Stimulation of adhesion molecule expression by *Helicobacter pylori* and increased neutrophil adhesion to human umbilical vein endothelial cells

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Abstract ***Helicobacter pylori*** **upregulates endothelial adhesion molecules but the pattern is unclear. Human umbilical vein endothelial cells (HUVEC) were exposed to control medium or *H. pylori* 60190. Binding of monoclonal antibodies against P-selectin, E-selectin, vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) was determined using enzyme-linked immunosorbent assay. Binding of polymorphonuclear leukocytes to HUVEC was determined on cells exposed as above. After 6 h exposure to *H. pylori*, there were 30%, 124%, 167% and 100% increases in P-selectin, E-selectin, VCAM-1 and ICAM-1 levels and a 400% increase in polymorphonuclear leukocyte adhesion in HUVEC exposed to *H. pylori*. Effects of incubation for other intervals between 0 and 18 h are also described. *H. pylori* exerts some of its effects on gastric mucosa via gastric vasculature. This study gives insight into the pattern of *H. pylori*-associated endothelial adhesion molecule upregulation.**

Key words: Endothelial; Selectin; Adhesion molecule; Neutrophil; *Helicobacter pylori*

1. Introduction

The urease-producing microorganism *Helicobacter pylori* plays a critical role in peptic ulcer disease, with up to 95% of duodenal ulcers and 80% of gastric ulcers associated with *H. pylori* infection [1]. *H. pylori* is also implicated in the development of certain gastric cancers, namely gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma [2]. The exact pathogenic mechanisms by which *H. pylori* exerts its effects on the stomach are still not entirely understood but there are certainly several factors to take into account. There is growing evidence that *H. pylori* exerts some of its effects on the stomach by actions on the gastric vasculature. Leukocyte–endothelial cell interactions play critical roles in inflammatory conditions of the gastrointestinal tract [3]. We have previously demonstrated upregulation of both cyclooxygenase-1 and -2 by *H. pylori* in an endothelial cell model [4]. *H. pylori* is a non-invasive organism and resides in the mucus layer overlying the gastric mucosa but it is felt that shed or secreted products may transmigrate and act, indirectly through other messengers or directly, to stimulate neutrophil activity and migration into the gastric mucosa [5–8]. During inflammation, recruitment of leukocytes to the endothelium is followed by migration to the gastric epithelial layer. This is a key factor involved in the gastroduodenal inflammation caused by *H. pylori* [9]. In vitro studies have shown that *H. pylori* extracts are chemotactic for monocytes and neutrophils [7,8]. In order for activated leukocytes to reach the gastric mucosa, they first need to adhere to the vascular endothelium [10,11]. Leukocyte adherence and emigration from the vasculature have been shown in mesenteric venules super-perfused with *H. pylori* [12]. There was also increased albumin leakage from vessels and platelet–leukocyte aggregation [12,13]. More recently, it has been shown that water extracts of *H. pylori* induce expression of CD11b/CD18 on granulocytes. However, it is still not entirely clear to which adhesion molecules on endothelial cells the activated neutrophils bind as the pattern of upregulation varies between studies [14,15].

There are several families of adhesion molecules that are expressed on the endothelial cell surface either constitutively or following endothelial cell activation. Selectins are a family of adhesive receptors on endothelial cells, platelets and leukocytes [16,17]. P-selectin is located either in the α-granules of platelets or in the Weibel–Palade bodies of endothelial cells and is translocated to the cell surface upon activation by inflammatory stimuli such as thrombin or histamine. L-selectin is also expressed on the surface of leukocytes following similar activation and E-selectin is expressed on endothelial cells following exposure to inflammatory cytokines. Vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are members of the immunoglobulin superfamily that are expressed following endothelial cell exposure to cytokines for several hours [18,19].

The aim of this study was to examine the early effects of *H. pylori* on the expression of vascular endothelial adhesion

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Abbreviations: HUVEC, human umbilical vein endothelial cells; PMN, polymorphonuclear leukocytes; ICAM, intercellular adhesion molecule; VCAM, vascular adhesion molecule; ANOVA, analysis of variance; IL, interleukin; MPO, myeloperoxidase; LPS, lipopolysaccharide; FBS, foetal bovine serum; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline

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molecules and to measure the subsequent leukocyte–endothelial interactions.

2. Materials and methods

All laboratory reagents were from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Culture medium (M199), foetal bovine serum (FBS), antibiotics, Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS) and HEPES were from Life Technologies (Paisley, UK).

2.1. Culture of human umbilical vein endothelial cells (HUVEC)

HUVEC were isolated and grown in culture as described by Jaffe et al. [20]. Briefly, cells were isolated from human umbilical vein cords by enzymatic digestion using collagenase type II (Worthington Chemical Company, Freehold, NJ, USA) and grown in M199 supplemented with 20% FBS and penicillin/streptomycin (100 units). Endothelial cell mitogen (Biogenesis, Poole, UK) was solubilised with heparin (1000 U) and added at a final concentration of 100 μg/ml. Cells were grown to confluence on 0.2% gelatin-coated 75-cm^2 flasks (Costar, Cambridge, MA, USA) in a humidified atmosphere at 37°C in 5% CO_2. Confluent primary cultures were routinely passaged by trypsin/EDTA or EDTA digestion and expanded through passages 2–3. Ethical approval for use of umbilical cords collected from Holles Street Maternity Hospital for isolation of HUVEC was given by the Royal College of Surgeons in Ireland ethics committee.

2.2. Enzyme-linked immunosorbent assays

Binding of monoclonal antibodies to HUVEC, that were either resting or stimulated with interleukin (IL) 1β or *H. pylori* 60190, was carried out according to the method of Murphy et al. [21]. Briefly, HUVEC were grown to confluence on 96-well microtitre plates and stimulated with IL-1β (100 U/ml) or *H. pylori* for 3, 6, 12 and 18 h. Wells were then emptied and washed three times with RPMI/2.5% FBS containing sodium azide (0.1%). HUVEC were then incubated with monoclonal antibodies directed against P-selectin, E-selectin, ICAM-1 and VCAM-1 (R&D Systems, Minneapolis, MN, USA) in RPMI/2.5% FBS for 1 h, washed again three times and added with a goat anti-mouse IgG/horseradish peroxidase conjugate in PBS. ABTS was used to detect bound monoclonal antibody.

2.3. Polymorphonuclear leukocyte (PMN) isolation

Routine isolation of PMN was performed under sterile conditions using Ficoll-Hypaque endotoxin-free Histopaque in a gradient density centrifugation technique followed by erythrocyte lysis [22]. Whole blood was drawn from donors on acid/citrate/dextrose, carefully layered on gradient and centrifuged at 2500 rpm for 25 min at room temperature. Lysing buffer (0.154 M ammonium chloride, 19 mM potassium bicarbonate, 1 mM EDTA) was added to the pellet containing erythrocytes and PMN for 10 min, centrifuged at 1500 rpm for 5 min and the PMN pellet washed three times with PBS. Cells were washed twice in PBS in RPMI for adhesion studies.

2.4. PMN–HUVEC adhesion assays

HUVEC were grown to confluence on 24-well plates and then added with M199/2.5% FBS in the presence or absence of IL-1β (100 U/ml) or *H. pylori* for 6 h. Isolated PMN were suspended in RPMI media at $0.5–1\times10^6$ cells/ml then added to each of the 24-well plates and incubated for 20–30 min at 37°C. Wells were then washed carefully three times to remove unbound PMN and bound cells lysed and assayed for myeloperoxidase (MPO) activity [23,24]. Lysed cells were added with HBSS (0.3 ml), phosphate buffer (pH 6.4, 0.2 ml), dimethoxybenzidine (*O*-dianisidine HCl (50 μl) and H_2O_2 (50 μl). The colour product was measured spectrophotometrically (Titertek, Multiscan) at 450 nm. Standard curves were plotted for each assay using PMN ranging between 0.03×10^5 and 2×10^5 cells/ml and the number of adherent cells determined from this. There is no MPO in HUVEC [24].

2.5. Statistics

Data were analysed using analysis of variance (ANOVA) followed by Dunnett multiple comparisons test if the ANOVA showed significant difference when comparing all columns. The data are expressed as mean ± S.E.M.

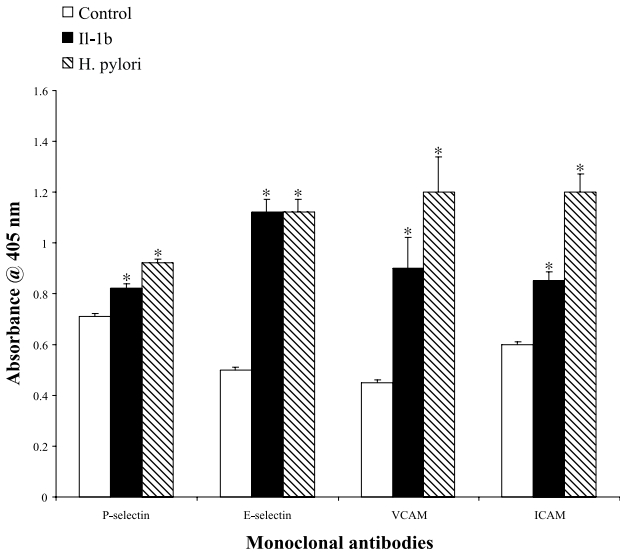

Fig. 1. *H. pylori* induces adhesion molecule expression in endothelial cells. HUVEC were seeded on 96-well tissue culture plates, grown to confluence and treated with IL-1β (100 U/ml) or *H. pylori* for 6 h. Antibodies directed against P-selectin, E-selectin, VCAM-1 and ICAM-1 (5 μg/ml) were then added and binding measured as described in Section 2. Binding was increased to varying degrees on all treated cells compared to resting cells. Data represent duplicate determinations from four independent experiments. $^*P<0.01$ against control for each of P-selectin, E-selectin, VCAM and ICAM.

3. Results

3.1. H. pylori induces endothelial cell adhesion molecule expression (Fig. 1)

HUVEC were exposed to *H. pylori* 60190 over a time course of 3, 6, 12 and 18 h. The maximum increase in P-selectin, E-selectin, and VCAM-1 after stimulation with *H. pylori* or IL-1β occurred at 3–6 h, other than for ICAM-1 where the maximum induction occurred at 12 h. Fig. 1 shows the results of the 6-h incubations. Repeated measures ANOVA comparing the effects of IL-1β and *H. pylori* after 6 h on adhesion molecule expression compared to unstimulated cells was performed for P-selectin ($P<0.0001$), E-selectin ($P<0.0001$), VCAM ($P=0.0004$) and ICAM ($P<0.0001$). There were 30% (±1.4%), 124% (±5%), 167% (±14%) and 100% (±7%) increases in the absorbance readings for P-selectin, E-selectin, VCAM-1 and ICAM-1 respectively in HUVEC exposed to *H. pylori* for 6 h relative to controls (Fig. 1). Dunnett multiple comparisons tests were performed for each of the adhesion molecules, and the effects of IL-1β ($n=4$, $P<0.01$ v. control) and *H. pylori* ($n=4$, $P<0.01$ v. control) were significant for each of the adhesion molecules. Fig. 2 summarises the patterns over the various time courses.

3.2. H. pylori-induced endothelial cell adhesion molecule expression mediates neutrophil adhesion (Fig. 3)

PMN were isolated from freshly drawn blood as described in Section 2 and layered onto HUVEC for 30 min. ANOVA comparing the effects of IL-1β and *H. pylori* on PMN adhesion against control cells was significant at $P=0.0018$. Dunnett multiple comparisons test showed that IL-1β significantly increased the adhesion of PMN to HUVEC ($n=3$, $P<0.05$ v. control). The difference between control cells and those ex-

posed to *H. pylori* was even more significant ($n = 3$, $P < 0.01$ v. control). There was a 250 ± 9% increase in PMN adhesion to IL-1β-treated HUVEC and an even more pronounced 400 ± 12% increase on the *H. pylori*-treated cells. The enhanced expression on the *H. pylori*-treated cells correlates well with the increase in adhesion molecule expression as seen in Fig. 1.

4. Discussion

Early studies using immunohistochemistry showed that lymphocyte infiltration in the gastric epithelium is important in the inflammatory response to *H. pylori* infection. There are several sources of evidence suggesting that some of the deleterious effects of *H. pylori* may be mediated through actions on the gastric microvasculature [3,12,25–27]. Several groups have shown that *H. pylori* promotes leukocyte adhesion to endothelial cells and a chemotactic response resulting in emigration of neutrophils and accumulation in the interstitium [7,8,13].

It is clear that endothelial adhesion molecules play a critical role in the neutrophil infiltration in *H. pylori*-associated inflammation [12,14]. We chose an in vitro model using HUVEC to determine the effects of *H. pylori* on the expression of endothelial P-selectin, E-selectin, VCAM-1 and ICAM-1. Previous work in this field has shown upregulation of a variety of adhesion molecules in endothelial cells but the pattern of upregulation varies significantly between studies [12,14]. It has also been suggested that the pattern of adhesion molecule expression varies between different inflammatory settings, for example *H. pylori*-associated gastritis and inflammatory bowel disease [15]. In this study, we found that *H. pylori* caused upregulation of endothelial E-selectin and P-selectin as well as VCAM-1 and ICAM-1.

In *H. pylori* models, some studies have highlighted the roles of VCAM-1 and ICAM-1 in neutrophil adhesion to endothelium [15,28]. ICAM-1 is known to be important in leukocyte adherence and emigration and is constitutively expressed on endothelial cells [18]. Activation of endothelial cells with lipopolysaccharide (LPS) causes upregulation of ICAM-1 expression [29,30]. Indeed, maximum levels of leukocyte adherence are associated with ICAM-1 expression [31]. The importance of induction of the constitutively expressed ICAM-1 molecule by *H. pylori* was confirmed in our study in keeping with previous work. VCAM-1 is also involved in adherence of leukocytes but is not present on resting HUVEC. However, bacterial LPS can elicit upregulation [32]. In our work, there was little baseline expression of VCAM-1 but marked upregulation with IL-1β and even greater induction with *H. pylori*.

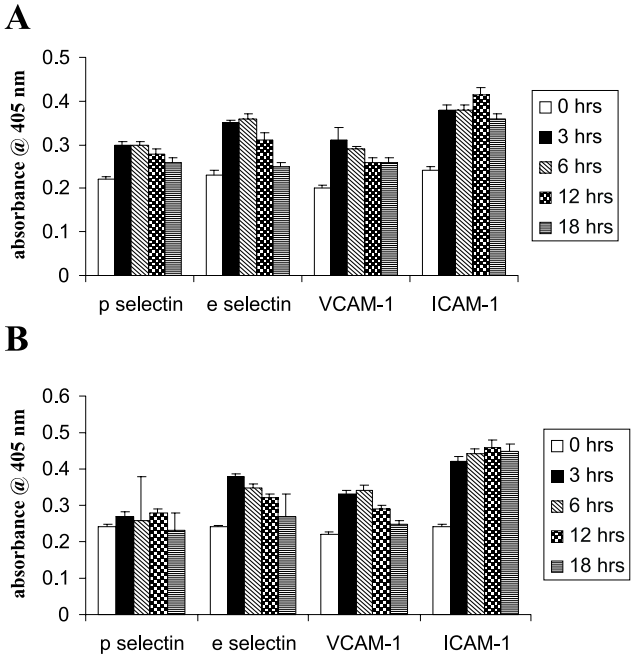

Fig. 2. Time course of effects of *H. pylori* (a) and IL-1β (b) on adhesion molecules.

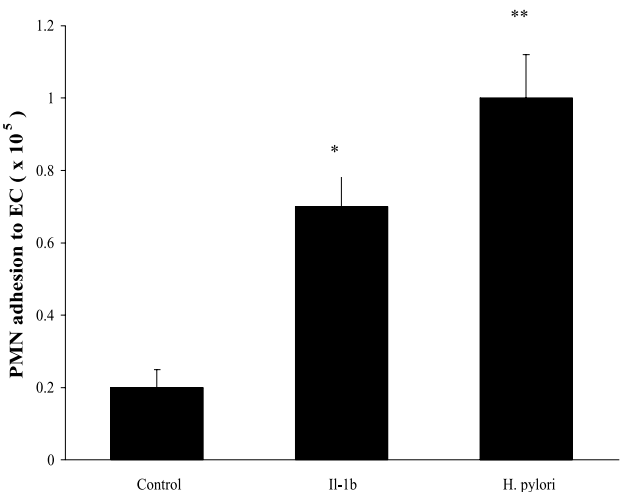

Fig. 3. *H. pylori*-induced endothelial cell adhesion molecule expression mediates neutrophil adhesion. HUVEC were seeded on 24-well tissue culture plates, grown to confluence and treated with IL-1β (100 U/ml) or *H. pylori* for 6 h. PMN were isolated and added to cells for 30 min, unbound cells washed off and the number of adhered cells quantified by measurement of MPO activity as described in Section 2. Data represent triplicate determinations from three independent experiments. $*P < 0.05$, $**P < 0.01$ against control.

Our findings in relation to the involvement of the selectin family of adhesion molecules are interesting. We found upregulation of both P- and E-selectin with *H. pylori*. Hatz et al. [15] suggest using immunohistochemical studies that *H. pylori* does not lead to upregulation of P-selectin. They did however comment that an enhanced steady state of P-selectin expression in *H. pylori*-associated gastritis may be missed by immunohistochemical methods. P-selectin is normally found in the α granules of platelets and Weibel–Palade bodies of endothelial cells [33]. These granules are translocated to the cell surface and expressed briefly upon activation with agents such as thrombin or histamine, before returning to basal levels within minutes [34]. Previous work has shown that, in addition to mobilisation of the preformed pool, bacterial endotoxin exerts transcriptional regulation upon P-selectin expression and is responsible for the more prolonged elevation of P-selectin [35]. Upregulation of P-selectin is also known to be important in *H. pylori* activation of platelets [36].

Unlike P-selectin, there is no E-selectin present normally on the surface of resting endothelial cells and neither is there a preformed pool [16]. Darveau et al. [37] showed in an in vitro study that intact *H. pylori* cell wall preparations or LPS of *H. pylori* did not upregulate E-selectin in a HUVEC model. However, they found that other bacteria such as *Escherichia*

coli did upregulate E-selectin. The suggestion was that lower gut bacteria such as *E. coli* may in part be responsible for the E-selectin expression seen in inflammatory conditions of the lower gastro-intestinal tract such as Crohn's disease. Several studies have shown that E-selectin is the first adhesion molecule upregulated in response to LPS or IL-1 but that its expression peaks at around 3 h and is near baseline levels by 8 h [38]. One conclusion from these studies was that E-selectin does not appear to be involved in continued leukocyte adherence to endothelium and rolling after the initiation of the inflammatory process and that it has no or little involvement in the inflammation associated with *H. pylori*. We found after exposure of endothelial cells to *H. pylori* for a 6-h period that there was an increase in E-selectin expression was significantly greater than that for P-selectin. Although we did not assess leukocyte rolling, we hypothesise that E-selectin may be involved in the continued rolling of leukocytes in *H. pylori*-associated inflammation and that E-selectin may be more important in this process than originally thought.

Although we did not confirm that the five-fold increase we observed in neutrophil adhesion to activated endothelial cells is dependent on adhesion molecule expression, previous studies have shown this to be the mechanism [12,14]. Neutrophil infiltration into the submucosa is a prominent feature of *H. pylori*-associated inflammation in the stomach.

In this study, we show that as well as VCAM-1 and ICAM-1, E-selectin and P-selectin also play important roles and that E-selectin may be more involved in the ongoing inflammatory process than originally thought. This pattern of adhesion molecule induction allows adherence to endothelium and subsequent transmigration of neutrophils. This supports the idea that *H. pylori* exerts some of its effects on the gastric mucosa through effects on the gastric vasculature as well as the direct injurious effects on the gastric mucosa previously described. Understanding the pattern of adhesion molecule expression in *H. pylori*-associated disease will allow further insight into the pathogenesis of *H. pylori*-associated inflammation and its progression to ulcer disease or gastric cancer.

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