Beverly, MA.), electrophoresed on 1% agarose gel, and then transferred onto a nylon membrane. The *cagA*, *cagE*, *cagG*, and *vacA* probes prepared as described above were labeled with digoxigenin (DIG) using a PCR DIG probe synthesis kit (Roche, Basel, Switzerland). The membrane was treated to hydridize with one of the labeled probes for 20 h at 42°C in DIG Easy Hyb (Roche). After being washed sequentially in 2x standard saline citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) and 0.2x SSC-0.1% SDS, the hybridized probes were examined using a DIG Nuleic Acid Detection Kit (Roche).

Preparation of Hp LPS and lipid A

LPS was prepared from six clinical strains by the hot-phenol-water method of Westphal and Jann (18) and subsequently treated with DNase 1, RNase A, and proteinase K, as described by Moran *et al.* (19). The treated LPS was ultracentrifuged and dialyzed against LPS-free water (Otsuka Pharmaceutical Co., Tokushima, Japan). LPS from *E. coli* K-235 strain was purchased from Sigma Chemical Co. (St. Louis, MO). The ketosidic linkage between core oligosaccharide and lipid A was decomposed by boiling LPS in 0.1 M

acetate buffer (pH 6.5) for 1 h (20). Lipid A was pelleted by centrifugation at 3,000 x *g* for 30 min. Precipitated lipid A was dissolved in LPS-free saline (Otsuka Pharmaceutical Co.). After the purified LPS and lipid A had been lyophilized, their dry weights and *Limulus* activities were measured using a Sartorius Supermicro (model S4 ; Sartorius, Göettingen, Germany) and by the *Limulus amebocyte* lysate assay, respectively.

Analyses of DNA fragmentation and nuclear morphology

Cells were cultured for 48 h and treated with LPS. Cells both floating and attached to dishes were lysed for 30 min at 4°C in 200 μl of 10 mM Tris-HCl buffer (pH8.0), containing 10 mM EDTA and 0.5% (vol/vol) Triton X-100. Fragmented DNA of these samples was isolated and analyzed as described previously (16). In separate experiments, cells were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature and were stained with the fluorescent dye Hoechst 33342 (Sigma Chemical Co.). Chromosomal condensation and fragmentation were examined using a fluorescence microscope, as described previously (16).

Detection of TLR4 transcript

Total RNA was isolated from guinea pig gastric mucosal cells by treating them with an acid guanidinium thiocyanate-phenol-chloroform mixture (21). Reverse transcriptase (RT)-PCR was carried out to detect the TLR4 transcript using the following PCR primer sets : 5'-TCACCTGATGCTTCTTGCTG-3' and 5'-AGTCGTCTCCAGAAGATGTG-3'. The resultant PCR products were separated on an agarose gel, purified and ligated into a pCR42-TOPO vector (Invitrogen, Carlsbad, CA.), and transformed into JM109 cells. Transformed plasmids containing the appropriate insert DNA were selected and sequenced with a DNA sequencer (model ABI 377 ; PE Biosystems Japan, Tokyo, Japan).

For measurement of the TLR4mRNA level, total RNA (8 μg per lane) was subjected to electrophoresis in 1% agarose gel and transferred to a nylon filter membrane. After prehybridization, the membrane was hybridized for 4 h at 60°C in a Rapid hyb buffer (Amersham Pharmacia) containing the amplified TLR4 cDNA or a cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ATCC57494 ; American Type Culture Collection, Rockville, MD). These probes were prelabeled with [α-^{32}P] deoxy-CTP using a random primer kit (Amersham Pharmacia). The membrane was washed twice with 2x SSC containing 0.5% SDS for 10 minutes at 65°C and then three times with 0.2x SSC containing 1% SDS. Bound probes were autoradiographed by exposure of Kodak X-Omat films for an appropriate time at-80°C.

Detection of TLR4 protein

An anti-TLR4antibody was raised by subcutaneous immunization of a rabbit with a synthetic peptide of the amino acid residues 183 to 199 of human TLR4 (22). The keyhole limpet hemocyanin-conjugated peptide was injected into the rabbit with Freund's complete adjuvant. Serum was collected and further purified by affinity chromatography with the synthetic peptide-conjugated agarose. A membrane fraction from guinea pig gastric mucosal cells was prepared as described previously (17). Each sample of 20 μg protein per lane was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride filter membrane. After blocking nonspecific binding sites with 4% purified milk casein, the membrane was incubated for 1 h at room temperature with the anti-TLR4 antibody. Bound antibodies were detected using an enhanced chemiluminescence system (Amersham Pharmacia, Piscataway, NJ). They were then removed by rinsing the membranes for 15 min at 50°C in 60 mM of Tris-HCl buffer, containing 0.1 mM 2-mercaptoethanol and 2% SDS. After washing with PBS, the membrane was again subjected to immunoblotting with an antibody against actin (Oncogene Res., Cambridge, MA).

Analysis of TLR4 signaling

For detection of transforming growth factor-β-activated kinase 1 (TAK1) and TAK1-binding protein 1 (TAB1), cellular proteins were prepared in the presence of inhibitors of both proteases and phosphatases as described previously (23). Phosphorylation of the proteins was also confirmed by treatment with 5 U of bacterial alkaline phosphatase as previously described (23). Each sample (20 μg protein) was separated by SDS-PAGE in 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride filter membrane. After blocking with 4% purified milk casein, the membrane was incubated for 1 h at room temperature with a 1 : 500 dilution of polyclonal antibody against 554 to 579 amino acid residues of mouse TAK1 or 480 to 500 residues of human TAB1 (from Dr. Matsumoto, Nagoya University, Nagoya, Japan). Bound antibodies were detected by the enhanced chemiluminescence system, and then actin was detected, as described above.

RESULTS

Effects of Hp LPS from clinical isolates on apoptosis of gasric mucosal cells

According to the results of PCR and Southern blot analyses, *Hps* 1, 2, and 3 were *vacA* (+) and *cag* PAI (+) strains (type I). *Hps* 5 and 6 were determined to be *vacA* (+) and *cag* PAI (-) strains (type II). *Hp* 4 was identified as a mutant with partial deletion of *cag* PAI (Table 1). As listed in Table 1, the specific *Limulus* activities of LPSs from *cag* PAI-negative strains (*Hps* 5 and 6) were 4,300-to 420,000-folds lower than those from the type I *Hps*, while *Hp* 4, a partial deletion mutant of *cag* PAI, had a similar *Limulus* activity to those of type I *Hp* strains (Table 1).

LPS was extracted from these clinical isolates by the hot-phenol-water method. Treatment of gastric mucosal cells with 42 EU/ml of *Hp* 1 LPS caused DNA ladder formation within 5 h (data not shown). When cells were incubated with different concentrations of *Hp* 1 LPS for 8 h, this LPS at a concentration of 42 EU/ml (385 ng/ml) or higher induced DNA fragmentation (Fig. 1A). Based on these findings, gastric mucosal cells were treated for 8 h with 42 EU/ ml of LPS from one of the clinical isolates. All of the type I *Hps* induced apoptotic DNA ladder formation (Fig. 1B) and increased the numbers of apoptotic cells with condensed and fragmented nuclei (Fig. 1C), while *Hps* 4-6 failed to stimulate these cellular responses.

Determination of active components of Hp LPS

The LPS extracted by the hot-phenol-water method might have contaminants, such as DNA, RNA, and denatured protein. *Hp* 1 LPS extracted by the hot-phenol-water

Table 1. Specific *Limulus* activities of LPS and lipid A of clinical isolates[a]

Organism	Genotypes	*Limulus* activity of LPS (EU/μg)
E. coli		344
Hp 1	*vacA* (+), *cagA* (+), *cagE* (+), *cagG* (+)	109
Hp 2	*vacA* (+), *cagA* (+), *cagE* (+), *cagG* (+)	73.3
Hp 3	*vacA* (+), *cagA* (+), *cagE* (+), *cagG* (+)	593
Hp 4	*vacA* (+), *cagA* (+), *cagE* (-), *cagG* (-)	157
Hp 5	*vacA* (+), *cagA* (-), *cagE* (-), *cagG* (-)	0.0014
Hp 6	*vacA* (+), *cagA* (-), *cagE* (-), *cagG* (-)	0.017

[a]*Hp* genotypes were determined by PCR and Southern blot analyses, as described in MATERIALS AND METHODS. LPS was purified from these *Hps* (1 through 6), as described in MATERIALS AND METHODS. The dry weight and *Limulus* activity were measured.

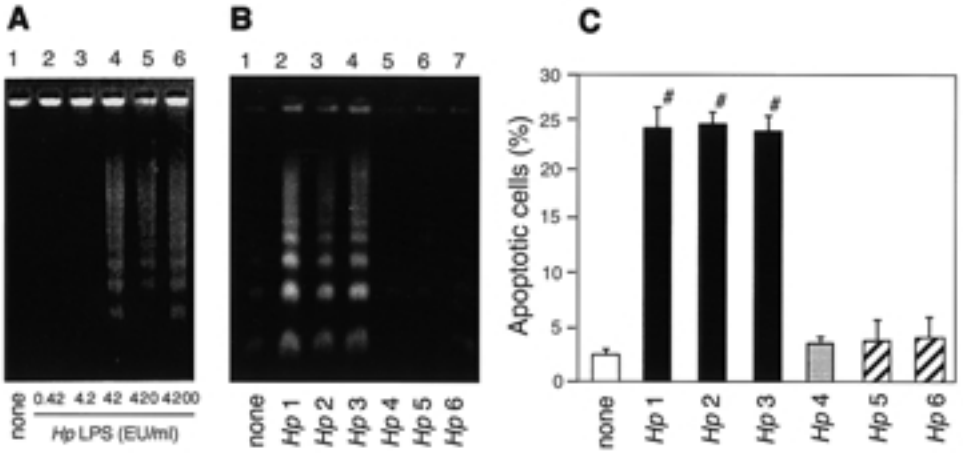

Fig. 1. Effects of *Hp* LPS from clinical isolates on apoptosis of gastric mucosal cells. After cultured cells were washed with saline, they were treated for 8 h with different concentrations of *Hp* 1 LPS (A) or 42 EU/ml of LPS from each clinical strain (B) in RPMI 1640 containing 0.1% FBS. These cells were harvested and analyzed for DNA ladder formation, as described in MATERIALS AND METHODS. Results were similar in three separate experiments. The cells were stained with Hoechst 33342 (C) as described in MATERIALS AND METHODS. Numbers of cells having condensed and fragmented nuclei were counted, and the percentages of apoptotic cells are shown. Values are the means±SD (n=8). #Significantly increased vs. vehicle-treated cells ($P<0.05$ by ANOVA and Scheffé's test).

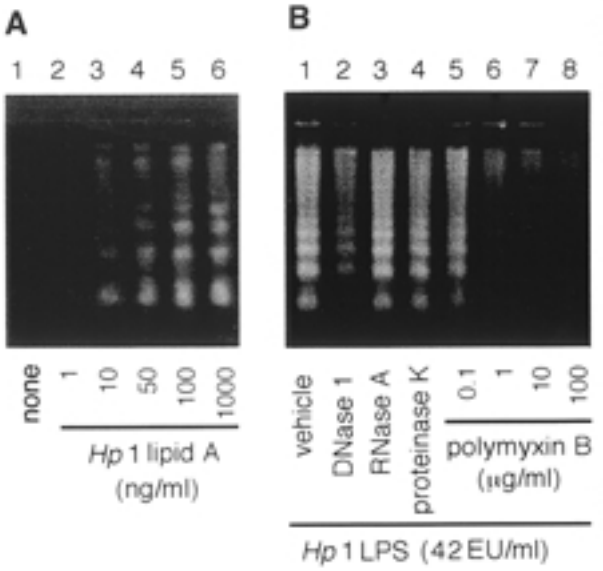

Fig. 2. Determination of active component of *Hp* LPS. Lipid A was isolated as described in MATERIALS AND METHODS. Cultured guinea pig gastric mucosal cells were incubated for 8 h with different concentrations of *Hp* 1 lipid A (A). *Hp* 1 LPS (42 x 10^3 EU/ml) extracted by the hot-phenol-water method was treated with 0.1 mg/ml DNase 1, 0.1 mg/ml RNase A, or 0.1 mg/ml proteinase K for 12 h, 4 h, or 4 h, respectively. Treated LPSs were boiled for 1 h to inactivate the enzymes. The gastric mucosal cells were incubated for 8 h at 37°C with saline (B, lane 1) or one of these LPSs (42 EU/ml) (B, lanes 2-4). After *Hp* 1 LPS (42 EU/ml) was incubated for 1 h at 37°C with different concentrations of polymyxin B, the gastric mucosal cells were treated for 8 h with 42 EU/ml of *Hp* 1LPS (B, lanes 5-8). The treated cells were harvested and analyzed for DNA ladder formation as described in the legend to Fig. 1. Results were similar in three separate experiments.

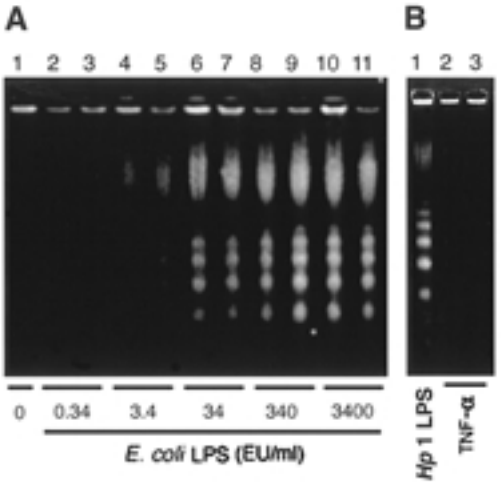

Fig. 3. Effect of *E. coli* LPS or TNF-α on DNA ladder formation. Cultured guinea pig gastric mucosal cells were treated with different concentrations of *E. coli* LPS (A), 42 EU/ml *Hp* 1 LPS (B, lane 1), or 50 ng/ml of recombinant mouse (B, lane 2) human (B, lane 3) TNF-α at 37°C for 8 h in RPMI 1640 containing 0.1% FBS. DNA fragmentation was analyzed as described in the legend to Fig. 1. Results were similar in three separate experiments.

method was treated with DNase 1, RNase A, or proteinase K, but none of these enzymes removed the activity of *Hp* 1 LPS (Fig. 2B, lanes 2-4). Polymyxin B inhibited the *Hp* LPS-induced ladder formation (Fig. 2B, lanes 5-8), and lipid A was confirmed to be a bioactive component of *Hp* LPS (Fig. 2 A).

Effects of E. coli LPS and TNF-α on apoptosis of gastric mucosal cells

We also tested whether the activity was specific for *Hp* LPS. For this purpose, we examined the effect of *E. coli* LPS from a commercial source. As shown in Fig. 3A, *E. coli* LPS (K-235 strain) similarly induced apoptotic DNA fragmentation as observed in *Hp*1. Next we examined the possibility that LPS could stimulate gastric epithelial cells or that a contaminating cell population induced TNF-α. It was previously reported that TNF-α subsequently induced apoptosis. Recombinant mouse or human TNF-α up to 50 ng/ml (Fig. 3B, lanes 2 or 3, respectively) did not stimulate apoptosis. Thus, we confirmed that LPS directly stimulated gastric mucosal cell apoptosis.

Expression of TLR4 in gastric mucosal cells

Among the TLR family members, TLR4 is now identified as the specific receptor for LPS *in vivo* and *in vitro* (24). Guinea pig gastric mucosal cells expressed low levels of the TLR4 transcript and detectable amounts of its protein (Fig. 4A). In response to *Hp* 1 LPS, gastric mucosal cells significantly increased the level of TLR4 protein (Fig. 4B) in association with an increase in the TLR4 mRNA level (Fig. 4A).

Stimulation of TLR4 signaling by Hp LPS

We also examined whether *Hp* LPS actually stimu-

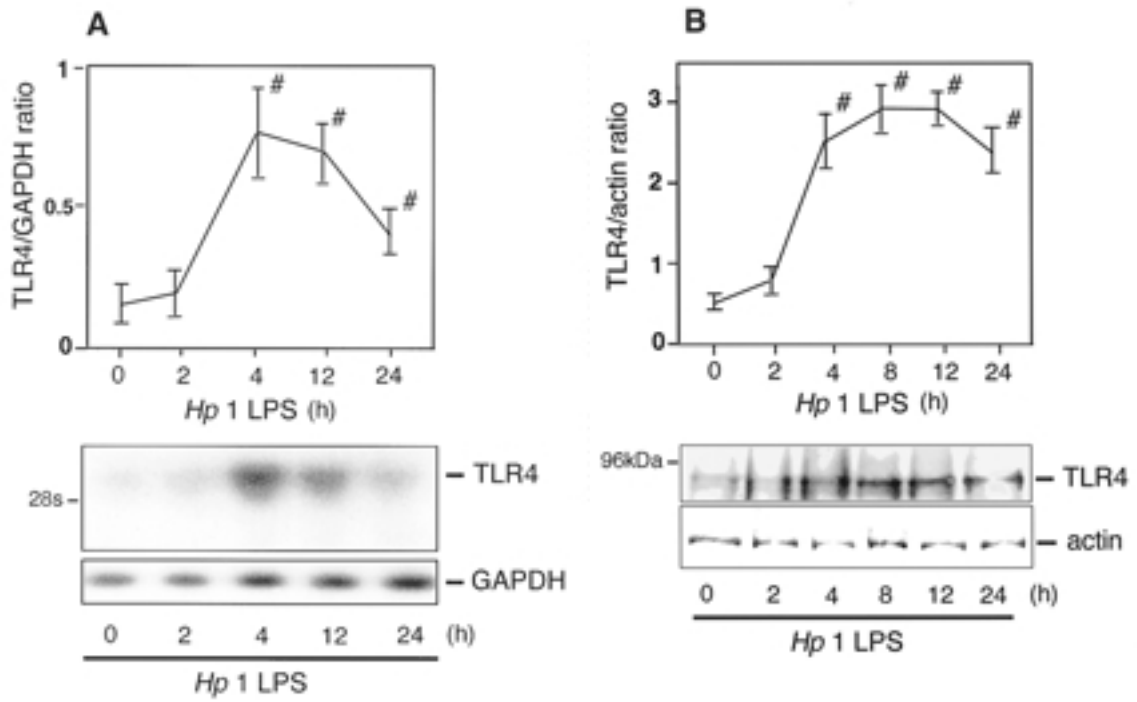

Fig. 4. Detections of TLR4 transcript and protein in gastric mucosal cells. Total RNA was isolated from cultured guinea pig gastric mucosal cells as described in MATERIALS AND METHODS. After cells had been treated with 42 EU/ml of *Hp* 1 LPS for the indicated times, total RNA (8 μg per lane) was separated in a 1% agarose gel. Northern hybridization with the cDNA probe for TLR4 or GAPDH was performed as described in MATERIALS AND METHODS. The levels of TLR 4 and GAPDH were quantified by laser densitometry, and the ratios of TLR4/GAPDH are expressed as means±SD (n=4) (A). Membrane proteins were prepared from the gastric mucosal cells as described in MATERIALS AND METHODS. Immunoblot analysis was performed with an antibody against TLR4. Bound antibodies were then removed by rinsing the membranes for 15 min at 50°C in 60 mM Tris-HCl buffer, containing 0.1 mM 2-mercaptoethanol and 2% SDS. The membrane was again subjected to immunoblotting with an antibody against actin. The levels of TLR 4 and membrane-associated actin were quantified by laser densitometry, and the ratios of TLR4/actin are expressed as the means±SD (n=4) (B). #Significantly increased vs untreated control cells (P<0.05 by ANOVA and Scheffé's test).

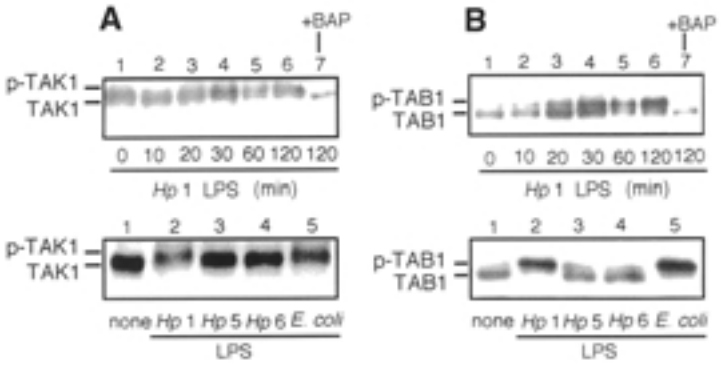

Fig. 5. Phosphorylation of TAK1 and TAB1 by LPS. Cultured cells were treated with 42 EU/ml of *Hp* 1 LPS for the times indicated (upper panels in A and B) or with 42 EU/ml of LPS from three *Hp* clinical strains (*Hps* 1, 5, and 6) and 34 EU/ml of *E. coli* for 60 min (lower panels in A and B). Whole-cell proteins were prepared from these cells as described in MATERIALS AND METHODS. Protein extracts were also prepared from the cells after treatment with 42 EU/ml of *Hp* 1 LPS for 2 h and were treated with bacterial alkaline phosphatase (BAP) (lane 7), as described in MATERIALS AND METHODS. Immunoblot analysis was performed with an antibody against TAK1 or TAB1, as described in MATERIALS AND METHODS. p-TAK1, phosphorylated TAK1 ; p-TAB1, phosphorylated TAB1. Similar results were obtained in three separate experiments.

lated TLR4 signaling. TAK1 is a mitogen-activated protein kinase kinase kinase that activates c-Jun N-terminal kinase-and NF-κB-cascades (25, 26). TAK1 is now recognized as one of the common signal transmission molecules for TLR4 and IL-1receptor signaling pathways (26, 27). TAB1 functions as an activator for the TAK1 (28). Treatment with *Hp* LPS rapidly phosphorylated TAK1 (Fig. 5A) and TAB1 (Fig. 5B). It should be noted that *Hp* 1 and *E. coli* LPSs activated these proteins, while Type II strains (*Hps* 5 and 6) did not phosphorylate them, suggesting that the *Hp cag* PAI genes are necessary for the synthesis of LPS that stimulates TLR4 signaling.

DISCUSSION

In this study, we showed that guinea pig gastric

mucosal cells expressed TLR4 and were sensitive to LPS of type I, but not to that of type II strains. The complete genome sequencing of *Hp* should provide a genetic basis for more fully understanding the biological processes. The proteins encoded within the *Hp cag* PAI genes contain the motifs found in bacterial proteins, including translocases, sensors, permeases, flagellum assembling proteins, and components of the type IV secretion machinery (10). Complete or partial deletion of or insertion of a new gene into *cag* PAI, or rearrangements of the genes within *cag* PAI are proposed as the basis for modified or reduced virulence (6-8).

Compared with these virulence factors, less attention has been paid to *Hp* LPS, since 1,000 to 10,000-folds higher concentrations of *Hp* LPS are required for activation of host spleen cells or macrophages than those of LPSs from other Gram-negative bacteria (13, 14). In addition to the lower immunological activities, *Hp* LPS contains Lewis blood antigens, and the molecular mimicry has been proposed to camouflage and allow colonization to persist chronically. *Hp* frequently changes its LPS molecule depending upon culture conditions (29) and the number of passages (19). The diversity of the *Hp* genomes, especially encoding the outer membrane proteins or enzymes for LPS synthesis, is known (30). However, these findings are limited to the biosynthesis of Lewis antigens in the O-polysaccharide regions, such as α3-fucosyltransferase gene (31). We demonstrated that lipid A, but not polysaccharides, a bioactive cell wall component of *Hp*, induced apoptosis, although the molecular basis for biosynthesis of *Hp* lipid A is not fully understood.

According to the information on synthetic *E. coli*-like lipid A, phosphate-patterns, the numbers of acyl chains, and fatty acid compositions are important for the full expression of a range of biological activities (29). For example, the lipid A composed of bisphosphates and hexaacyl chains is more toxic than that composed of monophosphate and tetraacyl chains (32). *Hp* synthesizes two types of lipid A molecules ; hexaacyl- and tetraacyl-lipid A (19, 29). Hexaacyl-lipid A has two phosphates or phosphorylethanolamines on the lipid A disaccharide backbone, while tetraacyl-lipid A contains only one phosphate. The potency of *Hp* tetraacyl-lipid A has about 4-fold lower toxicity on human monocytes than that of the hexaacyl-form (33). At present, there is no evidence that the *cag* PAI genes directly participate in the synthesis of bioactive lipid A molecule, and the genomic basis for the linkage between the *cag*-PAI genes and lipid A biosynthesis remains to be elucidated. However, it is possible that as observed in the known virulence genes, the *cag* PAI genes indirectly support the biosynthesis of endotoxin, LPS.

TLRs are now characterized as a family of mammalian homologs of *Drosophila* Toll. Among the TLR family, TLR4 confers responsiveness to LPS from Gram-negative bacteria, while TLR2 responds to the cell wall component from Gram-positive bacteria, such as a lipoprotein (34). The difference between type I and type II LPSs in the proapoptotic action coincided with the TLR4-stimulating action. Activation of TLR4 signaling also results in the activation of NF-κB, a crucial transcription activator of many inflammatory response genes, which was previously reported (16, 17). Accelerated apoptosis has been observed in gastric mucosal lesions caused by *Hp* infection, particularly in chronic, atrophic gastritis, while the molecular basis for this apoptosis is not known. At present, it is unclear whether TLR4-mediated signalings stimulated by type I *Hp* LPS actually participate in the development of gastric mucosal lesions. However, the present findings suggest that the diversity of the proapoptotic action of *Hp* LPS is one of the important determinants of virulence of Gram-negative bacteria.

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