BACTERIAL PATHOGENICITY

Induction of secretion of interleukin-8 from human gastric epithelial cells by heat-shock protein 60 homologue of Helicobacter pylori

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Escherichia coli cells expressing fusion proteins consisting of β-galactosidase and bacterial heat-shock protein (HSP) 60 of E. coli, Yersinia enterocolitica or Helicobacter pylori were constructed, and designated as HY1, HY2 or HY3, respectively. Fusion proteins prepared from HY2 and HY3 induced secretion of interleukin-8 (IL-8) from human gastric epithelial KATOIII cell cultures. On the other hand, the parent strain (E. coli pop2136), PEX (pop2136 transformed by vector) and fusion protein prepared from HY1 did not induce IL-8 secretion from KATOIII cells. Other human gastric (MKN45) and non-gastric cell lines (Int407 and A549) did not secrete IL-8 following treatment with these proteins. These results indicate that H. pylori HSP60 induces IL-8 secretion from human gastric cells and the levels of IL-8 differ among the various gastric cell lines, suggesting that HSP60 might be an important virulence factor associated with chronic gastric inflammation following H. pylori infection in man.

Introduction

Interleukin-8 (IL-8) is a potential mediator associated with gastric inflammation in patients with Helicobacter pylori infection [1, 2]. Rieder et al. reported that direct contact of H. pylori with epithelial cells was needed to induce IL-8 in vivo [3], indicating that a surface component of H. pylori might be important. An earlier study demonstrated that the heat-shock protein (HSP) 60 (Hsp B) homologue of H. pylori is located on the bacterial cell surface and is associated with adhesion of H. pylori to human gastric epithelial cells [4]. HSP60 conserved in prokaryotic cells and also eukaryotic cells is thought to facilitate the folding, unfolding and translocation of polypeptides as chaperonins and is associated with chronic inflammation [5, 6]. Therefore, HSP60 of H. pylori is a candidate virulence factor for chronic gastritis induced by H. pylori.

The present study examined whether recombinant H. pylori HSP60 expressed by Escherichia coli induces IL-8 secretion from cultured human cell lines.

Materials and methods

Human cell lines

Human gastric carcinoma cells (KATOIII, MKN45) and non-gastric cells (Int407, small intestinal epithelial cells; A549, lung cancer cells) were obtained from the Japanese Cancer Research Resources Bank (JCRB). They were grown at 37°C in RPMI1640 containing fetal calf serum 10% v/v in an atmosphere containing CO2 5%.

Monoclonal antibody (MAb)

MAb H9 (IgG2a), which reacted with H. pylori HSP60, was described previously [7]. The 60-kDa antigen derived from H. pylori strain TK1029 was partially purified by extraction from SDS-PAGE separating gel. BALB/c mice were immunised intraperitoneally (i.p.) with the antigen mixed with Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA), three times at an interval of 10 days. Ten days after the last i.p. injection, the mice were given the...
partially purified antigen intravenously. Then 3 days after the last immunisation, spleens were removed for cell fusion with mouse myeloma cells (P3-X63-Ag8-U1). The hybridoma cells producing MAb that reacted with the affinity-purified *H. pylori* HSP60 and the sonicated MKN45 cells (human gastric cancer cell line) in ELISA were collected. The hybridoma cells with apparent specific antibody production were cloned by limiting dilution. Cells 10^6 were inoculated i.p. into a BALB/c mouse pretreated i.p. with pristane (Wako Pure Chemical) 0.5 ml, 4 days before the inoculation of the cells and ascites fluids were obtained from the mouse 2 weeks later. The immunoglobulins in the ascites fluids were purified with the Immunoglobulin-Easy-Separation kit (Pharmacia Biotechnology, Tokyo, Japan). The purified MAb was used for affinity chromatography and immunoblotting analysis.

**Affinity-purified *H. pylori* HSP60**

HSP60 was affinity-purified with specific MAb recognising *H. pylori* HSP60 from *H. pylori* strain TK1029 (*cagA* and *vacA* positive) isolated from a patient with gastric ulcer as described above [8].

**Construction of E. coli expressing *H. pylori* HSP60**

DNA fragments encoding *E. coli* GroEL, *Yersinia enterocolitica* HSP60 or *H. pylori* HSP60 were amplified from bacterial strains by PCR as indicated in Table 1. The primer sets used were as follows: upstream 5'-GAATTCATGGCAAAAGAAATCAAATT-3', downstream 5'-GAATTCATGGCAAAAGAAATCAAATT-3' (*E. coli* and *Y. enterocolitica*); upstream 5'-GAATTCATGGCAAAAGAAATCAAATT-3', downstream 5'-GAATTCATGGCAAAAGAAATCAAATT-3' (*H. pylori*). The recognition sequences GAATTC with restriction endonuclease were added on both sides of 5'-upstream and 3'-downstream for subcloning the resultant cDNA into a plasmid pEX. The resultant cDNA was integrated into a plasmid pEX capable of producing a fused protein with β-galactosidase [9]. The constructed plasmids were transformed into *E. coli* pop2136. The bacteria were cultivated at 30°C and then shifted to 42°C for 2 h to induce the expression of the recombinant protein (fusion protein). Bacterial pellets were stored at -80°C until their use in SDS-PAGE.

**Preparation of fusion proteins**

The fusion proteins were prepared as described previously [10]. Each *E. coli* strain (HY1, HY2 or HY3) harbouring pHY1, pHY2 or pHY3 encoding *hsp60* of *E. coli*, *Y. enterocolitica* or *H. pylori*, respectively, was sonicated with a Sonifer 250 (Branson, NY, USA) and then insoluble fractions were collected by centrifugation at 10 000 g. The pellet (insoluble fraction containing fusion protein) was incubated in 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 1 mM DTT and 8 M urea for 1 h at 37°C. After the solutions containing fusion protein had been dialysed against PBS and sterilised by filtration, they were used for induction of IL-8 from cultured cell lines KATO III (human gastric cancer cells), MKN45 (human gastric cancer cells), Int407 (human small intestinal cells) and A549 (human lung cancer cells). Parental strains of *E. coli* pop2136 and PEX (pop2136 harbouring pEX) treated as described above were used as negative controls.

**SDS-PAGE and immunoblotting**

SDS-PAGE with acrylamide 8% w/v gels was performed as described by Laemmli [11]. Each bacterial pellet stored at -80°C (1 ml of cultured

<table>
<thead>
<tr>
<th>Plasmid or bacterial strain</th>
<th>Relevant characteristics or purpose for use</th>
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<tbody>
<tr>
<td>PEX</td>
<td>Protein expression vector</td>
</tr>
<tr>
<td>pHY1</td>
<td>groEL of <em>E. coli</em> cloned into pEX</td>
</tr>
<tr>
<td>pHY2</td>
<td><em>hsp60</em> of <em>Y. enterocolitica</em> cloned into pEX</td>
</tr>
<tr>
<td>pHY3</td>
<td><em>hsp60</em> of <em>H. pylori</em> cloned into pEX</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> ZM20 0:3</td>
<td>Used for purification of <em>H. pylori</em> HSP60 by affinity column with MAb* H20</td>
</tr>
<tr>
<td><em>E. coli</em> pop2136</td>
<td>Used for amplification of <em>Y. enterocolitica</em> HSP60-encoding gene by PCR</td>
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<tr>
<td>PEX</td>
<td>Parent strain for transformation</td>
</tr>
<tr>
<td>HY1</td>
<td><em>E. coli</em> harbouring pEX</td>
</tr>
<tr>
<td>HY2</td>
<td><em>E. coli</em> harbouring pHY1</td>
</tr>
<tr>
<td>HY3</td>
<td><em>E. coli</em> harbouring pHY2</td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em> harbouring pHY3</td>
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</tbody>
</table>

*MAb recognising *H. pylori* HSP60.
bacterial cells expressing fusion proteins) was suspended in 300 μl of PBS. The bacterial solutions were mixed with 300 μl of sample buffer (pH 6.8; 0.012 M Tris, glycerol 20% v/v, 0.015 M SDS and 0.4 mM 2-mercaptoethanol). The solutions were heated for 10 min at 100°C. Finally, 20 μl of the bacterial lysates were loaded into each lane of a separating 8% gel. Immunoblot analysis was done as described by Towbin et al. [12]. After electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) at 0.25A overnight. After blocking with phosphate-buffered saline (PBS; pH 7.4) containing skimmed milk 1% w/v (Yukijirushi Nyugyo, Sapporo, Japan) (PBS-S) for 1 h at room temperature, the membranes were treated for 1 h with MAb H9 diluted to 1 pg/ml with PBS-S. They were then incubated for 1 h with goat anti-mouse IgG peroxidase conjugate diluted 1 in 500 with PBS-S. Immunoblots were developed with 50 mM Tris-HCl buffer (pH 7.4) containing H2O2 0.12% v/v and 1 mM 3,3'-diaminobenzidine tetrahydrochloride (Dojinkagaku, Kumamoto, Japan).

Detection of IL-8 secretion

Established cultured human cells (3 × 10⁶) were incubated in RPMI1640 containing fetal bovine serum 10% v/v with fusion proteins or control cell lysate (1.25–150 μg/ml) for 24–96 h at 37°C. After cultivation, the amount of IL-8 in each culture supernate was measured by a sandwich-ELISA, with a commercially available ELISA kit (Cytoscreen Human IL-8; Bio Source International, CA, USA).

Statistics analysis

The statistical significance of the differences was assessed by the Friedman test.

Results

Profiles of the prepared fusion proteins

The profiles of the prepared fusion proteins were examined by SDS-PAGE and immunoblotting with MAb H9 directed to bacterial HSP60 [8]. As shown in Fig. 1, each E. coli (HY1, lane 4; HY2, lane 5; HY3, lane 6) expressing bacterial HSP60 was confirmed by SDS-PAGE as being constructed. All fusion proteins with mol. wts of c. 180 kDa (lanes 9–11) were recognised by MAb H9 directed to bacterial HSP60 [7]. The band corresponding to a 60-kDa molecule in all samples recognised by MAb H9 was thought to be native GroEL from the parent E. coli strain, and especially in lanes loaded with the fusion proteins, the intensity of bands was stronger than that in the other lanes. This phenomenon could be explained by the fact that the overexpression of foreign antigen such as the fusion protein in E. coli enhances production of native GroEL to eliminate the foreign protein [10].

Detection of IL-8 secretion from established human cell lines by recombinant H. pylori HSP60

As shown in Fig. 2, IL-8 secretion was induced from human gastric KATOIII cells by HY3 (fusion protein of H. pylori HSP60). The amounts of IL-8 detected after cultivation with 150 μg of fusion protein/ml for 24, 48, 72 and 96 h, were 2862, 2884, 3068 and
Fig. 2. IL-8 secretion from human gastric carcinoma cell line (KATO III) induced by fusion protein PEX, HY1, HY2 and HY3. Concentrations (μg/ml) of the prepared antigens used were 1.25 (a), 6 (b), 30 (c) and 150 (d) and IL-8 secretion was assayed after incubation for 24 (●), 48 (■), 72 (▲) and 96 (△) h.
4321 pg/ml, respectively. Similarly, HY2 (fusion protein of Y. enterocolitica HSP60) also induced a level of secretion of IL-8 as high as that induced by HY3. Furthermore, a significant dose-response was observed when HY2 and HY3 were used in IL-8 induction experiments (p < 0.01). Neither HY1 (fusion protein of E. coli GroEL), PEX nor E. coli pop2136 induced production of significant amounts of IL-8. In contrast, HY3 did not induce IL-8 secretion from other cell lines such as MKN45, Int407 and A549 cells (Table 2). These results indicate that recombinant H. pylori HSP60 induces IL-8 secretion from KATOIII cells, but not from other cell lines such as MKN45, Int407 and A549.

**Discussion**

The induction of IL-8 secretion from KATOIII cells with fusion proteins containing *H. pylori* or *Y. enterocolitica* HSP60, but not with *E. coli* GroEL, was observed in the present study. Amino-acid sequences of *H. pylori* HSP60, *Y. enterocolitica* HSP60 and *E. coli* GroEL have been reported previously [13–15]. The similarities of the amino-acid sequences among these junior proteins were as follows: 55.4% for *H. pylori* HSP60 versus *Y. enterocolitica* HSP60; 55.9% for *H. pylori* HSP60 versus *E. coli* GroEL; and 88.2% for *Y. enterocolitica* HSP60 versus *E. coli* GroEL. As shown in Fig. 3, the amino-acid regions that include an identical amino-acid position in *H. pylori* HSP60 and *Y. enterocolitica* HSP60 but not in *E. coli* GroEL might be associated with IL-8 induction from KATOIII cells.

The induction of IL-8 secretion from KATOIII cells but not from another gastric cell line (MKN45) by *H. pylori* HSP60 was observed. Aihara et al. [16] have reported that the secretion of IL-8 by live *H. pylori* was different in various human gastric cancer cell lines (MKN45, MKN1, MKN28, MKN74 and KATOIII). A previous study reported that *H. pylori* HSP60 was associated with adhesion of *H. pylori* to human gastric epithelial cells [4]. Huesca et al. reported that surface HSPs 60 and 70 of *H. pylori* are associated with attachment to human gastric epithelial cells by binding to the receptor, sulphated glycolipid [17]. These reports suggest that the different amounts of sulphated glycolipid on the various epithelial cells might also be associated with differences in IL-8 induction.

It has been reported that direct contact of *H. pylori* with epithelial cells was associated with the induction of IL-8 secretion [3]. Censini et al. reported an association of various Cag proteins encoded by the *cag* pathogenicity island of *H. pylori* with IL-8 secretion from gastric epithelial cells [18]. Huang et al. [19] have shown that water-soluble extract containing bacterial surface components such as urease, FlaAB, HSPs, membrane proteins and lipopolysaccharide (LPS) induced IL-8 secretion from gastric epithelial cell lines, AGS and KATOIII. They also indicated that a urease-negative mutant and a non-motive mutant did not differ from the parent *H. pylori* strain with respect to IL-8 induction [19]. Furthermore, it has been reported recently that *H. pylori* LPS did not induce IL-8 production from AGS cells [20]. It is possible that porin protein is involved in the induction of IL-8 production from gastric epithelial cells, as *H. pylori* porins have been reported to stimulate human monocytes and lymphocytes to release various cytokines such as TNF-α, IL-6 and IL-8 [21]. Recently, Galdero et al. [22] reported that GroEL and DnaK of *E. coli* induced both secretion of cytokines (TNF-α, IL-6 and IL-8) from human monocytes and lymphocytes and expression of adhesion molecule in endothelial cells.

The present study demonstrated for the first time that HSP60 or *H. pylori* induced IL-8 secretion from gastric epithelial cells, suggesting that HSP 60 might be an important virulence factor associated with chronic gastric inflammation following *H. pylori* infection. The mechanism by which IL-8 was induced by *H. pylori* HSP60 and the reason why the induction level of IL-8 was different depending on the gastric cells used are not yet understood.

### Table 2. Induction of IL-8 secretion from gastric and non-gastric epithelial cells by prepared antigens

<table>
<thead>
<tr>
<th>Prepared antigen added to cultures (150 μg/ml)†</th>
<th>Gastric epithelial cell line</th>
<th>Mean IL-8 secretion (pg/ml)* from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MKN45</td>
<td>Int407</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>pop2136</td>
<td>364</td>
<td>375</td>
</tr>
<tr>
<td>PEX</td>
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<td>680</td>
</tr>
<tr>
<td>HY1</td>
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<td>380</td>
</tr>
<tr>
<td>HY2</td>
<td>472</td>
<td>618</td>
</tr>
<tr>
<td>HY3</td>
<td>342</td>
<td>417</td>
</tr>
<tr>
<td>Medium</td>
<td>48</td>
<td>70</td>
</tr>
</tbody>
</table>

*NT, not tested.
†The results shown are the average of duplicate assays.
‡The antigens were prepared as described in the text.
Fig. 3. Homology of amino-acid sequences among *H. pylori* HSP60 (HpHSP60 AA), *Y. enterocolitica* HSP60 (YeHSP60 AA) and *E. coli* GroEL (EcGroEL AA). Homologous sequences are indicated by an asterisk. The positions of amino acids which are homologous between *H. pylori* HSP60 and *Y. enterocolitica* HSP60 but not *E. coli* GroEL are shown as boxes.
INDUCTION OF IL-8 BY *H. PYLORI* HSP60

References