

Effects of *Helicobacter pylori* water extract on expression of endothelial adhesion molecules

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The present study investigated whether *Helicobacter pylori* water extract induces the upregulation of intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin on human umbilical vein endothelial cells, using an ELISA. The nature of the substances mediating this upregulation was also analyzed. *H pylori* water extract derived from type strain (NCTC 11637) significantly upregulated intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin to the same extent as interleukin-1. Treatments with extracts from clinical strains showed no significant increases in expression of these adhesion molecules. In a fractionation study, approximately 7 kDa fraction showed peak activity. This activity was tolerant to heating and trypsin digestion. These results indicate that *H pylori* water extract contains water-soluble, low-molecular, nonprotein substances which induce upregulation of adhesion molecules on human umbilical vein endothelial cells, suggesting that products of *H pylori* may elicit gastric mucosal inflammation by promoting expression of endothelial adhesion molecules.

Key Words: *E-selectin*; *Helicobacter pylori*; *Human umbilical vein endothelial cells*; HUVEC; ICAM; *Intercellular adhesion molecule-1*; *Leukocyte-endothelial cell interactions*; *Vascular cell adhesion molecule-1*; VCAM

Helicobacter pylori infection, well known as the cause of chronic active gastritis and peptic ulcer, is associated with gastric mucosal injury. Mucosal infiltration by leukocytes is seen histologically in *H pylori* infection (1,2). Infiltration by leukocytes into gastric mucosa is associated with interactions between leukocytes and endothelial cells. This interaction is the initial important event in inflammation and is mediated by adhesion molecules expressed on both cell types (3). This interaction consists of three steps: rolling, sticking and transmigration (4). Leukocyte rolling on the vascular endothelium is mediated mainly by the selectins and selectin ligands (5). P-selectin and E-selectin on activated endothelial cells are involved in an initial slowing of leukocytes. Leukocyte sticking and transmigration are mediated by integrins on leukocytes and immunoglobulin superfamily on endothelial cells (6). Adhesion molecules in the immunoglobulin superfamily

Les effets de l'extrait aqueux de *Helicobacter pylori* sur l'expression des molécules d'adhésion endothéliale

Au moyen du test ELISA, la présente étude a évalué si les extraits aqueux de *Helicobacter pylori* induisent la régulation positive de l'adhésion intercellulaire de la molécule-1, l'adhésion vasculaire de la molécule-1 et l'E-sélectine sur les cellules endothéliales de la veine ombilicale humaine. La nature des substances qui assurent la médiation de cette régulation positive a également été analysée. L'extrait aqueux de *H. pylori* dérivé d'une souche type (NCTC 11637) provoque une importante régulation positive de l'adhésion intercellulaire de la molécule-1, de l'adhésion vasculaire de la molécule 1 et de l'E-sélectine, dans la même mesure que l'interleukine 1. Les traitements contenant des extraits de souches cliniques n'ont démontré aucune augmentation marquée de l'expression de ces molécules d'adhésion. Dans le cadre d'une étude de fractionnement, une fraction d'environ 7 kDa a révélé une activité de pointe. Cette activité était tolérante à la chaleur et à la digestion de trypsine. Ces résultats indiquent que l'extrait aqueux de *H. pylori* contient des substances hydrosolubles non protéiques à faible masse moléculaire qui induisent la régulation positive des molécules d'adhésion endothéliale de la veine ombilicale humaine, ce qui laisse supposer que les produits du *H. pylori* pourraient éliciter l'inflammation de la muqueuse gastrique en favorisant l'expression des molécules d'adhésion endothéliales.

include intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1, and vascular cell adhesion molecule-1 (VCAM-1). Although previous studies have demonstrated that leukocytes are chemoattracted by cytokines from epithelial cells (7) or activated by *H pylori* proteins (8-10), only a few authors have investigated the activation of endothelial cells, the initial important event in inflammation, in *H pylori*-induced mucosal injury in vivo (11,12). Furthermore, there are no reports examining whether products of *H pylori*, but not intact living *H pylori*, induce upregulation of adhesion molecules on endothelial cells in vitro. Because adherence between endothelial cells and *H pylori*, a noninvasive bacterium, cannot occur within gastric mucosa, it is very important to estimate activation of endothelial cells by the stimulation of some substance secreted or released from *H pylori*. In the present study, we examined whether *H pylori* water extract (HPE)

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TABLE 1
Lipopolysaccharide concentration in water extracts of
***Helicobacter pylori*, and *cagA* and *vacA* positivity of strains**

Strain	<i>cag A</i>	<i>vac A</i>	Lipopolysaccharide (EU/mL)
NCTC11637	+	+	0.65
H13	+	+	4.16
H16	+	+	1.29
930	+	+	0.65
933	+	+	1.58

induces the upregulation of three important adhesion molecules, ICAM-1, VCAM-1 and E-selectin, on cultured human umbilical vein endothelial cells (HUVEC). We also analyzed the nature of the substances which mediate such upregulation.

MATERIALS AND METHODS

HPE

HPE were prepared from a type strain (NCTC11637) and clinical strains (H13, H16, 930 and 933) of *H pylori* that were isolated from gastric antral biopsies of patients with gastric ulcers (13). These strains were tested for the presence of the *cagA* and *vacA* genes by a reverse transcription-polymerase chain reaction using specific primer pairs (14) (Table 1). The organism was grown on blood agar plates as previously described (15). The growth medium consisted of Bacto brain-heart infusion, with 0.5% Bacto (Difco, USA) yeast extract, 2.0% Bacto agar and 7% fresh horse blood. Plates were inoculated and the bacteria were grown for 12 passages in a CO₂ incubator with 12% CO₂ and 100% humidity for 48 h. Cells were harvested with sterile cotton swabs and suspended in distilled water using 1.0 mL per plate (10⁹ to 10¹⁰ bacteria). The cell suspension was kept at room temperature for 20 min before centrifugation at 12,000 rpm (17,000 g) for 15 min. The resulting supernatant, the initial water extract with no preservatives added, was stored at -20°C until needed. Before use, the extract was brought to room temperature and centrifuged at 18,000 rpm (38,700 g) for 20 min, and the pellet was discarded. The supernatant was then passed through a 0.2 micron Acrodisc (Gelman Science, USA) syringe-adapted filter. This procedure removed much of the high molecular weight complex material, which consisted mainly of membrane vesicles and intact flagellae.

Endothelial cells

HUVEC were harvested from umbilical cords by collagenase treatment as previously described (16). The cells were plated in Medium 199 (Gibco, USA) supplemented with 10% heat-inactivated fetal calf serum (JRH Biosciences, USA), thymidine (2.4 mg/L; Sigma Chemical Co, USA), glutamine (23 mg/L; Gibco, USA), heparin sodium (10 IU/mL; Mochida Pharmaceutical Co Ltd, Japan), penicillin G/streptomycin/Fungizone (Gibco, USA) and endothelial cell supplement: mitogen (80 mg/mL; Biomedical Technologies Inc, USA). The cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂ and expanded by brief trypsinization (0.25% trypsin in phosphate buffered saline (PBS) containing 0.02% EDTA). First through third passage HUVEC were seeded into gelatin-coated (0.1%), 96-well tissue culture plates and used when confluent.

Monoclonal antibodies

The monoclonal antibodies (mAbs) directed against ICAM-1 (LB-2) (17), VCAM-1 (E1/6) (18), and E-selectin (H18/7) (19) were supplied by Becton Dickinson, USA. These antibodies reportedly block the adhesive reactions of leukocytes and endothelial cells under static conditions.

ELISA

ELISA was used to assess the binding of mAbs to the endothelial cell monolayers. Confluent HUVEC monolayers prepared in 96-well plates were coincubated with interleukin (IL)-1 β (20 U/mL), *Escherichia coli* lipopolysaccharide (LPS) (0.74 EU/mL, Sigma Chemical Co, USA), or HPE (10%) for 6 h at 37°C in a CO₂ incubator. Immediately after pretreatment, the cells were fixed by the addition of 1% paraformaldehyde in PBS for 15 min at room temperature. After washing with the buffer (Hanks' balanced salt solution with 1% bovine serum albumin and 0.01% sodium azide), mAbs directed against ICAM-1, VCAM-1 or E-selectin were added and incubated for 30 min. After another wash, the cells were incubated for 15 min with biotinylated anti-rabbit and anti-mouse immunoglobulin in PBS containing carrier protein and 15 mM sodium azide (Dako Co, USA). Then the wells were washed, incubated in horseradish peroxidase-conjugated streptavidin (Dako Co, USA) (in PBS containing carrier protein and antimicrobial agents for 15 min, and were washed again in PBS. Finally, o-phenylenediamine dihydrochloride (0.4 mg/mL in citrate buffer, pH 5) with 0.012% hydrogen peroxide was added and incubated for 10 min, the reaction was stopped by the addition of 1.5 M sulfuric acid. The plates were then read at 492 nm in a Micro plate reader (Tosoh, Japan) to quantitate the amount of bound antibody. Data were expressed as a ratio of each sample optical density (OD) to control OD (IL-1 β).

Characterization of HPE

Gel filtration was used to estimate the molecular weight of the adhesion molecule-inducing factor in HPE from NCTC11637. Freeze-dried extract (10 mg) mixed with 1.5 mL of 150 mM NaCl was eluted through a HiPrep Sephacryl S-100 HR column (Pharmacia Fine Chemicals, USA), with collection of 2 mL fractions. Each fraction (10%) was estimated for adhesion molecule-induction activity. Data were expressed as a ratio of each sample OD to control OD (IL-1 β). To further characterize the adhesion molecule-inducing factor in HPE, the active fractions were heated (56°C for 30 min or 100°C for 10 min) in a water bath or incubated them with trypsin (250 mg/mL, final concentration) for 1 h at 37°C. Treated and untreated fractions were assayed for adhesion molecule-induction activity. Data were expressed as a ratio of each sample OD to control OD (IL-1 β). To test for a relationship between induction of adhesion molecules and the presence of LPS, LPS concentrations in the five HPE were measured by a turbidimetric *Limulus* amoebocyte lysate assay using the *Limulus* ES-J Test Wako (Wako Pure Chemical Industries Ltd, Japan) with a Toxinometer ET-301 BL (Wako Pure Chemical Industries Ltd, Japan).

Statistical analysis

All values were expressed as the mean \pm SE. Data were compared using analysis of variance (ANOVA) followed by Scheffe's test. $P < 0.05$ was considered statistically significant.

RESULTS

LPS concentrations in HPE, and *cagA* and *vacA* gene positivity of strains

LPS concentrations in the five HPEs ranged from 0.65 EU/mL to 4.16 EU/mL (Table 1). Final LPS concentrations in medium containing 10% HPE ranged from 0.065 EU/mL to 0.416 EU/mL. All strains were positive for both *cagA* and *vacA* genes.

Surface expression of ICAM-1, VCAM-1 and E-selectin on HUVEC exposed to HPE

Unstimulated HUVEC constitutively expressed ICAM-1 but little VCAM-1 and E-selectin (Figure 1). Coincubation with 20 U/mL of IL-1 β (positive control) for 6 h significantly ($P < 0.05$ versus unstimulated) upregulated surface expression of ICAM-1, VCAM-1 and E-selectin on HUVEC.

Ten per cent HPE from NCTC11637 significantly upregulated ICAM-1 ($P < 0.05$ versus unstimulated), VCAM-1 ($P < 0.05$ versus unstimulated) and E-selectin ($P < 0.05$ versus unstimulated). HPE from H13, H16, 930 and 933 did not induce any significant increase in expression of ICAM-1, VCAM-1 and E-selectin. Because the LPS concentrations in medium containing 10% HPE were less than 0.74 EU/mL as shown in Table 1, the inducing ability of 0.74 EU/mL of *E coli* LPS, which has more biological activities than *H pylori* LPS we examined (20,21). The results showed that 0.74 EU/mL of *E coli* LPS did not upregulate ICAM-1, VCAM-1 and E-selectin.

Characterization of HPE from NCTC11637

HPE from NCTC11637 was divided into 61 fractions (fraction 20 to 80) by gel filtration. As shown in Figure 2, fractions 58 to 65 (representing molecules of approximately 7 kDa) exhibited the peak of the inducing activity for the expression of ICAM-1, VCAM-1 and E-selectin.

The authors focused on fraction 62, which corresponded to the peak activity. As shown in Figure 3, the inducing activity of fraction 62 for ICAM-1 expression was not affected by heating (56°C for 30 min or 100°C for 10 min) or coincubation with 250 mg/mL of trypsin.

DISCUSSION

In the present study, we demonstrated that HPE derived from NCTC11637 induces upregulation of ICAM-1, VCAM-1 and E-selectin on HUVEC. These adhesion molecules play very important roles in leukocyte-endothelial cell interactions which are the initial steps in local inflammation (4). Inflammatory mediators or cytokines which are increased on tissues by various stimulants activate endothelial cells, and ICAM-1, VCAM-1, E-selectin and other adhesion molecules are expressed on endothelial cells (3). ICAM-1 has been proposed to mediate sticking and transmigration of neutrophils (6). VCAM-1 can mediate rolling, sticking and transmigration of mononuclear leukocytes (22). E-selectin plays an important role in rolling of leukocytes (5,19). We have reported that HPE induces adhesion between neutrophils and endothelial cells via upregulation of CD11/CD18, a ligand for ICAM-1, expressed on neutrophils in vitro and in vivo (15). This previous study (15) showed the importance of neutrophil activation in *H pylori*-induced gastric mucosal injury. However, there are only a few in vivo studies of the expression of adhesion molecules on endothelial cells in *H pylori*-infected gastric mucosa (11,12).

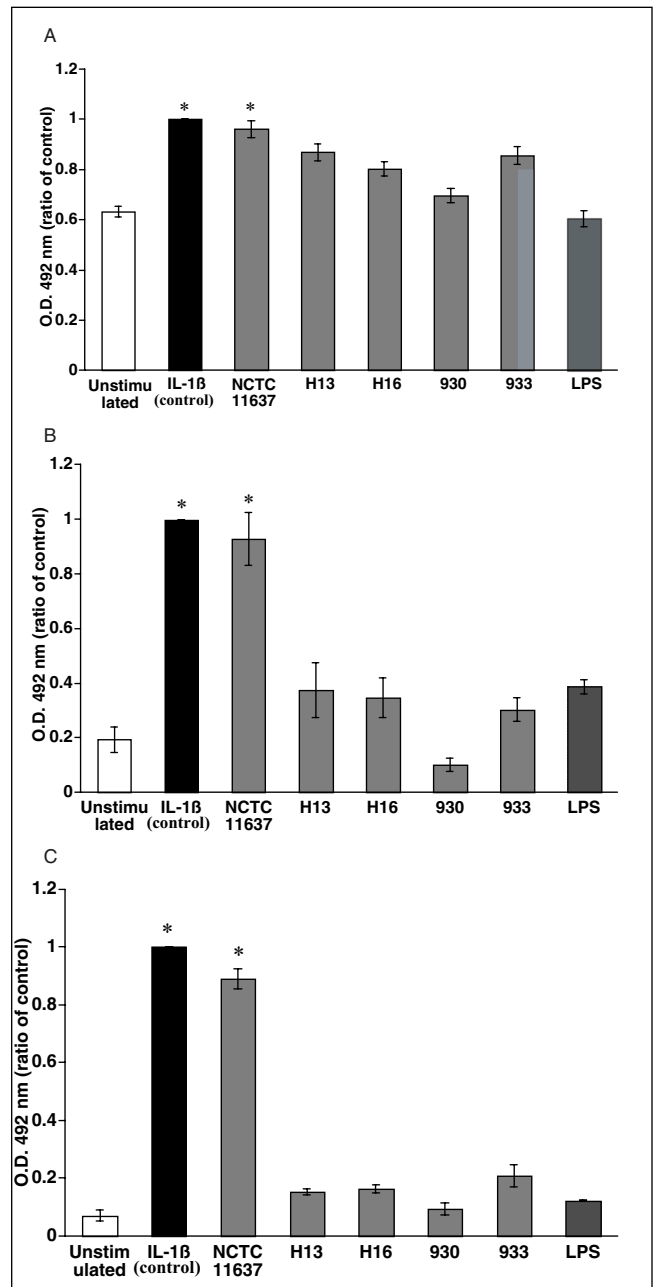


Figure 1 Surface expression of intercellular adhesion molecule (ICAM)-1 (A), vascular cell adhesion molecule (VCAM)-1 (B), and E-selectin (C) on human umbilical vein endothelial cells (HUVEC) stimulated by 20 U/mL of interleukin beta (IL-1 β), 10% *Helicobacter pylori* water extracts (from NCTC11637, H13, H16, 930, or 933), or 0.74 EU/mL of lipopolysaccharide (LPS). ICAM-1, VCAM-1, and E-selectin on HUVEC were assessed by enzyme-linked immunosorbent assay 6 h after stimulation. Data represent the mean \pm SE of three experiments in triplicate. * $P < 0.05$ compared with unstimulated HUVEC. OD Optical density

Recently, it has been reported that coincubation between the *H pylori* organism and cultured endothelial cells results in the increased expression of endothelial adhesion molecules (23,24). However, in the present study, because there is little opportunity for direct contact between bacteria and endothelial cells in gastric mucosa, we used HPE which contains water-soluble

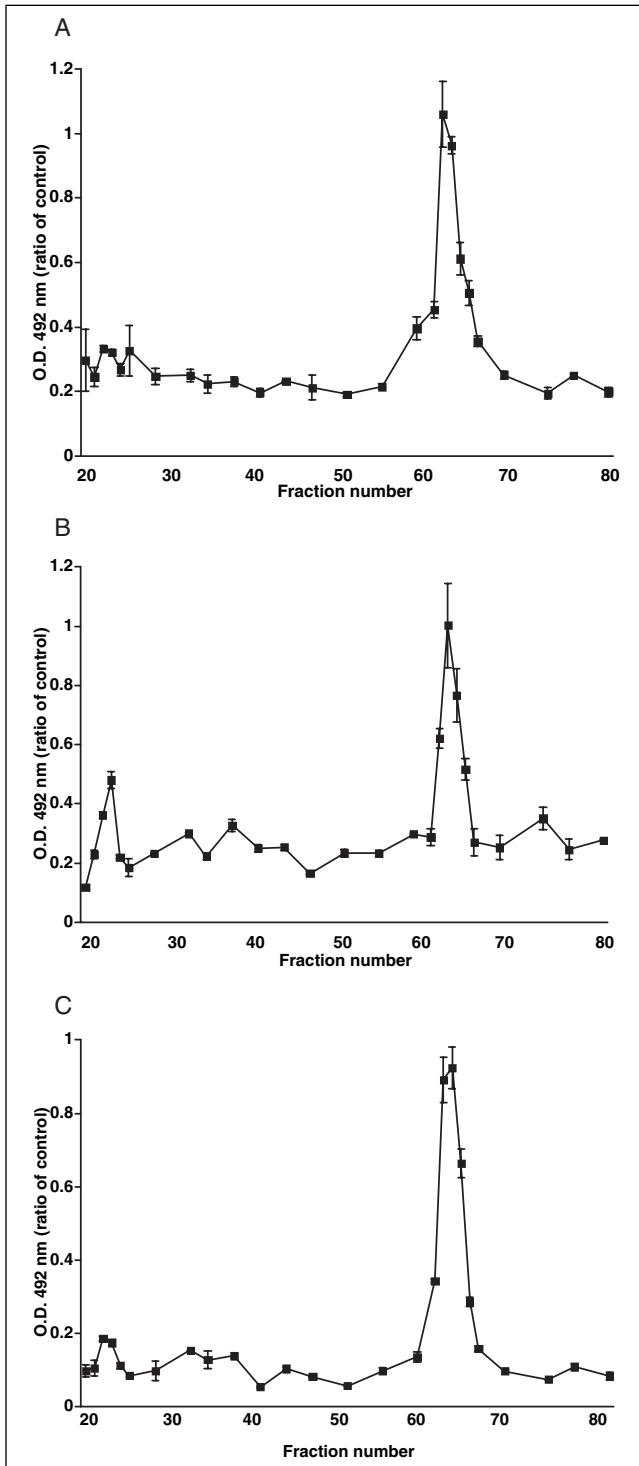


Figure 2) Surface expression of intercellular adhesion molecule (ICAM)-1 (A), vascular cell adhesion molecule (VCAM)-1 (B), and E-selectin (C) on human umbilical vein endothelial cells (HUVEC) stimulated by 10% of gel-filtrated fractions of Helicobacter pylori water extract from NCTC11637. ICAM-1, VCAM-1 and E-selectin on HUVEC were assessed by enzyme-linked immunosorbent assay 6 h after stimulation. Data represent the mean \pm SE of three experiments in duplicate. OD Optical density

components of the membrane or the cytosol. A recent report showed that *H pylori* alters the barrier properties of the epithelium in vitro (25). Therefore, HPE, especially low molecular

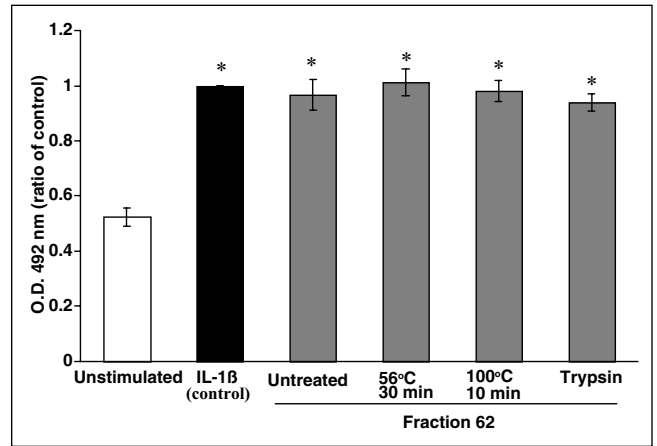


Figure 3) Effects of heat and trypsin treatment on the activity of fraction 62 for upregulation of intercellular adhesion molecule-1 on human umbilical vein endothelial cells (HUVEC). Treatment details are given in the text. Data represent the mean \pm SE of three experiments in duplicate. * $P < 0.05$ compared with unstimulated HUVEC. IL-1 β Interleukin beta; OD Optical density

substance in HPE, may permeate into the gastric mucosa and directly affect the endothelial cells. Our study is the first observation that products of *H pylori*, but not intact living bacteria, upregulates ICAM-1, VCAM-1 and E-selectin on endothelial cells. This suggests the following mechanism of *H pylori*-induced gastric mucosal injury: endothelial cells are activated by water-soluble substances released or secreted from the organism, ICAM-1, VCAM-1 and E-selectin are upregulated, and subsequently, leukocytes adhere to endothelial cells via increased adhesion molecules. Leukocytes adhering to endothelial cells emigrate into the interstitium by IL-8 released from gastric epithelial cells. Reactive oxygen species or proteases derived from leukocytes are implicated in the gastric mucosal injury associated with *H pylori*.

The *cagA* and *vacA* genes have been reported to be related to the pathogenicity of *H pylori* strains, especially with respect to cytokine production from epithelial cells (7,26,27). In the present study, though all strains had both genes, there was no relation between these genes and upregulation of adhesion molecules on HUVEC. In our fractionation study, low-molecular-weight fractions (approximately 7 kDa) showed peak adhesion molecule-inducing activity. This fraction showed a nonprotein character, resisting heat and trypsin incubation. This substance may be the same one as inducing IL-8 production from gastric epithelial cells, as previously described (28).

Some investigators have reported that water-soluble components derived from *H pylori* organisms activate leukocytes. Evans et al (8) purified a neutrophil-activating protein (150 kDa protein), which upregulated CD11b/CD18 on neutrophils. Tufano et al (10) have demonstrated that porins (30 kDa proteins in surface membranes) affect neutrophil chemotaxis and induce cytokine production by monocytes. Mai et al (29) have shown that a surface protein, urease, induces chemotactic activity in neutrophils and monocytes, and activates monocytes (30). Craig et al (31) have reported a low molecular (less than 3 kDa), heat-stable, acid-resistant factor that is chemotactic for monocytes and neutrophils. In the present study, we described a low molecular (approximately 7 kDa)

nonprotein substance. These results indicate that the adhesion molecule-inducing factor in HPE is neither neutrophil-activating protein, urease, nor the substance reported by Craig et al. Although LPS is present in membranes of Gram-negative bacteria such as *H pylori* and is an important nonprotein proinflammatory molecule, previous studies have shown that *H pylori* LPS has less biological activity than *E coli* LPS (20,21). We did not examine *H pylori* LPS directly, but 0.74 EU/mL of *E coli* LPS did not upregulate adhesion molecules on HUVEC. In addition, final concentrations of LPS in HPE derived from NCTC11637 were less than 0.74 EU/mL, and no correlation was found between adhesion molecule-inducing activities and LPS concentrations in HPE. Darveau et al (32) reported that *H pylori* LPS did not promote the E-selectin expression on HUVEC. Taken altogether, these findings

suggest that adhesion molecule-inducing substance in HPE may not be associated with LPS. Further studies are needed to identify the factor in HPE responsible for the production of endothelial adhesion molecules.

In summary, we have demonstrated that a water-soluble, low molecular, nonprotein substance in HPE induces upregulation of ICAM-1, VCAM-1 and E-selectin on HUVEC. These results suggest that *H pylori* infection may elicit endothelial-dependent interactions with leukocytes by promoting expression of endothelial adhesion molecules, and followed by gastric mucosal inflammation.

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