BACTERIAL PATHOGENICITY

Flow cytometric analysis of the heat shock protein 60 expressed on the cell surface of *Helicobacter pylori*

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The expression of a 60-kDa heat shock protein (HSP60) on the cell surface of *Helicobacter pylori* was analysed by flow cytometry with polyclonal antibody directed to HSP60. All 13 strains of *H. pylori* examined expressed HSP60 on the cell surface, although the intensity of expression was different among the strains and depended on culture conditions. There was a correlation between the intensity of HSP60 expressed on the cell surface and the rate of adherence to human gastric carcinoma cells (MKN45) by *H. pylori*, but not with urease activity and production of vacuolating toxin. By flow cytometric analysis with monoclonal antibody (MAb) 3C8 against HSP60, the reactive epitope in the HSP60 of *H. pylori* was detected on the surface of MKN45 cells. Furthermore, it was shown that gastric epithelial cells were positively stained with MAb 3C8 in one of two biopsy specimens examined. These results suggest that there is a common epitope showing homology between *H. pylori* HSP60 and human gastric epithelial cells.

Introduction

Helicobacter pylori is a gram-negative, spiral-shaped micro-aerophilic organism that colonises the human gastric mucosa [1, 2] and infection with *H. pylori* may be a permissive factor in peptic ulcer disease [3–5]. Various potential virulence factors have been identified in *H. pylori*, including flagella [6, 7], adhesin [8], urease [9, 10], vacuolating toxin [11, 12] and the toxin that inhibits secretion of gastric acid *in vitro* [5, 13]. However, the pathogenic mechanism by which *H. pylori* persists in the stomach is not fully understood.

Heat shock proteins (HSPs), induced by various environmental stresses such as temperature change, inflammation, viral infection, malignant transformation, irradiation, heavy metals, ethanol and anoxia, are highly conserved proteins found not only in prokaryotic but also in eukaryotic cells [14–16]. The HSP60 family of chaperonins such as GroEL of *Escherichia coli* and the 65-kDa immunodominant antigen of *Mycobacterium* spp. is thought to facilitate folding, unfolding and translocation of polypeptides, as well as the assembly and disassembly of oligomeric protein complexes [17–20]. *H. pylori* HSP60 is thought to be associated with urease, one of the putative virulence

factors of *H. pylori* [21, 22]. An understanding of the relationship between *H. pylori* HSP60 and pathogenicity of *H. pylori* in the induction of gastritis might be important for resolution of the mechanism by which *H. pylori* persists in the stomach [23, 24]. Recently, it has been demonstrated that human infection with *H. pylori* could stimulate an autoimmune response, possibly directed against HSPs expressed by stressed gastric epithelial cells [25, 26].

In the present study, the expression of HSP60 on the cell surface of various *H. pylori* strains was examined by flow cytometry and the relationships between the expression of the HSP60, adherence to human gastric carcinoma cells, urease activity and production of vacuolating toxin were also investigated. A cross-reaction between HSP60 of *H. pylori* and human gastric carcinoma cells was also analysed and human gastric biopsy specimens were used for immunostaining with monoclonal antibody (MAb) to HSP60.

Material and methods

Bacterial strains and growth conditions

H. pylori strains used in this study were: TK1025, TK1028, TK1029, TK1030, TK1042, TK1046, TK1054, TK1302, TK1308, TK1309, TK1311 and TK1313, which were isolated from gastric biopsy samples from patients as described previously [27];

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H. pylori NCTC 11638 was kindly provided by Dr T. Ito (Tokyo Metropolitan Research Laboratory of Public Health). For the analysis of the rate of HSP60 expression on the cell surface of *H. pylori*, bacteria were cultured either in Brain Heart Infusion (BHI) Broth (Difco) containing fetal calf serum (FCS; Wako Pure Chemical Ltd, Osaka, Japan) 5% or on BHI agar (Difco) with defibrinated horse blood 5% (BHI-blood plate) in an atmosphere consisting of O₂ 5%, CO₂ 10%, N₂ 85% for 4 days at 37°C. These cultures were centrifuged at 3000 *g* for 15 min and the cells were resuspended in Hanks's Balanced Salts Solution (HBSS; Gibco Laboratory, New York, USA) containing gelatin (Sigma) 0.1% (HGS) for flow cytometric analysis. The other part of the suspension was used for SDS-PAGE. For the analysis of cross-reaction between *H. pylori* HSP60 and human gastric carcinoma cells, bacteria cultured on BHI-blood agar were used. Table 1 shows the characteristics of the *H. pylori* isolates used in the present study.

Urease activity

Urease activity was measured by the method described previously by Kamiya *et al.* [28]. Urease activity was quantified by the ammonia-test WAKO (Wako Pure Chemical Ltd) according to the amount of ammonia produced. One hundred and seventy-five µl of sonicated bacteria (100 µg/ml) were added to 25 µl of 200 mM phosphate buffer (pH 7.4), 25 µl of 1 mM ethylenediamine tetraacetic acid, disodium salt (Dojin Ltd, Kumamoto, Japan), and 25 µl of substrate containing 4 M urea in the phosphate buffer with bovine serum albumin (Sigma) 2.5% w/v. The mixture was incubated at 25°C for 20 min. The reaction was stopped by the addition of 1.0 ml of sodium tungstate 5% w/v to the test sample and the mixture was then centrifuged

at 3000 rpm for 15 min. The amount of ammonia in the supernatant fluid was quantified by the commercially available ammonia assay.

Production of vacuolating toxin

Production of vacuolating toxin by the strains was measured by the method described previously by Kamiya *et al.* [12]. A rabbit kidney cell line (RK13) was used for the cytotoxicity assay to detect the vacuolating toxin. The serially diluted culture filtrate of *H. pylori* grown in BHI broth containing FCS 5% in a micro-aerophilic atmosphere for 4 days at 37°C was added into the culture medium for RK13 cell for 24 h at 37°C. The appearance of cytoplasmic vacuolation in RK13 cells was observed microscopically. The titre of vacuolating toxin was determined as the highest dilution of the culture filtrate that showed vacuolation of >10% of cells.

Cell culture

Human gastric carcinoma cells (MKN28, MKN45 and KATO III) were obtained from the Japanese Cancer Research Resources Bank (JCRB). They were grown at 37°C in RPMI 1640 containing FCS 10% in an atmosphere containing CO₂ 5%.

Preparation of antiserum against bacterial HSP60

Bacterial HSP60 was purified from *Yersinia enterocolitica* O3 (strain ZM20) by immuno-affinity chromatography with a specific MAbs, 1A4, against *Y. enterocolitica* HSP60 as reported previously [29]. Antibacterial HSP60 serum (anti-HSP60) was obtained from rabbits immunised with the purified HSP60 from *Y. enterocolitica*.

Table 1. Characteristics of *H. pylori* strains

Strain	Gastroendoscopic diagnosis	Urease activity (µg/dl)*		Vacuolating toxin [§] titre
		broth [†]	agar [‡]	
TK1025	Gi	86	463	ND
TK1028	DU	66	2187	ND
TK1029	GU, IM, At, SMT	177	347	8
TK1030	GP, At	77	384	4
TK1042	GU, DU, IM	157	697	ND
TK1046	GP	391	299	ND
TK1054	At	188	526	2
TK1302	DU	83	449	4
TK1308	GCa	188	583	16
TK1309	GU	97	659	1
TK1311	DU	68	411	ND
TK1313	GCa	120	286	2
NCTC11638	Gi	462	653	ND

Gi, gastritis; DU, duodenal ulcer; GU, gastric ulcer; IM, intestinal metaplasia; At, atrophy; SMT, submucosal tumor; GP, gastric polyp; GCa, gastric cancer; ND, not detected.

*Urease activity was quantified according to the amount of NH₃ produced as described in the text.

[†]Urease activity of the strains cultured in BHI broth containing FCS.

[‡]Urease activity of the strains cultured on BHI-blood agar.

[§]Vacuolating activity was measured by the cytotoxicity assay with RK13 cells as described in the text.

Monoclonal antibody

MAb 3C8 to *Y. enterocolitica* HSP60, showing reactivity with HSP60 from a wide range of bacteria and human cell lysate, was prepared as described previously [29].

Flow cytometric analysis

Flow cytometry was performed by the method described by Osaki *et al.* [30]. *H. pylori* strains (*c.* 5×10^7 cfu) were mixed with 200 μ l of either polyclonal anti-HSP60 antibody diluted 1 in 50 or MAb 3C8 (100 μ g/ml) in HGS, and then incubated for 30 min at 4°C. Similarly, the cultured cells (5×10^4) of MKN45, MKN28 or KATO III were mixed with 200 μ l of the solution of polyclonal antibody to HSP60 or MAb 3C8 (100 μ g/ml) in HGS followed by incubation for 30 min at 4°C. The cells were washed twice with HGS and fluorescent isothiocyanate (FITC)-conjugated goat anti-rabbit or mouse immunoglobulin G (Capel Research Products, Durham, USA) diluted to 1 in 20 in HGS was added. The cells were washed twice with HGS and resuspended in 300 μ l of PBS for flow cytometry. An EPICS-CS flow cytometer (Coulter Electronics, Hialeah, USA) was used for the measurement of fluorescence intensity. Fluorescence data were obtained in a logarithmic mode on a 256-channel scale. The mean fluorescence channel was calculated according to the fluorescence intensity of >3000 individual cells. The results were shown as the positive percentage of fluorescent cells calculated from fluorescence frequency distribution histograms.

Adherence assay

The adherence assay with human gastric carcinoma cells (MKN45) for *H. pylori* strains was performed by the method described by Osaki *et al.* [30]. MKN45 cells and *H. pylori* were incubated at 37°C for 1 h in a 1.5 ml tube with gentle shaking. Non-adherent bacteria were removed by centrifugation with 9 ml of sucrose 15% solution. The cells were washed once with HGS and then incubated for 30 min on ice with anti-*H. pylori* serum diluted 1 in 100. The cells were washed twice with HGS and stained with FITC-conjugated goat anti-rabbit IgG diluted in 1 in 20. The cells were washed twice with HGS and resuspended in 300 μ l of HGS for the flow cytometric adherence assay. An EPICS-CS flow cytometer was used to measure the number of bacteria adhering to MKN45 cells. The results were expressed as the mean channel of fluorescent cells showing the highest intensity into fluorescence frequency distribution histograms.

SDS-PAGE and immunoblotting

SDS-PAGE with acrylamide 10% w/v gel was performed as described by Laemmli [31]. Bacterial cells and human gastric carcinoma cells were harvested

and suspended in 100 μ l of the lysis buffer (0.4 mM 2-mercaptoethanol, Nonidet P-40 3.2% v/v), lysed by seven freeze-thaw cycles (frozen at -80°C and thawed at 37°C in a water-bath, each for 5 min). Cell lysates (100 μ l) were heated for 5 min at 100°C in sample buffer (0.00625 M Tris-HCl, pH 6.8, containing SDS 2% w/v, glycerol 5% v/v and 2-mercaptoethanol 5% v/v). Finally, 10 μ l of the cell lysates were loaded into each lane. Immunoblot analysis was performed as described by Towbin *et al.* [32]. After electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Schleier and Schuell, Dassel, Germany) at 0.25A overnight. After blocking with gelatin 3% w/v in Tris-buffered saline (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4), the membranes were treated for 1 h with either polyclonal antibody diluted 1 in 1000 or MAb 3C8 diluted to 100 μ g/ml with the blocking buffer. They were then incubated for 1 h with goat anti-mouse or anti-rabbit IgG peroxidase conjugate diluted 1 in 500 with Tris-buffered saline containing BSA 1% w/v. Immunoblots were developed with Tris-saline containing H₂O₂ 0.12% v/v and 1 mM *o*-dianisidine.

Immunohistochemical staining of gastric biopsy specimens with MAb 3C8 to bacterial HSP60

Frozen sections (4 μ m thick) obtained from two biopsy specimens were placed on slides. They were fixed in acetone for 5 min at 4°C, followed by washing with distilled water. Endogenous peroxidase was blocked by incubation with H₂O₂ 0.1% v/v for 10 min at room temperature. After washing with phosphate-buffered saline (PBS), pH 7.2, containing Tween 20 0.2% v/v (Wako Pure Chemical Ltd) (washing buffer), the sections were allowed to react with normal horse serum diluted 1 in 10 with PBS containing skimmed milk (Yukijirushi Nyugyo Co., Tokyo, Japan) 0.1% w/v (PBS-S) for 30 min to reduce non-specific staining. The sections were treated overnight at 4°C with either MAb 3C8 1 μ g/ml in PBS-S or the control MAb recognising glucose oxidase derived from *Aspergillus niger* (Dako Japan Co. Ltd, Kyoto, Japan). After the sections were treated with the washing buffer, they were incubated with biotinylated anti-mouse IgG secondary antibody (Dako Japan Co. Ltd) diluted 1 in 100 with PBS-S. Development by the peroxidase method with avidin-biotinylated enzyme complex and 3, 3'-diaminobenzidine, tetrahydrochloride was performed with the Vectastain ABC kit (Funakoshi Co. Ltd, Tokyo, Japan).

Results

Immunoblot analysis of *H. pylori* strains with anti-HSP60 polyclonal antibody

The reactivity of *H. pylori* strains with anti-HSP polyclonal antibody was examined by immunoblot analysis (Fig. 1). HSP60 was detected in all the cell lysates of strains TK1025, TK1028, TK1029, TK1030,

Fig. 1. Immunoblotting patterns of *H. pylori* strains with anti-HSP60 polyclonal antibody directed to *Y. enterocolitica* HSP60. Lanes 1–10 were loaded with cell lysates from the following *H. pylori* strains: 1, TK1025; 2, TK1028; 3, TK1029; 4, TK1030; 5, TK1042; 6, TK1046; 7, TK1054; 8, TK1302; 9, TK1308; 10, TK1309. Bar shows the 60-kDa protein, reacting with anti-HSP60.

TK1042, TK1046, TK1054, TK1302, TK1308 and TK1309 (lanes 1–10). HSP60 was also detected in the cell lysates of other strains (TK1311, TK1313 and NCTC11638; data not shown).

Expression of HSP60 on the cell surface of H. pylori

Expression of HSP60 on the surface of *H. pylori* was examined by flow cytometry. Fig. 2 shows the representative patterns of *H. pylori* strains with anti-HSP60, indicating that positive cell percentages of strains TK1029, TK1308 and TK1025 strains were 2.2, 32.8 and 80.6%, respectively. The expression of HSP60 on the cell surface of *H. pylori* was different among the strains used.

Fig. 3a shows the intensity of the expression of HSP60 on the cell surface of *H. pylori* strains cultured either on BHI-blood agar or in BHI broth containing FCS 5%. The expression of HSP60 on the cell surface of *H. pylori* was significantly different, depending on the culture conditions. In general, HSP60 was much more frequently expressed on the cell surface of *H. pylori* strains cultured on BHI-blood agar than in BHI broth containing FCS 5%, except for strain TK1313.

Correlation between urease activity, production of vacuolating toxin and expression of HSP60

Table 1 shows urease and vacuolating activities of strains used in the study. Urease activity of *H. pylori* cultured in BHI broth and on BHI-blood agar was measured; all strains gave positive results. Strain TK1028 cultured on BHI-blood plate had the highest

Fig. 2. Representative patterns showing the reactivities of *H. pylori* strains: a, TK1029; b, TK1308; c, TK1025 cultured on BHI-blood agar with anti-HSP60 by flow cytometry. ■, pattern of positive cells reacting with anti-HSP60; ▨, negative control *H. pylori* strain incubated with non-immune rabbit serum. The positive cell percentage of TK1029 (2.2%), TK1308 (32.8%) and TK1025 (80.6%) was calculated from the rate of the fluorescence frequency distribution between negatively and positively stained cells.

urease activity among the strains. However, the amount of HSP60 expressed on *H. pylori* strain TK1028 was not high. Although the urease activity of *H. pylori* grown on agar was higher than that grown in broth culture, there was no correlation between the expression of HSP60 on the cell surface and the urease activity of *H. pylori* strains. Vacuolating activity was detected in seven strains (TK1029, TK1054, TK1313, TK1308, TK1030, TK1302 and TK1025) of 13 strains examined. The toxin titre of strain TK1308 (16) was highest. Vacuolating activity was not related to the amount of HSP60 expressed on *H. pylori*.

Correlation between adherence rate to MKN45 cells and expression of HSP60

Fig. 3b shows the rate of adherence to human gastric carcinoma MKN45 cells by *H. pylori* strains cultured on BHI-blood agar. All strains adhered to MKN45 cells, although the intensity of adherence of *H. pylori*

Fig. 3. Expression of HSP60 on the cell surface of *H. pylori* strains and their adherence to human gastric carcinoma cells: **a**, patterns showing the reactivities of *H. pylori* strains, cultured either on BHI-blood agar or in BHI broth containing FCS 5%, with anti-HSP60 in flow cytometry; □, intensity of the expression of HSP60 on the cell surface of *H. pylori* strain cultured on BHI-blood agar; ▨, intensity of expression in BHI broth containing FCS 5%, **b**, □, adherence of *H. pylori* strains to human gastric carcinoma (MKN45) cells estimated by flow cytometry.

to MKN45 cells was different among the strains studied. Strain TK1029 had the lowest adherence rate, with a mean channel of 132; on the other hand, strain TK1025 had the highest adherence rate with a mean channel of 175. There was a significant correlation between the adherence rate and the expression of HSP60 (correlation index (r) = 0.68).

Cross-reaction between H. pylori HSP60 and human gastric carcinoma cells

The possibility of a cross-reaction between *H. pylori* HSP60 and gastric carcinoma cells was investigated by flow cytometry with MAb 3C8 that recognised both bacterial HSP60 and surface antigen of a human B cell

line [29]. Immunoblotting had shown that MAb 3C8 reacted with *H. pylori* HSP60 (data not shown). The percentages of human gastric cancer cells of MKN45, KATOIII and MKN28 reacting with MAb 3C8 in the flow cytometric analysis were 62.4, 18.1 and 4.9%, respectively, indicating that MAb 3C8 reacted strongly with MKN45 cells (Table 2). Osaki *et al.* [30] reported previously that the MKN45 cell line is more sensitive in terms of adherence by *H. pylori* than KATOIII or MKN28 cells.

Immunohistochemical staining of gastric biopsy specimens with MAb 3C8

To observe the profile of human gastric epithelial cells that show cross-reactivity with *H. pylori* HSP60, immunohistochemical staining of gastric biopsy specimens with MAb 3C8 directed to bacterial HSP60 was performed (Fig. 4). Two gastric biopsy samples were obtained from patients with gastric ulcer. Both rapid urease test and isolation of *H. pylori* were positive for these two biopsies. Gastric epithelial cells of one gastric biopsy were positively stained with MAb 3C8 (Fig. 4a), but not with the negative control MAb directed against glucose oxidase derived from *Aspergillus niger* (Fig. 4b). In contrast, the other biopsy specimen failed to stain positively with MAb 3C8 or the MAb to glucose oxidase (data not shown). These results suggested that some gastric epithelial cells express HSP60 homologue on the surface following *H. pylori* infection.

Discussion

The present study reports the expression of HSP60 on the cell surface of *H. pylori* analysed by flow cytometry. Many investigators previously reported the

Table 2. Reactivity of *H. pylori* and human gastric carcinoma cells with MAb 3C8 against bacterial HSP60 by flow cytometry*

Target antigens	Positive cells (%) [†]
Human gastric carcinoma cells	
MKN 45	62.4
KATO III	18.1
MKN 28	4.9
<i>H. pylori</i> strains [‡]	
TK1025	16.5
NCTC11638	14.4
TK1302	13.5
TK1311	10.3
TK1309	7.7
TK1029	7.4
TK1046	3.9
TK1313	2.6
TK1308	2.4
TK1028	2.3
TK1054	1.7
TK1042	1.7
TK1030	0.8

*Flow cytometry was performed as described in the text. MAb 3C8 used for the analysis of epitope homology between *H. pylori* HSP60 and human gastric carcinoma cells recognised bacterial HSP60 and human HSP60.

[†]The positive cell percentage, calculated from the fluorescence frequency distribution between negative and positive cells, shows the intensity of the reactivities with MAb 3C8.

[‡]The strains were cultured on BHI-blood agar.

detection of bacterial surface antigens by various methods [33–35] but not flow cytometry. However, these methods were very complex and the estimation of the results was relatively difficult. As shown in the present study, flow cytometry seems to be useful for the detection of a bacterial surface antigen such as HSP60.

By means of flow cytometry with the polyclonal antibody directed to *Y. enterocolitica* HSP60, the HSP60 expressed on the cell surface of *H. pylori* was detected in all strains studied. On the other hand, the

Fig. 4. Immunohistochemical analysis of a gastric biopsy specimen with: A, MAb 3C8 to bacterial HSP60; B, control MAb to glucose oxidase of *A. niger*. Magnification, $\times 200$.

values of the positive percentage showing the intensity of expression of *H. pylori* HSP60 on the cell surface of various strains differed, between strains and with different culture conditions. However, in the patterns of immunoblotting with the polyclonal antibody, the HSP60 was equally expressed in all bacterial strains. These results suggest the possibility that the mechanism for expression of HSP60 on the cell surface of *H. pylori* might be different among different strains. The expression of HSP60 on the cell surface in all strains except for TK1313 was higher when they were cultured on BHI-blood agar than when they were cultured in BHI broth. Similarly, urease activity of *H. pylori* strains cultured on BHI blood agar was higher than that of those grown in BHI broth. These results suggest that both expression of HSP60 on the cell surface and production of urease might be influenced by cultural conditions. Haemagglutinating activity expressed by *H. pylori* depends on culture conditions; haemagglutinin was produced when organisms were grown on solid medium but not liquid medium [8]. The difference in the expression of the HSP60 on the cell surface of *H. pylori* strains does not seem to be due to the leakage of HSP60 derived from bacterial cells, as the expression rates of *H. pylori* HSP60 were not significantly different between 2-day and 4-day cultures of *H. pylori* strains – not only on BHI-blood agar but also in BHI broth (data not shown).

It seems that HSP60 of *H. pylori* is a chaperone associating with *H. pylori* urease [21, 22]. It has been reported recently that HSP60 (GroEL) of *H. pylori* stimulates uptake of Ni^{2+} into the apoenzyme of urease in co-operation with HSP10 (GroES), resulting in an increase in urease activity [24]. There is a possibility that the amount of urease expressed on a bacterial cell might be associated with the amount of HSP60 expressed on the cell surface of *H. pylori*. However, the results of urease assays indicate that there might be no correlation between the HSP60 expressed on the bacterial cell surface and the amounts of urease produced by *H. pylori*. Furthermore, there was no correlation between the expression of HSP60 and production of vacuolating toxin. However, a significant correlation between the expression of HSP60 and adherence rate of *H. pylori* to MKN45 cells was demonstrated. Further studies are required to clarify whether the bacterial HSP60 acts as an adhesin, or whether the HSP60 is associated with some adhesin molecules.

The present study demonstrated that there was a homologous epitope between *H. pylori* HSP60 and human gastric carcinoma MKN45 cells by flow cytometry with 3C8 MAb. The MAb 3C8 reacted not only with bacterial HSP60, including *H. pylori* HSP60, but also with several molecules in human cells [29]. MAb 3C8 was used for flow cytometry in the present study as expression of HSP60 following *H. pylori* infection may stimulate an autoimmune re-

sponse in gastric epithelial tissue. The expression of HSP60 on the surface of various carcinoma cells was also examined with polyclonal antibody to HSP60. The degree of positivity in MKN45, KATOIII and MKN28 cells by flow cytometry as 11.9, 2.2 and 14.9%, respectively (data not shown). These results indicate that an epitope homologous to HSP60 molecule is expressed on the surface of gastric carcinoma cells. In addition to cultured gastric carcinoma cells, MAb 3C8 reacted with the epithelial cells in one of two gastric biopsy specimens obtained from two patients with gastric ulcer in an immunohistochemical assay (Fig. 4). Recently, it has been reported that *H. pylori* infection stimulates an autoimmune response in man, possibly directed against HSPs expressed by stressed gastric epithelial cells [25, 26]. Therefore, we suppose that the homologous epitope detected between *H. pylori* HSP60 and human gastric cells is an important factor in the induction of gastroduodenal diseases by *H. pylori*, and its pathological significance remains to be clarified.

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