Flow-conditioned HUVECs support clustered leukocyte adhesion by coexpressing ICAM-1 and E-selectin

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Burns, Michael P., and Natacha DePaola. Flow-conditioned HUVECs support clustered leukocyte adhesion by coexpressing ICAM-1 and E-selectin. Am J Physiol Heart Circ Physiol 288: H194–H204, 2005. First published August 26, 2004; doi:10.1152/ajpheart.01078.2003.—Endothelial sequestration of circulating monocytes is a key event in early atherosclerosis. Hemodynamics is proposed to regulate monocyte-endothelial cell interactions by direct cell activation and establishment of flow environments that are conducive or prohibitive to cell-cell interaction. We investigated fluid shear regulation of monocyte-endothelial cell adhesion in vitro using a disturbed laminar shear system that models in vivo hemodynamics characteristic of lesion-prone vascular regions. Human endothelial cell monolayers were flow conditioned for 6 h before evaluation of monocyte adhesion under static and dynamic flow conditions. Results revealed a distinctive clustered cell pattern of monocyte adhesion that strongly resembles in vivo leukocyte adhesion in early- and late-stage atherosclerosis. Clustered monocyte cell adhesion correlated with endothelial cells coexpressing intercellular adhesion molecule-1 (ICAM-1) and E-selectin as result of a flow-induced, selective up-regulation of E-selectin expression in a subset of ICAM-1-expressing cells. Clustered monocyte cell adhesion assay under static conditions exhibited a spatial variation in size and frequency of occurrence, which demonstrates differential regulation of endothelial cell adhesiveness by the local flow environment. Dynamic adhesion studies conducted with circulating monocytes resulted in clustered cell adhesion only within the disturbed flow region, where the monocyte rate of motion is sufficiently low for cell-cell interaction. These studies provide evidence and reveal mechanisms of local hemodynamic regulation of endothelial adhesiveness and endothelial monocyte interaction that lead to localized monocyte adhesion and potentially contribute to the focal origin of arterial diseases such as atherosclerosis.

A KEY EVENT IN EARLY ATHEROSCLEROSIS is monocyte adhesion and extravasation at predictable vascular sites (13, 27, 30). Hemodynamics may contribute to atherogenesis, as lesion-prone vascular regions are associated with low wall-shear stresses, temporal and spatial shear-stress gradients, and flow recirculation (3, 44). The fluid dynamics characteristic of lesion-prone regions may favor monocyte adhesion by direct alteration of endothelial cell adhesiveness and by establishment of monocyte transport environments conducive to arterial wall adhesion.

Adhesion molecules mediate endothelial cell sequestration of circulating leukocytes by physically supporting adhesive interactions and participating in cell signaling (34, 35). The endothelial cell adhesion molecules of interest in adhesion with leukocytes are intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin. VCAM-1 has long been proposed to be a principal endothelial cell adhesion molecule in the selective recruitment of monocytes and lymphocytes to atherosclerotic lesions (6), because the integrin ligand of VCAM-1, very late antigen (VLA)-4, is expressed by monocytes and lymphocytes but not neutrophils. However, present knowledge of the leukocyte-adhesion cascade suggests that the endothelium uses selectin- and cytokine-mediated cell signaling pathways in addition to adhesion-molecule pairing to obtain specificity in leukocyte recruitment (33, 43).

There is supporting evidence that the endothelium uses ICAM-1 and selectin coexpression in its selective sequestration of circulating monocytes. Histological evaluation of human atherosclerosis consistently reports the colocalized expression of ICAM-1 and E-selectin at the borders of developing lesions (17, 41), and this coexpression has been correlated with recent monocyte infiltrates (11, 17). Growing evidence indicates that selectins are also involved in intracellular signaling and potentiate cell-cell interactions between leukocytes and endothelial cells. Leukocyte adhesion to E-selectin activates β2-integrin-dependent adhesion in leukocytes (19, 33) and triggers the mitogen-activated protein kinase signaling pathway in endothelial cells (15). Likewise, ICAM-1 ligation also induces cell signaling in leukocytes and endothelial cells (42). Interestingly, neutrophil adhesion to activated endothelial cells has been shown to induce the colocalization of ICAM-1 and E-selectin in endothelial cell plasma membranes, and the cytoplasmic domains of these focal adhesion complexes are associated with Src, a molecule involved in cell signaling (38, 39).

Numerous in vitro studies have shown that fluid shear stress upregulates human endothelial cell adhesion molecule expression. Increased ICAM-1 expression has been detected as early as 6 h and is sustained for up to 48 h (26, 28). Fluid shear modulation of E-selectin expression remains unclear; increased expression was reported for oscillatory laminar shear stress (5), whereas unidirectional laminar shear did not alter E-selectin expression (26, 28). In the present study, we report that endothelial cell expression of E-selectin is significantly increased in a selective, small group of cells after 6 h of exposure to laminar shear.

Previous in vitro investigations have reported that endothelial adhesiveness for leukocytes increases after exposure to fluid shear stress (5, 14, 25, 26, 28) due to elevated expression of ICAM-1 (5, 26, 28) or VCAM-1 (5, 14, 25). However, the...
heterogeneous nature of leukocyte adhesion to flow-conditioned endothelial layers has never before been addressed. The present study is to our knowledge the first detailed characterization of heterogeneous leukocyte adhesion to flow-conditioned endothelial layers. We developed a novel method of quantifying leukocyte adhesion that allows the two patterns of cell adhesion (single and clustered) to be characterized.

Our experimental model (9, 10) permits endothelial layers to be exposed to a well-defined flow field in which a portion of the layer is exposed to disturbed flows, with wall shear stresses and shear-stress gradients similar to lesion-prone regions of the human carotid sinus while adjacent areas of the layer experience a flow field characteristic of lesion-resistant regions. The adhesion assays were performed with U-937 cells, which express β1- and β2-integrins, sialylated Lewis x antigen, and L-selectin (29). This array of adhesion molecules allows U-937 cells to bind ICAM-1, VCAM-1, E-selectin, and P-selectin. We evaluated leukocyte adhesion under both static and flow conditions to assess the functional adhesiveness of the flow-conditioned endothelial layer. The findings reported here provide an important contribution to understanding fluid shear modulation of endothelial adhesiveness for leukocytes and its potential role in the development of vascular lesions.

MATERIALS AND METHODS

Cell culture. Single-donor human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA) and maintained with manufacturer's endothelial growth medium-2 supplemented with 10% fetal bovine serum (GIBCO; Green Island, NY). HUVECs were grown in tissue culture-treated polystyrene flasks (Corning; Corning, NY) coated with 0.1% (vol/vol) aqueous gelatin solution (Sigma) and kept in an incubator that provided a humidified, 37°C, 95% air-5% CO2 atmosphere. Confluent HUVEC layers were subcultured at a 1:3 ratio using 0.025% trypsin-0.01% EDTA (Clonetextics) to detach cells from their substratum and were used for experiments up to the sixth passage. For shear experiments, HUVECs were seeded onto glass slides coated with a 0.5% gelatin subbing solution that contained 0.05% potassium chromate sulfate and, cell layers were used within 12 h of reaching confluency. Control monolayers were similarly seeded and maintained. U-937 cells (37) were obtained from American Type Culture Collection (Rockville, MD) and were maintained with RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated FBS (GIBCO), 1 mM MEM sodium pyruvate (GIBCO), and 2.5 mg/ml d-glucose (Fisher Scientific; Pittsburgh, PA). U-937 cell suspensions were kept at a density of 0.5–2.0 × 10⁶ cells/ml by periodic dilution with fresh growth medium.

Flow system. The parallel-plate flow chamber used to expose HUVEC layers to disturbed laminar shear has been described previously (9). Briefly, glass slides with HUVEC monolayers were assembled into a parallel-plate flow chamber, and shear stress was imposed on the HUVECs by viscous medium flowing through the chamber. A flow disturbance was created by a 0.5-mm-thick rectangular step (0.5 × 2 × 25 mm) fixed to the glass slide upstream of the HUVEC monolayer. The flow rate of the circulating fluid was adjusted so that the shear stress distribution imposed on the HUVEC layer was similar to the lesion-prone region of the lateral wall of the internal carotid sinus (44). Figure 1 illustrates the four distinct hemodynamic regions that were investigated within the disturbed laminar shear environment imposed on the HUVEC layer: flow recirculation (A), a region where the flow rate recovers to uniform laminar shear and wall shear stress ranges between +6 and +10 dyn/cm²; flow reattachment (B), a narrow region near flow stagnation where shear stress gradients are high and wall shear stress ranges between −7 and +6 dyn/cm²; flow recovery (C), a region where flow recovers to uniform laminar shear and wall shear stress ranges between +6 and +10 dyn/cm²; and laminar flow (D), the region of fully recovered flow, where the wall shear stress is spatially uniform and of highest magnitude (+11 dyn/cm²). The locations and dimensions of each of the flow zones relative to the step disturbance and the corresponding shear stress values are summarized in Table 1. Fluid was circulated through the chamber by connecting the chamber to a flow loop that consisted of a fluid reservoir, a variable-speed peristaltic pump (Cole-Parmer; Vernon Hills, IL), and a trapped-air flow damper that removes pressure fluctuations created by peristaltic pumping. The circulating medium was maintained at 37°C in a humidified 95% air-5% CO2 atmosphere. After 6 h of flow exposure, glass slides were removed from the flow chamber and processed for in situ immunocytochemical evaluation of adhesion molecule expression or were used in static adhesion assays with U-937 cells. In some studies, functional U-937 cell adhesion was assessed under flow conditions by injecting leukocytes into the circulating medium and visually monitoring U-937 cell adhesion to the flow-conditioned endothelial layer. A detailed description of static and dynamic U-937 cell adhesion assays is presented (see Monocyte cell adhesion assay).

In situ immunocytochemistry. HUVEC surface expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin was evaluated by in situ immunocytochemistry using the following antibodies: 1) mouse monoclonal IgG1 anti-human ICAM-1 (BBA3 from R&D Systems or MAB1379 from Chemicon), 2) mouse monoclonal IgG1 anti-human VCAM-1 (BB5 from R&D Systems or NCL-VCAM from Novocastra Laboratories), and 3) mouse monoclonal IgG1 anti-human E-selectin (CBL-180 or MAB2150 from Chemicon). ICAM-1 binding was blocked using MAB1379, which recognizes the D1 domain of ICAM-1 and blocks binding of leukocyte function-associated antigen-1 to ICAM-1. E-selectin binding was blocked using CBL-180, which has been shown to inhibit neutrophil adhesion to E-selectin when used as F(ab')₂ fragments (manufacturer's information). Simultaneous staining of ICAM-1 and E-selectin expression was done using a goat polyclonal anti-human ICAM-1 antibody (BBA17; R&D Systems), which was visualized using a Texas red-labeled rabbit anti-goat secondary antibody (Zymed Laboratories; San Francisco, CA).

Immunocytochemistry was performed using two techniques. HUVEC layers were either fixed in 100% methanol (10 min at −20°C) or stained live (unfixed). For both methylanol-fixed and live HUVEC layers, the immunostaining protocol was as follows: HUVEC layers were blocked with PBS that contained 3% BSA, incubated with 15 μg/ml of the respective primary antibody (1 h at 37°C), incubated with 15 μg/ml biotinylated rabbit anti-mouse IgG antibody (Zymed Laboratories) for 30 min at room temperature, incubated with 25 μg/ml Alexa 488-conjugated streptavidin (Molecular Probes; Eugene, OR) for 30 min at room temperature, and coverslipped with Vectashield (Vector Laboratories; Burlingame, CA). Immunoblocking studies were done by performing the live immunostaining protocol before the U-937 cell adhesion assay.

Monocyte cell adhesion assay. U-937 cells, which comprise a human histiocytic lymphoma cell line (37) that expresses ligands for ICAM-1, VCAM-1, and E-selectin (29), were used as a model of peripheral blood monocytes. U-937 cells were fluorescently labeled by centrifuging (500 g for 5 min) the cells and incubating the resuspended pellet with 40 μM 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM in 1% BSA-PBS (pH 7.4) for 45 min at 37°C. Control adhesion assays were performed with both labeled and unlabeled U-937 cells to verify that centrifugation and BCECF-AM incubation did not affect U-937 cell adhesion. For the static U-937 cell adhesion assay, flow-conditioned or control HUVEC layers were briefly rinsed with PBS and then incubated with labeled U-937 cells (5 × 10⁶ cells/ml) under static conditions for 45 min at 37°C. Unbound U-937 cells were removed by gently submerging the glass slide in warm RPMI 1640 medium that contained 5% FBS and...
slowly moving the slide back and forth in the direction parallel to the long dimension of the step disturbance. The layer was visualized by phase-contrast microscopy to assess the amount of unbound leukocytes remaining on top of the layer. If necessary, washing was repeated until the unbound leukocytes were removed. On average, two washes were required to remove leukocytes that were not adhered to the endothelial layer. Wash removal of leukocytes was also performed by gravity detachment. For this method of wash removal, the glass slide was flipped over and rested inverted on a rack submerged in RPMI 1640 medium that contained 5% FBS. The layer was left on the rack for 15 min to allow unbound leukocytes to fall away. Similar leukocyte adhesion data were obtained with manual washing and the gravity detachment method. The leukocyte adhesion results reported here were obtained from studies using the manual washing procedure.

HUVEC layers with bound U-937 cells were fixed overnight in 1% glutaraldehyde-PBS at 4°C. U-937 cell adhesion was quantified by cell counting. Digital images of the HUVEC layer were systematically acquired using a Cooke Sensicam cooled digital camera connected to the side port of an Olympus IX70 fluorescence microscope with the movement of the microscope stage controlled by an H107 motorized stepper stage (Prior Scientific; Rockland, MA). Series of digital images were taken along lines parallel to the direction of flow, with the first image of each series being located immediately downstream of the step (flow disturbance) and each successive image located downstream of the preceding image. For each sample, four to six series of images were taken. Each series contained 20 images, which resulted in an average of 100 images per sample. In this way, a continuous documentation of U-937 cell adhesion to the HUVEC layer was obtained, and the spatial location of each image was known. For each digital image, the following information was recorded: 1) the number of U-937 cells that adhered as single cells, 2) the number of U-937 cells involved in each clustered cell adhesion, and 3) the distance of the image from the downstream edge of the step. The total number of U-937 cell adhesions was determined by adding single and clustered cell adhesions. A cluster of U-937 cells was defined to be any group of four or more cells that attached to the HUVEC layer within 10 μm of another U-937 cell.

For the dynamic U-937 cell adhesion assay, the HUVEC layers were preconditioned for 6 h as previously described, before the incorporation of monocytes to the circulating medium. Standard cell culture medium (with viscosity three times lower than blood viscos-
FLOW-INDUCED CLUSTERED-LEUKOCYTE ADHESION

Table 1. Investigated flow regions

<table>
<thead>
<tr>
<th>Flow Region</th>
<th>Fig. 1 Image</th>
<th>Location Downstream, mm</th>
<th>Shear Stress Range, dyn/cm²</th>
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<tbody>
<tr>
<td>Recirculation</td>
<td>A</td>
<td>0.6–2.4</td>
<td>−7 to −4</td>
</tr>
<tr>
<td>Reattachment</td>
<td>B</td>
<td>2.4–3.0</td>
<td>−7 to +6</td>
</tr>
<tr>
<td>Recovery</td>
<td>C</td>
<td>3.0–4.8</td>
<td>+6 to +10</td>
</tr>
<tr>
<td>Laminar shear</td>
<td>D</td>
<td>&gt; 6.0</td>
<td>+11</td>
</tr>
</tbody>
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See Fig. 1 for corresponding images. Location is measured from the downstream edge of the step disturbance. Each region is 25 mm long in the direction parallel to the long axis of the step disturbance. Negative shear stress indicates direction of the local flow opposite to the bulk flow in the flow chamber.

ity) was used as perfusion fluid in the preconditioned phase of the studies to recreate physiological shear stresses on the endothelial monolayer.

Once the monocytes were added to the circulating medium, the perfusion rate was adjusted (reduced) to achieve a physiological near-wall velocity (10 μm from wall) of 0.3 cm/s (44) for the study of cell-cell interactions between monocytes and endothelial cells. As the flow rate was reduced from the preconditioning value, the recirculation region became smaller (one-third the length of the vortex during preconditioning), thereby redefining the dynamic conditions within the four flow zones of interest. Only the recirculation and fully recovered laminar flow zones can be accurately recreated with the present experimental model to evaluate monocyte adhesion under physiologically relevant dynamic conditions. In the recirculation region, monocytes circulating within the smaller vortex (recirculation region) interacted with endothelial cells that were also preconditioned under flow recirculation. In the fully recovered flow region, monocytes circulating within unidirectional laminar flow interacted with endothelial cells also preconditioned with unidirectional laminar flow at a physiological wall-shear stress. In contrast, in both the flow-recovery and the flow-reattachment regions, circulating monocytes interacted with endothelial cells preconditioned under flow recirculation, because the reduced flow rate positions these zones within the preconditioned recirculation region. Therefore, for the analysis of dynamic U-937 cell adhesion, the endothelial layers were divided into two regions only: the “region of flow disturbance” that included recirculation, reattachment, and recovery, and the “recovered laminar shear region” that was located far downstream from the flow disturbance.

The concentration of U-937 cells in the circulating medium was 10⁶ cells/ml. U-937 cells were allowed to circulate over the flow-conditioned layer for 45 min and were continuously visualized by phase-contrast microscopy. Digital images of the endothelial layer were periodically taken with the time and location of each image recorded. Dynamic adhesion assays were concluded by gently flushing the flow chamber with monocyte-free medium and processing the endothelial layers for in situ immunocytochemistry as described (see In situ immunocytochemistry).

Statistical analysis. U-937 cell static adhesion data was obtained from five independent shear experiments. In some experiments, multiple endothelial layers were simultaneously exposed to shear. A total of 14 shear samples and 10 control samples were analyzed and reported in this study. Significant differences in cell adhesion were determined using Tukey’s multiple-comparison test (α = 0.05) to compare respective adhesion types between various flow regions. Static control samples (containing a flow disturbance step but not exposed to flow) were similarly evaluated and found to have no spatial variation in U-937 cell adhesion; thus static control adhesion is represented by a single value in the analysis.

RESULTS

Flow-induced clustered leukocyte cell adhesion. U-937 cells adhere to all regions of the flow-conditioned HUVEC monolayer when cell-cell interaction is allowed to occur under static conditions (Fig. 1). As can be seen in Fig. 1, U-937 cells adhere to flow-conditioned HUVECs in two distinct patterns: as single U-937 cells or as a cluster of U-937 cells. U-937 cell adhesion to control endothelial cell layers (Fig. 1E) appears similar to the region of the layer exposed to flow reattachment (Fig. 1B) and is characterized by a low number of U-937 cell adhesions, with most adhesions occurring as single U-937 cells. The pattern of U-937 cell adhesion in the region of the layer exposed to recovered laminar shear (Fig. 1D) shows the most difference when compared to control layers. Adhesion in the region of recovered laminar shear is characterized by a large number of adherent cells; clustered U-937 cell adhesions occur more frequently and involve more U-937 cells than any other region in the monolayer. These findings indicate that flow conditioning increases the adhesiveness of the endothelial layer. Furthermore, this fluid shear-induced response appears to be sensitive to the local characteristics of the preconditioning flow environment.

Fluid shear alteration of endothelial cell adhesiveness was evaluated by quantifying static U-937 cell adhesion for each region of the flow-conditioned HUVEC layer (Fig. 2). Significant differences in adhesion were evaluated by comparing respective adhesion types of various regions using Tukey’s multiple comparison test (α = 0.05). Clustered U-937 cell adhesion is significantly higher in the region of laminar shear compared with all other regions except the region of flow recirculation. Single U-937 cell adhesion is consistently higher in flow-conditioned layers compared with control layers, but not significantly so, and does not appear to be influenced by the preconditioning environment. These data suggest that the observed fluid shear alteration of HUVEC adhesiveness has two components. One component is shear magnitude dependent and supports clustered cell adhesion, whereas the other component is a general response of the layer to flow and supports single cell adhesion.

![Fig. 2. Spatial distribution of static U-937 cell adhesion. Number of adhered U-937 cells per square centimeter of HUVEC layer is shown for the flow regions marked in Fig. 1 (schematic flow diagram). Total cell adhesion (light gray bars) is the sum of single cell adhesion (dark gray bars) and clustered cell adhesion (black bars). Values are means ± SE; n = 5. Significant differences were evaluated using Tukey’s multiple-comparison test. *α = 0.05, significant differences compared with static control; †α = 0.05, significant differences compared with both reattachment and recovery regions.](http://ajpheart.physiology.org/ by 10.22033.4 on July 9, 2017)
U-937 cell clusters vary in the number of cells participating in the cluster formation. Figure 3 shows the distribution of cluster sizes in flow-conditioned and control HUVEC layers. This plot was used to classify the U-937 cell clusters in three sizes, which are defined as small (4–6 U-937 cells), medium (7–10 U-937 cells), and large (>10 U-937 cells). The spatial distribution of each cluster size is shown for flow-conditioned and control HUVEC layers in Fig. 4. The region of laminar shear has a significantly higher number of medium-sized U-937 cell clusters compared with all other regions and also has more large-sized U-937 cell clusters compared with flow-reattachment and static control (Fig. 4A). U-937 cell adhesion in the fully recovered laminar shear region of layers conditioned with disturbed flow is indistinguishable from that of HUVEC layers conditioned with uniform laminar shear (no step disturbance) of the same magnitude (data not shown).

The occurrence of small clusters is consistently higher in flow-conditioned layers compared with control layers, but not significantly so, and it does not appear to be significantly influenced by the preconditioning environment (Fig. 4B). This finding suggests that small clusters are not indicative of the same alteration in endothelial cell adhesiveness that supports medium and large cluster formations. Thus, from now on, we refer to cluster formations to designate only clusters involving more than six U-937 cells.

**Fluid shear selectively induces E-selectin expression.** Unfixed endothelial layers were immunostained for ICAM-1, VCAM-1, and E-selectin to investigate which adhesion molecules modulate the observed flow-induced leukocyte adhesion. Although E-selectin is not detected in control layers, flow-conditioned HUVEC monolayers reveal a significant upregulation of E-selectin expression (Fig. 5, A and B). ICAM-1 expression is present in both control and flow-conditioned layers, and the level of expression appears unchanged after 6 h of flow conditioning (Fig. 5, C and D). VCAM-1 expression was not detected in control or flow-conditioned HUVEC layers (Fig. 5, E and F) but could be induced by TNF-α stimulation (10 ng/ml for 6 h), which indicates that the HUVECs used in these experiments are capable of expressing VCAM-1 but do not do so in response to the flow stimulus used in this study. Simultaneous staining of E-selectin and ICAM-1 (Fig. 6) shows that flow-induced E-selectin expression always colocalizes with existing ICAM-1 expression, but not all ICAM-1-expressing cells express E-selectin. Thus flow selectively upregulates E-selectin expression in a subset of endothelial cells already expressing noticeable levels of ICAM-1. The number of endothelial cells that upregulate their E-selectin expression is very small (~5% of the population), and no statistically significant spatial variation in E-selectin expression was found when the number of immunoreactive cells was quantified in situ by visual cell counting (data not shown). E-selectin-expressing endothelial cells were observed in each of the four flow regions and were most numerous in the laminar shear region; however, the quantification data did not support any statistically significant difference in the number of E-selectin-expressing cells between any of the various flow regions. Endothelial cell adhesion molecule expression in HUVEC layers conditioned with 11 dyn/cm² uniform laminar shear (no step disturbance) was similar to the fully recovered laminar shear region of layers conditioned with disturbed flow.
**U-937 cell clusters adhere to HUVECs coexpressing ICAM-1 and E-selectin.** Immunoblocking ICAM-1 or E-selectin alone does not inhibit cluster formation but indirectly indicates that U-937 cell clusters associate with HUVECs coexpressing ICAM-1 and E-selectin (Fig. 7, A and B). Simultaneously immunoblocking flow-conditioned layers for both ICAM-1 and E-selectin does inhibit clustered cell adhesion (Fig. 7C). Furthermore, when ICAM-1 alone is blocked, immunoreactivity is sometimes unassociated with cluster formations (Fig. 7A), whereas E-selectin immunoreactivity is always associated with cluster formation (Fig. 7B). This agrees with our immunocytochemical findings that flow-induced E-selectin upregulation occurs only in a subset of ICAM-1-expressing cells and that only coexpressing cells support cluster cell adhesion. Dual staining of HUVEC layers for ICAM-1 and E-selectin after performance of U-937 cell adhesion assays (under both static and dynamic conditions) directly indicates that cluster adhesions are associated exclusively with endothelial cells that coexpress ICAM-1 and E-selectin (Fig. 8). In this way, U-937 cells can be viewed as reporters of endothelial ICAM-1/E-selectin coexpression.

**Functional U-937 cell adhesion under dynamic conditions.** Under dynamic conditions, functional U-937 cell adhesion to flow-conditioned endothelial layers is limited to the disturbed flow region (Fig. 9A). Circulating monocytes adhere within the region of flow recirculation and the vicinity of flow reattachment in the same clustered cell pattern observed in static adhesion assays. The laminar shear region does not support...
U-937 cell adhesion under flow conditions (Fig. 9B). Adhesion in areas of lowest but not highest shear supports the idea of a near-wall velocity threshold for cell-cell interaction. Control layers, which have a step disturbance but were not preconditioned with flow, do not support leukocyte adhesion under dynamic conditions in any region of the layer. These results demonstrate that arrest and adhesion of circulating monocytes is determined by shear stress regulation of endothelial adhesiveness and by the local flow dynamics that regulate monocyte transport at the interface with the endothelial layer.

**DISCUSSION**

The present study is the first report of a detailed characterization of flow-induced endothelial cell adhesiveness for leukocytes to demonstrate that shear stress selectively induces the coexpression of ICAM-1 and E-selectin in a limited number of endothelial cells supporting clustered leukocyte adhesion. This cluster pattern of adhesion is not uniformly distributed in the monolayer, as shear stress upregulates E-selectin expression only in a subset of ICAM-1-expressing cells. Flow-mediated

![Image](image1.jpg)

Fig. 6. Flow-induced E-selectin expression colocalizes with ICAM-1 expression. Flow-conditioned HUVECs (6 h) were fixed in methanol and stained for ICAM-1 (red) and E-selectin (green). Phase-contrast (A) and corresponding fluorescent (B) micrographs are shown. Red and green fluorescence is superimposed with cells coexpressing ICAM-1 and E-selectin, which appear yellow. Magnification, ×100.

![Image](image2.jpg)

Fig. 7. Simultaneous ICAM-1 and E-selectin immunoblocking inhibits cluster adhesion. Before a static U-937 cell adhesion assay was performed, flow-conditioned HUVEC layers were immunoblocked for either ICAM-1 alone (A), E-selectin alone (B), or both ICAM-1 and E-selectin simultaneously (C). U-937 cell clusters associate with immunoreactivity when either ICAM-1 or E-selectin is blocked independently (A and B). Clusters do not form when both ICAM-1 and E-selectin are blocked simultaneously (C). Magnification, ×200.
E-selectin expression is never observed in the absence of ICAM-1. These findings support the idea of a naturally heterogeneous endothelium in which individual cells or small groups of cells selectively respond to the local flow environment and potentially contribute to the focal origin of arterial diseases such as atherosclerosis.

Clustered leukocyte adhesion has been reported in vivo in humans with advanced atherosclerosis and in hypercholesterolemic animal models (7, 30) but never before in vitro. The leukocyte adhesion patterns in the in vivo studies are remarkably similar to those observed in our in vitro flow-preconditioned HUVEC layers, which suggests that the mechanisms of adhesion may also be similar. The observed clustered leukocyte adhesion is not a consequence of leukocyte aggregation but is clearly a localized adhesion of individual leukocytes to an adhesive cell or group of cells in the endothelial layer. As mentioned above, our results demonstrate that clustered leukocyte adhesion is supported by a flow-mediated, localized, coexpression of the adhesion molecules ICAM-1 and E-selectin.

Colocalized selectin and ICAM-1 expression has interesting implications with respect to the leukocyte adhesion cascade. It is well recognized that selectins mediate leukocyte capture and rolling adhesion, whereas ICAM-1 is associated with monocyte arrest and firm adhesion (4, 21, 23, 24, 34, 35). Relevant synergistic effects of selectin and ICAM-1 have been reported in animal models, which demonstrate that ICAM-1 expression optimizes selectin-mediated rolling and results in lower leukocyte rolling velocity (36). Selectin-mediated rolling has also been reported to activate leukocyte β2-integrins to bind ICAM-1 in vitro (33), and most recently it was shown that E-selectin and ICAM-1 cluster in endothelial plasma membrane lipid rafts after leukocyte adhesion to these molecules (38, 39). Furthermore, the cytoplasmic regions of these selectin-ICAM-1 adhesion complexes are associated with cortactin and Src, which suggests potential involvement in cell signaling mechanisms.

To our knowledge, the present study is the first in vitro evaluation of in situ ICAM-1 and E-selectin coexpression in flow-conditioned endothelial cells. The immunocytochemistry presented here is a detailed in situ investigation of fluid-shear effects on endothelial adhesion molecule expression, which allows for the identification of localized small amounts of protein expression heterogeneously distributed on the monolayer. Previous in vitro work investigating flow-induced changes in endothelial adhesion molecule expression removed cells from the monolayer and used flow cytometry to quantify protein expression (5, 14, 26, 28). Results from those studies were often reported in the form of percent changes relative to baseline expression without accounting for any heterogeneity in the monolayer response. The detailed in situ immunocytochemistry presented in this study revealed that in response to flow, select ICAM-1-expressing cells upregulate their E-selectin expression. However, the number of cells that upregulate E-selectin expression in response to flow is small (<5%), which may explain the difficulties in detecting it using flow cytometry, because a minimal shift in the mean fluorescence would be expected and may pass unnoticeable. In this sense, flow cytometry is an effective and sensitive technique for detecting homogeneous cell responses in protein expression but it is less able to detect heterogeneous ones.

Fig. 8. HUVECs coexpressing ICAM-1 and E-selectin support cluster adhesions. Flow-conditioned HUVECs were incubated with U-937 cells under static conditions and then immunostained for ICAM-1 and E-selectin expression. U-937 cells viewed in phase contrast (A) adhere to HUVEC layer as cluster formations (yellow box) or as single cells (black arrowheads). U-937 cell cluster adhesion is found exclusively on endothelial cells that coexpress E-selectin (green fluorescence; B) and ICAM-1 (red fluorescence; C). Magnification, ×200; scale bar, 50 μm.
The observed flow-mediated, clustered leukocyte adhesion and underlying cellular mechanisms potentially involved in the synergistic effects of selectin-ICAM-1 that are associated with this phenomenon are not simple to explore or explain. Our results demonstrate that only cells that coexpress ICAM-1 and E-selectin support leukocyte adhesion in the form of clusters, and that simultaneous immunoblocking of ICAM-1 and E-selectin is required to inhibit clustered cell adhesion in flow-conditioned monolayers. Interestingly, immunoblocking of E-selectin or ICAM-1 in the ICAM-1/E-selectin-coexpressing cells is not sufficient to inhibit clustered leukocyte adhesion, yet flow-conditioned cells that express ICAM-1 alone are not associated with cluster formations. Both clustered leukocyte cell adhesion and endothelial coexpression of ICAM-1 and E-selectin are absent in control (no flow) HUVEC layers. In fact, no detectable amounts of E-selectin expression were found by immunocytochemistry in control layers.

In interpreting the results of our immunoblocking studies, we suggest that blocking both ICAM-1 and E-selectin removes both ligands that leukocytes can use to bind our flow-conditioned endothelial cells, and thus adhesion is inhibited. Our finding that blocking of ICAM-1 alone does not inhibit clustered leukocyte adhesion can be explained by the ability of leukocytes to bind flow-conditioned endothelial cells using an E-selectin ligand.

On the other hand, our finding that immunoblocking E-selectin alone does not inhibit clustered leukocyte adhesion is a difficult scenario to explain, because endothelial cells that express only ICAM-1 (in both flow-conditioned and static control layers) are not associated with clustered cell adhesion. One possible explanation for this phenomenon is that flow conditioning also selectively induces chemokine expression in these same cells that express ICAM-1 and E-selectin and thereby offers a pathway for activating leukocyte β2-integrins, which are then able to bind ICAM-1. In this scenario, blocking E-selectin alone in flow-conditioned endothelial layers may only remove one leukocyte ligand, while leukocyte β2-integrins could still become activated by endothelial secreted chemokine and thus bind endothelial cells by ICAM-1. Because leukocyte β2-integrins require activation to bind ICAM-1, endothelial cells that express ICAM-1 but not E-selectin would presumably not be secreting chemokine and thus would not support leukocyte adhesion. This argument once again supports the idea of a heterogeneous endothelium in which individual cells or small groups of cells selectively respond to shear stress.

This proposed explanation that flow-mediated chemokine secretion activates leukocyte β2-integrins is supported by earlier studies demonstrating fluid shear modulation of HUVEC mRNA levels and protein expression of both IL-8 (18) and monocyte chemoattractant protein-1 (31). Although IL-8 was suppressed by laminar shear (7 dyn/cm²), monocyte chemoattractant protein-1 demonstrated a biphasic response whereby mRNA levels initially increased in response to laminar shear before returning to basal levels after 4 h of flow conditioning. The evaluative techniques used in those studies (ELISA, Northern blot) provide no information about the potential heterogeneity of the flow-induced endothelial cell response.

Another potential mechanism that may offer pathways for activating the leukocyte β2-integrins is flow-mediated E-selectin signaling. It has been demonstrated that incubation of leukocytes with E-selectin increases the affinity of β2-integrins for ICAM-1 (33). In our static adhesion assay, monocytes were in contact with endothelial cells expressing E-selectin, which may have potentially activated the β2-integrins and contributed to the observed localized clustered adhesion. However, as indicated by our immunoblocking findings, E-selectin-mediated cell signaling cannot be the only pathway for activating...
Leukocyte β2-integrins. Adhesion studies with immobilized ICAM-1 substrates have demonstrated that leukocyte β2-integrins can be activated by prolonged contact with ICAM-1 (32). This is unlikely to be the mechanism in the clustered adhesion we report here, because it would not explain the lack of clustered cell adhesion in cells expressing only ICAM-1 in both flow-conditioned and static control layers. We have linked ICAM-1 and E-selectin coexpression to our observed flow-induced leukocyte adhesion, whereas VCAM-1 expression was not detected using either immunocytochemistry or Western blot analysis (data not shown). VCAM-1 involvement in leukocyte adhesion is well accepted. In vivo, VCAM-1 expression has been reported in early atherogenesis (6, 8, 11, 16). In vitro, although flow modulation of VCAM-1 expression has been extensively investigated, the findings are not in full agreement (2, 5, 14, 25, 28, 40). In the present study, exposure of endothelial layers to flow fields representative of lesion-prone hemodynamics did not induce VCAM-1 expression. VCAM-1 expression could be induced by cytokine stimulation (TNF-α), but flow regulation of adhesion molecule expression was found to be limited to E-selectin. Fluid dynamic modulation of cell-cell interactions between leukocytes and endothelial cells has been demonstrated in this study by direct alteration of endothelial adhesiveness resulting in clustered leukocyte adhesions similar to those reported in association with early- and late-stage atherosclerosis. The data revealed that fluid shear alteration of HUVEC adhesiveness is shear magnitude dependent, which demonstrates that leukocyte adhesion patterns are influenced by characteristics of the local fluid dynamic environment imposed on the endothelial layer during flow conditioning. We also demonstrated that hemodynamics regulate monocyte-endothelial cell interactions by establishing flow environments that may be conducive or prohibitive to cell-cell interaction. In the disturbed flow model investigated, monocytes circulating under flow-conditioned endothelial layers adhered only within the recirculation region and in the vicinity of flow reattachment. Monocytes in the flow-recirculation region benefit from an increased residence time for interaction with the endothelial layer. In the vicinity of flow reattachment, the flow streamlines are directed almost perpendicular to the endothelial layer surface, thereby enhancing monocyte transport to that region. In both recirculation region and vicinity of flow reattachment, the near-wall velocity (10 μm from wall), which is representative of the speed of motion of the circulating monocytes near the endothelial layer, is lower than in the downstream undisturbed flow region. The adhesion of circulating monocytes was not supported in the laminar shear region despite the high adhesiveness of the underlying endothelial layer. This is the region of highest wall-shear stress and near-wall velocity in the experimental model which, although representative of physiological values, appears to be beyond the near-wall velocity threshold for cell-cell interaction leading to adhesion. Here, our dynamic adhesion results are consistent with earlier studies (12, 20) that reported leukocyte adhesion through selectins is sensitive to hydrodynamic shear levels. The cluster cell pattern of monocyte adhesion reported in this study does not appear to involve L-selectin-mediated secondary (leukocyte-leukocyte) capture mechanisms. This is evidenced by the fact that the clusters we report can form under static conditions where L-selectin-mediated secondary capture is not expected to occur, because L-selectin bond formation requires a threshold hydrodynamic shear (12, 20). Furthermore, under flow conditions, we recognize cluster-formation mechanisms that are distinct from those reported for L-selectin-mediated secondary capture (1, 22). We observe cluster formation that does not always involve leukocyte-leukocyte contact. Rather, leukocyte accumulation appears to be associated with particular endothelial cells as demonstrated by the fact that often leukocyte detachment from an endothelial cell is followed by the arrival and arrest of another leukocyte on the same endothelial cell. This mechanism of adhesion is distinct from L-selectin-mediated secondary capture, which has been reported to involve an initial homotypic leukocyte contact followed by a rolling transfer of the leukocyte to the substrate (1, 22).

The studies reported here provide an important contribution to understanding fluid shear modulation of endothelial adhesiveness and endothelial-monocyte interaction leading to localized monocyte adhesion. Our in vitro findings, which parallel in vivo observations, demonstrate the value of a model system that may significantly aid in unraveling the mechanisms underlying the focal origin of arterial diseases and early atherosclerotic lesion development.

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REFERENCES

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FLOW-INDUCED CLUSTERED-LEUKOCYTE ADHESION


