P-selectin mediates neutrophil adhesion to endothelial cell borders

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Abstract: During an acute inflammatory response, endothelial P-selectin (CD62P) can mediate the initial capture of neutrophils from the free flowing bloodstream. P-selectin is stored in secretory granules (Weibel-Palade bodies) and is rapidly expressed on the endothelial surface after stimulation with histamine or thrombin. Because neutrophil transmigration occurs preferentially at endothelial borders, we wished to determine whether P-selectin-dependent neutrophil capture (adhesion) occurs at endothelial cell borders. Under static or hydrodynamic flow (2 dyn/cm^2) conditions, histamine (10^{-4} M) or thrombin (0.2 U/mL) treatment induced preferential ($\geq$75%) neutrophil adhesion to the cell borders of endothelial monolayers. Blocking antibody studies established that neutrophil adhesion was completely P-selectin dependent. P-selectin surface expression increased significantly after histamine treatment and P-selectin immunostaining was concentrated along endothelial borders. We conclude that preferential P-selectin expression along endothelial borders may be an important mechanism for targeting neutrophil migration at endothelial borders. *J. Leukoc. Biol.* 65: 299–306; 1999.

Key Words: *histamine · leukocytes · inflammation · adhesion molecules*

INTRODUCTION

Endothelial P-selectin (CD62P) plays critical roles in the early inflammatory response. It is stored in specialized granules known as Weibel-Palade (WP) bodies and, after activation with inflammatory mediators such as histamine, thrombin, or complement proteins, WP bodies fuse with the plasma membrane and P-selectin is rapidly mobilized to the endothelial apical surface [1–5]. *In vitro*, histamine-induced P-selectin expression is maximal by 10 min, but then declines toward baseline values by 30 min [3]. Down-regulation is mediated by an endocytotic process that involves the interaction of the cytoplasmic domain of P-selectin with clathrin-coated pits [3, 6]. Experiments using human umbilical vein endothelial cell (HUVEC) monolayers, a commonly used model for studying leukocyte interactions, show that neutrophil adhesion after histamine activation is prevented by anti-P-selectin antibody [7]. The interaction of P-selectin with clathrin-coated pits apparently enhances neutrophil adhesion presumably through P-selectin clustering that leads to an increased avidity for P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils (and other leukocytes) [6]. Indeed, Lawrence et al. showed that higher P-selectin site densities favor neutrophil adhesion *in vitro* [8].

In vivo models of inflammation reveal a substantial contribution of P-selectin at sites where extravasation of neutrophils is evident. Observations of leukocytes in postcapillary venules in vascular beds such as hamster cheek pouch or mouse cremaster revealed significant increases in leukocyte rolling after introduction of an inflammatory stimulus [9, 10]. This phenomenon in many postcapillary venules is now known to be a principal function of P-selectin. In such vessels, leukocytes also exhibit firm adhesion before transendothelial migration, and in the absence of rolling (e.g., in P-selectin-deficient mice or blocking with anti-P-selectin antibodies), firm adhesion and leukocyte emigration are significantly delayed [10–12].

Early electron microscopic studies revealed that transendothelial migration of neutrophils occurs prominently at the borders of endothelial cells [13, 14]. We have recently begun to investigate the events that immediately precede the process of transmigration and here address the question of whether flowing and rolling neutrophils firmly arrest at random locations on the endothelium or whether they arrest at endothelial borders. Our initial observations contained in this report involve endothelial interactions where P-selectin is a major determinant of rolling.

MATERIALS AND METHODS

Reagents

Histamine was obtained from Sigma (St. Louis, MO). Thrombin was obtained from Ortho Diagnostics Systems Inc. (Raritan, NJ). Dulbecco's phosphate-

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Abbreviations: WP, Wiebel-Palade; HUVEC, human umbilical vein endothelial cell; PSGL-1, P-selectin glycoprotein ligand-1; DPBS, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; ECGS, endothelial cell growth supplement; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

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buffered saline + glucose (DPBS) buffer, M199 and Dulbecco's modified Eagle's medium (DMEM), and Hanks' balanced salt solution (HBSS) were obtained from GIBCO Laboratories (Grand Island, NY).

Endothelial cell culture

HUVECs were routinely harvested from 5–10 umbilical veins by collagenase perfusion according to Huang and colleagues [15]. Pooled cells were seeded in T75 flasks (Corning) containing M199 medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc.) and 10% bovine calf serum (Hyclone Laboratories, Inc.), 1% penicillin-streptomycin (GIBCO-BRL), 1% Fungizone (GIBCO-BRL), 1% HEPES buffer (GIBCO-BRL), 1 μg/mL heparin (Sigma), and 50 μg/mL endothelial cell growth supplement (ECGS; Collaborative Biomedical Products, Bedford, MA). Four or five days after seeding, confluent cultures were detached with HBSS containing 0.05% trypsin/5 mM EDTA (GIBCO-BRL) and seeded on 25-mm glass coverslips (Belco) or 35-mm polystyrene tissue culture dishes (Corning) and used for experiments at 4 days post-confluence. Before seeding, the glass coverslips and Petri dishes were coated with glutaraldehyde cross-linked gelatin as previously described in detail [16].

Human lung microvascular endothelial cells at passage 7 (HLMVEC; a generous gift from Dr. J. Bruce Sundstrom, Division of Viral and Rickettsial Disease, Atlanta, GA) were cultured in EGM-2MV (Clonetics). HLMVEC were subcultured on glutaraldehyde cross-linked gelatin 25-mm glass coverslips (Belco) as described above and used at 4 days post-confluence.

Neutrophil isolation

Human peripheral blood neutrophils were isolated by two different methods, each of which yielded neutrophils of similar purity and viability (purity >95% and, as determined by trypan blue exclusion, >97% viable). For static adherence assays, citrate anti-coagulated venous blood was sedimented in 6% dextran (mol. wt. 250,000; Spectrum, Gardena, CA) and centrifuged over a gradient of 6.07% Ficoll® (mol. wt. 400,000; Sigma) and 10% Hypaque® (Sanofi Winthrop Pharmaceuticals, New York, NY) at room temperature. For hydrodynamic flow assays, neutrophils were isolated using a simplified procedure that did not require dextran sedimentation. Anti-coagulated (heparin, 10 U/mL) venous blood was centrifuged over a Ficoll-Hypaque gradient (Mono-Poly resolving medium, Flow Laboratories Inc., McLean, VA) as previously described [17] and kept at 4°C in calcium-free 30 mM HEPES buffer (containing 10 mM KCl, 110 mM NaCl, 10 mM glucose, and 1 mM $MgCl_2$; pH 7.35). Neutrophils could be isolated in less time using this simplified method and they behaved similarly in the flow assay when compared to neutrophils isolated by the dextran/Ficoll-Hypaque protocol. Isolated neutrophils were kept at room temperature in DPBS for up to 2 h before being used in static or flow assays. Before each assay, the neutrophils were resuspended in room temperature DPBS (containing 10 mM glucose, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). The use of HUVEC monolayers and human neutrophils has been approved by the Baylor College of Medicine Human Use Committee. Human subjects donating blood gave their informed consent.

Monoclonal and polyclonal antibodies

The following mouse mAbs and rabbit polyclonal antibodies were used for immunofluorescence microscopy: anti-P-selectin (WAPS 12.2) from Zymed Laboratories (San Francisco, CA) and anti-von Willebrand Factor (vWF; rabbit polyclonal) from DAKO (Glostrup, Denmark). Nonspecific fluorescence was assessed by substituting the primary antibody with non-immune mouse IgG1 (MOPC 21) or non-immune rabbit serum from Sigma.

The following inhibitory mouse monoclonal antibodies (mAbs) were used in the neutrophil adhesion assay: anti-CD18 (mAb R15.7) was kindly supplied by Dr. R. Rothlein (Boehringer-Ingelheim Pharmaceuticals, Inc., Ridgefield, CT), anti-ICAM-1 $F(ab')_2$ and anti-ICAM-2 $F(ab')_2$ were obtained from Caltag Laboratories (Burlingame, CA), and anti-E-P-selectin and anti-E-selectin were kindly supplied as humanized mouse mAbs [mouse Fc domains substituted with human IgGγ4 Fc domains, which have low affinity for neutrophil Fc receptors (CD16 and CD32)] by Dr. Ellen Berg (Protein Design Labs, Inc., Plymouth, MA).

Cell-surface ELISA

ICAM-1, ICAM-2, P-selectin, and MHC I surface expression on HUVEC monolayers was evaluated using a cell surface enzyme-linked immunosorbent assay (ELISA) and the antibodies described above. In addition, E-selectin expression was examined using mAb ENA1 from Monosan (Uden, The Netherlands). Briefly, first-passage HUVEC monolayers were seeded in 96-well Corning® plastic plates. At 4 days post-confluence, the HUVEC monolayers were treated for 5 min at 37°C with DPBS alone or DPBS containing histamine (10^{-4} M). Some monolayers were treated with interleukin-1β (IL-1β; 10 U/mL) for 4 h before challenge with DPBS or DPBS containing histamine. After the 5-min challenge period, the monolayers were fixed for 20 min at room temperature in PBS buffer containing 0.25% paraformaldehyde. Fixed monolayers were blocked overnight at 4°C with DPBS containing 1% bovine serum albumin (BSA) and then labeled in duplicate with a saturating (10 μg/mL) amount of antibody. Binding was assessed by secondary detection with alkaline phosphatase conjugated to goat anti-mouse IgG (Sigma). The plates were read at 405 nm by an automatic microplate reader (Cambridge Technology, Inc., Waterford, MA). Nonspecific binding was assessed by substituting the primary antibody with anti-CD43 (mAb DFT1) from BioDesign (Kennebunk, ME), which binds to leukocytes but not to endothelial cells.

Immunofluorescence microscopy

To study the cytoplasmic distribution of WP bodies containing vWF and the surface expression of P-selectin, untreated HUVEC monolayers were incubated with DPBS or DPBS containing histamine (10^{-4} M) for various periods of time (1–10 min) at 37°C. The monolayers were then fixed in DPBS containing 1% paraformaldehyde (20 min, room temperature), rinsed in DPBS, and immunolabeled with saturating concentrations (5–10 μg/mL) of primary antibody diluted in DPBS containing 1% BSA. For intracellular staining of WP bodies the monolayers were permeabilized (10 min with Triton X-100, 0.1% in DPBS) before the addition of the primary antibody. BODIPY-conjugated goat anti-mouse IgG or Texas Red-conjugated goat anti-rabbit IgG were obtained from Molecular Probes (Eugene, OR) and used for secondary detection. Nonspecific fluorescence was assessed by substituting the primary antibodies with the appropriate non-binding isotype-matched antibody [mouse IgG1 (MOPC 21) or rabbit serum from Sigma]. In some cases, DAPI (Molecular Probes) was used to visualize cell nuclei and BODIPY-conjugated phalloidin (Molecular Probes) was used to visualize filamentous actin. All coverslips were mounted in Gelvatol (Monsanto Corp., St. Louis, MO) and examined with a Leitz Diaplan epifluorescence microscope equipped with a digital camera and digital capture device (Snappy V.2; Play Inc., Rancho Cordova, CA).

Computer-assisted image analysis was used to quantify P-selectin fluorescence on endothelial cells. Grayscale images were analyzed using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, TX and available from the Internet by anonymous ftp from ftp://maxrd6.uthscsa.edu). The median pixel intensity (possible range from black = 0 to white = 255) for P-selectin fluorescence over the endothelial cell border was compared with that over the cell body. The border area was defined as a 1.5-μm-wide zone encompassing the perimeter of the cell, whereas the residual area was defined as the cell body.

Neutrophil adherence to endothelial borders

Neutrophil adherence to endothelial cell borders under static conditions was examined using coverslips bearing HUVEC monolayers or HLMVEC monolayers. Coverslips were rinsed in DPBS, placed in dry 35-mm polystyrene tissue culture dishes, and neutrophils (1×10^6/mL) were layered onto the monolayer surface in 250 μL of DPBS alone or DPBS containing histamine (10^{-4} M) or thrombin (0.2 units/mL) at 37°C. After 3 min of interaction, the monolayers were fixed in glutaraldehyde (0.05%) and the endothelial cell borders stained with silver [16].

Neutrophil adherence to endothelial cell borders under hydrodynamic flow conditions was examined using a parallel plate flow chamber maintained at 37°C as previously described [18]. The chamber produces a well-defined laminar flow over HUVEC monolayers grown in 35-mm tissue culture dishes. Isolated neutrophils were diluted (4×10^6/mL) in DPBS alone or DPBS containing histamine (10^{-4} M) and perfused through the chamber at a shear stress of 2 dyn/cm^2. Adhesive interactions between neutrophils and endothelial monolayers were observed by phase-contrast microscopy (Diaphot-TMD; Nikon Inc., Garden City, NY) and videotaped. Four minutes after the neutrophils entered the chamber, the monolayers were fixed in glutaraldehyde (0.05%) and the endothelial cell borders stained with silver as above.

Quantitation of neutrophil adherence in the presence of blocking antibodies

Neutrophil adherence to HUVEC monolayers under static conditions in the presence of antibodies directed against adhesion determinants was examined using Muntz adhesion chambers [19, 20]. Coverslips bearing HUVEC monolayers were rinsed in DPBS, placed in the adhesion chamber, and covered with a plain glass coverslip that was separated from the lower coverslip by a rubber O-ring. Within this closed compartment a suspension of neutrophils (1×10^6/mL) in DPBS was introduced through a 25-gauge needle connected to a 1-mL syringe. All experiments were conducted at 37°C. Under phase-contrast optics (Diaphot-TMD; Nikon Inc.), the numbers of neutrophils that settled and contacted the endothelial monolayer during an initial 210-s observation period were determined. The adhesion chamber was then inverted for an additional 120 s; nonadherent neutrophils fell away from the HUVEC monolayer and the percentages of adherent neutrophils were determined. To study the effects of blocking antibodies on β_2 integrins (CD18), the neutrophils were pre-mixed with antibody for 20 min at room temperature before injection. To study the effect of blocking antibodies on endothelial determinants (ICAM-1, ICAM-2, E-selectin, P-selectin), HUVEC monolayers were pre-incubated with antibodies for 20 min at room temperature before beginning the assay.

Scanning electron microscopy

Guinea pigs ($n = 2$) were killed and their lungs fixed using a perfusion technique that has been previously described in detail . Briefly, under deep anesthesia (intraperitoneal urethane) catheters were placed in the internal left jugular vein and right carotid artery. Krebs buffer containing histamine (10^{-10} or 10^{-9} M) was perfused through the jugular vein at 50 cmH_2O pressure for 10 min and then the perfusion was switched to 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde and 0.01% toluidine blue (to monitor the perfusion process) for 15 min. The lungs were removed, sliced in 2-mm-thick pieces, fixed with osmium, dehydrated through ethanol, and transferred to amyl acetate. The slices were critical point dried in CO_2 and then sputter coated with gold-palladium in a Balzers FDU 010 unit. Specimens were examined and photographed in a Cambridge T150 scanning electron microscope.

Data presentation and statistical analysis

Data are presented as means ± SEM unless otherwise stated, with n being the number of separate experiments. Statistical assessments were made using a one-way analysis of variance followed by a Student-Newman-Keuls test for multiple comparisons. Statistical significance was set at $P \leq 0.05$.

RESULTS

Histamine increases P-selectin expression on endothelial borders

Under the culture conditions used, P-selectin was minimally detected by ELISA on resting HUVEC monolayers, but increased fourfold within 5 min after histamine stimulation (**Fig. 1**). There was no significant change in the levels of ICAM-1 or ICAM-2. E-selectin was not detected on resting or histamine-stimulated HUVEC, but was observed 4 h after stimulation of monolayers with IL-1 (data not shown).

Immunofluorescence provided a visual image of the locations of WP bodies (identified by vWF-specific antibody). In resting HUVEC monolayers the WP bodies appeared more numerous near the borders of endothelial cells (**Fig. 2A**). Histamine stimulation (10^{-4} M) produced rapid changes in the distribution of WP bodies in these cells. Within 1 min WP bodies were found clustered along the endothelial borders (Fig. 2B). Immunofluorescence observations of non-permeabilized monolayers labeled with anti-P-selectin revealed very little detectable staining on resting monolayers (**Fig. 3A**), but 2 min after the addition of histamine, P-selectin-specific fluorescence was markedly increased (Fig. 3B). Nonspecific staining was absent when primary antibody was substituted with isotype-matched control antibody (MOPC 21, mouse IgG_1) or control rabbit serum (data not shown).

Quantitative image analysis of the distribution of P-selectin fluorescence showed that the median intensity was not uniform over the endothelial cell surfaces. Median pixel intensity over the endothelial cell borders was threefold greater ($P \leq 0.01$)

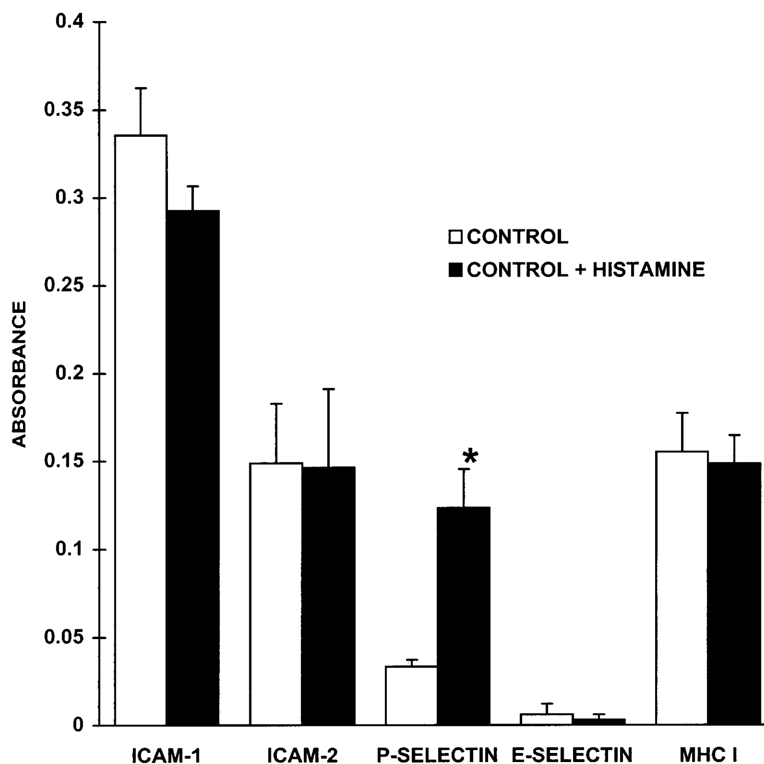

Fig. 1. Adhesion molecule surface expression on HUVEC monolayers before (Control) and after treatment with 10^{-4} M histamine for 5 min (Control + Histamine). Monolayers were fixed in paraformaldehyde and adhesion molecule expression was determined by cell-surface ELISA using specific mAbs (see Materials and Methods). Absorbance was measured at 405 nm. Data are means ± SEM of duplicate determinations from three independent experiments. $*P \leq 0.05$ compared to matched control.

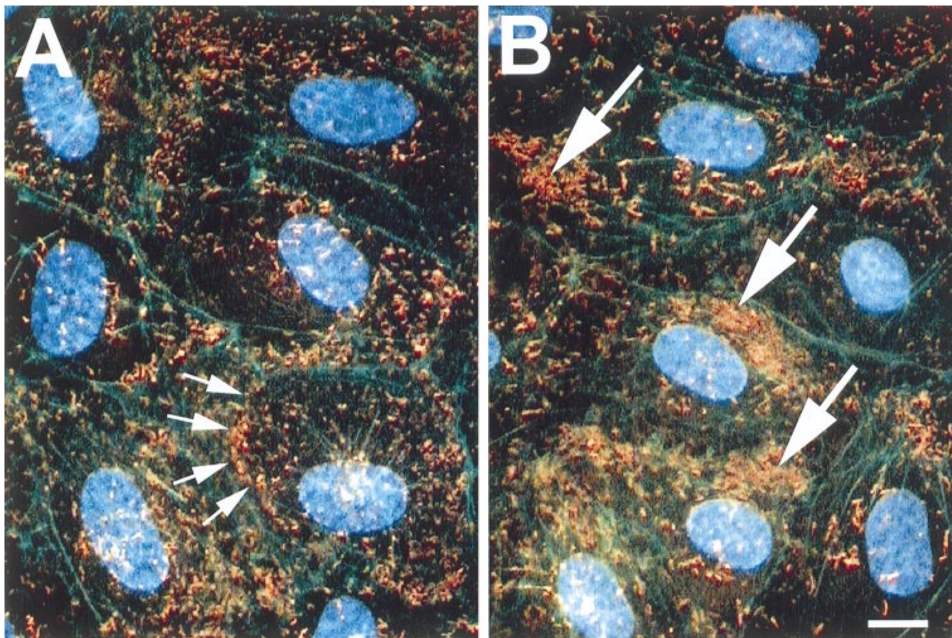

Fig. 2. Immunostaining for WP bodies in confluent HUVEC monolayers treated without (A) or with histamine (B; 10^{-4} M histamine for 1 min). WP bodies were localized using a rabbit polyclonal against von Willebrand Factor. Cell nuclei were stained with DAPI (blue) and F-actin with phalloidin (green). WP bodies in control endothelial cells appear more numerous near the cell borders (A, arrows). After histamine treatment, marked clustering of WP bodies was apparent (B, arrows; bar, 10 µm).

than that over the cell bodies (48.3 ± 6.8 SD vs. 17.3 ± 3.2, respectively).

Localized adhesion of neutrophils

Silver-stained monolayers were used to reveal the location of adherent neutrophils with reference to the interendothelial junctions. Baseline neutrophil adhesion without histamine or thrombin stimulation was not observed on silver-stained monolayers. After histamine or thrombin stimulation, adhesion markedly increased. As shown in **Figure 4**, **A** and **B**, >85% of the neutrophils were on endothelial cell borders. Similar results were obtained with human lung microvascular endothelial cell (HLMVEC) monolayers. In these preparations, >85% of the neutrophils were adherent to endothelial cell borders (Fig. 4, C and D).

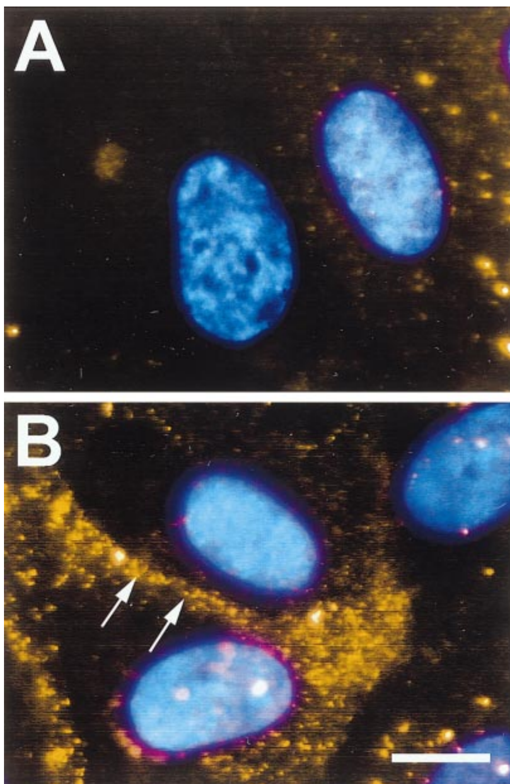

Fig. 3. Immunostaining for P-selectin on the surface of confluent HUVEC monolayers treated without (A) or with histamine (B; 10^{-4} M histamine for 2 min). Control endothelial cells expressed very little P-selectin on the surface (A), but after histamine treatment P-selectin-specific fluorescence increased markedly along cell borders (arrows; bar, 10 µm).

The effects of hydrodynamic shear on this localization of neutrophils was assessed at a wall shear stress of 2 dyn/cm^2. In the absence of histamine, neutrophils did not interact with the monolayer (data not shown). However, 1 min after addition of histamine, neutrophils began to interact and by 4 min large numbers of neutrophils rolled on the monolayer, and ≈4% arrested (i.e., were stationary for >3 s). Monolayers fixed after 4 min of histamine stimulation revealed that >75% of attached neutrophils were at endothelial cell borders (Fig. 4, E and F).

Adhesion molecule contribution to this localized adhesion was examined using blocking antibodies against CD18, ICAM-1, ICAM-2, P-selectin, and E-selectin (**Fig. 5**). Only mAb E-P (a humanized murine antibody against E- and P-selectin) was effective. This mAb entirely prevented the increase in adhesion caused by histamine stimulation. This was apparently a direct effect of blocking P-selectin and not E-selectin because anti-E-selectin was ineffective, and histamine-stimulated monolayers failed to express E-selectin (see Fig. 1).

Neutrophil adherence to endothelial cell borders *in vivo*

We investigated whether histamine would induce leukocyte adherence to endothelial borders in the pulmonary vasculature of guinea pigs ($n = 2$). Histamine was infused for 10 min and the lungs were fixed by vascular perfusion (this preserves the lung and flushes out nonadherent leukocytes). By scanning electron microscopy, adherent leukocytes were not observed in control guinea pig pulmonary vessels. After histamine infusion, 13 adherent leukocytes were observed from 5 different blood

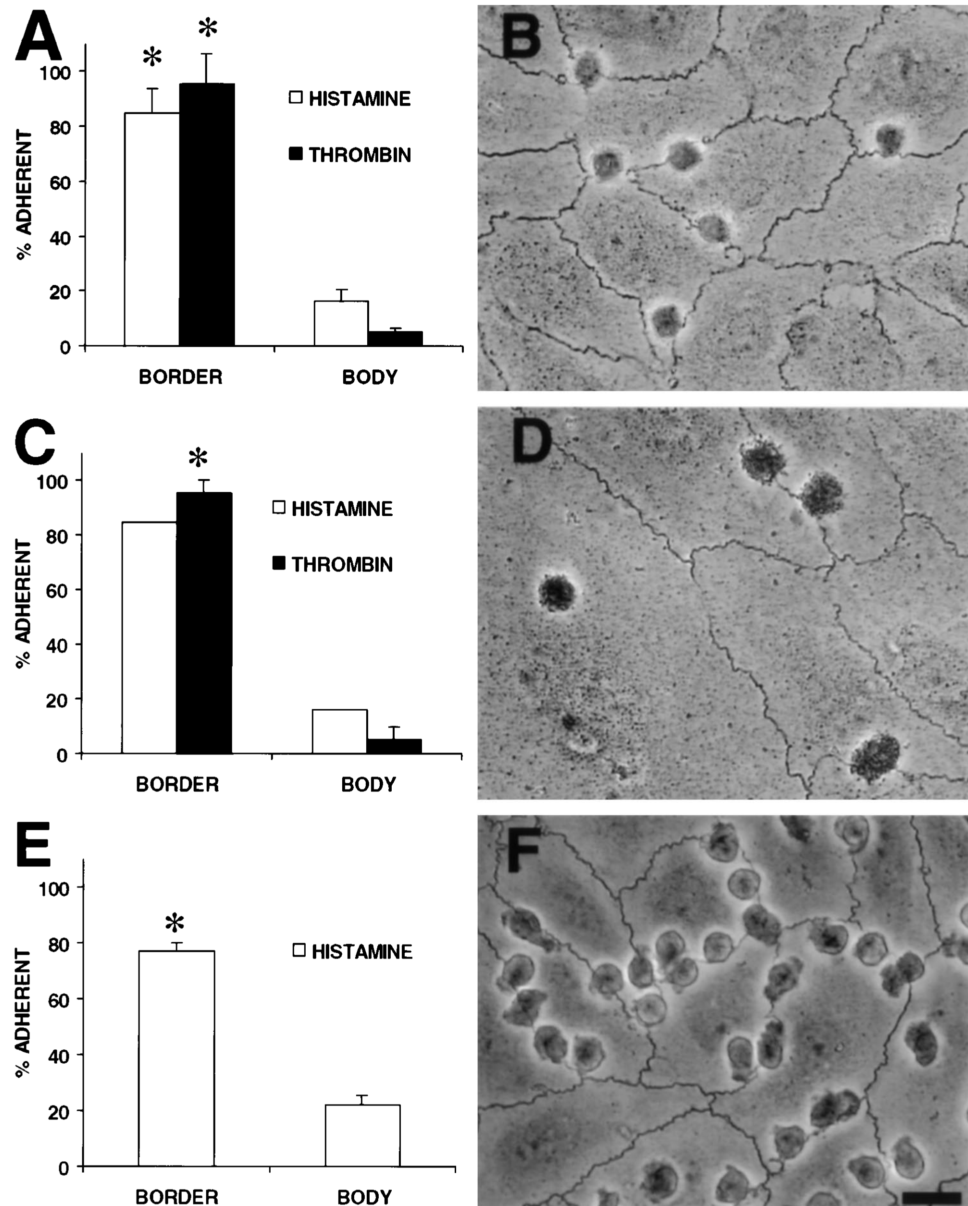

Fig. 4. Neutrophil adherence to HUVECs (A, B, E, F) or human lung microvascular endothelial cells (C, D) after treatment with histamine (10^{-4} M) or thrombin (0.2 U/mL). Endothelial cells and neutrophils were co-stimulated for 3 min (static adhesion; A–D) or for 4 min [hydrodynamic flow (2 dyn/cm^2); E, F] and silver stained to enhance the cell border appearance. Panels A, C, and E show that >75% of the interacting neutrophils adhered to cell borders (Border) rather than cell bodies (Body) Panels B, D, and F are representative light micrographs showing neutrophil adherence after histamine (B, F) or thrombin (D) stimulation. Baseline neutrophil adhesion without histamine or thrombin stimulation was not observed on silver-stained monolayers. $*P \leq 0.05$ compared to matched adherence to cell body (bar, 10 μm).

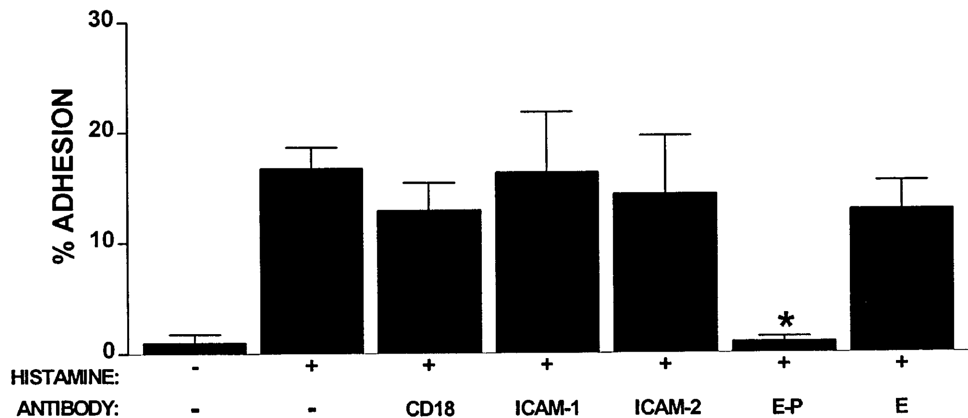

Fig. 5. Effect of blocking antibodies against leukocyte β_2-integrins (CD18) and endothelial ICAM-1, ICAM-2, E-selectin, and P-selectin on histamine (10^{-4} M) stimulated neutrophil adhesion to HUVECs. Adhesion was determined in a static adhesion chamber (see Materials and Methods for details). Antibodies were used as either whole mouse mAbs (anti-CD18), mouse F(ab')$_2$ fragments (anti-ICAM-1 and anti-ICAM-2), or humanized mouse mAbs (anti-E-P blocks E- and P-selectin-mediated adhesion; anti-E blocks E-selectin-mediated adhesion only). $*P \leq 0.05$ compared to control (i.e., no histamine and no antibody).

vessels. The location of the endothelial border relative to the location of the leukocytes was clearly defined for 9 of the 13 leukocytes. These 9 adherent leukocytes were within 1 cell (leukocyte) diameter (< 6 μm) of an endothelial border (**Fig. 6**). This observation is consistent with our *in vitro* findings that histamine induces preferential neutrophil adherence to pulmonary endothelial cell borders (Fig. 4, C and D).

DISCUSSION

WP bodies containing P-selectin fuse rapidly with the endothelial plasma membrane on stimulation with secretagogues such as histamine and thrombin, resulting in increased P-selectin surface expression. An important function of P-selectin is its ability to mediate neutrophil adherence (tethering and rolling) on inflamed endothelium. This study extends these observations and demonstrates for the first time that P-selectin surface expression on histamine-treated endothelial monolayers is greatest along cell borders. Neutrophil adhesion to histamine- or thrombin-treated endothelium was P-selectin dependent and $\geq$75% of the interacting neutrophils adhered to cell borders with the rest distributed randomly over the cell body. The physiological significance of enhanced P-selectin receptor density along endothelial borders mediating preferential neutrophil adherence remains to be determined. However, we suggest it may facilitate the targeting of neutrophils to sites of preferred transendothelial migration (i.e., borders). *In vitro*, neutrophil transmigration on HUVEC monolayers occurs exclusively at endothelial borders [16].

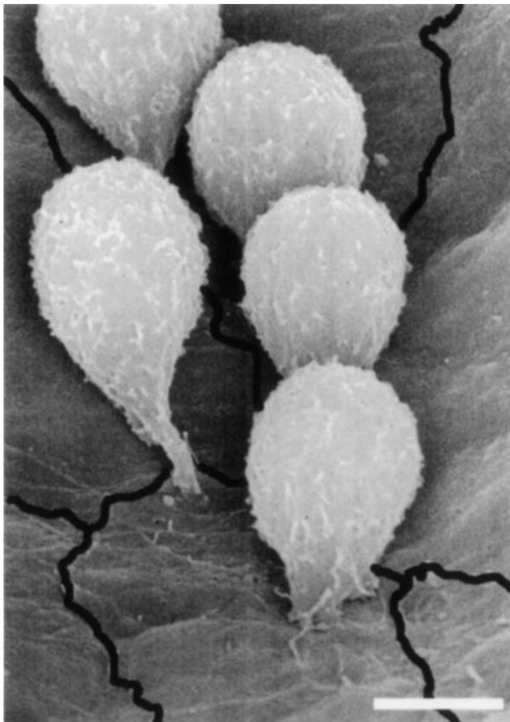

Fig. 6. Scanning electron micrograph of leukocytes adhering near endothelial borders (black lines) in a guinea pig pulmonary vessel after histamine perfusion. The animal was anesthetized and the lung perfused through the jugular vein with histamine (10^{-9} M) for 10 min before tissue fixation and processing (see Materials and Methods for details). Five adherent leukocytes can be seen, each positioned within one cell (leukocyte) diameter (< 6 μm) of an endothelial border (bar, 5 μm).

A consistent finding in this study is that neutrophil adhesion to endothelial cell borders is solely dependent on P-selectin. Histamine and thrombin have been reported to up-regulate the expression of surface-bound platelet-activating factor (PAF) and co-expression of P-selectin and PAF lead to juxtacrine activation of CD18-dependent neutrophil adhesion [21]. In this study, using blocking mAbs, we were unable to detect any contribution of CD18 or its endothelial ligands ICAM-1 and ICAM-2, to neutrophil adhesion under static conditions. In a previous study we did observe that under hydrodynamic flow, a small fraction of neutrophils ($<$3%) firmly adhered (for at least 3 s) to the monolayer and this adhesion was mediated by CD18 [7]. However, because CD18-dependent adhesion was preceded by P-selectin-dependent rolling at typical venous shears, it seems likely that P-selectin is responsible for the initial targeting of neutrophils to endothelial cell borders.

It has recently been reported that the lifetime of a neutrophil tether via a single bond to P-selectin under shear is $<$1 s. Continuous reformation of tethering bonds is dependent on the cell surface distribution and site density of P-selectin [22]. For example, the cytoplasmic tail of P-selectin interacts with coated pits and it has been suggested that the clustering of P-selectin that occurs just before internalization may increase the avidity of P-selectin for the P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils through increased bond formation [6]. Our study suggests that there is another mechanism for enhancing P-selectin avidity. As early as 1 min after treatment with histamine we observed by immunofluorescence microscopy that P-selectin expression along endothelial cell borders was threefold greater than non-border regions (i.e., the cell body). Greater than 75% of the interacting neutrophils adhered to the cell borders under static conditions, a significant finding given that there is ~8-fold more membrane area making up the cell body (assuming a border width of 1.5 μm and a cell perimeter distance of 140 μm) [16]. Blocking antibody studies confirmed that neutrophil adhesion was entirely mediated by P-selectin. This direct correlation between site distribution and adhesive function underscores the importance of considering the surface distribution of adhesion molecules when assessing their known functions in neutrophil adhesion.

The studies of Lawrence and Springer [8] support our interpretation that higher P-selectin site densities along endothelial borders mediate preferential neutrophil adhesion. Using artificial lipid bilayers, these authors were able to show that neutrophil rolling velocity is inversely proportional to the site density of P-selectin. Neutrophils roll more slowly at any given shear stress (0–8 dyn/cm^2) and in significantly greater numbers (10-fold at 2 dyn/cm^2) at P-selectin densities of 200 sites/μm^2 compared to 50 sites/μm^2 (0–8 dyn/cm^2) [8]. From iodinated antibody binding data collected by Hattori and colleagues [3], Lawrence et al. estimated that 5 min after histamine treatment, the average P-selectin site density on HUVEC monolayers increases from 20 to 50 sites/μm^2 [8]. The cell surface ELISA data in the present study compare favorably with these values and show that P-selectin expression increased fourfold after 5 min of histamine exposure. Because P-selectin surface expres-

sion is threefold greater at cell borders, we estimate that the average P-selectin density on cell borders is at least 150 sites/μm². Whether similar site densities are present over endothelial borders *in vivo* is unknown.

Hydrodynamic factors may also influence the path line of a rolling neutrophil under shear. Barbee and colleagues have shown that the gradient of shear stress is highest toward the raised center of the endothelial cell and lowest at the borders [23]. However, the present study shows that neutrophil adhesion in the static chamber occurs in the absence of significant shear stress. Moreover, the preference shown for adhesion to endothelial borders is identical under static or hydrodynamic flow conditions. These observations further support the concept that higher P-selectin site densities along the border mediate preferential neutrophil adhesion.

The mechanism by which P-selectin is targeted to cell borders is currently unknown but may be related to the cytoplasmic positioning of WP bodies. Cultured endothelial cells viewed on edge taper from a height of approximately 2 μm at the nucleus to <0.5 μm at the cell borders [16, 23, 24]. There is very little intervening cytoplasm between the cell nucleus and the apical plasma membrane and this probably accounts for the observation that WP bodies tend to be positioned in the peripheral regions of the endothelial cytoplasm away from the nucleus. Immunostaining for vWF, a constituent of WP bodies, revealed that histamine induces rapid (within 20 s) WP body clustering and aggregation. The rapidity of WP body clustering suggests that it precedes WP body fusion with the plasma membrane and this may explain why P-selectin expression is greater on some regions of the cell border than others.

That histamine or thrombin increases neutrophil adhesion to HUVEC as well as HLMVEC borders *in vitro* suggests that this phenomenon may be common to venous endothelia from different tissues. Scanning electron microscopic observations of a small number of leukocytes ($n = 9$) suggest this phenomenon may occur *in vivo* because leukocytes adhered on or very near endothelial borders in guinea pig pulmonary vessels after histamine infusion (see Fig. 6). The physiological significance of enhanced P-selectin expression and increased neutrophil adhesion along endothelial borders is not known. Using HUVEC monolayers, we determined previously that neutrophil transmigration occurs exclusively at cell borders [16]. Taken together with our current observations, it is likely that P-selectin plays a role in targeting neutrophils, and other leukocytes, to potential sites of emigration along endothelial borders. *In vivo*, this may be extremely important at sites of allergic and chronic inflammation where endothelial P-selectin surface expression is up-regulated [10].

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