

Endothelial Cell E- and P-Selectin and Vascular Cell Adhesion Molecule-1 Function as Signaling Receptors

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Abstract. Previous studies have shown that polymorphonuclear leukocyte (PMN) adherence to endothelial cells (EC) induces transient increases in EC cytosolic free calcium concentration ($[Ca^{2+}]_i$) that are required for PMN transit across the EC barrier (Huang, A.J., J.E. Manning, T.M. Bandak, M.C. Ratau, K.R. Hanser, and S.C. Silverstein. 1993. *J. Cell Biol.* 120:1371–1380). To determine whether stimulation of $[Ca^{2+}]_i$ changes in EC by leukocytes was induced by the same molecules that mediate leukocyte adherence to EC, $[Ca^{2+}]_i$ was measured in Fura2-loaded human EC monolayers. Expression of adhesion molecules by EC was induced by a pretreatment of the cells with histamine or with *Escherichia coli* lipopolysaccharide (LPS), and $[Ca^{2+}]_i$ was measured in single EC after the addition of mAbs directed against the EC adhesion proteins P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), or platelet/endothelial cell adhesion molecule-1 (PECAM-1). Both anti-P- and anti-E-selectin mAb, as well as anti-VCAM-1 mAb, induced transient increases in EC $[Ca^{2+}]_i$ that were comparable to those induced by 200 μ M histamine. In contrast, no effect was obtained by mAbs directed against the endothelial ICAM-1 or PECAM-1. PMN adherence directly stimulated increases in $[Ca^{2+}]_i$ in histamine- or LPS-treated EC. mAbs directed against leukocyte CD18 or PECAM-1, the leukocyte counter-receptors for endo-

thelial ICAM-1 and PECAM-1, respectively, did not inhibit PMN-induced EC activation. In contrast, mAb directed against sialyl Lewis x (sLe^x), a PMN ligand for endothelial P- and E-selectin, completely inhibited EC stimulation by adherent PMN. Changes in EC $[Ca^{2+}]_i$ were also observed after adherence of peripheral blood monocytes to EC treated with LPS for 5 or 24 h. In these experiments, the combined addition of mAbs to sLe^x and VLA-4, the leukocyte counter-receptor for endothelial VCAM-1, inhibited $[Ca^{2+}]_i$ changes in the 5 h-treated EC, whereas the anti-VLA-4 mAb alone was sufficient to inhibit $[Ca^{2+}]_i$ changes in the 24 h-treated EC. Again, no inhibitory effect was observed with an anti-CD18 or anti-PECAM-1 mAb. Of note, the conditions that induced changes in EC $[Ca^{2+}]_i$, i.e., mAbs directed against endothelial selectins or VCAM-1, and PMN or monocyte adhesion to EC via selectins or VCAM-1, but not via ICAM-1 or PECAM-1, also induced a rearrangement of EC cytoskeletal microfilaments from a circumferential ring to stress fibers. We conclude that, in addition to their role as adhesion receptors, endothelial selectins and VCAM-1 mediate endothelial stimulation by adhering leukocytes.

Key words: endothelial • adherence • signaling • selectin • VCAM-1

THE process of leukocyte extravasation at sites of inflammation involves two main events: (1) adherence of circulating leukocytes to the endothelial wall of post-capillary venules, and (2) passage (diapedesis) of leukocytes across the endothelium. Endothelial cells (ECs)¹ play an active role in both events. Signals generated at the in-

flammatory site induce expression of endothelial adhesion molecules that promote leukocyte rolling and sticking, and expression by EC of various combinations of adhesion molecules in response to particular cytokines accounts in part for the recruitment of leukocyte subtypes during an inflammatory or immune reaction (Bevilacqua and Nelson, 1993; Carlos and Harlan, 1994; Austrup et al., 1997). Leukocyte adhesion to EC is then followed by leukocyte penetration at inter-endothelial cell junctions, thus permitting leukocyte migration into the subendothelial space.

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1. *Abbreviations used in this paper:* EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; ICAM, PECAM, VCAM, intercellular,

platelet/endothelial, vascular cell adhesion molecule; LPS, lipopolysaccharide; PMN, polymorphonuclear leukocyte; sLe^x, sialyl Lewis x.

Hixenbaugh et al. (1997) reported that neutrophil adhesion to EC monolayers causes myosin light chain phosphorylation and generation of isometric tension in the monolayers. Since myosin phosphorylation was associated with the formation of gaps between neighboring EC (Garcia et al., 1995), it was proposed that leukocyte adherence to endothelial cells triggers intracellular signals that, in turn, lead to opening of the junctions.

Endothelial stimulation by adhering leukocytes was investigated by Huang et al. (1993). They found that there was a transient increase in endothelial $[Ca^{2+}]_i$ during transendothelial migration of polymorphonuclear leukocytes (PMNs). Moreover, these authors demonstrated that, by inducing an increase in endothelial cell $[Ca^{2+}]_i$, PMN were able to affect the integrity of junctions between EC, and that rises in $[Ca^{2+}]_i$ were required for PMN transit across EC monolayers. Further studies confirmed that a rise in EC $[Ca^{2+}]_i$ may result from the adherence to EC of PMNs and monocytes (Ziegelstein et al., 1994) and natural killer lymphocytes (Pfau et al., 1995). As to the mechanism(s) whereby leukocytes transduce signals to EC, recent studies implicate leukocyte-endothelial cell adhesion molecules. Yoshida et al. (1996) suggested that E-selectin, an inducible endothelial adhesion molecule that binds to complex carbohydrate ligands on leukocytes, may have a role in outside-in signaling. Leukocyte adherence to EC was shown to induce association of E-selectin with the endothelial cytoskeleton. Similarly, cytoskeletal association of E-selectin was observed after clustering induced by specific antibodies. Durieux-Trautmann et al. (1994) demonstrated that clustering of intercellular adhesion molecule-1 (ICAM-1), the endothelial ligand for the leukocyte CD11/CD18, induced tyrosine phosphorylation of cytoskeletal proteins potentially involved in the assembly of adherence junctions.

In an effort to analyze comprehensively the involvement of endothelial adhesion proteins in transmembrane signaling, we examined the role of E-selectin, P-selectin, ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and platelet/endothelial cell adhesion molecule-1 (PECAM-1) in mediating intracellular changes resulting from leukocyte adherence. Because of its functional relevance in leukocyte diapedesis (Huang et al., 1993), $[Ca^{2+}]_i$ was studied. In addition, we investigated potential rearrangements of endothelial cytoskeleton. Dramatic changes in endothelial morphology have been shown to accompany leukocyte adherence to and migration across endothelium (Marchesi and Florey, 1960; Furie et al., 1987). Similar changes in endothelial cell shape (Laposata et al., 1982), and a typical rearrangement of actin microfilaments from a circumferential ring to stress fibers were shown to occur in ECs exposed to soluble mediators, such as thrombin (Morel et al., 1990; Kolodney and Wysolmerski, 1992). Of note, as with leukocyte adherence, endothelial stimulation by thrombin was shown to produce an increase in endothelial $[Ca^{2+}]_i$ (Goligorsky et al., 1989). Therefore, we questioned whether engagement of endothelial adhesion receptors by leukocytes would provoke cytoskeletal changes similar to those induced when thrombin binds to its receptor, and whether these would account for changes in EC shape during leukocyte diapedesis.

We show here that mAbs directed against the endothe-

lial adhesion receptors E-selectin, P-selectin, and VCAM-1, but not ICAM-1 and PECAM-1, induce changes in EC $[Ca^{2+}]_i$ and formation of stress fibers. Moreover, we show that $[Ca^{2+}]_i$ changes and stress fiber formation induced by the adherence of PMNs or monocytes to EC are inhibited by mAbs directed against the leukocyte counter-receptors for E-selectin, P-selectin, or VCAM-1, but not against the leukocyte counter-receptors for ICAM-1 and PECAM-1. Our results indicate that endothelial selectins and VCAM-1 function as signaling as well as adhesion receptors.

Materials and Methods

Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment of the vessels as described elsewhere (Jaffe et al., 1973) and maintained in HUVEC medium (medium 199 buffered with 10 mM HEPES and supplemented with 20% newborn bovine serum [HyClone Laboratories Inc., Logan, UT], 50 μ g/ml endothelial cell growth supplement [prepared from bovine brain, as described by Maciag et al., 1979], 90 μ g/ml heparin [Sigma Chemical Co., St. Louis, MO], 50 U/ml penicillin, and 50 μ g/ml streptomycin). For experiments, first-passage HUVEC were grown on gelatin-coated 22-mm glass coverslips placed on the bottom of 35-mm Petri dishes. The monolayers reached confluence within 2 d, as evidenced by prominent immunostaining for PECAM-1 along the cell borders (Muller et al., 1989; Albelda et al., 1990). The cells were used in assays at 4–8 d after plating.

Leukocytes

Human PMN were isolated from acid-citrate-dextrose (ACD)-anticoagulated blood by Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway) gradient centrifugation, 3% dextran (Pharmacia Biotech AB, Uppsala, Sweden) sedimentation, and hypotonic saline lysis of contaminating erythrocytes (Boyum, 1968). The entire procedure was conducted at 4°C. Purified PMN were suspended in PBS (Sigma Chemical Co.) containing 1% BSA (Sigma Chemical Co.) and held on ice (<3 h) until use.

Human monocytes were isolated from ACD-anticoagulated blood following the method of Peri et al. (1990), modified as follows. After erythrocyte sedimentation in 1.5% dextran, the white cell-rich plasma was washed once with PBS containing 13 mM sodium citrate and 0.5% BSA. The cell pellet was then resuspended in isotonic Percoll (Pharmacia Biotech AB) containing 13 mM sodium citrate and 0.5% BSA, pH 7.4. The density of the Percoll suspension was 1.0635 ± 0.0003 g/ml, as measured at 20°C by a DMA density meter (A. Paar, Graz, Austria), and Percoll osmotic value was 290 ± 2 mosM, as measured by a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany). PBS containing 0.5% BSA was layered over Percoll and the cell suspension was centrifuged at 1,000 g for 20 min at 20°C. The cell ring formed at the PBS-Percoll interface was collected, washed twice, and then suspended in ice-cold PBS containing 1% BSA. The resulting cell suspension contained >90% (generally 95%) monocytes, as assessed by morphology and peroxidase-staining, and cell viability was >98%, as determined by trypan blue dye exclusion. Monocytes were then held on ice until use (<3 h).

In experiments involving a pretreatment of leukocytes with mAbs, before addition to HUVEC monolayers, PMN or monocytes were incubated on ice in the presence of mAb (20 μ g/ml) for at least 1 h. The cells were then washed twice at room temperature with PBS containing 1% BSA, 5 mM glucose, and 0.7 mM $CaCl_2$, resuspended in the same medium, and immediately added to the HUVEC monolayers at a leukocyte/HUVEC ratio of 6–8:1.

Calcium Measurements

Cytosolic-free calcium ($[Ca^{2+}]_i$) was monitored in single HUVEC by using the fluorescent calcium probe Fura2-AM as described by Huang et al. (1993), with minor modifications. $[Ca^{2+}]_i$ measurements were performed on a digital fluorescence-imaging microscopy system built around a Zeiss Axiovert 135 (Oberkochen, Germany) as already described (Lorenzon et al., 1997). The excitation light at 340 and 380 nm was provided by a modified dual wavelength microfluorimeter (CAM-230; Jasco Intl. Co. Ltd., Tokyo, Japan) and the fluorescence images were collected by a low light

level CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). The camera output was fed into a digital image processor where video frames were digitized and integrated at real time. The image acquisition was performed at two frames/s. Determination of ratio and $[Ca^{2+}]_i$ calculation were performed pixel by pixel on pairs of corresponding 340 and 380 images according to Grynkiewicz et al. (1985). R_{max} and R_{min} were determined using ionomycin (40 μ M) with calcium (30 mM) or a stoichiometric excess of EGTA, respectively. Thus, R_{max} represented the ratio of 340 nm/380 nm signal under saturating conditions of calcium, whereas R_{min} represented the same ratio in the absence of calcium. Values used for calibration included $R_{max} = 650$, $R_{min} = 200$, $K_d = 224$ nM, and $f380_{min}/f380_{max}$ (ratio of fluorescence at high calcium to that at low calcium) = 2. Temporal plots were calculated from ratio images and each temporal plot indicated the mean value of variation of the fluorescent signal at the single cell level.

Endothelial monolayers grown for 3–5 d on gelatin-coated glass coverslips were loaded with 5 μ M Fura2-AM (Sigma Chemical Co.) for 30 min. Cell loading was performed at room temperature to avoid compartmentalization of the dye. The monolayers were then washed twice and incubated in Hepes (10 mM) buffered medium 199 supplemented with 20% newborn bovine serum (M199-H/NBS) for an additional 20 min at room temperature to allow de-esterification of Fura2-AM. For experiments involving a pretreatment of the cells with *Escherichia coli* lipopolysaccharide (LPS), the monolayers were first incubated for 5 or 24 h at 37°C with 0.5 μ g/ml LPS, and then loaded with Fura2, as described above. Tissue culture dishes were then mounted on the temperature-monitored stage of the Axiovert 135, and bathed with M199-H/NBS. To minimize spontaneous $[Ca^{2+}]_i$ spiking by HUVEC during the assay, the monolayers were maintained at 37°C for 30 min before starting $[Ca^{2+}]_i$ recordings. When indicated, the cells were further incubated 5 min in the presence of histamine (50 μ M), washed three times with prewarmed M199-H/NBS medium, and then incubated in the same medium for another 5 min at 37°C. The homogeneous distribution of the fluorescent dye was carefully checked before starting $[Ca^{2+}]_i$ recordings. Agonists (mAbs or leukocytes) were pipetted onto the monolayers 2–3 min after starting the recordings, and HUVEC $[Ca^{2+}]_i$ was then monitored for 15 min unless otherwise indicated. As a control of variability among HUVEC, a maximal increase in HUVEC $[Ca^{2+}]_i$ (Jacob et al., 1988) was induced by the addition of 200 μ M histamine by the end of each recording. By calculating the ratio between the integral value of the area covered by $[Ca^{2+}]_i$ spike(s) and the level of the spike induced by histamine, a numeric value was obtained for each cell. This value allowed to compare changes in HUVEC $[Ca^{2+}]_i$ obtained under different experimental conditions.

Immunofluorescence

HUVEC for immunofluorescence assay were grown on gelatin-coated glass coverslips following the same procedure used to prepare the cells for $[Ca^{2+}]_i$ measurements. Since preliminary experiments indicated that endothelial stress fibers almost completely disappeared in long-term cultures, we performed these assays on monolayers grown for only 6–8 d after seeding. On the basis of preliminary experiments, care was also taken to change HUVEC medium every day. When indicated, *E. coli* LPS (0.5 μ g/ml) was present in the cell medium during the last 5 or 24 h of incubation. Agonists (mAbs or leukocytes) were then added to the monolayers and the cells were further incubated at 37°C for 10 min. The monolayers were then fixed with freshly made 3% formaldehyde (Merck, Darmstadt, Germany) in PBS for 5 min at room temperature and permeabilized with 0.5% Tx-100 (Merck) for 5 min at 0°C, as described by Dejana et al. (1987). After rinsing in PBS, coverslips to be examined for actin morphology were incubated in the presence of 0.2 μ M fluorescein-labeled phalloidin (NBD-phalloidin; Molecular Probes Europe BV, Leiden, The Netherlands) in PBS for 20 min at room temperature, as suggested by the manufacturer. In contrast, cells to be examined for PECAM-1 or ICAM-1 immunostaining were incubated for 20 min at room temperature in the presence of fluorescein-labeled rabbit anti-mouse IgG, F(ab')₂ fragment. The slides were then mounted in glycerol/PBS and observations were carried out in a Zeiss Axiophot microscope equipped with epifluorescence. Fluorescence images were recorded on Fuji N1600 professional (Fuji Photo Film Co., Ltd., Tokyo, Japan) with a constant exposure of 40 s.

ELISA

First-passage HUVEC were plated onto gelatin-coated 96-well tissue culture plates (Costar Cluster, Cambridge, MA) and grown to confluence in HUVEC medium. Histamine (50 μ M) or LPS (0.5 μ g/ml) or medium was

then added to the monolayers, and the cells were further incubated at 37°C for the indicated time periods. The monolayers were then washed with a three-well volumes of medium and treated for 1 h at 4°C with mAbs to adherence molecules (5 μ g/ml). For the detection of P-selectin, the cells were fixed with 1% PFA in PBS for 15 min at room temperature in the dark before exposure to the relevant mAb. The cell monolayers were then washed three times with PBS containing 1% BSA, incubated for 1 h at 4°C in the presence of alkaline phosphatase-labeled affinity-purified antibodies from goat to mouse IgG (Sigma Chemical Co.), and further washed three times with PBS containing 1% BSA. The reaction product was generated by the addition of *para*-nitrophenyl-phosphate (Sigma Chemical Co.) at the concentration of 1 mg/ml in 0.1 M glycine buffer, pH 10.4, containing 1 mM MgCl₂ as substrate for the enzyme. Quantification of reaction product was performed in a Multiscan 340 apparatus (Flow Laboratories Inc., Milan, Italy) at 405 nm.

Adherence Assay

First-passage HUVEC were plated onto gelatin-coated 96-well tissue culture plates (Costar Cluster) and grown to confluence in HUVEC medium. LPS (0.5 μ g/ml) or medium was then added to the monolayers, and the cells were further incubated for 24 h at 37°C. Subsequently, the monolayers were washed with three-well volumes of medium and untreated monocytes, or monocytes that were pretreated for 60 min with mAb (20 μ g/ml), were added to the monolayers (70 μ l/well). Monocytes and HUVEC were then incubated for 15 min at 37°C. After incubation, the monolayers were washed with a two-well volume of PBS to remove non-adherent monocytes. A colorimetric assay was then used to detect the leukocytes adhering to the monolayers with tetramethyl benzidine (Sigma Chemical Co.) as the peroxidase substrate, as described previously in detail (Dobrina et al., 1991).

Antibodies

Anti-P-selectin/CD62P mAb CBL474 and anti-E-selectin/CD62E mAb CBL180 were from Cymbus Bioscience Ltd. (Southampton, UK). Anti-ICAM-1/CD54 mAb RR1/1 (Dustin et al., 1986) was obtained through the courtesy of R. Rothlein (Boehringer Ingelheim, Ridgefield, CT) and anti-ICAM-1/CD54 mAb MCA532 was obtained from Serotec Ltd. (Kidlington, Oxford, UK). Anti-VCAM-1/CD106 mAb 4B9 is a blocking mAb (Carlos et al., 1991). Anti-PECAM-1/CD31 mAb M89D3 inhibits leukocyte migration across HUVEC monolayers (Zocchi et al., 1996). mAb 60.3 recognizes a functional epitope on CD11/CD18 (Beatty et al. 1983). mAb CSLEX1 (Becton Dickinson, San Jose, CA) recognizes a functional epitope on sialyl Lewis x(sLe^x)/CD15s. mAb HP2/1 binds to a functional epitope on the CD49d (α chain) of leukocyte CD49d/CD29 (VLA-4) integrin and was a gift of Dr. F. Sanchez Madrid (Servicio de Immunologia, Hospital de la Princesa, Madrid, Spain; Sanchez Madrid et al., 1986). Preliminary observations confirmed that mAb M89D3 inhibited PMN and monocyte transendothelial migration and that all other mAbs used in this study inhibited PMN and/or monocyte adherence to HUVEC. Migration and adherence assays were performed as described previously (Dobrina et al., 1991; Zocchi et al., 1996).

Results

mAbs Directed Against P-selectin, E-selectin, and VCAM-1 Increase Endothelial Cytosolic Free Calcium Concentration

To examine the potential involvement of adhesion proteins in endothelial stimulation, $[Ca^{2+}]_i$ was measured in Fura2-loaded HUVEC. After incubation of EC in the presence or absence of histamine or LPS, two agents known to induce expression of adhesion molecules on HUVEC (Schleimer and Rutledge, 1986; Lorant et al., 1991), EC were exposed to leukocyte adherence-blocking mAbs directed against endothelial P- and E-selectins, ICAM-1, VCAM-1, and PECAM-1. In HUVEC stimulated with agents such as histamine or thrombin, expression of P-selectin is known to peak by 10 min and return to

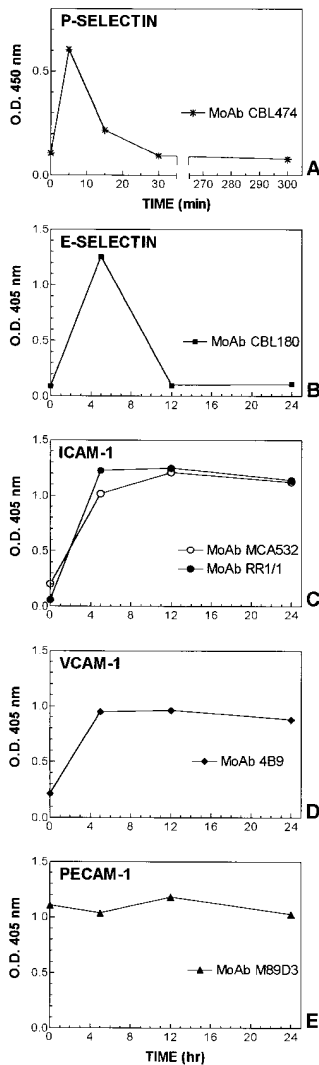


Figure 1. Time-course of induction of P-selectin, E-selectin, ICAM-1, VCAM-1, and PECAM-1 expression by HUVEC exposed to histamine or LPS. HUVEC monolayers were treated with medium containing 50 μ M histamine (A) or 0.5 μ g/ml *E. coli* LPS (B–D), or medium alone (E). Cells were harvested at the indicated times and examined for adhesion molecule expression by ELISA. The results are means of duplicate samples in a single experiment that is representative of two similar experiments performed with different cell isolates.

baseline within 20–30 min (Hattori et al., 1989). In contrast, in LPS- or cytokine-activated HUVEC expression of E-selectin is known to peak by 4–5 h and to return to baseline within 8–12 h, while ICAM-1 and VCAM-1 peak at 5–10 h and decline by 48–72 h (Pober et al., 1986; Tedesco et al., 1997). Finally, PECAM-1 is strongly expressed at the intercellular junctions of unstimulated HUVEC (Muller et al., 1989). In preliminary experiments we confirmed this pattern of expression of adhesion molecules in HUVEC, by using mAb CBL474 to P-selectin, mAb CBL180 to E-selectin, mAb RR1/1 or mAb MCA532 to ICAM-1, mAb 4B9 to VCAM-1, and mAb M89D3 to PECAM-1 (Fig. 1). Thereafter, we performed $[Ca^{2+}]_i$ measurements after treatment of the cells for 5 min with histamine or for 5 h or 24 h with LPS. As shown in Fig. 2, the anti-P-selectin mAb induced an increase in $[Ca^{2+}]_i$ in the histamine-treated HUVEC, but not in untreated HUVEC. Similarly, the anti-E-selectin mAb and the anti-VCAM-1 mAb caused $[Ca^{2+}]_i$ changes in HUVEC treated with LPS for 5 or 24 h, but not in untreated HUVEC. Notably, the mAb-induced response, when present, was comparable in magnitude to the increase in $[Ca^{2+}]_i$ caused by

the addition of 200 μ M histamine. In contrast, no change in $[Ca^{2+}]_i$ was observed after the addition of the anti-ICAM-1 mAb RR1/1 to HUVEC treated with LPS for 5 or 24 h or after the addition of the anti-PECAM-1 mAb to unstimulated cells.

Further experiments with the anti-ICAM-1 mAb MCA532 confirmed the negative results obtained with RR1/1. The anti-PECAM-1 mAb also failed to activate histamine-treated HUVEC or LPS-treated HUVEC. Similarly, we were unable to induce $[Ca^{2+}]_i$ changes in HUVEC that were treated first with the anti-ICAM-1 mAb or anti-PECAM-1 mAb, and then washed and incubated with a goat anti-mouse IgG polyclonal antibody to induce cell surface clustering of ICAM-1 or PECAM-1 (results not shown).

Anti-sLe^x mAb and Anti-VLA-4 mAb Inhibit the Increase in HUVEC $[Ca^{2+}]_i$ Induced by the Adherence of PMNs and Monocytes

Previous studies have shown that PMN adherence to EC elicits transient increases in endothelial $[Ca^{2+}]_i$ (Huang et al., 1993; Ziegelstein et al., 1994). Having established that stimulation of HUVEC with mAb directed against EC adhesion proteins was similarly effective in causing transient changes in the cell $[Ca^{2+}]_i$, we investigated whether these molecules were actually involved in the transduction of signals generated upon PMN or monocyte adherence to HUVEC. PMN are known to adhere to HUVEC via sLe^x, CD11/CD18, and PECAM-1, counter-receptors for endothelial P- and E-selectins, ICAM-1 and PECAM-1, respectively. Hence, HUVEC were stimulated with histamine for 5 min to induce expression of P-selectin, or with LPS for 5 h to induce expression of E-selectin and ICAM-1. HUVEC $[Ca^{2+}]_i$ was then monitored after the addition of PMN to the monolayers. PMN were unable to induce $[Ca^{2+}]_i$ changes in untreated HUVEC, but actively stimulated $[Ca^{2+}]_i$ increases both in histamine- and in LPS-treated monolayers (Fig. 3). Recordings of HUVEC $[Ca^{2+}]_i$ were then repeated using PMN that had been preincubated in the presence of an adherence-blocking mAb directed against the sLe^x, CD18, or PECAM-1. As reported in Table I, pretreatment of PMN with the anti-sLe^x mAb (CSLEX1) led to a marked inhibition (>80%) of their capacity to induce $[Ca^{2+}]_i$ changes in both histamine- and LPS-treated HUVEC. In contrast, $[Ca^{2+}]_i$ changes in histamine- and LPS-treated HUVEC were not significantly inhibited by a pretreatment of PMN with an anti-CD18 mAb (60.3) or an anti-PECAM-1 mAb (M89D3).

The same experimental approach was then followed to investigate the effect of monocyte adhesion to HUVEC. Monocytes are known to share with PMN the counter-receptors for endothelial E-selectin, ICAM-1, and PECAM-1. In addition, monocytes bind to endothelial VCAM-1 via VLA-4. To induce the expression on HUVEC of E-selectin, ICAM-1, and VCAM-1, the monolayers were treated with LPS. E-selectin was assumed to be maximally expressed on HUVEC after 5 h of incubation with LPS, whereas expression of ICAM-1 and VCAM-1 was assumed to be induced after 24 h as well as 5 h of incubation with LPS. The cells were then loaded with Fura2, and $[Ca^{2+}]_i$ recordings were performed upon addition of

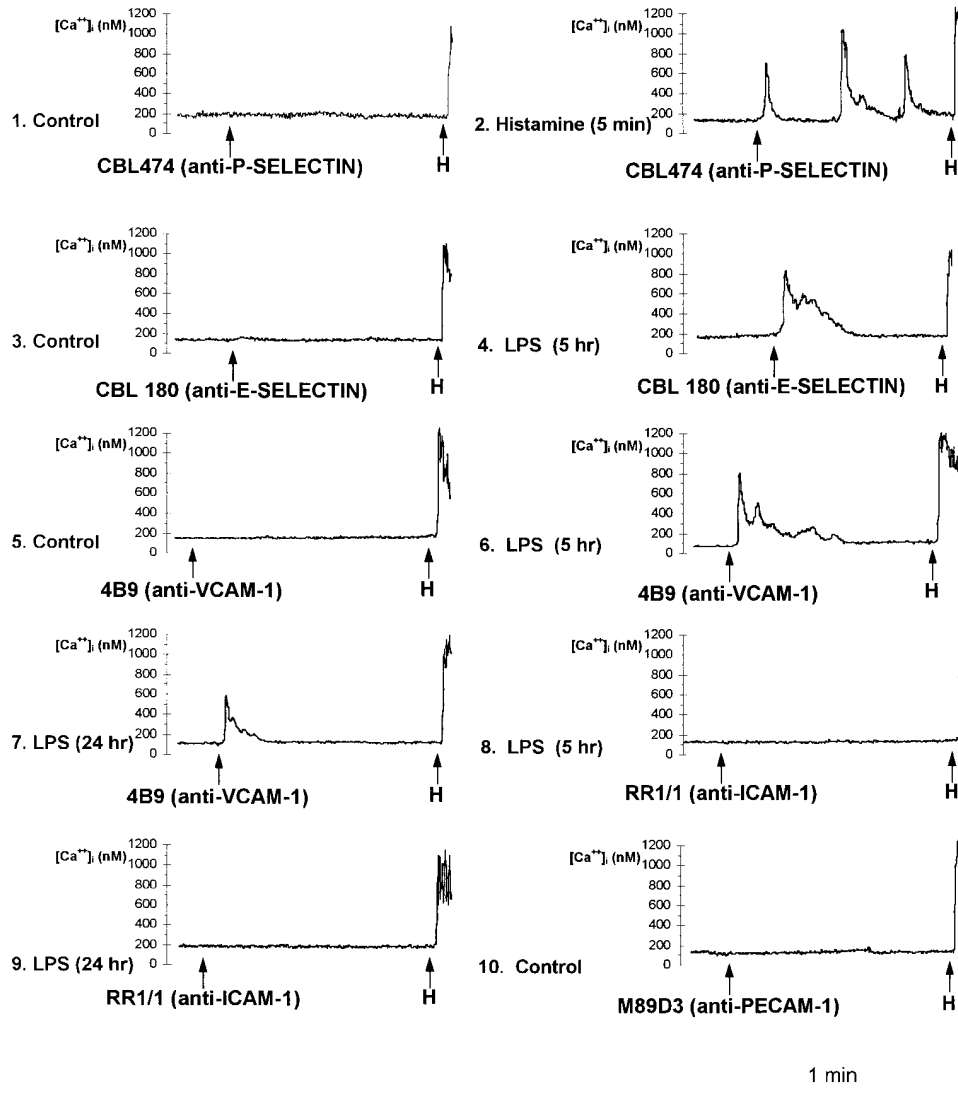


Figure 2. HUVEC $[Ca^{2+}]_i$ in response to mAbs directed against endothelial adhesion receptors for leukocytes. Representative tracings are shown of HUVEC $[Ca^{2+}]_i$ measured in single cells in Fura2-loaded endothelial monolayers. Surface expression of adhesion receptors was induced by incubating the HUVEC monolayers for 5 min in the presence of histamine (50 μ M) or for 5 h or 24 h in the presence of *E. coli* LPS (0.5 μ g/ml). $[Ca^{2+}]_i$ was measured for 15 min after the addition (arrows) of the indicated mAb. To control for variability among HUVEC, a maximal increase in $[Ca^{2+}]_i$ was induced by the addition of 200 μ M histamine (H) at the end of each recording. Each curve is representative of an average of thirty recordings obtained in a minimum of three independent experiments.

monocytes to the monolayers. As with PMN, monocytes failed to induce $[Ca^{2+}]_i$ changes in untreated HUVEC. However, they actively stimulated $[Ca^{2+}]_i$ changes in HUVEC treated with LPS for 5 and 24 h (Fig. 4). Table II shows the inhibitory effect of preincubation of monocytes incubation with adherence-blocking mAb on their capacity to stimulate $[Ca^{2+}]_i$ changes in HUVEC monolayers. Pretreatment of monocytes with the anti-sLe^x mAb CSLEX1 alone, the anti-CD18 mAb 60.3 alone, the anti-VLA-4 mAb HP2/1 alone, the anti-PECAM-1 mAb M89D3 alone, or pretreatment of monocytes with combinations of these anti-CD18, anti-VLA-4, and anti-PECAM-1 mAbs, was ineffective in inhibiting monocyte-induced $[Ca^{2+}]_i$ changes in 5 h LPS-treated HUVEC. However, $[Ca^{2+}]_i$ changes were markedly reduced when monocytes were pretreated with a combination of anti-sLe^x mAb and anti-VLA-4 mAb. Since sLe^x and VLA-4 are ligands of endothelial selectins and VCAM-1, respectively, it appears that HUVEC treated with LPS for 5 h are stimulated by mono-

cytes primarily via E-selectin and VCAM-1. In HUVEC treated with LPS for 24 h, treatment of monocytes with mAb to sLe^x alone or to CD18 alone did not inhibit monocyte-induced $[Ca^{2+}]_i$ changes. In contrast, pretreatment of monocytes with the anti-VLA-4 mAb HP2/1 alone was sufficient to inhibit strongly monocyte-induced $[Ca^{2+}]_i$ changes in HUVEC, indicating that in this setting the VLA-4/VCAM-1 interaction plays a major role in monocyte stimulation of EC.

Finally, we addressed the question whether the results obtained with mAb CSLEX1-treated PMN or with mAb CSLEX1- and/or mAb HP2/1-treated monocytes could be accounted for by some interference of these mAbs with leukocyte binding to HUVEC, thus preventing other leukocyte molecules from engaging HUVEC adhesion proteins. This possibility could not be excluded in our experiments with histamine-treated HUVEC, since Lorant et al. (1991) demonstrated that PMN adherence to thrombin-treated HUVEC was abolished by adherence-blocking

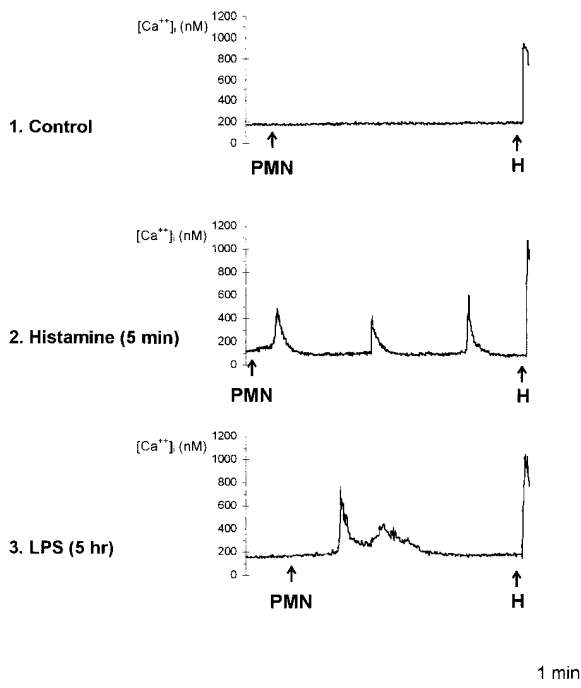


Figure 3. Effect of PMN adherence on HUVEC $[Ca^{2+}]_i$. HUVEC monolayers were treated for 5 min with medium alone or medium containing 50 μ M histamine, or for 5 h with medium containing 0.5 μ g/ml *E. coli* LPS. PMN were then added (arrows) to the monolayers (PMN/HUVEC ratio = 6–8:1) and fluorescence changes were recorded for 15 min. To control for variability among HUVEC, a maximal increase in $[Ca^{2+}]_i$ was induced by the addition of 200 μ M histamine (H) at the end of each recording. Each curve is representative of an average of thirty recordings obtained in four independent experiments.

mAb directed against P-selectin. In contrast, in a previous report we showed that PMN adherence to HUVEC treated with LPS for 4 h was only partially (by 50%) inhibited by the presence of an adherence-blocking mAb directed against E-selectin (Dobrina et al., 1991). Similarly,

Table I. Effect of mAbs to Leukocyte Adhesion Molecules on PMN-induced Changes in HUVEC $[Ca^{2+}]_i$

	Percent inhibition			
	CSLEX1 (anti-sLe ^x)	60.3 (anti-CD18)	M89D3 (anti-PECAM-1)	60.3 + M89D3
PMN treatment				
HUVEC treatment				
Hist 5 min	86.6 \pm 2.1*	16.3 \pm 5.9	5.9 \pm 3.2	ND
LPS 5 h	84.5 \pm 5.8*	3.1 \pm 5.2	3.4 \pm 9.6	2.1 \pm 3.4

PMN were treated for 60 min in the presence or absence of mAb CSLEX1, mAb 60.3 and mAb M89D3 (20 μ g/ml). HUVEC monolayers were treated for 5 min with 50 μ M histamine, or for 5 h with 0.5 μ g/ml *E. coli* LPS. PMN were then added to the monolayers (PMN/HUVEC ratio = 6–8:1) and fluorescence changes were recorded for 15 min in single Fura2-loaded HUVEC maintained in intact monolayers. Inhibitions were calculated by comparing the areas delineated by fluorescence spikes induced by mAb-treated and -untreated (control) PMN. To make recordings comparable to each other, a maximal increase in HUVEC $[Ca^{2+}]_i$ was induced by the addition of 200 μ M histamine at the end of each recording. Results are means \pm SE of 3–4 independent experiments, with 10–15 replicate recordings in each experiment. * $P < 0.005$ (paired *t* test). The other results were not significantly different from control values. ND, not determined.

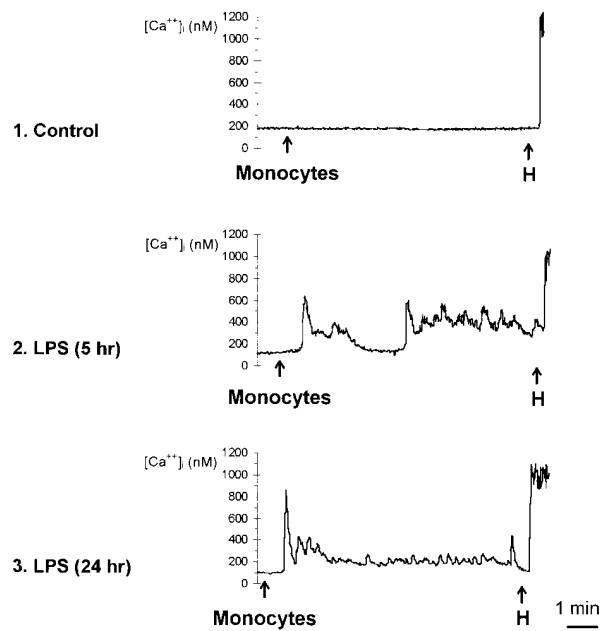


Figure 4. Effect of monocyte adherence on HUVEC $[Ca^{2+}]_i$. HUVEC monolayers were pretreated for 5 or 24 h with medium alone or medium containing 0.5 μ g/ml *E. coli* LPS. Monocytes were then added (arrows) to the monolayers (monocyte/HUVEC ratio = 6–8:1) and fluorescence changes were recorded for 15 min. To control for variability among HUVEC, a maximal increase in $[Ca^{2+}]_i$ was induced by the addition of 200 μ M histamine (H) at the end of each recording. Each curve is representative of an average of 30 recordings obtained in four independent experiments.

we showed that monocyte adherence to HUVEC was only minimally (\sim 15%) altered in the presence of anti-E-selectin mAb and/or anti-VLA-4 mAb (Carlos et al., 1991). These observations indicate that PMN and monocytes maintain their ability to adhere to HUVEC even in the presence of mAbs, which interfere with the sLe^x/E-selectin and/or the VLA-4/VCAM-1 pathways. As an additional control, we studied the adherence of monocytes to HUVEC treated with LPS for 24 h. As shown in Fig. 5, whereas pretreatment of monocytes with a combination of anti-CD18 and anti-VLA-4 mAbs significantly inhibited binding to the LPS-treated HUVEC, there was no inhibition when monocytes were pretreated with either mAb alone. Hence, it seems unlikely that the potent inhibition of monocyte-induced changes in HUVEC $[Ca^{2+}]_i$ by the anti-VLA-4 mAb HP2/1 was accounted for solely by its potential inhibitory effect on monocyte adherence.

mAbs Directed Against P-selectin, E-selectin, and VCAM-1 Induce Changes in Endothelial F-Actin Distribution

Previous studies have shown that stimulation of EC with soluble agonists such as histamine and thrombin, which increase endothelial $[Ca^{2+}]_i$, also induces rearrangements in endothelial actin morphology. Having established that an

Table II. Effect of mAbs to the Leukocyte Adhesion Molecules on Monocyte-induced Changes in HUVEC $[Ca^{2+}]_i$

Monocyte treatment	Percent inhibition							
	CSLEX1 (anti-sLe ^x)	60.3 (anti-CD18)	HP2/1 (anti-VLA-4)	M89D3 (anti-PECAM-1)	CSLEX1 + HP2/1	HP2/1 + M89D3	60.3 + HP2/1	60.3 + M89D3
HUVEC treatment								
LPS 5 h	5.7 ± 8.4	1.7 ± 8.6	3.1 ± 5.8	1.8 ± 5.8	80.1 ± 7.1*	0.4 ± 12.8	1.49 ± 3.42	7.9 ± 9.4
LPS 24 h	4.3 ± 2.06	7.9 ± 7.4	81.8 ± 8.0*	13.9 ± 12.7	ND	ND	ND	ND

HUVEC monolayers were treated for 5 or 24 h with 0.5 µg/ml *E. coli* LPS. Monocytes were treated for 60 min in the presence or absence of mAb CSLEX1, mAb 60.3, mAb HP2/1, and mAb M89D3 (20 µg/ml). Monocytes were then added to the monolayers (monocyte/HUVEC ratio = 6–8:1) and fluorescence changes were recorded for 15 min in single Fura2-loaded HUVEC maintained in intact monolayers. Inhibitions were calculated by comparing the areas delineated by fluorescence spikes induced by mAb-treated and -untreated (control) monocytes. To make recordings comparable to each other, a maximal increase in HUVEC $[Ca^{2+}]_i$ was induced by the addition of 200 µM histamine at the end of each recording. Results are means ± SE of 3–4 independent experiments, with 10–15 replicate recordings in each experiment. **P* < 0.005 (paired *t* test). The other results were not significantly different from control values. ND, not determined.

increase in HUVEC $[Ca^{2+}]_i$ results upon engagement of endothelial P-selectin, E-selectin, and VCAM-1, we sought evidence for a relationship between rearrangement of cytoskeletal actin filaments and endothelial stimulation via selectins or VCAM-1. Monolayers prepared identically to those used for $[Ca^{2+}]_i$ measurements were treated with *E. coli* LPS to induce expression of adhesion proteins. The cells were then treated with various mAbs to adhesion molecules, fixed, permeabilized, and then stained with fluorescein-labeled phalloidin. Fig. 6 A illustrates the F-actin distribution in a control monolayer treated with LPS for 5 h. Individual cells were clearly delineated by a rim of fluorescent staining. The central regions of the cells were devoid of prominent filament bundles. However, rare and randomly oriented actin fibers were present in some cells. When LPS-treated HUVEC were exposed to mAb CBL180 to E-selectin (Fig. 6 B), the rim of fluorescent staining at the cell periphery was less distinct, and the actin network was reorganized into thick cables mostly aligned parallel to the long axis of the cell (stress fibers). Similar results were obtained by treating the cells with mAb 4B9 to VCAM-1 (Fig. 6 C). Since not all of the cells responded similarly to the stimuli, we analyzed the percentage of stress fiber-positive and -negative cells under the various conditions. As shown in Table III, >50% of cells were positive for stress fibers after treatment of the monolayers with mAb CBL 180 to E-selectin or mAb 4B9 to VCAM-1. In contrast, we were unable to induce the appearance of

stress fibers by incubating the cells with mAb RR1/1 or mAb MCA532 to ICAM-1, or with mAb M89D3 to PECAM-1. Similarly, there was no evidence of stress fiber formation when HUVEC monolayers that had not been pretreated with LPS were exposed to any of the above mAb to endothelial adhesion molecules or to the anti-P-selectin mAb CBL474. Finally, treatment of the cells with histamine to induce P-selectin expression caused formation of prominent stress fibers directly, thus preventing us from testing the effect of the anti-P-selectin mAb on activated HUVEC.

Anti-sLe^x mAb and Anti-VLA-4 mAb Inhibit Rearrangement of Endothelial Actin Morphology Induced by PMN and Monocyte Adherence

We next investigated whether the adherence of PMNs or monocytes to EC would induce changes in the organization of endothelial cytoskeleton. Therefore, leukocytes were added either to unstimulated HUVEC monolayers or to monolayers that had been pretreated with *E. coli* LPS to induce expression of adhesion molecules on their surface. Fig. 7 depicts the effect of PMN–HUVEC interaction on endothelial actin morphology. When PMN were added to unstimulated HUVEC, F-actin distribution on the periphery of EC remained unchanged and we could not detect the appearance of stress fibers (Fig. 7 A). In contrast, addition of PMN to monolayers that had been

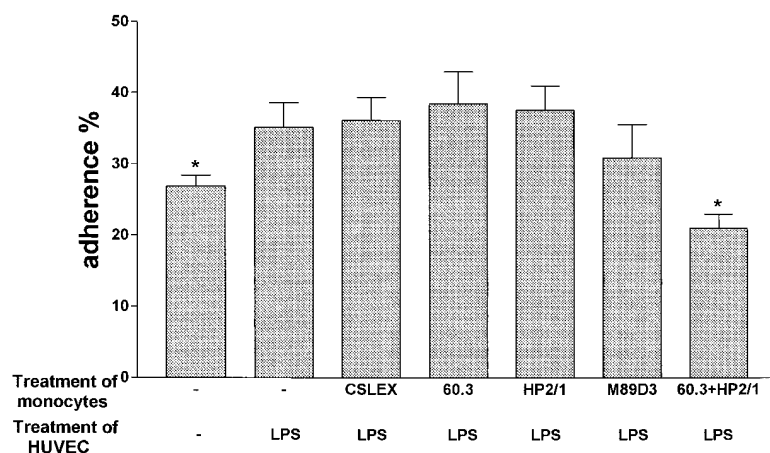


Figure 5. Effect of mAbs to leukocyte adhesion molecules on monocyte adherence to HUVEC. Endothelial monolayers were pretreated for 24 h with medium alone or medium containing 0.5 µg/ml *E. coli* LPS. Monocytes were treated for 60 min in the presence or absence of mAb CSLEX1, mAb 60.3, mAb HP2/1, or mAb M89D3 (20 µg/ml). Monocytes were then added to the monolayers (monocyte/HUVEC ratio = 6–8:1). Percent monocyte adherence was determined after a 15 min incubation at 37°C. Values are means ± SD of three experiments with 3–4 replicate wells in each experiment. **P* < 0.05 (paired *t* test) compared with adherence of untreated monocytes to LPS-treated HUVEC. The other results were not significantly different from control values.

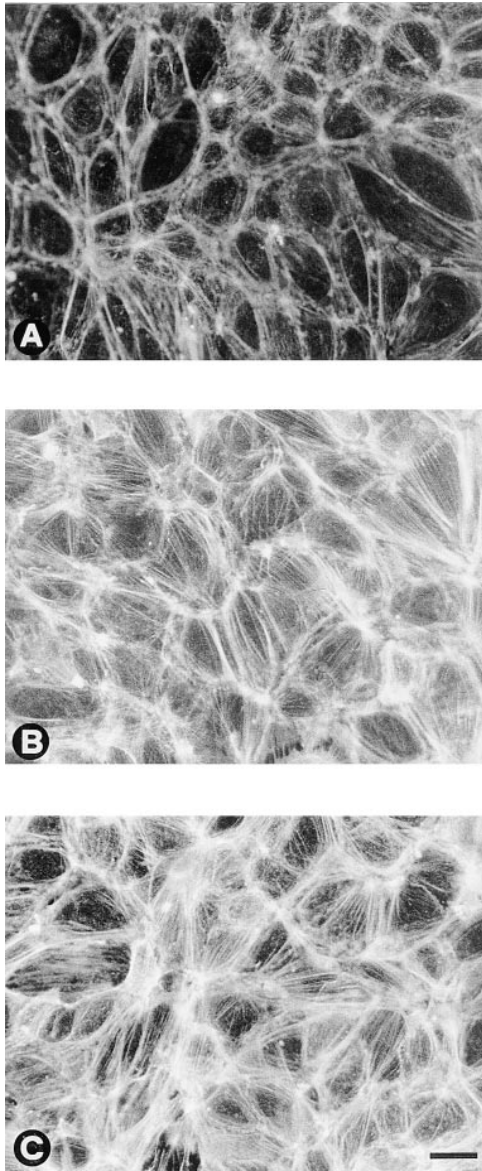


Figure 6. Fluorescence micrographs of F-actin distribution in HUVEC monolayers treated with mAbs directed to endothelial adhesion receptors for leukocytes. Surface expression of adherence receptors by HUVEC was induced by pretreating the cells for 5 h with medium containing *E. coli* LPS (0.5 $\mu\text{g}/\text{ml}$). mAb or medium (control) was then added to the monolayers and the cells were incubated for 10 min at 37°C. The monolayers were then fixed and stained for F-actin. HUVEC margins are delineated by a rim of fluorescent staining in control monolayers (A). Rare actin filaments appear within the cytoplasm. In contrast, a promi-

pretreated for 5 h with LPS caused the formation of prominent stress fibers in the large majority of the cells (Fig. 7 B). As shown in Table IV, pretreatment of PMN with the anti-sLe^x mAb CSLEX1 completely abolished the capacity of PMN to induce the formation of stress fibers in LPS-treated HUVEC, thus implicating endothelial selectins in EC stimulation. No inhibitory effect was observed with preincubation of PMN with the anti-CD18 mAb 60.3 or the anti-PECAM-1 mAb M89D3, suggesting that endothelial ICAM-1 and PECAM-1 were not involved in this response. Monocytes were used to explore the potential involvement of the endothelial VCAM-1 in leukocyte-EC interactions leading to formation of stress fibers. As in the experiments with PMN, monocytes were first added to unstimulated HUVEC. Under these conditions, we found no evidence of stress fiber formation in the monolayers (Fig. 8 A). To induce maximal expression of ICAM-1 and VCAM-1 on the endothelial surface, the monolayers were then incubated with LPS for 24 h. Under these conditions, the addition of monocytes actively stimulated a reorganization of endothelial actin filaments into stress fibers (Fig. 8 B). As shown in Table IV, formation of stress fibers was still induced by monocytes that had been pretreated with the adherence-blocking anti-sLe^x mAb CSLEX1, the anti-CD18 mAb 60.3, or the anti-PECAM-1 mAb M89D3. However, pretreatment of monocytes with the anti-VLA-4 mAb HP2/1 prevented changes endothelial actin morphology, thus confirming an involvement of VCAM-1 in EC stimulation by adhering monocytes.

Discussion

Our results demonstrate a role in outside-in signaling for endothelial P-selectin, E-selectin, and VCAM-1 adhesion proteins. mAbs directed against these receptors induced a rise in endothelial $[\text{Ca}^{2+}]_i$ and rearrangements of the endothelial cytoskeleton. Moreover, adherence-blocking mAbs directed against the leukocyte counter-receptors of P-selectin, E-selectin, and VCAM-1 inhibited both endothelial $[\text{Ca}^{2+}]_i$ changes and cytoskeletal changes induced by the adhering PMNs or monocytes.

Yoshida et al. (1996) previously demonstrated a role for E-selectin in outside-in signaling. They observed that clustering of E-selectin on the endothelial surface caused its anchoring to the actin cytoskeleton. In their study, treat-

ment network of actin filaments occupies the cytoplasm of monolayers treated with anti-E-selectin mAb (CBL180) (B), and anti-VCAM-1 mAb (4B9) (C). Bar, 10 μm .

Table III. Effect of mAbs to Adhesion Molecules on F-Actin Distribution in HUVEC

	Stress fiber-positive HUVEC (%)						
	Medium (control)	CBL180 (anti-E-selectin)	4B9 (anti-VCAM-1)	RR1/1 (anti-ICAM-1)	MCA532 (anti-ICAM-1)	M89D3 (anti-PECAM-1)	CBL474 (anti-P-selectin)
Unstimulated HUVEC	15.1 \pm 2.1	13.1 \pm 2.1	6.2 \pm 3.1	14.1 \pm 1.8	11.6 \pm 3.6	16.1 \pm 2.7	15.2 \pm 2.7
LPS-treated HUVEC	7.3 \pm 1.8	58.5 \pm 5.6*	67.2 \pm 6.8*	12.9 \pm 4.0	14.6 \pm 2.7	12.3 \pm 2.7	ND

Endothelial monolayers were pretreated for 5 h with medium or medium containing 0.5 $\mu\text{g}/\text{ml}$ *E. coli* LPS. mAb or medium (control) was then added to the monolayers and the cells were incubated for 10 min at 37°C. The monolayers were then fixed and stained for F-actin. Coverslips were analyzed for HUVEC actin distribution by two observers. An average of 300–400 cells were examined on each coverslip. Values are means \pm SE of three experiments. * $P < 0.01$ (paired *t* test) compared to controls. ND, not determined.

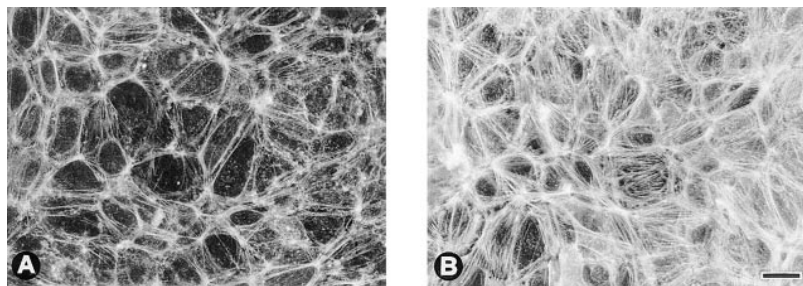


Figure 7. Fluorescence micrographs of HUVEC F-actin distribution in the presence of PMN. Before the assay, HUVEC monolayers were treated at 37°C for 5 h with medium alone (A) or medium containing 0.5 µg/ml *E. coli* LPS (B). PMN were then incubated with HUVEC for 10 min at 37°C. PMN adherence caused the appearance of actin filaments in the cytoplasm of LPS-treated HUVEC. Bar, 10 µm.

ment of HUVEC with a mAb to a functional epitope in the extracellular domain was not alone sufficient to cause association of E-selectin with the cytoskeleton; cross-linking by a secondary polyclonal antibody was required. In contrast, by using another adherence-blocking mAb (CBL180) to E-selectin, we were able to induce $[Ca^{2+}]_i$ changes and formation of stress fibers in HUVEC without a secondary cross-linking antibody. These results suggest that the outside-in signaling function of E-selectin leading to $[Ca^{2+}]_i$ changes and formation of stress fibers is not linked to its association to the cytoskeleton. Moreover, our results establish the participation of the E-selectin epitope recognized by mAb CBL180 in outside-in signaling. Similarly, the leukocyte adherence-blocking mAbs to P-selectin (mAb CBL474) and VCAM-1 (mAb 4B9) were by themselves effective in triggering endothelial $[Ca^{2+}]_i$ changes and formation of stress fibers, indicating that they identify functional epitopes in outside-in signaling. It is important to note that virtually all leukocyte populations are endowed with adherence mechanisms recognizing E-selectin, P-selectin, or VCAM-1 (Carlos and Harlan, 1994), thus emphasizing the potential role of outside-in signaling by these receptors in regulating the leukocyte traffic across the endothelial barrier. It is also worth noting that the overlapping function of P- and E-selectin with respect to outside-in signaling is consistent with recent observations in gene-targeted animals with E- and/or P-selectin deficiency. Neutrophil extravasation into inflamed peritoneum was minimally altered in either the P- or the E-deficient mice, whereas it was markedly reduced in animals deficient in both selectins (Frenette et al., 1996; Munoz et al., 1997; Subramaniam et al., 1997).

Huang et al. (1993) were the first to demonstrate that leukocyte adherence to HUVEC induced a transient increase in endothelial $[Ca^{2+}]_i$. Since $[Ca^{2+}]_i$ changes were induced by leukocytes equally well in the presence and in

the absence of serum, these authors concluded that oxidants and/or proteases secreted by leukocytes were unlikely to be the effectors of endothelial stimulation. Rather, they noted that the changes in endothelial $[Ca^{2+}]_i$ coincided temporally with leukocyte transit across the endothelial monolayers, suggesting that endothelial stimulation was due in part to adhesive interactions of endothelial cells with diapedesing leukocytes. Recently, Muller et al. (1993) demonstrated that the homophilic interaction of leukocyte and endothelial PECAM-1 is crucial to the process of PMN and monocyte migration through intercellular junctions of endothelial cells. Therefore, in investigating the potential involvement of adhesion molecules in endothelial activation by leukocytes we expected that PECAM-1 would be involved. However, treatment of HUVEC with the anti-PECAM-1 mAb M89D3, which inhibits leukocyte migration across HUVEC monolayers (Zocchi et al., 1996), did not cause $[Ca^{2+}]_i$ changes nor induce the formation of stress fibers. In addition, endothelial $[Ca^{2+}]_i$ changes and the formation of stress fibers induced by the addition of PMN and monocytes to the EC monolayers were not inhibited by a pretreatment of the leukocytes with mAb M89D3. Since changes in surface expression of PECAM-1 were described in HUVEC exposed to various cytokines (Ferrero et al., 1996; Rival et al., 1996), we also tested the anti-PECAM-1 mAb on LPS-treated HUVEC, but again observed no effect. Similarly, negative results were obtained when clustering of PECAM-1 was induced by a secondary antibody. Nevertheless, we cannot exclude a potential contribution of PECAM-1 to signaling since: (1) the epitope recognized by M89D3, although functionally relevant in leukocyte-HUVEC adhesive interactions, might be distinct from the epitope(s) involved in outside-in signaling, and (2) the stimulation of the EC monolayers by PMN or monocytes pretreated with the anti-PECAM-1 mAb is likely dependent upon the interac-

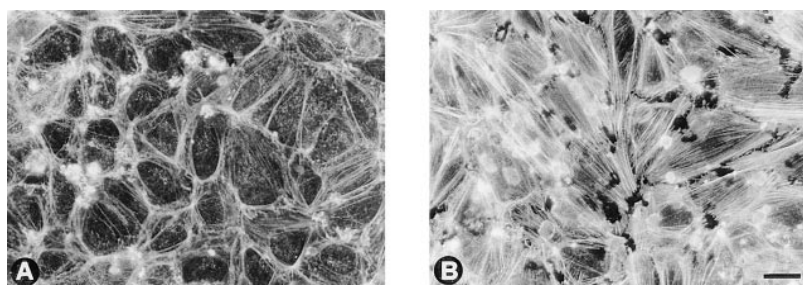


Figure 8. Fluorescence micrographs of HUVEC F-actin distribution in the presence of monocytes. Before the assay, HUVEC monolayers were pretreated at 37°C for 24 h with medium alone (A) or medium containing 0.5 µg/ml *E. coli* LPS (B). Monocytes were then incubated with HUVEC for 10 min at 37°C. Monocyte adherence causes the appearance of actin bundles in the cytoplasm of LPS-treated HUVEC. Monocytes adherent to HUVEC were also detected by F-actin staining. Bar, 10 µm.

Table IV. Effect of mAb to Leukocyte Adhesion Molecules on Leukocyte-induced Changes in HUVEC F-Actin Morphology

PMN treatment	Stress fiber–positive HUVEC (%)				
	Medium (control)	CSLEX1 (anti-sLe ^x)	60.3 (anti-CD18)	M89D3 (anti-PECAM-1)	
LPS-treated HUVEC (5 h)	64.3 ± 5.8	16.7 ± 2.5*	58.4 ± 6.1	60.9 ± 6.6	
Monocyte treatment	Medium (control)	CSLEX1 (anti-sLe ^x)	60.3 (anti-CD18)	M89D3 (anti-PECAM-1)	HP2/1 (anti-VLA-4)
LPS-treated HUVEC (24 h)	61.3 ± 4.4	56.2 ± 3.7	68.3 ± 4.6	71.2 ± 7.1	20.1 ± 2.1*

Before the assay, HUVEC monolayers were treated for 5 or 24 h with 0.5 µg/ml *E. coli* LPS. Leukocytes (PMN or monocytes) were treated for 60 min in the presence or absence of mAb CSLEX1, mAb 60.3, mAb M89D3, or mAb HP2/1 (20 µg/ml). Leukocytes were then added to the monolayers (leukocyte/HUVEC ratio = 6–8:1) and the cells were incubated for 10 min at 37°C. The monolayers were then fixed and stained for F-actin. Coverslips were analyzed for HUVEC actin distribution by two observers. An average of 300–400 cells were examined on each coverslip. Values are means ± SE of three experiments. **P* < 0.05 (paired *t* test) compared to controls.

tion of the leukocytes with endothelial receptors other than PECAM-1 (e.g., selectins and/or VCAM-1), thereby obscuring any effect of the leukocyte pretreatment.

In contrast to PECAM-1, the inhibitory effect of leukocyte pretreatment with the anti-sLe^x or anti-VLA-4 mAb provides convincing evidence for the participation of their endothelial counter-structures in outside-in signaling. Notably, these treatments did not inhibit leukocyte adherence to HUVEC by the CD11/CD18-ICAM-1 adherence mechanism, nor leukocyte diapedesis. Regardless, with these mAbs leukocytes were unable to induce [Ca²⁺]_i changes or formation of stress fibers in the endothelial monolayers, thus excluding any major role in [Ca²⁺]_i signaling for PECAM-1 as well as for other molecules at the inter-endothelial junctions. A recent report by Gurubhagavatula et al. (1998) showed that an anti-PECAM-1 antibody (mAb 4G6) induced a slow but sustained increase in HUVEC [Ca²⁺]_i. Notably, the kinetics of the responses to mAb 4G6 were quite different from those induced by thrombin. In contrast, in all published reports to date, leukocyte adherence was shown to induce rapid, large changes in HUVEC [Ca²⁺]_i, comparable to those induced by agents such as histamine or thrombin. Hence, the study by Gurubhagavatula et al. (1998) supports our contention that endothelial molecules other than PECAM-1 function as signaling receptors for adhering leukocytes. Since anti-PECAM antibodies inhibit leukocyte migration across EC at the level of the junctions (Muller et al., 1993), our results suggest that changes in endothelial [Ca²⁺]_i and actin morphology are necessary, but not sufficient, to facilitate leukocyte diapedesis.

Four groups of investigators already investigated the involvement of ICAM-1, the endothelial ligand for CD11a/CD18 and CD11b/CD18, in endothelial outside-in signaling. Durieux-Trautmann et al. (1994) reported that clustering of ICAM-1-induced tyrosine phosphorylation of cytoskeletal proteins potentially involved in the arrangement of inter-endothelial junctions, such as cortactin and src. More recently, the mechanisms leading to opening of endothelial adherence junctions during leukocyte diapedesis were investigated by Del Maschio et al. (1996) who did not observe changes in the junctional structures when ICAM-1 was clustered on the endothelial surface. Ziegelstein et al. (1994) and Pfau et al. (1995) examined the effect of anti-CD18 mAb, and anti-ICAM-1 mAb, respectively, on changes in endothelial [Ca²⁺]_i induced by the adherence of leukocytes. Ziegelstein et al. (1994) found that [Ca²⁺]_i changes induced by PMN adherence were partially

(~50%) inhibited by anti-CD18 mAb. Similarly, Pfau et al. (1995) observed that changes in HUVEC [Ca²⁺]_i caused by the adherence of natural killer lymphocytes were partially inhibited by an anti-ICAM-1 mAb. In the present study, we observed some degree of inhibition of the changes in HUVEC [Ca²⁺]_i induced by leukocytes by anti-ICAM-1 mAb only when PMN were added to histamine-stimulated HUVEC. However, the inhibition was weak and inconsistent, and did not achieve statistical significance. It should be noted that leukocytes treated with anti-sLe^x and/or anti-VLA-4 mAb were unable to induce endothelial [Ca²⁺]_i changes or stress fibers formation, even though CD11a/CD18 and CD11b/CD18 were free to interact with endothelial ICAM-1. From this result we suggest that the interaction of leukocyte CD11/CD18 with endothelial ICAM-1 acts primarily as a co-stimulus for endothelial [Ca²⁺]_i changes.

Formation of stress fibers was described in ECs undergoing mechanical stress (Sato and Ohshima, 1994; Cucina et al., 1995), or ECs exposed to soluble inflammatory agonists, such as histamine and thrombin (Rotrosen and Gallin, 1986; Kolodney and Wysolmerski, 1992). A novel finding in the present study is that PMN or monocyte adherence also induces rearrangements of endothelial actin filaments, leading to formation of stress fibers. Gross changes in endothelial morphology were described to occur during leukocyte adherence to and passage across the endothelial lining of blood vessels (Marchesi and Florey, 1960). More recently, Hixenbaugh et al. (1997) demonstrated that neutrophil adherence to HUVEC induces endothelial myosin light chain phosphorylation and contraction of the monolayers. However, the mechanism(s) by which adhering leukocytes induce endothelial contraction have not been well characterized. Our evidence linking adhesion receptors, [Ca²⁺]_i changes, and formation of stress fibers contributes to our understanding of these mechanisms.

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References

- Albelda, S.M., P.D. Oliver, L.H. Romer, and C.A. Buck. 1990. EndoCAM: A novel endothelial cell-cell adhesion molecule. *J. Cell Biol.* 110:1227-1237.
- Austrup, F., D. Vestweber, E. Borges, M. Lohning, R. Brauer, U. Herz, H. Renz, R. Hallman, A. Scheffold, A. Radbruch, and A. Hamann. 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissue. *Nature.* 385:81-83.
- Beatty, P.G., J.A. Ledbetter, P.G. Martin, T.H. Price, and J.A. Hansen. 1983. Definition of a common leukocyte cell-surface antigen (Gp95-150) associated with diverse cell-mediated immune function. *J. Immunol.* 131:2913-2918.
- Bevilacqua, M.P., and R.M. Nelson. 1993. Selectins. *J. Clin. Invest.* 91:379-387.
- Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl.):77-81.
- Carlos, T.M., N.L. Kovach, B.R. Schwartz, M. Rosa, B. Newman, E. Wayner, C. Benjamin, L. Osborn, R. Lobb, and J.M. Harlan. 1991. Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1. *Blood.* 77:2266-2271.
- Carlos, T.M., and J.M. Harlan. 1994. Leukocyte-endothelial adhesion molecules. *Blood.* 84:2068-2101.
- Cucina, A., A.V. Sterpetti, G. Pupelis, A. Fragale, S. Lepidi, A. Cavallaro, Q. Giustiniani, and L. Santoro-D'angelo. 1995. Shear stress induces changes in the morphology and cytoskeleton organization of arterial endothelial cells. *Eur. J. Vasc. Endovasc. Surg.* 9:86-92.
- Dejana, E., S. Colella, L.R. Languino, G. Balconi, G.C. Corbascio, and P.C. Marchisio. 1987. Fibrinogen induces adhesion, spreading, and microfilament organization of human endothelial cells in vitro. *J. Cell Biol.* 104:1403-1411.
- Del Maschio, A., A. Zanetti, M. Corada, Y. Rival, L. Roco, M.G. Lampugnani, and E. Dejana. 1996. Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. *J. Cell Biol.* 135:497-510.
- Dobrina, A., R. Menegazzi, T.M. Carlos, E. Nardon, R. Cramer, T. Zacchi, J.M. Harlan, and P. Patriarca. 1991. Mechanisms of eosinophil adherence to cultured vascular endothelial cells. Eosinophils bind to the cytokine-induced ligand vascular cell adhesion molecule-1 via the very late activation antigen-4 integrin receptor. *J. Clin. Invest.* 88:20-26.
- Durieux-Trautmann, O., N. Chaverot, S. Cazaubon, A.D. Strosberg, and P.O. Couraud. 1994. Interleukin-1 activation induces tyrosine phosphorylation of the cytoskeleton-associated protein cortactin in brain microvessel endothelial cells. *J. Biol. Chem.* 269:12536-12540.
- Dustin, M.L., R. Rothlein, A.K. Bhan, C.A. Dinarello, and T.A. Springer. 1986. Induction by IL-1 α and interferon- γ : tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol.* 137:245-254.
- Ferrero, E., A. Villa, M.E. Ferrero, E. Toninelli, J.R. Bender, R. Pardi, and M.R. Zocchi. 1996. Tumor necrosis factor alpha-induced vascular leakage involves PECAM-1 phosphorylation. *Cancer Res.* 56:3211-3215.
- Frenette, P.S., T.N. Mayadas, H. Rayburn, R.O. Hynes, and D.D. Wagner. 1996. Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. *Cell.* 84:563-574.
- Furie, M.B., B.L. Naprstek, and S.C. Silverstein. 1987. Migration of neutrophils across monolayers of cultured microvascular endothelial cells. An vitro model of leukocyte extravasation. *J. Cell Sci.* 88:161-175.
- Garcia, J.G., H.W. Davis, and C.E. Patterson. 1995. Regulation of endothelial gap formation and barrier dysfunction: role of myosin light chain phosphorylation. *J. Cell Physiol.* 163:510-522.
- Goligorsky, M.S., D.N. Menton, A. Laszlo, and H. Lum. 1989. Nature of thrombin-induced sustained increase in cytosolic calcium concentration in cultured endothelial cells. *J. Biol. Chem.* 264:16771-16775.
- Grynkiewicz, G.M., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450.
- Gurubhagavatula, I., Y. Amrani, D. Pratico, F.L. Ruberg, S.M. Albelda, and R.A. Panettieri, Jr. 1998. Engagement of human PECAM-1 (CD31) on human endothelial cells increases intracellular calcium ion concentration and stimulates prostacyclin release. *J. Clin. Invest.* 101:212-222.
- Hattori, R., K.K. Hamilton, R.D. Fugates, R.P. McEver, and P.J. Sims. 1989. Stimulate secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. *J. Biol. Chem.* 264:7768-7771.
- Hixenbaugh, E.A., Z.M. Goeckeler, N.N. Papaiya, R.B. Wysolmerski, S.C. Silverstein, and A.J. Huang. 1997. Stimulated neutrophils induce myosin light chain phosphorylation and isometric tension in endothelial cells. *Am. J. Physiol.* 273:981-988.
- Huang, A.J., J.E. Manning, T.M. Bandak, M.C. Ratau, K.R. Hanser, and S.C. Silverstein. 1993. Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. *J. Cell Biol.* 120:1371-1380.
- Jacob, R., J.E. Merritt, T.J. Hallam, and T.J. Rink. 1988. Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. *Nature.* 335:40-45.
- Jaffe, E.A., R.L. Nachman, C.G. Becker, and R.C. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745-2756.
- Kolodney, M.S., and R.B. Wysolmerski. 1992. Isometric contraction by fibroblasts and endothelial cells in tissue culture: a quantitative study. *J. Cell Biol.* 117:73-82.
- Laposata, M., D.K. Dohnarsky, and H.S. Shin. 1982. Thrombin-induced gap formation in confluent endothelial cell monolayers in vitro. *Blood.* 62:549-556.
- Lorant, D.E., K.D. Patel, T.M. McIntyre, R.P. McEver, S.M. Prescott, and G.A. Zimmerman. 1991. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J. Cell Biol.* 115:223-234.
- Lorenzon, P., A. Giovannelli, D. Ragozzino, F. Eusebi, and F. Ruzzier. 1997. Spontaneous and repetitive calcium transients in C2C12 mouse myotubes during in vitro myogenesis. *Eur. J. Neurosci.* 9:800-808.
- Maciag, T., J. Cerundolo, S. Ilsley, and P.R. Forand. 1979. An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. *Proc. Natl. Acad. Sci. USA.* 76:5674-5678.
- Marchesi, V.T., and H.W. Florey. 1960. Electron micrographic observation on the emigration of leukocytes. *Q. J. Exp. Physiol.* 45:343-348.
- Morel, N.M., P.P. Petruzzo, H.B. Hechtman, and D. Shepro. 1990. Inflammatory agonist that increase microvascular permeability in vivo stimulated cultured pulmonary microvessel endothelial cell contraction. *Inflammation.* 14:571-583.
- Muller, W.A., S.A. Weigl, X. Deng, and D.M. Phillips. 1993. Pecam-1 is required for transendothelial migration of leukocytes. *J. Exp. Med.* 178:449-460.
- Muller, W.A., C.M. Ratti, S.L. McDonnell, and Z.A. Cohn. 1989. A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. *J. Exp. Med.* 170:399-414.
- Munoz, F.M., E.P. Hawkins, D.C. Bullard, A.L. Beaudet, and S.L. Kaplan. 1997. Host defense against systemic infection with *Streptococcus pneumoniae* is impaired in E-, P-, and E-/P-selectin-deficient mice. *J. Clin. Invest.* 100:2099-2106.
- Peri, G., F. Chiaffarino, S. Bernasconi, I. Martin-Padura, and A. Mantovani. 1990. Cytotoxicity of activated monocytes on endothelial cells. *J. Immunol.* 144:1444-1448.
- Pfau, S., D. Leitenberg, H. Rinder, B.R. Smith, R. Pardi, and J.R. Bender. 1995. Lymphocyte adhesion-dependent calcium signaling in human endothelial cells. *J. Cell Biol.* 128:969-978.
- Pober, J.S.M., M.A. Gimbrone, Jr., L.A. Lapierre, D.L. Mendrick, W. Fiers, R. Rothlein, and T.A. Springer. 1986. Overlapping patterns of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J. Immunol.* 137:1893-1896.
- Rival, Y., A. Del Maschio, M.J. Rabiet, E. Dejana, and A. Duperray. 1996. Inhibition of platelet endothelial cell adhesion molecule-1 synthesis and leukocyte transmigration in endothelial cells by the combined action of TNF- α and IFN- γ . *J. Immunol.* 157:1233-1241.
- Rotrosen, D., and J.I. Gallin. 1986. Histamine type I receptor occupancy increases endothelial cytosolic calcium, reduces F-actin, and promotes albumin diffusion across cultured endothelial monolayers. *J. Cell Biol.* 103:2379-2387.
- Sanchez Madrid, F., M.O. De Londazuri, G. Morago, M. Cebrian, A.L. Acevedo, and C. Bernabeu. 1986. VLA-3: a novel polypeptide association with the VLA molecular complex: cell distribution and biochemical characterization. *Eur. J. Immunol.* 16:1343-1349.
- Sato, M., and N. Ohshima. 1994. Flow-induced changes in shape and cytoskeletal structure of vascular endothelial cells. *Biorheology.* 31:143-153.
- Schleimer, R.P., and B.K. Rutledge. 1986. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters. *J. Immunol.* 136:649-654.
- Subramaniam, M., S. Saffaripour, L. Van De Water, P.S. Frenette, T.N. Mayadas, R.O. Hynes, and D.D. Wagner. 1997. Role of endothelial selectins in wound repair. *Am. J. Pathol.* 150:1701-1709.
- Tedesco, F., M. Pausa, E. Nardon, M. Introna, A. Mantovani, and A. Dobrina. 1997. The cytolytically inactive terminal complement complex activates endothelial cells to express adhesion molecules and tissue factor procoagulant activity. *J. Exp. Med.* 185:1619-1627.
- Yoshida, M., W.F. Westlin, N. Wang, D.E. Ingber, A. Rosenzweig, N. Resnick, and M.A. Gimbrone, Jr. 1996. Leukocyte adhesion to vascular endothelium induces E-selectin linkage to the actin cytoskeleton. *J. Cell Biol.* 133:445-455.
- Ziegelstein, R.C., S. Corda, R. Pili, A. Passaniti, D. Lefer, J.L. Zweier, A. Fraticelli, and M.C. Capogrossi. 1994. Initial contact and subsequent adhesion of human neutrophils or monocytes to human aortic endothelial cells releases an endothelial intracellular calcium store. *Circulation.* 90:1899-1907.
- Zocchi, M.R., E. Ferrero, B.E. Leone, P. Rovere, E. Bianchi, E. Toninelli, and R. Pardi. 1996. CD31/PECAM-1-driven chemokine-independent transmigration of human T lymphocytes. *Eur. J. Immunol.* 26:759-767.