Helicobacter pylori heat-shock protein 60 induces inflammatory responses through the Toll-like receptor-triggered pathway in cultured human gastric epithelial cells

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Contact between *Helicobacter pylori* and gastric epithelial cells results in activation of NF- κ B followed by secretion of interleukin (IL)-8. However, host-cell receptor(s) and their ligands involved in *H. pylori*-related IL-8 production have yet to be fully defined. In this study, the interaction between Toll-like receptors (TLRs), which are host receptors for pathogens involved in the innate immune response, and heat-shock protein (HSP) 60, an immune-potent antigen of *H. pylori*, was examined during *H. pylori*-induced IL-8 secretion *in vitro*. Recombinant *H. pylori* HSP60 (rHpHSP60) was prepared and added to cultured KATO III human gastric epithelial cells with or without pre-incubation with mouse monoclonal anti-TLR2 or anti-TLR4 antibodies. IL-8 mRNA expression and IL-8 protein release were analysed by Northern blotting and immunoassay. Involvement of NF- κ B activation was analysed immunocytochemically by anti-NF- κ B p65 antibody and ammonium pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF- κ B-mediated transcriptional activation. rHpHSP60 induced IL-8 mRNA expression and IL-8 secretion in a dose-dependent manner in KATO III cells. Anti-TLR2 antibody inhibited rHpHSP60-induced IL-8 secretion by 75 %, and anti-TLR4 antibody inhibited it by 30 %. rHpHSP60 induced nuclear translocation of NF- κ B p65, which was inhibited by pretreatment with anti-TLR2 antibody. Treatment with PDTC significantly decreased the secretion of IL-8 induced by rHpHSP60. These findings suggest that *H. pylori* HSP60 activates NF- κ B and induces IL-8 production through TLR-triggered pathways in gastric epithelial cells. Thus, it is possible that *H. pylori* HSP60 and TLR interaction in host cells contributes to the development of gastric inflammation caused by *H. pylori* infection.

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INTRODUCTION

Chronic infection with *Helicobacter pylori* is recognized as the cause of chronic active gastritis, peptic ulcer diseases and mucosa-associated lymphoid tissue lymphoma, and is an important risk factor in gastric carcinoma development (Blaser, 1990; Parsonnet *et al.*, 1991, 1994). Binding of *H. pylori* to gastric epithelial cells, in particular through the blood group antigen-binding adhesin, and the binding of strains expressing the *cag* pathogenicity island results in the production of proinflammatory chemokines such as

interleukin (IL)-8 (Crabtree *et al.*, 1995a; Rad *et al.*, 2002). NF- κ B and AP-1 function as intracellular messengers in this process (Meyer-ter-Vehn *et al.*, 2000; Sharma *et al.*, 1998).

The 60 kDa heat-shock protein (HSP60), an immune-potent antigen of *H. pylori* (Sharma *et al.*, 1997), induces IL-8 secretion from human gastric epithelial cells (Yamaguchi *et al.*, 1999). We have reported that humoral immune responses to HSP60 are closely associated with gastric inflammation and play a role in the pathogenesis of mucosa-associated lymphoid tissue lymphoma (Hayashi *et al.*, 1998; Kawahara *et al.*, 1999). With regard to host-cell receptors for HSP60, sulfated glycolipids are reportedly involved (Huesca *et al.*, 1996), but the host-cell receptor(s) involved in HSP60-induced IL-8 production have not been fully elucidated. Some studies have shown that Toll-like receptors (TLRs) act as receptors for bacterial structures

Abbreviations: FCS, fetal calf serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HSP, heat-shock protein; IL, interleukin; PDTC, ammonium pyrrolidinedithiocarbamate; PMB, polymyxin B; rHpHSP60, recombinant *H. pylori* HSP60; TLR, toll-like receptor; TNF- α , tumour necrosis factor α .

such as LPS and HSP60 (Kawahara *et al.*, 2001; Ohashi *et al.*, 2000; Sasu *et al.*, 2001; Vabulas *et al.*, 2001) in the development of an innate immune response against bacterial infection. Among the TLR family, TLR2 and/or TLR4 (Ohashi *et al.*, 2000; Sasu *et al.*, 2001; Vabulas *et al.*, 2001) are reportedly important in the recognition of HSP60 in human cells and chlamydia. TLR2 and TLR5 are reported to be required for *H. pylori*-induced NF- κ B activation and chemokine expression by epithelial cells (Smith *et al.*, 2003).

In the present study, to investigate the involvement of the interaction between bacterial HSP60 and host TLRs in inflammatory responses against *H. pylori* infection, we reacted cultured KATO III human gastric epithelial cells with *H. pylori* HSP60 or recombinant *H. pylori* HSP60 (rHpHSP60) in the presence or absence of anti-TLR antibodies. The effects on IL-8 production and NF- κ B activation were measured.

METHODS

Bacterial cells and culture. *H. pylori* (ATCC 43504) was cultured on *Brucella* agar supplemented with 7% horse blood at 37 °C under microaerophilic conditions. After 5 days incubation, bacteria were harvested in RPMI 1640 with or without 10% fetal calf serum (FCS) to an OD₆₀₀ of 1.0, corresponding to approximately 1×10^8 c.f.u. ml⁻¹.

KATO III human gastric cell lines (obtained from the Japanese Collection at Research Bioresources, Tokyo, Japan) were grown in RPMI 1640 supplemented with 10% FCS, 50 U penicillin G sodium ml⁻¹ and 50 µg streptomycin sulfate ml⁻¹ at 37 °C in an atmosphere of 5% CO₂ and 99% humidity. KATO III cells were used at a final concentration of 5×10^5 cells ml⁻¹.

Preparation of rHpHSP60. rHpHSP60 was prepared as described by Ishii *et al.* (2001). In brief, the ORF of HSP60 taken from *H. pylori* ATCC 43504 genomic DNA was amplified and cloned into the vector pGEX-5X3 (Amersham Biosciences). The resultant plasmid was transformed into *Escherichia coli* DH5 α . Cells were cultured in Luria-Bertani broth containing 2% glucose and ampicillin (100 µg ml⁻¹), harvested by centrifugation and disrupted by sonication on ice for 5 min with a probe sonicator (Astron). Soluble rHpHSP60 was purified by glutathione-Sepharose 4B affinity chromatography (Amersham Biosciences) according to the manufacturer's instructions.

Protein production in KATO III cells (ELISA). KATO III cells were co-cultured with various amounts of intact *H. pylori* or various concentrations of rHpHSP60. Cell-culture supernatants were collected and IL-8 was measured by ELISA (IL-8 ELISA Development kit; Techne). To evaluate the interaction between rHpHSP60 and TLRs in IL-8 production, cells were preincubated with 10 µg TL2.1 ml⁻¹ (anti-human TLR2 mouse mAb; Sanbio), 10 µg HTA125 ml⁻¹ (anti-human TLR4 mouse mAb; Sanbio) or 10 µg control irrelevant mouse mAb ml⁻¹ (anti-botulinum toxin type C), which we had prepared previously (Mahmut *et al.*, 2002), for 30 min before stimulation under FCS-free conditions. In addition, KATO III cells were pretreated with ammonium pyrrolidinedithiocarbamate (PDTTC) (Sigma-Aldrich), an inhibitor of NF- κ B-mediated transcriptional activation (Kawai *et al.*, 1995; Ziegler-Heitbrock *et al.*, 1993), for 1 h before being reacted with intact *H. pylori* (1×10^7 c.f.u. ml⁻¹) or rHpHSP60 (50 µg ml⁻¹). Cell-culture supernatants were collected and IL-8 was measured as described above.

To exclude the possible effects of trace amounts of LPS on IL-8 secretion induced by rHpHSP60 preparations, 20 µg polymyxin B ml⁻¹ (PMB) (Wako Pure Chemical Industries), a potent LPS antagonist (Stokes *et al.*, 1989), was added when KATO III cells were reacted with rHpHSP60, and *E. coli* LPS (Sigma-Aldrich) was used as control. In addition, a PMB-agarose gel column (Pierce) was used to remove LPS and LPS-associated molecules in rHpHSP60 preparations. The endotoxin activity of rHpHSP60 and PMB-agarose column fractions was determined by using the LAL assay kit (BioWhittaker) according to the manufacturer's instructions. IL-8 secretion from KATO III cells that had been reacted with the original rHpHSP60 preparation was compared with that from cells reacted with the PMB-agarose column pass-through fractions.

NF- κ B activation in KATO III cells that had been reacted with rHpHSP60 was detected and quantified by the use of Trans AM NF- κ B (Active Motif) according to the manufacturer's instructions. Briefly, nuclear extracts were prepared and incubated for 1 h on a 96-well plate with immobilized oligonucleotide containing the NF- κ B consensus site (5'-GGGACTTTCC-3'). After incubation with antibody to p65 (RelA), a subunit of the activated NF- κ B dimer, horseradish peroxidase-conjugated secondary antibody was added, followed by developing solution included in Trans AM NF- κ B. The optical density was read at 450 nm with a reference wavelength of 655 nm on a spectrophotometer. Recombinant tumour necrosis factor α (TNF- α) (Sigma-Aldrich) was used as a positive control.

Detection of rHpHSP60 in KATO III cells with bacterial or rHSP stimulation. To measure the rHpHSP60 concentration when live or heat-killed bacteria contacted and stimulated KATO III cells, the culture supernatants and cells were analysed by Western blotting. KATO III cells (5×10^6 cells ml⁻¹) were reacted with several concentrations of rHpHSP60 (100–1 µg ml⁻¹), or intact or heat-killed *H. pylori* [from OD₆₀₀ = 0.5 (approx. 5×10^7 c.f.u. or cells ml⁻¹) to OD₆₀₀ = 0.01 (approx. 1×10^5 c.f.u. or cells ml⁻¹)]. After incubation for 8 h, supernatants were gently collected. Cells were resuspended in 1 ml PBS. Aliquots of 10 µl of supernatants and cell suspensions were taken for SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were reacted with 1:3000-diluted anti-*H. pylori* HSP60 polyclonal antibody prepared as described previously (Yunoki *et al.*, 2000). After washing with PBS, the membranes were reacted with peroxidase-conjugated anti-rabbit immunoglobulins (Dako). The reactions were detected by ECL Western blotting detection reagents (Amersham Pharmacia). The luminol reaction was visualized by a LAS-1000mini Bio-Imaging Analyser System (Fuji).

mRNA expression in KATO III cells (RT-PCR and Northern blotting). Using Northern blotting analysis, we analysed IL-8 mRNA expression in KATO III cells treated with *H. pylori* and rHpHSP60. Total RNA was isolated from KATO III cells using RNAzol-B (Tel-Test Inc.), and IL-8 cDNA was prepared by RT-PCR using ReverTra Dash (Toyobo) with the following oligonucleotide primer set for IL-8 (5'-AAGGAACCATCTCACTG-3' and 5'-GAT-TCTTGATACACAGAG-3'; Crabtree *et al.*, 1994) and labelled with horseradish peroxidase by an ECL direct nucleic acid labelling and detection system (Amersham Biosciences). Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was also prepared, as a control, with a primer set in an RT-PCR kit (ReverTra Dash) according to the manufacturer's protocol. After treatment with rHpHSP60 (50 µg ml⁻¹), total RNA was isolated from KATO III cells and separated by electrophoresis (15 µg per lane) through a 1% agarose gel containing formaldehyde and transferred to a nylon membrane (Hybond-N+; Amersham Biosciences) in 20 × SSC (1 × SSC is 0.15 M sodium chloride/15 mM sodium citrate). The membrane was baked for 30 min at 80 °C and cross-linked by a UV cross-linker (Stratagene). After pre-hybridization, the membrane was

hybridized with labelled IL-8 or G3PDH cDNA probes for 12 h at 42 °C. The membrane was washed twice with $0.5 \times$ SSC containing 0.4% SDS for 10 min at 55 °C and then washed twice more with $2 \times$ SSC for 5 min at room temperature. RNA in the blots was detected by using the ECL direct nucleic acid labelling and detection system and analysed with the LAS-1000mini Bio-Imaging Analyser System.

TLR mRNA and protein expression in KATO III cells was examined by RT-PCR. Total RNA was isolated from KATO III cells by using RNAzol-B. After DNase I (Invitrogen) treatment, RT-PCR was performed using ReverTra Dash. PCR amplification was performed for 35 cycles at 95 °C for 10 s, 53 °C for 2 s and 74 °C for 30 s using the following oligonucleotide primer sets: for TLR2, 5'-CTTATCCAGCACACGA-ATACACAG-3' (sense 1455–1478) and 5'-TGGAGAGTCACACAG-GTAATTTGC-3' (antisense 1807–1830); and for TLR4, 5'-CTCCA-GGTTCTTGATTACAGTCTC-3' (sense 1800–1823) and 5'-TAGCT-CATTCTTACCCAGTCTC-3' (antisense 2218–2240). These primer sets were designed by using GENETYX-MAC (Genetyx Corporation) based on the DNA sequences of TLR2 and TLR4 from the GenBank Database. As a housekeeping gene for the control reaction, PCR was performed simultaneously with G3PDH primers (ReverTra Dash). Appropriate negative controls, including amplification of a non-specific RT-PCR reaction product, were included in each run. PCR products were resolved on 2% agarose gels and DNA fragments were visualized under UV light by staining with ethidium bromide.

Real-time PCR for mRNA of TLR2 and 4. Total RNA was isolated from KATO III cells using RNAzol-B (Tel-Test Inc.). After DNase I (Invitrogen) treatment, cDNA was prepared using ReverTra

Dash. Quantitative PCR analysis was performed on a LightCycler using the LightCycler Primer set of human TLR2, TLR4 and G6PDH. Real-time PCR was done in a 24 µl volume consisting of 10 µl cDNA sample, 10 µl H₂O, 2 µl LightCycler primer set and 2 µl LightCycler FastStart DNA master SybrGreen I. The reaction mixtures were loaded into capillary tubes and thermal cycling was carried out as follows: activation of hotstart *Taq* DNA polymerase, 95 °C for 10 min; cycling, 95 °C for 10 s, 68 °C for 10 s, 72 °C for 16 s for 40 cycles. The DNA concentration was calculated by LightCycler software and the ratio of TLR2 or TLR4/GAPDH was also calculated.

Immunocytochemistry of TLR and NF-κB in KATO III cells.

KATO III cells were seeded on chamber slides and fixed in 70% methanol. After incubation with TL2.1 (anti-human TLR2 antibody) or HTA125 (anti-human TLR4 antibody) as primary antibodies, the cells were incubated with tetraethylrhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulins (Sigma–Aldrich). As negative controls, irrelevant mouse mAb (anti-botulinum toxin type C) and PBS were used instead of the primary antibodies. The stained cells were observed under a confocal laser-scanning microscope (LSM510; Carl Zeiss).

For NF-κB activation analysis, KATO III cells were seeded in poly-L-lysine (Wako Pure Chemicals Industries)-treated chamber slides 3 days before the experiment. After pretreatment with TL2.1 (anti-human TLR2 antibody), HTA125 (anti-human TLR4 antibody) or medium control for 30 min, cells were stimulated with rHpHSP60 or intact *H. pylori* for 1 h and fixed in methanol (70%) containing

Fig. 1. IL-8 protein release from and mRNA expression in KATO III cells by *H. pylori* and rHpHSP60. KATO III cells were treated for 24 h with the indicated numbers of intact *H. pylori* (a) or concentrations of rHpHSP60 (b) and harvested. IL-8 protein levels in the culture supernatant were measured by immunoassay. Both *H. pylori* and rHpHSP60 induced IL-8 release in a dose-dependent manner. Data are expressed as means $\pm$ SD ($n=3$). *, $P<0.01$ vs control. After treatment of KATO III cells with intact *H. pylori* (c) or rHpHSP60 (50 µg ml⁻¹) (d) for the indicated time periods, Northern blotting analysis was performed with IL-8 and G3PDH cDNA probes. IL-8 mRNA increased and reached a maximum 3 h after rHpHSP60 stimulation and returned to its original level 6 h after the stimulation.

glycerol (30%) at -20°C for 30 min. After washing, cells were treated with 0.1% Triton X100 for 4 min, incubated with rabbit anti-p65 antibody (Santa Cruz Biotechnology) and then reacted with fluorescein isothiocyanate-conjugated F(ab')_2 fragments of goat anti-rabbit immunoglobulins (Sigma-Aldrich). The stained cells were observed under a confocal laser-scanning microscope.

Statistical analysis. Results were expressed as means $\pm$ SD. Data were compared using Scheffé's multiple comparison test and differences were considered significant at P values <0.05 .

RESULTS

rHpHSP60 increased the protein release and mRNA expression of IL-8 in KATO III cells

rHpHSP60, as well as intact *H. pylori*, significantly increased IL-8 release from KATO III gastric epithelial cells in a dose-dependent manner (Fig. 1a, b). Recently, release of TNF- α from murine macrophages that had been

reacted with recombinant human HSP60 was found due to LPS contamination of the human HSP60 preparations (Gao & Tsan, 2003). To exclude the possibility that the release of IL-8 induced by our rHpHSP60 similarly was caused by contaminating LPS, we reacted KATO III cells with LPS or rHpHSP60 in the presence or absence of PMB; PMB did not inhibit IL-8 release, whereas LPS-induced IL-8 secretion was inhibited by PMB (Fig. 2a). We also searched for endotoxin activities in our rHpHSP60 preparations (Fig. 2b). Even though the endotoxin activity of the original rHpHSP60 preparation was negligible, we further purified rHpHSP60 by passing it through a PMB-agarose column. No endotoxin activity was detected in fractions passed once (PMB-1) or twice (PMB-2) through the column (Fig. 2b), yet both these fractions induced as much IL-8 release from KATO III cells as the original rHpHSP60 preparation (Fig. 2c). These results indicate that LPS was not responsible for IL-8 secretion induced by rHpHSP60.

Fig. 2. Analysis of the possible involvement of contaminating LPS in IL-8 release induced by rHpHSP60. (a) Effect of PMB treatment. KATO III cells were pretreated with PMB at a dose of $20 \mu\text{g ml}^{-1}$ before being reacted with rHpHSP60 ($50 \mu\text{g ml}^{-1}$) or LPS ($1 \mu\text{g ml}^{-1}$). The treatment with PMB inhibited release of IL-8 induced by LPS, but not by rHpHSP60. Data are expressed as means $\pm$ SD ($n=3$). *, $P<0.05$ vs LPS without PMB pretreatment. (b) Endotoxin activity in rHpHSP60 and fractions passed through a PMB-agarose column. Endotoxin activity of the rHpHSP60 ($50 \mu\text{g ml}^{-1}$) preparation is negligible when compared with that of LPS ($1 \mu\text{g ml}^{-1}$), and no endotoxin activity is detected in fractions passed once (PMB-1, $50 \mu\text{g ml}^{-1}$) or twice (PMB-2, $50 \mu\text{g ml}^{-1}$) through a PMB-agarose column. Endotoxin activity was estimated by use of an LAL assay kit. (c) IL-8 release induced by rHpHSP60 compared with IL-8 release induced by the rHpHSP60 fractions, PMB-1 and PMB-2. KATO III cells were treated with PMB-1 or PMB-2 at the indicated concentrations and IL-8 release was measured by immunoassay. The PMB-1 or PMB-2 fractions induced the same level of IL-8 release as that induced by the original rHpHSP60 preparation. Data are expressed as means $\pm$ SD ($n=3$). *, $P<0.001$ vs control without stimulation; †, $P<0.01$ vs control without stimulation. (d) The concentration of rHpHSP60 in KATO III during *H. pylori* stimulation of the cells was measured by Western blotting.

The concentrations of rHpHSP60 in KATO III cells while *H. pylori* stimulates the cells were measured by Western blotting (Fig. 2d). The rHpHSP60 concentration of the co-culture with intact or heat-killed *H. pylori* (1×10^7 cells ml^{-1}) and KATO III cells was the same as that of culture supernatant following stimulation with rHpHSP60 ($50 \mu\text{g ml}^{-1}$). rHpHSP60 was detected in supernatants stimulated at 100 and $50 \mu\text{g rHpHSP60 ml}^{-1}$; however, it was not detected in KATO III cells taken from co-culture with rHpHSP60. In supernatant collected from intact *H. pylori* stimulation, rHpHSP60 was detected at 1×10^6 bacterial cells; however, it was not detected following stimulation with heat-killed *H. pylori*.

Expression of IL-8 mRNA in KATO III cells stimulated with *H. pylori* (Fig. 1c) or rHpHSP60 (Fig. 1d) was examined by Northern blotting analysis. IL-8 mRNA expression increased and reached a maximum level at 3 h after rHpHSP60 stimulation.

Involvement of TLRs in rHpHSP60-induced IL-8 release from KATO III cells

We next examined whether interaction of rHpHSP60 with TLRs was involved in IL-8 secretion induced by rHpHSP60. First, we confirmed the expression of TLR2 and TLR4 in KATO III cells. TLR2 and TLR4 mRNAs were constitutively expressed in the cells, irrespective of the presence of rHpHSP60 stimulation, as demonstrated by RT-PCR (Fig. 3a). Also, TLR2 and TLR4 proteins were identified immunocytochemically along the cell surface of KATO III cells (Figs 4b and 3c). Real-time PCR was performed to confirm the expression of TLR2 or TLR4 mRNA. The number of PCR product (copies) are shown in Fig. 3(d) and the TLR2 or TLR4/G6PDH ratio is shown in Fig. 3(e). rHSP60 did not enhance TLR mRNA expression.

When we pretreated KATO III cells with TL2.1 (anti-TLR2 mAb) or HTA125 (anti-TLR4 mAb) before reaction

Fig. 3. TLR2 and TLR4 expression in KATO III cells. (a) KATO III cells were incubated in the presence or absence of rHpHSP60 and TLR mRNA was detected by RT-PCR. PCR bands of TLR2 and TLR4 mRNAs are present irrespective of rHpHSP60 treatment. (b, c) Immunocytochemical staining of KATO III cells with TL2.1 anti-TLR2 antibody (b) or HTA125 anti-TLR4 antibody (c). TLR2 (b) and TLR4 (c) are stained on the surface of KATO III cells. (d) Increased numbers of PCR products of TLR2 (left) and TLR4 (right) were detected in real-time PCR. Filled diamonds, 0 h; open squares, 1 h; filled triangles, 3 h; crosses, 6 h; stars, 9 h. (e) The ratios of TLR2 or TLR4/G6PDH after 35 cycles were calculated.

Fig. 4. The effect of pretreatment of KATO III cells with anti-TLR antibodies on rHpHSP60-induced IL-8 release. KATO III cells were pretreated with 10 µg TL2.1 anti-TLR2 antibody ml⁻¹ or 10 µg HTA125 anti-TLR4 antibody ml⁻¹ before the addition of intact *H. pylori* (1×10^7 c.f.u. ml⁻¹) (a) or rHpHSP60 (50 µg ml⁻¹) (b); IL-8 release was measured by immunoassay. Treatment with anti-TLR2 or anti-TLR4 antibody significantly inhibited *H. pylori*-induced IL-8 release, by approximately 50 and 40%, respectively (a). In the experiments with rHpHSP60, treatment with anti-TLR2, but not anti-TLR4 antibody significantly decreased IL-8 release (75%) (b). Results are shown as means $\pm$ SD ($n=3$). *, $P<0.01$ vs intact *H. pylori* stimulation (a) or vs rHpHSP60 stimulation (b).

with intact *H. pylori*, IL-8 secretion from KATO III was significantly inhibited by approximately 50 or 40%, respectively (Fig. 4a). Also in rHpHSP60-induced IL-8 secretion, a significant decrease (75%) was observed after treatment with TL2.1, but not after treatment with HTA125 (Fig. 4b).

NF- κ B activation in KATO III cells reacted with rHpHSP60

Next, we investigated NF- κ B activation in KATO III cells reacted with rHpHSP60. An increase of intranuclear NF- κ B was observed 10 min after rHpHSP60 stimulation and reached a maximum level 1 h after treatment (Fig. 5a). This reaction was significantly inhibited by anti-TLR2 antibody (TL2.1) treatment (Fig. 5b). Immunocytochemically, p65 (RelA), a subunit of the activated NF- κ B dimer, was

Fig. 5. NF- κ B activation in KATO III cells treated with rHpHSP60. NF- κ B in nuclear extracts was measured after treatment of KATO III cells with rHpHSP60. The amounts of intranuclear NF- κ B increased and reached a maximum level 1 h after treatment (a). Treatment with anti-TLR2, but not anti-TLR4 antibody significantly inhibited NF- κ B activation (b). Data are shown as means $\pm$ SD ($n=3$). *, $P<0.05$ vs control without stimulation.

localized exclusively in the cytoplasm before reaction with intact *H. pylori* or rHpHSP60 (Fig. 6a). After reaction with intact *H. pylori* (Fig. 6b) or rHpHSP60 (Fig. 6c), p65 was translocated into the nucleus. The HSP60-induced translocation of p65 was inhibited by pretreatment with TL2.1 anti-TLR2 antibody (Fig. 6d).

When we pretreated KATO III cells with PDTC, an inhibitor of NF- κ B-mediated transcriptional activation, the release of IL-8 induced by reaction with intact *H. pylori* or rHpHSP60 stimulation was significantly inhibited (Fig. 7a, b).

DISCUSSION

In this study, we focused on the possibility that *H. pylori* HSP60 is a bacterial ligand in the host inflammatory response to *H. pylori* infection. We demonstrated in KATO III human gastric cells that rHpHSP60 induces the secretion

Fig. 6. Immunocytochemical analysis of NF- κ B nuclear translocation in KATO III cells treated with rHpHSP60. Cells were treated with medium alone (a), intact *H. pylori* (b), rHpHSP60 (c) or rHpHSP60 after pre-incubation with TL2.1 anti-TLR2 antibody (d). The cells were then reacted with anti-p65 (RelA) antibody. Before stimulation, p65 is present in the cytoplasm only (a). After stimulation with intact *H. pylori* (b) or rHpHSP60 (c), p65 is translocated into the nucleus. This rHpHSP60-induced translocation of p65 was inhibited by pretreatment with TL2.1 anti-TLR2 antibody (d).

of the inflammatory cytokine IL-8 and increases the expression of IL-8 mRNA. Also, we established that TLRs, especially TLR2, are host receptors for *H. pylori* HSP60 that are likely to play an important role in the secretion of IL-8, and that the interaction between rHpHSP60 and TLR2 induced IL-8 production via the signalling pathway involving NF- κ B activation.

HSPs are a family of proteins induced in prokaryotic and eukaryotic cells by environmental stress. These proteins function as chaperones to facilitate folding, unfolding and translocation of intracellular polypeptides (Ellis, 1990; Young, 1990). HSP60 has been shown to play a role in the adherence and attachment of *H. pylori* to gastric epithelium (Huesca *et al.*, 1996; Yamaguchi *et al.*, 1997) and

Fig. 7. The effect of PDTC on rHpHSP60-induced IL-8 release from KATO III cells. KATO III cells were pretreated with the indicated amounts of PDTC, an inhibitor of NF- κ B-mediated transcriptional activation, 1 h before reaction with intact *H. pylori* or rHpHSP60; IL-8 release was measured by immunoassay. PDTC treatment significantly inhibited IL-8 release induced by either intact *H. pylori* (a) or rHpHSP60 (b). Data are shown as means $\pm$ SD ($n=3$). *, $P<0.001$ vs *H. pylori* (a) and rHpHSP60 (b) stimulation without PDTC pretreatment. †, $P<0.01$ vs rHpHSP60 stimulation without PDTC pretreatment.

to induce IL-8 secretion from KATO III human gastric epithelial cells (Yamaguchi *et al.*, 1999). The results of the present study, which confirmed that rHpHSP60 can induce IL-8 release from KATO III cells and further found that IL-8 secretion was accompanied by an increase of IL-8 mRNA, indicate that *H. pylori* HSP60 is a bacterial virulence factor that can induce host inflammatory responses.

It has been reported that commercially available recombinant human HSP60 induced TNF- α release from murine macrophages in a manner similar to that of LPS, but Gao & Tsan (2003) reported that the TNF- α -inducing activity of this HSP60 was due to the presence of contaminating LPS and LPS-associated molecules. In the present study, three experiments excluded the possibility that LPS contamination of our rHpHSP60 preparation was responsible for the release of IL-8: (1) the addition of PMB did not inhibit rHpHSP60-induced IL-8 release; (2) endotoxin activities in the rHpHSP60 preparation were negligible;

and (3) fractions of rHpHSP60 further purified by passage through a PMB-agarose column induced as much IL-8 release from KATO III as did the original rHpHSP60 preparation.

Sulfated glycolipid is reportedly a host-cell receptor for *H. pylori* HSP60 (Huesca *et al.*, 1996), and HSP60s of human cells and chlamydia bind to TLR2 or TLR4 (Ohashi *et al.*, 2000; Sasu *et al.*, 2001; Vabulas *et al.*, 2001). In *H. pylori* infection, TLR4 involvement is reported in the activation of mitogen oxidase 1 by LPS of *H. pylori* in gastric pit cells (Kawahara *et al.*, 2001). Other investigators reported that human gastric epithelial cells recognize and respond to *H. pylori* at least in part via TLR2 and TLR5 (Smith *et al.*, 2003). Recently, *H. pylori* HSP60 was reported to mediate IL-6 production by macrophage via TLRs independent mechanism (Gobert *et al.*, 2004). However, the interaction of the TLRs with *H. pylori* HSP60 on IL-8 production by human gastric epithelial cells had not been studied. In the present study, we demonstrated that treatment of KATO III cells with anti-TLR antibodies, especially anti-TLR2, inhibited HSP60-induced IL-8 secretion. This result is persuasive evidence that TLRs are host-cell receptors for *H. pylori* HSP60 in the induction of initial inflammatory responses.

In vertebrates, TLRs play a key role in the recognition of infectious pathogens and initiate innate immunity as a first line of defence (Aderem & Ulevitch, 2000). For this purpose, it is reasonable that TLRs should be directed to specific motifs on pathogens. Because HSPs are highly conserved proteins with significant molecular mimicry among prokaryotic and eukaryotic cells, the recognition of *H. pylori* HSP by TLRs is a likely mechanism for the initiation of innate immunity and the concurrent host inflammatory response against this bacterium. In support of this possibility, we found that anti-TLR2 antibody significantly inhibited IL-8 secretion. However, the inhibition was incomplete, even when anti-TLR4 antibody was added. Thus, receptors other than TLR2 and TLR4 may also recognize *H. pylori* HSP60. At present it remains unclear whether the observed induction of IL-8 is specific to HSP60 from *H. pylori*. However, preliminary data suggest that production of this cytokine from monocytes involves the less conserved regions of the protein.

Activation of the TLR signalling pathway leads to NF- κ B translocation to the nucleus and transactivation (Aderem & Ulevitch, 2000; Akira & Hemmi, 2003). In the present study, we demonstrated that nuclear translocation of RelA (p65) was induced by the addition of rHpHSP60 and inhibited by pretreatment with the anti-TLR antibody. Involvement of NF- κ B activation was further confirmed by the observation that IL-8 secretion was inhibited after treatment with PDTC, an inhibitor of NF- κ B-mediated transcriptional activation. However, the possible involvement of other signalling pathways, such as the mitogen-activated protein kinase pathway, which is also triggered after TLR ligation (Aderem & Ulevitch, 2000; Akira & Hemmi, 2003), deserves consideration.

Adherence and attachment of *H. pylori* to gastric epithelial cells cause host responses, including the synthesis of various inflammatory mediators and cytokines that lead to the development of gastric injury. The *cag* pathogenicity island of *H. pylori* is reportedly important in the production of these cytokines through NF- κ B activation (Covacci *et al.*, 1999; Crabtree *et al.*, 1995b; Sharma *et al.*, 1998). Other reports have indicated that not only *cag* pathogenicity island, but also flagella, LPS, outer-membrane protein and HSP60 of *H. pylori* are associated with IL-8 induction from epithelial cells or monocytes (Bhattacharyya *et al.*, 2002; Cunningham *et al.*, 2000; Lee *et al.*, 2003; Yamaguchi *et al.*, 1999; Yamaoka *et al.*, 2002). These non-*cag* antigens are located on the bacterial surface and make direct contact with host cells. The *cag*-independent pathway of cytokine induction may also involve induction of mucosal inflammation. The results of the present study, which indicate that *H. pylori* HSP60 acts as a bacterial virulence factor for the induction of host inflammatory responses, acting through binding to TLRs, further characterize the mechanisms by which *H. pylori* can cause disease.

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REFERENCES

- Aderem, A. & Ulevitch, R. J. (2000). Toll-like receptors in the induction of the innate immune response. *Nature* **406**, 782–787.
- Akira, S. & Hemmi, H. (2003). Recognition of pathogen-associated molecular patterns by TLR family. *Immunol Lett* **85**, 85–95.
- Bhattacharyya, A., Pathak, S., Datta, S., Chattopadhyay, S., Basu, J. & Kundu, M. (2002). Mitogen-activated protein kinases and nuclear factor-kappaB regulate *Helicobacter pylori*-mediated interleukin-8 release from macrophages. *Biochem J* **368**, 121–129.
- Blaser, M. J. (1990). *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. *J Infect Dis* **161**, 626–633.
- Covacci, A., Telford, J. L., Del Giudice, G., Parsonnet, J. & Rappuoli, R. (1999). *Helicobacter pylori* virulence and genetic geography. *Science* **284**, 1328–1333.
- Crabtree, J. E., Wyatt, J. I., Trejdosiewicz, L. K., Peichl, P., Nichols, P. H., Ramsay, N., Primrose, J. N. & Lindley, I. J. (1994). Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa. *J Clin Pathol* **47**, 61–66.
- Crabtree, J. E., Covacci, A., Farmery, S. M., Xiang, Z., Tompkins, D. S., Perry, S., Lindley, I. J. & Rappuoli, R. (1995a). *Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. *J Clin Pathol* **48**, 41–45.
- Crabtree, J. E., Xiang, Z., Lindley, I. J., Tompkins, D. S., Rappuoli, R. & Covacci, A. (1995b). Induction of interleukin-8 secretion from gastric epithelial cells by a *cagA* negative isogenic mutant of *Helicobacter pylori*. *J Clin Pathol* **48**, 967–969.
- Cunningham, M. D., Shapiro, R. A., Seachord, C., Ratcliffe, K., Cassiano, L. & Darveau, R. P. (2000). CD14 employs hydrophilic regions to 'capture' lipopolysaccharides. *J Immunol* **164**, 3255–3263.
- Ellis, R. J. (1990). The molecular chaperone concept. *Semin Cell Biol* **1**, 1–9.
- Gao, B. & Tsan, M. F. (2003). Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor alpha from murine macrophages. *J Biol Chem* **278**, 22523–22529.
- Gobert, A. P., Bambou, J. C., Werts, C., Balloy, V., Chignard, M., Moran, A. P. & Ferrero, R. L. (2004). *Helicobacter pylori* heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. *J Biol Chem* **279**, 245–250.
- Hayashi, S., Sugiyama, T., Yokota, K., Isogai, H., Isogai, E., Oguma, K., Asaka, M., Fujii, N. & Hirai, Y. (1998). Analysis of immunoglobulin A antibodies to *Helicobacter pylori* in serum and gastric juice in relation to mucosal inflammation. *Clin Diagn Lab Immunol* **5**, 617–621.
- Huesca, M., Borgia, S., Hoffman, P. & Lingwood, C. A. (1996). Acidic pH changes receptor binding specificity of *Helicobacter pylori*: a binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. *Infect Immun* **64**, 2643–2648.
- Ishii, E., Yokota, K., Sugiyama, T. & 7 other authors (2001). Immunoglobulin G1 antibody response to *Helicobacter pylori* heat shock protein 60 is closely associated with low-grade gastric mucosa-associated lymphoid tissue lymphoma. *Clin Diagn Lab Immunol* **8**, 1056–1059.
- Kawahara, Y., Yokota, K., Mizuno, M. & 7 other authors (1999). Antibodies to human gastric epithelial cells and heat shock protein 60 in *Helicobacter pylori* positive mucosa associated lymphoid tissue lymphoma. *Gut* **45**, 20–23.
- Kawahara, T., Teshima, S., Oka, A., Sugiyama, T., Kishi, K. & Rokutan, K. (2001). Type I *Helicobacter pylori* lipopolysaccharide stimulates toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. *Infect Immun* **69**, 4382–4389.
- Kawai, M., Nishikomori, R., Jung, E. Y., Tai, G., Yamanaka, C., Mayumi, M. & Heike, T. (1995). Pyrrolidine dithiocarbamate inhibits intercellular adhesion molecule-1 biosynthesis induced by cytokines in human fibroblasts. *J Immunol* **154**, 2333–2341.
- Lee, S. K., Stack, A., Katzowitsch, E., Aizawa, S. I., Suerbaum, S. & Josephans, C. (2003). *Helicobacter pylori* flagellins have very low intrinsic activity to stimulate human gastric epithelial cells via TLR5. *Microbes Infect* **5**, 1345–1356.
- Mahmut, N., Inoue, K., Fujinaga, Y. & 7 other authors (2002). Characterisation of monoclonal antibodies against haemagglutinin associated with *Clostridium botulinum* type C neurotoxin. *J Med Microbiol* **51**, 286–294.
- Meyer-ter-Vehn, T., Covacci, A., Kist, M. & Pahl, H. L. (2000). *Helicobacter pylori* activates mitogen-activated protein kinase cascades and induces expression of the proto-oncogenes *c-fos* and *c-jun*. *J Biol Chem* **275**, 16064–16072.
- Ohashi, K., Burkart, V., Flohe, S. & Kolb, H. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol* **164**, 558–561.
- Parsonnet, J., Friedman, G. D., Vandersteen, D. P., Chang, Y., Vogelstein, J. H., Orentreich, N. & Sibley, R. K. (1991). *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* **325**, 1127–1131.
- Parsonnet, J., Hansen, S., Rodriguez, L., Gelb, A. B., Warnke, R. A., Jellum, E., Orentreich, N., Vogelstein, J. H. & Friedman, G. D. (1994). *Helicobacter pylori* infection and gastric lymphoma. *N Engl J Med* **330**, 1267–1271.
- Rad, R., Gerhard, M., Lang, R., Schoniger, M., Rosch, T., Schepp, W., Becker, I., Wagner, H. & Prinz, C. (2002). The *Helicobacter pylori*

blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. *J Immunol* **168**, 3033–3041.

Sasu, S., LaVerda, D., Qureshi, N., Golenbock, D. T. & Beasley, D. (2001). *Chlamydia pneumoniae* and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ Res* **89**, 244–250.

Sharma, S. A., Miller, G. G., Peek, R. J., Jr, Perez-Perez, G. & Blaser, M. J. (1997). T-cell, antibody, and cytokine responses to homologs of the 60-kilodalton heat shock protein in *Helicobacter pylori* infection. *Clin Diagn Lab Immunol* **4**, 440–446.

Sharma, S. A., Tummuru, M. K., Blaser, M. J. & Kerr, L. D. (1998). Activation of IL-8 gene expression by *Helicobacter pylori* is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. *J Immunol* **160**, 2401–2407.

Smith, M. F., Jr, Mitchell, A., Li, G., Ding, S., Fitzmaurice, A. M., Ryan, K., Crowe, S. & Goldberg, J. B. (2003). Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem* **278**, 32552–32560.

Stokes, D. C., Shenep, J. L., Fishman, M., Hildner, W. K., Bysani, G. K. & Rufus, K. (1989). Polymyxin B prevents lipopolysaccharide-induced release of tumor necrosis factor-alpha from alveolar macrophages. *J Infect Dis* **160**, 52–57.

Vabulas, R. M., Ahmad-Nejad, P., da Costa, C., Miethke, T., Kirschning, C. J., Hacker, H. & Wagner, H. (2001). Endocytosed

HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J Biol Chem* **276**, 31332–31339.

Yamaguchi, H., Osaki, T., Kurihara, N., Taguchi, H., Hanawa, T., Yamamoto, T. & Kamiya, S. (1997). Heat-shock protein 60 homologue of *Helicobacter pylori* is associated with adhesion of *H. pylori* to human gastric epithelial cells. *J Med Microbiol* **46**, 825–831.

Yamaguchi, H., Osaki, T., Kurihara, N., Kitajima M., Kai, M., Takahashi, M., Taguchi, H. & Kamiya, S. (1999). Induction of secretion of interleukin-8 from human gastric epithelial cells by heat-shock protein 60 homologue of *Helicobacter pylori*. *J Med Microbiol* **48**, 927–933.

Yamaoka, Y., Kikuchi, S., el-Zimaity, H. M., Gutierrez, O., Osato, M. S. & Graham, D. Y. (2002). Importance of *Helicobacter pylori* *oipA* in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. *Gastroenterology* **123**, 414–424.

Young, D. B. (1990). Chaperonins and the immune response. *Semin Cell Biol* **1**, 27–35.

Yunoki, N., Yokota, K., Mizuno, M. & 7 other authors (2000). Antibody to heat shock protein can be used for early serological monitoring of *Helicobacter pylori* eradication treatment. *Clin Diagn Lab Immunol* **7**, 574–577.

Ziegler-Heitbrock, H. W., Sternsdorf, T., Liese, J., Belohradsky, B., Weber, C., Wedel, A., Schreck, R., Bauerle, P. & Strobel, M. (1993). Pyrrolidine dithiocarbamate inhibits NF-kappa B mobilization and TNF production in human monocytes. *J Immunol* **151**, 6986–6993.