Cell adherence-promoted activity of *Plesiomonas shigelloides* GroEL

Hitoshi Tsugawa, Humie Ito, Miho Ohshima and Yoshio Okawa

Correspondence

Yoshio Okawa

okawa@tohoku-pharm.ac.jp

Department of Infection and Host Defense, Tohoku Pharmaceutical University,
4-4-1 Komatsushima, Sendai Aoba-ku, Miyagi 981-8558, Japan

Previously, it has been demonstrated that the invasion of Caco-2 cells by *Plesiomonas shigelloides* induces apoptotic cell death. Therefore, the attachment to and colonization of eukaryotic intestinal host cells by *P. shigelloides* are important steps in causing pathogenicity. In this study, the participation of *P. shigelloides* GroEL in the attachment of *P. shigelloides* was examined. The *groESL* operon of *P. shigelloides* was isolated by PCR. The nucleotide sequence of the *groESL* operon of *P. shigelloides* revealed two ORFs of 294 nucleotides for *groES* and 1647 nucleotides for *groEL*. Cell fractionation and immunostaining experiments suggested that the GroEL of *P. shigelloides* was associated with the bacterial cell surface. The expression of the *groEL* gene was upregulated during the attachment and apoptosis-induction stages, and the expression of the protein was also induced during the attachment stage. Furthermore, GroEL efficiently promoted the attachment of *P. shigelloides* to Caco-2 cells, as measured by a FACSCalibur flow cytometer. These results demonstrated that GroEL has a positive influence on the attachment of *P. shigelloides* to Caco-2 cells.

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INTRODUCTION

Plesiomonas shigelloides is recognized as a causative organism of diarrhoeal disease (Brenden *et al.*, 1998; Saraswathi *et al.*, 1983). Several potential and putative virulence factors that could play a role in the pathogenicity of *P. shigelloides* have been examined (Falcon *et al.*, 2003; Janda & Abbott, 1993; Okawa *et al.*, 2004; Theodoropoulos *et al.*, 2001). Recently, we have demonstrated the mechanism of invasion of *P. shigelloides* into Caco-2 cells and that the invasion of *P. shigelloides* induces apoptotic cell death (Tsugawa *et al.*, 2005). These results show that an interaction between the bacteria and the host cells is important for the pathogenicity of *P. shigelloides*. Therefore, the analysis of factors which are involved in the interaction between *P. shigelloides* and host cells is important to understand the infection mechanism of the bacteria. In this study, we focused on *P. shigelloides* GroEL, known to be a chaperone or heat-shock protein (HSP).

HSPs have been grouped into families according to molecular mass, and members of each family have common features (Goulhen *et al.*, 1998). Some HSPs function as chaperones, and may help in transporting proteins across cell membranes or in protein folding (Goulhen *et al.*, 1998). The production of HSPs is greatly

enhanced by stress stimuli such as heat, acid or osmotic shock, and iron deprivation (Hennequin *et al.*, 2001a; Retzlaff *et al.*, 1994). The main function of HSPs is preservation of essential cellular proteins and their functions. HSPs are predominantly located in intracellular compartments, but recent studies suggest that some HSPs can be expressed on the bacterial surface and secreted extracellularly (Garduno *et al.*, 1998a; Paju *et al.*, 2000; Phadnis *et al.*, 1996). Recent studies using immunocytochemical experiments have indicated that GroEL is involved in microbial pathogenicity: the 66 kDa HSP of *Salmonella typhimurium*, the GroEL of *Haemophilus ducreyi*, HSP60 of *Legionella pneumophila* and the GroEL of *Clostridium difficile* are involved in interactions between the bacteria and host cells (Ensgraber & Loos, 1992; Frisk *et al.*, 1998; Garduno *et al.*, 1998b; Hennequin *et al.*, 2001b). Furthermore, it has also been demonstrated that HSPs of *Escherichia coli* increase cytokine and adhesion molecule expression (Galdiero *et al.*, 1997; Marcatili *et al.*, 1997; Retzlaff *et al.*, 1994).

Based on these experimental results, we have investigated the possibility that GroEL is involved in the initial stage (attachment) of *P. shigelloides* infection. First, we isolated and characterized the *P. shigelloides groESL* operon, and demonstrated the localization of the GroEL protein in the whole bacterial cell. Next, in order to understand the role of GroEL at the point of bacterial attachment, we examined the adhesion of the bacteria to Caco-2 cells stimulated with GroEL by using a flow cytometer. Finally, we showed that

Abbreviations: HSP, heat-shock protein; ICAM-1, intercellular adhesion molecule-1.

The GenBank/EMBL/DDBJ accession number for the *groESL* operon sequence of *P. shigelloides* is AB251936.

Fig. 1. Structure and cloning strategy of the *P. shigelloides* P-1 strain *groESL* operon. The *Sse8387* I fragment of *P. shigelloides* genomic DNA, as shown by Southern blot analysis, was ligated into the *Sse8387* I site of the pGEM-3Z vector. FP1/2 and RP1/2 denote the PCR primers; the combination of FP1 and RP1 was used for cloning the first fragment of *groEL*, RP2 and vector-specific primer SP6 for the upstream region of *groEL*, including *groES* and the promoter region, and FP2 and vector-specific primer SP6 for the downstream region of *groEL*.

Caco-2 cells stimulated with GroEL induced the expression of intercellular adhesion molecule-1 (ICAM-1).

METHODS

***P. shigelloides* strains and growth conditions.** The *P. shigelloides* P-1 strain used in this study was a clinical isolate from the Osaka Prefectural Institute of Public Health (Tsukamoto *et al.*, 1978). The bacteria were grown in brain heart infusion (BHI) broth (Difco) for 18 h at 37 °C with shaking.

DNA isolation and cloning of the *P. shigelloides* *groESL* operon. The bacterial cell pellets from 12 h culture were sonicated and centrifuged. The supernatant was loaded to Sephacryl S-100 HR, and then subjected to antibody affinity chromatography. In order to determine the molecular mass and the N-terminal amino acid sequence, the fraction containing GroEL was separated on SDS-PAGE and transferred to a PVDF membrane (Problott, Applied Biosystems). The N-terminal amino acid sequence of the blotted protein was determined by Edman degradation. It was identified that the purified protein was a GroEL from the N-terminal amino acid sequence. The genomic DNA of *P. shigelloides* was extracted using a Wizard Genomic DNA Purification kit (Promega). The *groESL* operon of *P. shigelloides* was amplified by PCR in three different fragments using three sets of primers that included FP1/2 and RP1/2 (Fig. 1). Primers used in the PCR reactions are shown in Table 1. The first set of primers, FP1 and RP1, was designed based on the *P. shigelloides* GroEL N-terminal amino acids (AAKDVKFG) and the GroEL motif sequence, respectively. For 50 µl of the PCR reaction, 10 ng *P. shigelloides* genomic DNA was used. Thermal cycling conditions were initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 63 °C for 30 s and 72 °C for 1.5 min, and a final extension at 72 °C for 5 min using Ex *Taq* DNA polymerase (TaKaRa). The PCR products (600 bp) were purified by a QIAquick gel extraction kit (Qiagen). The purified PCR products were directly sequenced by a CEQ DTCS Quick Start kit (Beckman Coulter) and an automatic DNA sequencer (CEQ8000XL, Beckman Coulter). In the next step, using the first PCR products (600 bp) as a probe, genomic Southern blot analysis of the chromosomal DNA, which had been digested with *Sse8387* I, was performed. The *Sse8387* I fragment shown by Southern blot analysis was ligated into the *Sse8387* I site of the pGEM-3Z Vector (Promega) and transformed into *Escherichia coli* DH5α (TaKaRa). The second fragment was PCR-amplified from the plasmid DNA using the vector-specific primer SP6 and RP2 (based on the sequence determined for the first fragment). Finally, the third fragment was PCR-amplified from the plasmid DNA using the vector-specific primer SP6 and FP2 (based on the GroEL motif sequence). The second (560 bp) and third (900 bp) fragments were cloned into the pCR4-TOPO vector and transformed into TOP10 chemically competent *E. coli* cells

(Invitrogen). The plasmid DNAs were purified using a Wizard Plus SV Minipreps DNA purification system (Promega), and the inserts were sequenced using the same conditions as mentioned for the first fragments. The sequences of all three fragments of the *P. shigelloides* *groESL* operon were combined and aligned using Genetyx version 7 (Software Development). The complete *groESL* operon of *P. shigelloides* was PCR-amplified and cloned into the pCR4-TOPO vector. The sequence of the *groESL* operon and the deduced amino acid sequence were analysed using Genetyx version 7.

Protein expression and purification of the *P. shigelloides* GroEL. Two oligonucleotide primers, Ex-FP and Ex-RP, incorporating *Nde*I and *Spe*I sites in pET-41a(+), respectively, shown in Table 1 (lower-case italic type), were synthesized and used to PCR-amplify the full-length coding region of the *groEL* of *P. shigelloides*. The PCR products (1600 bp) were cloned into the pCR4-TOPO vector and transformed into TOP10 chemically competent *E. coli* cells (Invitrogen). The plasmid DNA was purified and digested with *Nde*I and *Spe*I, and the inserts were cloned into pET-41a(+), which had been digested with *Nde*I and *Spe*I, and transformed into *E. coli*

Table 1. Primers used in PCR reactions

Primer	Primer sequence (5'→3')
Cloning primers	
FP1	GCAGCTAAAGACGTAAAATTCGG
RP1	GGCTTGTTGATGAAGTAAGGAGA
FP2	ACTACGGTTACAACGCAGC
RP2	CTACGTTACGGCCTTTTGGACC
Expression primers	
Ex-FP	<i>catatg</i> GCAGCTAAAGACG
Ex-RP	<i>actagt</i> TACATCATGCCGCC
One-step RT-PCR primers	
<i>P. shigelloides</i> -16S rRNA FP	GTTTGATCATGGCTCAGATTGAAC
<i>P. shigelloides</i> -16S rRNA RP	TTACCTTGTTACGACTTCACCCC
<i>P. shigelloides</i> - <i>groEL</i> FP	ATGGCAGCTAAAGACGTAAAATTCG
<i>P. shigelloides</i> - <i>groEL</i> RP	TTACATCATGCCGCCCA
Caco-2 Actin FP	TACGCCAACACAGTGCTGTCT
Caco-2 Actin RP	TACTCCGCTTGCTGATCCACAT
Caco-2 ICAM-1 FP	CATGCAGCACCTCCTGTGAC
Caco-2 ICAM-1 RP	AGGGAGGCGTGGCTTGTG

BL21 (DE3) (Novagen). The transformed bacteria were cultured in Luria-Bertani broth (Invitrogen) supplemented with kanamycin ($30 \mu\text{g ml}^{-1}$), and expression of the GroEL was induced with 1 mM IPTG at 37°C . The bacterial cells were harvested by centrifugation and lysed by a BugBuster HT (Novagen). Inclusion bodies were harvested and washed three times with RIPA buffer [25 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 % (w/v) SDS, 1 % (v/v) Triton X-100, 1 % (w/v) deoxycholic acid]. Inclusion bodies were solubilized in 10 % (w/v) SDS, and then refolded by dialysis.

Anti-GroEL antibody production. A rabbit polyclonal, monospecific GroEL antiserum was prepared. Purified protein (300 μg) was injected together with Freund's complete adjuvant (Difco) into Japanese White rabbits (SLC), followed by boosters with 300 μg protein in Freund's complete adjuvant at days 7, 21 and 28. The rabbits were killed and bled 14 days after the last injection. The antiserum was pooled and stored at -20°C until use.

Western immunoblot. The protein concentration in the samples was determined by a BCA Protein Assay Reagent kit (Pierce) using BSA as a standard. SDS-PAGE was done using the PhastSystem (Amersham Bioscience) with a 10–15 % gradient PhastGel. Western immunoblotting was performed as described previously by Okawa *et al.* (2004), using rabbit antisera against the *P. shigelloides* GroEL protein (1:1000 dilution). Following 1 h incubation with this first antibody, the nitrocellulose membrane was washed three times with PBS. The nitrocellulose membrane was then reacted with goat anti-rabbit antibody coupled to horseradish peroxidase (1:1000 dilution; Biosource) for 1 h, and the membrane was washed three times with PBS and then developed with the 3,3'-diaminobenzidine (DAB) colour development reagent. The intensity of GroEL signals was analysed by densitometric scanning of the membranes using a gel video system (GelDoc EQ, Bio-Rad) and image analysis software (Quantity One, Bio-Rad).

Fractionation of bacterial cells. Fractionation was performed as previously described by Goulhen *et al.* (1998). Bacterial cells were exposed to osmotic shock with ice-cold distilled water for 10 min, the suspension was centrifuged, and the supernatant contained the periplasmic material. The pellet was sonicated, centrifuged at 6000 *g* for 15 min, and the supernatant was centrifuged at 200 000 *g* for 2 h. The resulting supernatant was dialysed against distilled water and contained the cytoplasmic material. The pellet was resuspended in 2 % (v/v) Triton X-100 containing 10 mM MgCl_2 . The latter suspension was centrifuged at 200 000 *g* for 1 h. The supernatant contained the cytoplasmic-membrane-rich cell envelope, and the pellet resuspended in distilled water contained the outer-membrane-rich cell envelope. All fractions were kept at -20°C prior to further analysis.

Stress cultivations. Heat shock was induced in the stationary phase of growth by transferring cultures grown at 37°C to a 43°C water bath and incubating for 60 min. To investigate the effect of iron starvation, bacteria were cultured at 37°C in iron-limited medium prepared by adding 200 μM 2,2'-dipyridyl (Nacalai tesque) to BHI medium. To investigate the effect of osmolarity, bacteria were cultivated at 37°C in BHI medium containing 150 mM NaCl.

RT-PCR analysis of gene expression during infection. The RNA of *P. shigelloides* during Caco-2 cell infection was extracted using an SV Total RNA Isolation system (Promega). The resulting RNAs of *P. shigelloides* at different stages during Caco-2 cell infection were amplified using the specific primers listed in Table 1. The *P. shigelloides* 16S rRNA gene (Martinez-Murcia *et al.*, 1992) was used as the internal control for RT-PCR.

The RT-PCR reaction was carried out with the SuperScript III One-Step RT-PCR system with Platinum *Taq* High Fidelity (Invitrogen), in a 50 μl mixture containing 1 μg RNA template, 0.5 μM of the primers

Fig. 2. Presence of the GroEL protein in various cell fractions of *P. shigelloides* P-1 strain. (a) Western immunoblotting detection of the GroEL protein in fractions of *P. shigelloides* P-1. Each lane contained 0.4 μg protein. Lanes: 1, culture supernatant; 2, periplasm; 3, cytoplasm; 4, cytoplasmic membrane; 5, outer membrane. (b) Densitometric analysis of GroEL. The GroEL bands were quantified by the densitometry function of the Quantity One software. Results are means $\pm$ SD of three independent assays.

P. shigelloides-groEL FP and *P. shigelloides*-groEL RP (Table 1), and 2 $\times$ Reaction Mix, with a reaction mixture formulated according to the manufacturer's instructions. The cDNA synthesis step was performed at 55°C for 30 min and a pre-denaturation step at 94°C for 2 min, followed by 60 cycles at 94°C for 15 s, 54°C for 30 s and 68°C for 3 min, and a final extension at 68°C for 5 min. The amounts of *groEL* product were analysed by densitometric scanning of gels using a gel video system (GelDoc EQ) and image analysis software (Quantity One).

Adherence promotion by GroEL. Measurement of Caco-2 cells with *P. shigelloides* attached was conducted using a FACSCalibur flow cytometer (Becton Dickinson). Direct flow cytometry was done using *P. shigelloides* labelled with FITC (Sigma). The labelling of *P. shigelloides* with FITC was carried out by the method of Tsugawa *et al.* (2005) as follows. *P. shigelloides* was suspended in 500 μl PBS

Fig. 3. Effect of several stresses on synthesis of *P. shigelloides* GroEL. Western immunoblot analysis is shown of GroEL present in total protein extracts of *P. shigelloides* detected with antibodies against GroEL. Lanes: 1, normal conditions; 2, heat treatment (43°C) for 60 min of normally cultured cells; 3, iron-deprived conditions; 4, elevated osmolarity conditions. Each lane contained proteins from equivalent numbers of bacteria.

containing 5 µl FITC solution (30 mg FITC ml⁻¹ in DMSO) and incubated at room temperature for 2 h. The labelled bacteria were washed eight times in PBS and suspended in 500 µl PBS. The Caco-2 cells were seeded at a density of 10⁶ per well in a 24-well tissue-culture plate and incubated for 18 h in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO₂. The labelled bacteria (5.0 × 10⁸) were added to each well containing a monolayer of Caco-2 cells, and the mixture was incubated at 37 °C in 5% CO₂ for 10 min. The monolayer was washed three times with PBS, and the fluorescence intensity of the cells was then measured by the FACSCalibur flow cytometer.

An adherence-promoting assay for GroEL was conducted using the purified GroEL protein and a FACSCalibur flow cytometer as follows. First, the monolayer of Caco-2 cells was incubated with 10, 20 and 30 µg of GroEL or BSA (control) in DMEM with 10% (v/v) FBS for 60 min at 37 °C under a 5% CO₂ atmosphere. After that, the FITC-labelled bacteria (5.0 × 10⁸) were infected for 10 min. The monolayer was washed three times with PBS and the fluorescence intensity of the cells was then measured by a FACSCalibur flow cytometer. Analysis of the data was performed using the CELLQuest software program (Becton Dickinson). The assays were repeated three times.

Expression of ICAM-1 on Caco-2 cells stimulated with GroEL. The expression of ICAM-1 on Caco-2 cells which had been incubated with GroEL for 60 min at 37 °C in 5% CO₂ was evaluated by two different methods. (i) RT-PCR analysis of ICAM-1 gene expression, and (ii) flow cytometer studies that used mouse anti-human ICAM-1 conjugated with FITC (Biosource) to determine ICAM-1 expression on the cell surface.

For the RT-PCR analysis of ICAM-1 gene expression, the RNA of the Caco-2 cells which had been incubated with GroEL or BSA (control) for 60 min at 37 °C in 5% CO₂ with 10, 20 and 30 µg of protein were extracted using an SV Total RNA isolation system. The resulting RNAs of Caco-2 cells stimulated with GroEL or BSA were amplified using the specific primers listed in Table 1. The RT-PCR reaction was carried out with the SuperScript III One-Step RT-PCR system with Platinum *Taq* High Fidelity, in a 50 µl mixture containing 1 µg RNA template, 0.5 µM of the primers Caco-2 ICAM-1 FP and Caco-2 ICAM-1 RP (Table 1), and 2 × Reaction Mix, with a reaction mixture formulated according to the manufacturer's instructions. The cDNA synthesis step was performed at 55 °C for 30 min, and a predenaturation step at 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 57 °C for 30 s and 68 °C for 3 min, and a final extension of 68 °C for 5 min. For the Caco-2 β-actin, the Caco-2 Actin FP and Caco-2 Actin RP primers shown in Table 1 were used as the internal control for RT-PCR (Wang *et al.*, 2000).

For the flow cytometry, 30 µg GroEL or 30 µg BSA (control) were added to each well containing the monolayer of Caco-2 cells, and incubated at 37 °C in 5% CO₂ for 60 min. The monolayer was washed three times with PBS, followed by 1 h incubation with mouse anti-human ICAM-1 conjugated with FITC (1:100 DMEM dilutions). The monolayer was washed three times with PBS, and the fluorescence intensity of the cells was then measured by a FACSCalibur flow cytometer. Analysis of the data was performed using CELLQuest software. Each experiment was repeated four times.

RESULTS AND DISCUSSION

Molecular cloning and sequence analysis of the *groESL* operon of *P. shigelloides*

The GroEL protein of the *P. shigelloides* P-1 strain was purified as described in Methods. The molecular mass of the

Fig. 4. Kinetics of GroEL expression during *in vitro* *P. shigelloides* P-1 strain infection. (a) Various stages during *P. shigelloides* infection, such as attachment, internalization and apoptosis induction. (b) The transcription of *groEL* in Caco-2 cells at various stages during *P. shigelloides* infection was determined by one-step RT-PCR. Amplified products were electrophoresed on 0.8% agarose gels stained with ethidium bromide. The *P. shigelloides* 16S rRNA was used as an endogenous control to confirm an equivalent quantity of template loading. The size of the amplified fragment is indicated on the left. Un, RNA isolated from bacteria that had not been in contact with Caco-2 cells; 10 min, 60 min and 24 h, RNA isolated from bacteria that had been in contact with Caco-2 cells. (c) Western immunoblotting detection of GroEL in *P. shigelloides*-infected Caco-2 cells at the attachment stage during *P. shigelloides* infection. Un, proteins isolated from bacteria that had not been in contact with Caco-2 cells; 10 min, proteins isolated from bacteria that had been in contact with Caco-2 cells. Each lane contained proteins from equivalent numbers of bacteria. Data were from a representative experiment repeated three times.

GroEL was 64 kDa from the results of SDS-PAGE and Western immunoblotting (data not shown). We determined that the N-terminal sequence of the GroEL was AAKDVKFGNDARVKMLXXVNV (X is an unidentified amino acid) by automatic Edman degradation. The *groESL* operon of the *P. shigelloides* P-1 strain was cloned and sequenced as described in Methods. We cloned a 2.7 kb DNA fragment containing the *groESL* operon of *P. shigelloides* by PCR in three different fragments (Fig. 1). The nucleotide sequence of the *groESL* operon of *P. shigelloides* has been submitted to GenBank under accession number AB251936. The sequence analysis of the *groESL* operon (2292 bp) of *P. shigelloides* revealed two ORFs of 294 nucleotides (97 amino acids) for *groES* and 1647 nucleotides (548 amino acids) for *groEL*. Only *groES* was preceded by a potential promoter sequence, -35

Fig. 5. Representative patterns of the effect of GroEL on attachment promotion using a flow cytometer. Dotted lines, control Caco-2 cells alone; solid lines, Caco-2 cells infected with FITC-labelled *P. shigelloides* for 10 min; bold lines, Caco-2 cells pretreated with 10, 20 and 30 µg of GroEL (a) or BSA (b), which were added to the cells for 60 min prior to the addition of FITC-labelled *P. shigelloides*. The amounts of GroEL and BSA are indicated at the left. Data were from a representative experiment repeated three times.

(TTGAAT) and -10 (TATCAT) (data not shown). Compared with other chaperonins, the putative *P. shigelloides* GroEL protein displayed 99 and 98 % identity to those of *E. coli* and *Salmonella typhimurium*, respectively. The putative *P. shigelloides* GroES protein exhibited 100 and 90 % identity, respectively, to *E. coli* and *S. typhimurium* homologues.

Localization of and effect of several stresses upon GroEL

P. shigelloides P-1 strain was fractionated as described in Methods. The proteins in each fraction were separated by SDS-PAGE and analysed for GroEL proteins by Western immunoblotting (Fig. 2a), and the band intensity of GroEL protein by Western immunoblotting was quantified using the densitometry function of the Quantity One software (Fig. 2b). GroEL protein was found in culture supernatant and in both the soluble (cytoplasmic and periplasmic) and membrane fractions (Fig. 2a). The GroEL protein was detected principally in the outer-membrane, secondly in the periplasm and cytoplasmic membrane, and thirdly in the culture supernatant and cytoplasm (Fig. 2a, b). The amount of detected GroEL protein in the outer membrane was about twice that in the periplasm and cytoplasmic membrane, and it was about 12 times that in the culture supernatant and cytoplasm (Fig. 2b). The results obtained by fractionation of the bacterial cells were further corroborated by indirect immunostaining investigations of whole bacteria, using the

anti-GroEL antibody and preimmune serum. The former yielded strong colouring under stress cultivation (high osmolarity medium), whereas the preimmune serum did not react with these cells (data not shown).

The production of *P. shigelloides* GroEL was induced by cultivation under stresses such as heat, iron deprivation and osmotic shock (Fig. 3). The surface association of GroEL was also induced by stress cultivation (osmotic shock) (data not shown).

How the GroEL of *P. shigelloides* ends up on the bacterial surface is unclear, since the protein does not carry a classical signal peptide. The GroEL of *P. shigelloides* was secreted in the culture supernatant after 4 h cultivation, as measured by ELISA and Western immunoblotting (data not shown). Therefore, it can be assumed that the protein is released by autolysis, or secreted by type III or IV secretion machinery, or by a simple system such as an ABC transporter in the extracellular space, and is then absorbed on adjacent bacteria.

Kinetics of *groEL* expression during *in vitro* *P. shigelloides* infection

Next in this study we characterized the *P. shigelloides* *groEL* transcriptional profiles during *P. shigelloides* infection of Caco-2 cells. RT-PCR was performed on synthesized cDNA from samples collected during specific stages of the *P. shigelloides* infection, such as attachment, internalization

Fig. 6. RT-PCR and flow-cytometric analysis of ICAM-1 expressed by GroEL-stimulated Caco-2 cells. (a) The transcription of ICAM-1 in Caco-2 cells stimulated with 10, 20 and 30 μ g GroEL or BSA was determined by one-step RT-PCR. Amplified products were electrophoresed on 0.8% agarose gels stained with ethidium bromide. The β -actin of Caco-2 cells was used as an endogenous control to confirm an equivalent quantity of template loading. The size of the amplified fragment is indicated on the left. (b) The ICAM-1 expression of the Caco-2 cell surface stimulated with 30 μ g GroEL or BSA was determined by flow-cytometric analysis. Data were from a representative experiment repeated three times.

and apoptosis induction (shown diagrammatically in Fig. 4a). When the samples were assayed for expression of the *P. shigelloides* 16S rRNA gene, amplification was observed for all samples, confirming that *P. shigelloides* RNA was present in sufficient quantity and quality to allow detection (Fig. 4b). Expression of *groEL* was upregulated at two points: during the attachment stage and the apoptosis-induction stage. After attachment, during the internalization stages, the *groEL* transcriptional level decreased (Fig. 4b). Densitometric scanning of the upregulated amplified products revealed a two–threefold increase in GroEL expression in comparison with uninfected Caco-2 cells (Un). By Western immunoblotting, the expression of GroEL protein was also induced during the attachment stage (Fig. 4c).

We speculate that host-cell contact could send a signal to *P. shigelloides*, the result of which is increased expression of GroEL followed by the induction of the surface association of GroEL. There is therefore a possibility that GroEL is involved in *P. shigelloides* infection, for example during the attachment and apoptosis-induction stages.

GroEL plays a role in cell attachment and ICAM-1 induction

Next we examined the role of GroEL at the attachment stage. Because chaperone proteins of the GroEL family have an essential function in bacteria, we did not attempt to inactivate the *groEL* gene. Consequently, the Caco-2 cells were incubated with 10, 20 and 30 μ g of GroEL or BSA (control) for 60 min. After that, they were infected with FITC-labelled *P. shigelloides* for 10 min and the fluorescence intensity of the cells was then measured by a FACSCalibur flow cytometer. The mean fluorescence intensities of Caco-2 cells uninfected (Fig. 5, dotted lines) and infected with FITC-labelled *P. shigelloides* for 10 min (Fig. 5, solid lines) were 2.23 and 49.85, respectively. An increase in fluorescence intensity was caused by the adhesion of the bacteria (Tsugawa *et al.*, 2005). Furthermore, the mean fluorescence intensities of Caco-2 cells pretreated with 10, 20 and 30 μ g GroEL were 73.81, 150.93 and 231.64, respectively, and this was related to GroEL in a dose-dependent manner (Fig. 5a, bold lines). On the other hand, the mean fluorescence intensities of Caco-2 cells pretreated with 10, 20 and 30 μ g BSA were 49.23, 50.07 and 53.89, respectively, and in comparison with GroEL, these changed little with the different doses (Fig. 5b, bold line). These results showed that the adhesion of the bacteria to the Caco-2 cells was stimulated efficiently by GroEL.

How does GroEL of *P. shigelloides* promote the attachment of organisms to the Caco-2 cells? Two hypotheses can be proposed. First, the reactivity of the cell is altered by GroEL to promote the attachment of *P. shigelloides*, for example by the overexpression of a receptor involved in attachment. In this paper, we focused on ICAM-1 and examined whether GroEL of *P. shigelloides* was able to increase the cell surface expression of ICAM-1. In fact, the expression of the ICAM-1 gene was upregulated in the Caco-2 cells stimulated with GroEL (Fig. 6a). Furthermore, as detected by the FACSCalibur flow cytometer, stimulation with 30 μ g GroEL increased the fluorescence intensity, which was shown by the expression level of ICAM-1 on the Caco-2 cell surface. It was found that the fluorescence intensity of Caco-2 cells was increased by the GroEL stimulus 1.8 times in comparison with the BSA (control) stimulus (Fig. 6b). These results showed that stimulation with GroEL increased the cellular mRNA levels of ICAM-1, and that as a result the expression of ICAM-1 on the Caco-2 cell surface was upregulated. The adhesion molecule ICAM-1 has been characterized as present in the Caco-2 human intestinal epithelial cell line and identified as a rhinovirus receptor (Kaiserlian *et al.*, 1991). Recent studies have indicated that *Haemophilus influenzae* adheres to Chinese hamster ovary (CHO) cells by binding of P5 fimbriae to ICAM-1 (Avadhanula *et al.*, 2006). However, we did not observe that the attachment promoted with GroEL was completely inhibited with anti-human ICAM-1 antibody, and the percentage inhibition was about 13 %, based on the decrease of the fluorescence intensity of the adhesion assay using a

FACSCalibur flow cytometer (data not shown). Thus, this hypothesis does not appear to be valid for *P. shigelloides*.

As a second hypothesis, it is possible that GroEL binds to a Caco-2 cell surface protein after secretion and then interacts with a bacterial cell surface protein, forming a bridge between *P. shigelloides* and the Caco-2 cells, as described for *Vibrio cholerae* GbpA protein (Kirn *et al.*, 2005). This hypothesis is supported by the fact that the GroEL was detected both in the culture supernatant and at the bacterial cell surface (Fig. 2). We will have to examine this hypothesis.

In conclusion, we demonstrated that the expression of *P. shigelloides* GroEL is induced by contact with host cells, and that the surface association of GroEL is also induced. The GroEL of *P. shigelloides* efficiently promoted the attachment of the bacteria, which is an important function for establishing infection.

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