BACTERIAL PATHOGENICITY

Heat-shock protein 60 homologue of *Helicobacter pylori* is associated with adhesion of *H. pylori* to human gastric epithelial cells

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A previous study reported a relationship between the expression of heat-shock protein 60 (HSP60) by *Helicobacter pylori* and its adhesion to human gastric carcinoma (MKN45) cells. To examine whether the HSP60 homologue of *H. pylori* is associated with the adhesion of *H. pylori* to human gastric epithelial cells, an inhibition assay of adhesion of *H. pylori* to MKN45 cells was performed by flow cytometric analysis with monoclonal antibody (MAb) designated as H20 recognising HSP60 of *H. pylori*. The rate of adhesion of *H. pylori* pretreated with MAbH20 to MKN45 cells was lower than that of untreated *H. pylori*. Primary human gastric epithelial cells from a patient with gastric cancer were also prepared for comparison in the inhibition assay with MAbH20. *H. pylori* adhered to the primary human gastric epithelial cells, and this adhesion was significantly inhibited by MAbH20. These results suggest that the *H. pylori* HSP60 homologue recognised by MAbH20 might be associated with the adhesion of *H. pylori* to primary human gastric epithelial cells as well as to cultured gastric cancer cells.

Introduction

Helicobacter pylori is associated with chronic gastritis, gastroduodenal ulcer and gastric adenocarcinoma [1–3]. Although virulence factors such as flagella [4, 5], adhesin [6], urease [7, 8], vacuolating toxin [9, 10] and the toxin that inhibits secretion of gastric acid *in vitro* [11, 12] have been identified, the relationship between these *H. pylori* antigens and the mechanism by which *H. pylori* persists in the stomach is not clear.

A study of bacteria-epithelial cell adhesion, the first step in *H. pylori* infection, is important for any understanding of the mechanism of induction of tissue inflammation in the stomach by *H. pylori* to emerge. Several ligand–receptor interactions between *H. pylori* and gastric epithelial cells have been reported: N-acetylneuraminylactose-binding fibrillar haemagglutinin [13], haemagglutination activities with strain specificity [13, 14], binding to GM3 ganglioside and sulphatides [15, 16], and a laminin-binding protein [14, 17] have been demonstrated. These indicate that

the adhesion of *H. pylori* to human gastric epithelial cells might be multifactorial.

A recent study demonstrated that there is a correlation between the adhesion of *H. pylori* to human gastric carcinoma cells and the expression of heat-shock protein 60 (HSP60) on the cell surface [18]. Huesca *et al.* [19] reported that HSP60 of *H. pylori* localised on the bacterial surface, which mediates sulphatide recognition, might be related to the adhesion of *H. pylori* to gastric epithelial cells. HSP60, conserved not only in prokaryotic cells but also in eukaryotic cells, is thought to facilitate folding, unfolding and translocation of polypeptides as chaperonins [20–25]. Accordingly, the interaction between HSP60 of *H. pylori* and the adherence characteristics of *H. pylori* is important for an understanding of the mechanism of persistent infection with *H. pylori*.

In the present study, in order to clarify whether *H. pylori* HSP60 is associated with adhesion to human gastric epithelial cells, an inhibition assay of adhesion to human gastric carcinoma cells (MKN45) was performed. These experiments were repeated with primary human gastric epithelial cells from a patient with gastric cancer.

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Materials and methods

Bacterial strains and culture conditions

H. pylori strain TK1029 used in this study was isolated from gastric biopsy material of a patient as described previously [26]. The bacteria were cultured on Brain Heart Infusion (BHI) Agar (Difco) with defibrinated horse blood 5% in an atmosphere of O₂ 5%, CO₂ 10%, N₂ 85% for 4 days at 37°C. Cells of *H. pylori* TK1029 were centrifuged at 3000 *g* for 15 min and resuspended in Hanks's Balanced Salts Solution (Gibco) containing gelatin 0.1% (Sigma) (HGS) for flow cytometric analysis.

Cell line

Human gastric carcinoma (MKN45) cells were obtained from the Japanese Cancer Research Resources Bank (JCRB). They were grown at 37°C in RPMI 1640 (Gibco) containing fetal calf serum (FCS; Wako Pure Chemical Ltd, Osaka, Japan) 10% in an atmosphere of air with CO₂ 5%. The cells were resuspended in HGS for flow cytometric analysis.

Preparation of primary human gastric epithelial cells

For the preparation of primary human gastric epithelial cells, a specimen of gastric mucosa (*c.* 5 cm square, 50 g) was obtained from a patient undergoing surgery for gastric carcinoma. The specimen was negative in tests for both rapid urease and isolation of *H. pylori*. The specimen was stored at 4°C in RPMI 1640 medium containing FCS 10% (RF-10). The specimen was then shaken for 60 min at 37°C with 50 ml of pronase solution (10 mg/ml; Pronase MS, Kaken Seiyaku Co. Ltd, Tokyo, Japan) which is a gastric mucinolytic agent. The cell suspension was centrifuged at 1200 rpm for 10 min, and the cell pellet was suspended in RF-10 with Percoll (Sigma) 10% followed by centrifugation at 1800 rpm for 18 min. Subsequently, the cell pellet was suspended again in RF-10 with Percoll 44% and the prepared suspension was put on to RF-10 with Percoll 70%. After centrifugation at 1800 rpm for 18 min, the whole cells on the Percoll 70% were recovered and washed twice with RF-10. The cells formed the preparation of primary human gastric epithelial cells.

Preparation of monoclonal antibodies

The monoclonal antibody (MAb) was prepared by the methods described previously [27]. BALB/c mice were immunised intraperitoneally (i.p.) with partially purified 60-kDa antigen derived from *H. pylori* TK1029, which had been extracted from the separating gel of SDS-PAGE. These inoculations in Freund's complete adjuvant (Difco) were made at intervals of 10 days. Ten

days after the last injection, the mice were given an intravenous injection of the partially purified antigen and 3 days later the spleens were removed for fusion of spleen cells and mouse myeloma cells (P3-X63-Ag8-U1). The hybridoma producing MAb which reacted with the partially purified antigen, purified HSP60 from *Yersinia enterocolitica* [28] and the sonicated MKN45 cells were collected. The hybridoma with apparent specific antibody production was cloned by limiting dilution and designated as MAbH20 (class: IgM). The hybridoma cells (10⁶ cells) were inoculated i.p. into a BALB/c mouse pre-treated intraperitoneally with 0.5 ml of pristane (Wako Pure Chemical) 4 days before the inoculation of the cells. About 2 weeks later, ascites fluid was obtained from the mouse. The immunoglobulins in the ascitic fluid were purified with an Immunoglobulin-Easy-Separation kit (Pharmacia Biotech. Co., Tokyo, Japan). The purified MAbs were used for flow cytometric analysis.

Microsequencing of protein

To examine whether the molecule of *H. pylori* recognised with MAbH20 was HSP60, microsequencing of the N-terminal amino acids of the protein was performed. The whole proteins of *H. pylori* TK1029 were separated by SDS-PAGE and transferred on to an Immobilon membrane (Millipore, Bedford, MA, USA). The band corresponding to the protein recognised by MAbH20 was excised from the membrane and sequenced on a protein sequencer (473A; Applied Biosystems, Foster City, CA, USA).

Flow cytometric analysis

The inhibition assay of *H. pylori* adhesion to MKN45 and primary human gastric epithelial cells was performed by the method described by Osaki *et al.* [29]. Lipophilic dye PKH-2 (Zynaxis Cell Sciences, Phoenixville, PA, USA) was used to label *H. pylori* as described by Taguchi *et al.* [30]. *H. pylori* labelled with PKH-2 and MKN45 cells were pre-treated with 200 µg/ml, 40 µg/ml and 8 µg/ml of MAbH20. PKH-2-labelled *H. pylori* with or without treatment with MAbH20 were incubated with either MKN45 cells or primary gastric epithelial cells 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. After the cells were washed four times with HGS, they were resuspended in 300 µl of HGS for flow cytometric adherence assay. A flow cytometer (FACSVantage Coulter Electronics, Hialeah, USA) was used for the measurement of fluorescence intensity. Fluorescence data were obtained in a logarithmic mode on a 1024 channel scale. The results were shown as peak fluorescence intensity and mean (SD) channel indicating the fluorescence frequency distribution histograms.

Results

Preparation of primary human gastric epithelial cells

The profiles containing side and forward scatter of the prepared cells by flow cytometry are shown in Fig. 1. The group of large cells (arrow) was thought to comprise human gastric epithelial cells. In addition, cell morphology was also ascertained for these large cells (Fig. 2). The viability of these cells was determined with propidium iodide (Wako Pure Chemical), and was found to be 95%. This group of cells was gated, and the gated cells were analysed in the inhibition assay of adhesion of *H. pylori* to primary human gastric epithelial cells.

*Sequencing of N-terminal amino acids of the 60-kDa antigen of *H. pylori* recognised with MAbH20*

The N-terminal amino acid sequence of the *H. pylori* 60-kDa antigen recognised with MAbH20 was analysed. The sequence, ****IKFSVYAMKLLFEGV (* denotes unsuccessful determination), of the first 19 amino acids was determined. Eleven amino acids (underlined) corresponded to the N-terminal amino acids of *H. pylori* HSP60 previously reported by Macchia *et al.* [31].

*Inhibition of adhesion of *H. pylori* to human gastric carcinoma MKN45 cells by MAbH20*

Fig. 3 shows the representative patterns of the inhibition of *H. pylori* adhesion to MKN45 cells by MAbH20, directed to *H. pylori* HSP60. In *H. pylori* pre-treated with MAbH20, the fluorescence intensity of

the peaks that indicate the adhesion rate of *H. pylori* to MKN45 were significantly decreased (Fig. 3c). The decrease of the adhesion rate was dependent on the concentrations of MAbH20 used (8, 40 or 200 $\mu\text{g/ml}$). Each adhesion rate, indicated as mean channel (SD) when treated with MAbH20, was 182.3 (86.8), 185.9 (51.3) and 42.2 (25.4) for the results in Fig. 3a, b and c, respectively. There was a significant difference in adhesion when *H. pylori* was pre-treated with MAb and untreated organisms (Mann-Whitney U test, $p < 0.0001$) (Table 1). However, there was no significant difference in the adhesion rates of *H. pylori* when MKN45 cells were pre-treated with MAbH20 (8–40 $\mu\text{g/ml}$), in comparison to non-pre-treated control. These results indicate that *H. pylori* HSP60 might mediate the adhesion of *H. pylori* to human gastric carcinoma MKN45 cells.

*Inhibition of adhesion to *H. pylori* to primary human gastric epithelial cells*

Human gastric epithelial cells from a patient with gastric cancer were prepared for use in the inhibition assay with MAbH20. As shown in Figs. 1 and 2, the prepared cells contained a large population of human gastric epithelial cells. The relative proportion of the population of gastric cells which were epithelial was *c.* 25%. The mean channel number of the gastric epithelial cells adhered with *H. pylori* TK1029 was 188.1 (SD 51.3), and that of negative control of the epithelial cells without *H. pylori* was 3.1 (1.0) (Fig. 4). The results indicate that *H. pylori* TK1029 could adhere to the primary human gastric epithelial cells. When *H. pylori* was pre-treated with each concentration (125–1000 $\mu\text{g/ml}$) of MAbH20, the adhesion rates of *H. pylori* to the primary epithelial cells were

Fig. 1. Flow cytometry analysis of primary human gastric epithelial cells from gastric tissue of a patient with gastric cancer. Forward scatter (x axis) indicates cell size and side scatter (y axis) indicates molecular density in the cell. The cell group with greater size and density (arrow) is thought to comprise human gastric epithelial cells.

Fig. 2. Morphological features of the prepared human gastric cells ($\times 200$).

significantly decreased compared with the positive control, depending on the concentration of the MAb (Fig. 4). These results suggest that MAbH20 directed to *H. pylori* HSP60 could inhibit the adhesion of *H. pylori* not only to cultured MKN45 cells but also to primary human gastric epithelial cells.

Discussion

A recent report showed that there is a significant correlation between the expression of *H. pylori* HSP60 on the cell surface and the adhesion of *H. pylori* to human gastric carcinoma MKN45 cells [18]. Phadnis *et*

al. [32] have shown that *H. pylori* HSP60 exists on the cell surface and adheres to extracellular antigen. Huesca *et al.* [19] have also reported that *H. pylori* HSP60 localising on the bacterial surface, which mediates sulphatide recognition, might be related to the adhesion of *H. pylori* to gastric epithelial cells. These findings suggest that *H. pylori* HSP60 might be an adhesin and could be directly associated with the adhesion of *H. pylori* to gastric epithelial cells.

The present study established that MAbH20 is directed against *H. pylori* HSP60 and also demonstrated that the MAb inhibits the adhesion of *H. pylori* to MKN45 cells. The results indicate that *H. pylori* HSP60 could

Fig. 3. Representative patterns in flow cytometric analysis of adherence inhibition of *H. pylori* to MKN45 cells by MAbH20. **a**, MKN45 cells were pre-treated with MAbH20 at 4°C for 1 h, and incubated with *H. pylori*; **b**, *H. pylori* was incubated with MKN45 cells at 4°C for 1 h in the presence of MAbH20; **c**, *H. pylori* was pre-treated with MAbH20 at 4°C for 1 h and incubated with MKN45 cells. MAb H20 diluted to 8 µg/ml was used; —, shows the results of the inhibition assay with the MAb; ···, shows the negative control, indicating the fluorescence intensity of MKN45 cells only; ----, shows the positive control, indicating the fluorescence intensity of MKN45 to which *H. pylori* has adhered in the absence of the MAb.

be related to the adhesion of *H. pylori* to human gastric carcinoma MKN45 cells.

As MKN45 cells are not thought to be natural cells derived from human gastric tissue, primary human gastric epithelial cells were prepared from a patient

with gastric cancer. As shown in Fig. 4, *H. pylori* adhered to the primary human gastric epithelial cells. Osaki *et al.* [29] previously reported that the adhesion of *H. pylori* to three human gastric carcinoma cell lines (MKN45, KATOIII and MKN28) differed, showing that the cell line MKN45 was the most

Table 1. Inhibitory effect of MAbH20 on adhesion of *H. pylori* to MKN45 cells

Concentration of MAbH20 ($\mu\text{g/ml}$)	Mean (SD) fluorescence intensity*		
	Pre-treatment of <i>H. pylori</i>	Pre-treatment of MKN45	No pre-treatment
0	NT	NT	186.6 (92.0)†
8	42.2 (25.4)‡	185.9 (51.3)	182.3 (86.8)
40	27.8 (16.5)‡	175.9 (81.3)	170.6 (83.8)
200	24.6 (15.1)‡	161.4 (73.1)	115.6 (54.9)§

NT, not tested.

*Mean fluorescence intensity of negative control of MKN45 cells was 2.7 (SD 0.9).

†Mean $\pm$ SD of the mean.‡Statistically significant ($p < 0.0001$) in comparison to control without MAbH20.§Statistically significant ($p < 0.001$) in comparison to control without MAbH20.

sensitive to *H. pylori* adhesion. The results indicate that unknown host factors are associated with the adhesion of *H. pylori* to gastric epithelial cells.

The adhesion of *H. pylori* to the primary human gastric epithelial cells was inhibited by MAbH20 directed against *H. pylori* HSP60. These findings indicate that *H. pylori* HSP60 might be associated with adhesion of *H. pylori* not only to MKN45 but also to human primary gastric epithelial cells.

However, the inhibition of adhesion of *H. pylori* to MKN45 and the prepared human gastric epithelial cells by MAbH20 was not complete. The reason for these phenomena might be explained by the fact that the association between the adhesin(s) of *H. pylori*

Fig. 4. The inhibitory effect of MAbH20 on the adhesion of *H. pylori* to primary human gastric epithelial cells. Adhesion was evaluated by flow cytometric analysis when *H. pylori* was pre-treated with MAbH20 (0–1000 $\mu\text{g/ml}$). Mean fluorescence intensity of primary human gastric epithelial cells only as negative control was 3.1 (SD 1.0). *Statistically significant ($p < 0.001$) in comparison to MAbH20-untreated control.

and host receptors is multifactorial [13–17] (Fig. 5). In addition, it is speculated that the intensity of the expression of the receptor for *H. pylori* HSP60 on the surface is different in prepared human gastric epithelial cells and in established human gastric

Fig. 5. Hypothetical scheme to explain the adhesion of *H. pylori* to human gastric epithelial cells and human gastric cancer MKN45 cells.

cancer MKN45 cells (Fig. 5). Further studies are in progress to determine whether *H. pylori* HSP60 is directly associated with the adhesion of *H. pylori* to human gastric epithelial cells.

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References

- Blaser MJ. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori* induced inflammation. *Gastroenterology* 1992; **102**: 720–727.
- Parsonnet J, Friedman GD, Vandersteen DP *et al.* *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127–1131.
- Parsonnet J, Hansen S, Rodriguez L *et al.* *Helicobacter pylori* infection and gastric lymphoma. *N Engl J Med* 1994; **330**: 1267–1271.
- Eaton KA, Morgan DR, Krakowka S. *Campylobacter pylori* virulence factors in gnotobiotic piglets. *Infect Immun* 1989; **57**: 1119–1125.
- O'Toole PW, Kostrzynska M, Trust TJ. Non-motile mutants of *Helicobacter pylori* and *Helicobacter mustelae* defective in flagellar hook production. *Mol Microbiol* 1994; **14**: 691–703.
- Evans DG, Evans DJ, Moulds JJ, Graham DY. N-acetylneuraminylactose-binding fibrillar hemagglutinin of *Campylobacter pylori*: a putative colonization factor antigen. *Infect Immun* 1988; **56**: 2896–2906.
- Eaton KA, Krakowka S. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. *Infect Immun* 1994; **62**: 3604–3607.
- Segal ED, Shon J, Tompkins LS. Characterization of *Helicobacter pylori* urease mutants. *Infect Immun* 1992; **60**: 1883–1889.
- Leunk RD, Johnson PT, David BC, Kraft WG, Morgan DR. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J Med Microbiol* 1988; **26**: 93–99.
- Kamiya S, Kai M, Ozawa A *et al.* Characteristics of vacuolating toxin produced by *Helicobacter pylori*. *Eur J Gastroenterol Hepatol* 1994; **6** Suppl 1: S23–S27.
- Cave DR, Vargas M. Effect of a *Campylobacter pylori* protein on acid secretion by parietal cells. *Lancet* 1989; **2**: 187–189.
- Peterson WL. *Helicobacter pylori* and peptic ulcer disease. *N Engl J Med* 1991; **324**: 1043–1048.
- Lelwala-Guruge J, Ljungh Å, Wadström T. Haemagglutination patterns of *Helicobacter pylori*: frequency of sialic acid-specific and non-sialic acid-specific haemagglutinins. *APMIS* 1992; **100** Suppl 28: 908–913.
- Moran AP, Kuusela P, Kosunen TU. Interaction of *Helicobacter pylori* with extracellular matrix proteins. *J Appl Bacteriol* 1993; **75**: 184–189.
- Saito T, Natomi H, Zhao W *et al.* Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining. *FEBS Lett* 1991; **282**: 385–387.
- Slomiany BL, Piotrowski J, Samanta A, Vanhorn K, Murty VLN, Slomiany A. *Campylobacter pylori* colonization factor shows specificity for lactosylceramide sulfate and GM3 ganglioside. *Biochem Int* 1989; **19**: 929–936.
- Trust TJ, Doig P, Emody L, Kienle Z, Wadstrom T, O'Toole P. High-affinity binding of the basement membrane proteins collagen type IV and laminin to the gastric pathogen *Helicobacter pylori*. *Infect Immun* 1991; **59**: 4398–4404.
- Yamaguchi H, Osaki T, Taguchi H, Hanawa T, Yamamoto T, Kamiya S. Flow cytometric analysis of the heat shock protein 60 expressed on the cell surface of *Helicobacter pylori*. *J Med Microbiol* 1996; **45**: 270–277.
- Huesca M, Borgia S, Hoffman P, Lingwood CA. 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* 1996; **64**: 2643–2648.
- Born W, Happ MP, Dallas A *et al.* Recognition of heat shock proteins and gamma-delta cell function. *Immunol Today* 1990; **11**: 40–43.
- Kaufmann SHE, Schoel B, Wand-Wurtenburger A, Steinhoff U, Munk ME, Koga T. T-cells, stress proteins and pathogenesis of mycobacterial infections. *Curr Top Microbiol Immunol* 1990; **155**: 125–141.
- Young DB. Chaperonins and the immune response. *Semin Cell Biol* 1990; **1**: 27–36.
- Creighton TE. Unfolding protein folding. *Nature* 1991; **352**: 17–18.
- Ellis RJ. The molecular chaperone concept. *Semin Cell Biol* 1990; **1**: 1–10.
- Hemmingsen SM, Woolford C, van der Vies SM *et al.* Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* 1988; **333**: 330–334.
- Kamiya S, Taniguchi I, Yamamoto T *et al.* Evaluation of rapid urease test for detection of *Helicobacter pylori* in gastric biopsy specimens. *Eur J Epidemiol* 1993; **9**: 450–452.
- Yamaguchi H, Yamamoto T, Taguchi T, Ogata S. *Yersinia enterocolitica* immunodominant 60 kDa antigen, common to a broad range of bacteria, is a heat-shock protein. *J Gen Microbiol* 1990; **136**: 1091–1097.
- Yamaguchi H, Taguchi H, Katura T, Kumada J, Uekusa T, Ogata S. Purification of cross-reacting protein antigen shared by *Yersinia enterocolitica* and other gram-negative bacteria with monoclonal antibody. *Microbiol Immunol* 1989; **33**: 683–688.
- Yamamoto-Osaki T, Yamaguchi H, Taguchi H, Ogata S, Kamiya S. Adherence of *Helicobacter pylori* to cultured human gastric carcinoma cells. *Eur J Gastroenterol Hepatol* 1995; **7** Suppl 1: S89–S92.
- Taguchi H, Osaki T, Yamaguchi H, Kamiya S. Flow cytometric analysis using lipophilic dye PKH-2 for adhesion of *Vibrio cholerae* to intestine 407 cells. *Microbiol Immunol* 1995; **39**: 891–894.
- Macchia G, Massone A, Burrone D, Covacci A, Censin S, Rappuoli R. The Hsp60 protein of *Helicobacter pylori*: structure and immune response in patients with gastroduodenal diseases. *Mol Microbiol* 1993; **9**: 645–652.
- Phadnis SH, Parlow MH, Levy M *et al.* Surface localization of *Helicobacter pylori* urease and a heat shock protein homolog requires bacterial autolysis. *Infect Immun* 1996; **64**: 905–912.