

# Avidity Modulation Activates Adhesion under Flow and Requires Cooperativity among Adhesion Receptors

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**ABSTRACT** An early step in activation of leukocyte adhesion is a release of integrins from cytoskeletal constraints on their diffusion, leading to rearrangement and, consequently, increased avidity. Static adhesion assays using purified ligand as a substrate have demonstrated that very low doses of cytochalasin D disconnect  $\beta_2$ -integrins from their cytoskeletal links, allowing rearrangement and activating adhesion. The adhesion process in blood vessels is poorly simulated by these assays, however, for two reasons: leukocyte adhesion to endothelium 1), occurs in the presence of blood flow and 2), involves the simultaneous interactions of multiple sets of adhesion molecules. We investigated the effect of cytochalasin D, at concentrations that increase integrin diffusion but do not alter leukocyte shape and surface features, on adhesion of leukocytes to endothelial cells under flow. Cytochalasin D increased the number of rolling cells, the number of firmly adherent cells, and the duration of both rolling and firm adhesion. These effects required endothelial cell expression of ICAM-1, the ligand for leukocyte  $\beta_2$ -integrins. The  $\beta_2$ -integrin–ICAM-1 interaction alone was not sufficient, however. Experiments using purified substrates demonstrated that avidity effects on activation of adhesion under flow require functional cooperativity between integrins and other adhesion receptors.

## INTRODUCTION

The physiology of inflammatory reactions depends on the recruitment of leukocytes into tissues, which depends on regulated adhesion of leukocytes to the blood vessel wall. A key point in the regulation of adhesion is control of leukocyte integrin function. Adhesion via integrins is regulated by two mechanisms: 1), changes in the ligand-binding affinity of the individual integrin molecule and 2), rearrangement of the integrins, which increases the avidity of the interaction. Recent advances in understanding conformational changes associated with integrin activation have helped to elucidate the role of affinity modulation (Lu et al., 2001a,b; Shimaoka et al., 2002, 2003; Takagi et al., 2001; Hantgan et al., 1999, 2001; Vinogradova et al., 2000, 2002; Salas et al., 2002). The role of integrin rearrangement, however, remains less well understood.

Rearrangement of integrins on leukocytes upon cell activation occurs not by directed movement, but rather by diffusion, which occurs after release of cytoskeletal constraints on integrin motion (Kucik et al., 1996; Yauch et al., 1997; Sigal et al., 2000). Integrin rearrangement increases avidity both by allowing receptors to move to areas of high ligand density (Kucik et al., 1996), and also by receptor clustering (Lub et al., 1997). Theory predicts that clustering alone can lead to increased avidity, due to cooperative effects on resistance to bond breakage (Ward et al., 1994). Direct

evidence that the cooperative binding associated with clustering increases adhesion has been provided by atomic force microscopy (Chen and Moy, 2000).

So far, the only adhesion molecules shown to adjust their avidity on a rapid timescale by rearrangement are the  $\beta_2$ -integrins, which are specialized for leukocyte adhesion. Both  $\alpha_L\beta_2$  (Lub et al., 1997) and  $\alpha_M\beta_2$  (Zhou and Li, 2000; Zhou et al., 2001; Jones et al., 1998) increase avidity by diffusion and rearrangement. The principal ligand for both of these leukocyte integrins is intercellular adhesion molecule 1 (ICAM-1) (Diamond et al., 1990; Lub et al., 1996).

Physiologically, activation of integrin-mediated adhesion occurs in the context of activation of the whole leukocyte. Leukocyte activation affects many functions, including adhesion, production of cytokines, and proliferation. To understand the effects of integrin rearrangement, therefore, control of integrin diffusion must be separated experimentally from other effects of cell activation. The phorbol ester PMA (phorbol 12-myristate 13-acetate) is often used to activate integrin adhesion experimentally, but PMA has myriad effects, including changes in surface morphology and induction of cell spreading. To isolate the effects of integrin rearrangement from other effects of cell activation, cytochalasin D, at a concentration that has minimal effects on cytoskeletal architecture, has proven to be a useful tool. We showed previously that a low dose of cytochalasin D (0.3–1  $\mu\text{g/ml}$ ), too small to grossly disrupt the structure of the actin cytoskeleton, is able to release integrins from cytoskeletal constraints on their motion, as measured directly by single particle tracking (SPT) (Kucik et al., 1996). This treatment caused integrin rearrangement and activated lymphocyte adhesion. Several studies from a number of laboratories have demonstrated that low-dose cytochalasin D increases

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diffusion of  $\beta_2$ -integrins on a variety of leukocyte types (van Kooyk and Figdor, 1997; Yauch et al., 1997; Stewart et al., 1998; van Kooyk et al., 1999), including the WEHI 274.1 cell line (Zhou and Li, 2000), with little effect on other cell functions. The utility of low-dose cytochalasin D in increasing integrin avidity has since been confirmed in a number of systems (Lub et al., 1997; Stewart et al., 1998; Yauch et al., 1997; Zhou and Li, 2000; van Kooyk et al., 1999). Although cytochalasin D effects on integrin diffusion and clustering are well documented, there is no evidence that cytochalasin D has any effect on integrin conformation (Kucik, 2002).

The biphasic effect of cytochalasin D on adhesion (activation at low concentrations, inhibition at higher concentrations) has been explained as follows (Kucik et al., 1996). At low doses, cytochalasin D activates  $\beta_2$ -integrin-mediated adhesion through its effects on integrin diffusion. These low doses sever the link between  $\beta_2$ -integrins and actin filaments, without major disruption of the cytoskeleton, while high doses prevent reinforcement of nascent contacts. (Kucik et al., 1996). Because no specific integrin-mobilizing drugs have yet been identified, cytochalasin D is still the best tool available to increase integrin diffusion.

Although it is well established that rearrangement of integrins activates adhesion to purified ICAM-1 under static conditions, its role in leukocyte adhesion to endothelial cells in the presence of shear is less clear. Shear changes the biophysics of adhesion, requiring rapid formation of adhesive bonds to resist the forces that would pull a leukocyte free of the endothelium. Static assays of adhesion, therefore, poorly simulate the adhesion that occurs in blood or lymph vessels. The relative importance of affinity changes and integrin rearrangement in the presence of shear stress has not been worked out.

Similarly, although much is known about the role of integrin rearrangement in activating adhesion to purified ICAM-1, less is known regarding diffusion-mediated receptor rearrangement in the context of simultaneous binding of multiple sets of adhesion receptors. When leukocytes bind to vascular endothelium, a number of adhesion receptors and their ligands are available for binding. The importance of cooperativity among endothelial selectins,  $\beta_2$ -integrins, and other adhesion receptors is increasingly becoming appreciated (Steeber et al., 1998, 1999; Henderson et al., 2001).

To address the role of integrin rearrangement in adhesion under flow and the contribution of cooperative binding among different classes of adhesion molecules, we used a laminar flow adhesion assay system. We measured the effect of cytochalasin D mobilization of integrins on adhesion of a leukocyte cell line to both cultured endothelium and purified ligand substrates. We chose the WEHI 274.1 cell line, a well-established monocyte-like cell line used to study monocyte adhesion (Warner et al., 1979; Yue et al., 2000; Zhou and Li, 2000). In previous work, we demonstrated that the adhesive properties of this cell line on TNF- $\alpha$  stimulated

endothelium were similar to those of whole blood monocytes (Kevil et al., 2001). In addition, it has been established that low doses of cytochalasin D increase  $\beta_2$ -integrin diffusion on the WEHI 274.1 cell line (Zhou and Li, 2000) and that this increased diffusion activates adhesion in static assays (Zhou and Li, 2000; Zhou et al., 2001). In this study, we found that receptor rearrangement has consequences for both monocyte rolling and firm adhesion, and that these effects are enhanced by cooperative interactions among classes of adhesion molecules. This confirms that receptor rearrangement by diffusion can play an important role in adhesion of leukocytes to vascular endothelium under flow. In addition, these findings suggest that cooperativity between the ICAM-1-integrin receptor-ligand pair and other sets of adhesion molecules may be more important in the adhesion process than previously supposed.

## MATERIALS AND METHODS

### Cells and treatments

Endothelial cells purified from mouse aorta were isolated as previously described (Kevil and Bullard, 2001) and cultured in dishes precoated with 1% gelatin (Sigma, St. Louis, MO). The cells were maintained for seven days in MCDB 131 media (Gibco, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT), 10 mg/L bovine brain extract (Biomedical Technologies, Stoughton, MA), 1000 units/L heparin (American Pharmaceutical Partners, Los Angeles, CA), 1 mg/L hydrocortisone (Sigma, St. Louis, MO), and 10 ml penicillin (Gibco). The cells were seeded in 8-cm<sup>2</sup> tissue culture plastic dishes (Corning, Corning, NY) coated with 1% gelatin (Sigma). The cells formed confluent monolayers 6–7 days after seeding and were used for experiments within 2–3 days.

WEHI 274.1 cells were obtained from ATCC and were cultured in DMEM (Gibco) containing 10% FBS and 4  $\mu$ l 2-mercaptoethanol (Sigma).

### In vitro rolling and adhesion assay

Cells of the WEHI 274.1 monocyte-like cell line were loaded with fluorescent dye by 20 min incubation at room temperature with 1  $\mu$ M BCECF (2', 7' -bis-carboxyethyl-5-carboxyfluorescein; Molecular Probes, Eugene, OR) and suspended in Hanks' balanced salt solution (HBSS) (Sigma) at a concentration of  $0.5 \times 10^6$ /ml. The cells were then treated with cytochalasin D (Sigma) at 0, 0.3, 1.0, or 3.0  $\mu$ g/ml for 30 min at 37°C, washed once in HBSS, resuspended in HBSS at  $10^6$  cells/ml, and loaded into a 25-ml syringe.

Eight hours before the flow chamber experiment, a confluent monolayer of endothelial cells in tissue culture dishes were treated with 10 ng/ml of murine TNF- $\alpha$  (Sigma) to activate the endothelium. For flow experiments, a GlycoTech (Rockville, MD) flow chamber insert and gasket were inserted into the dish to form a laminar flow chamber that can be viewed on a microscope. Cells were injected into the flow chamber in HBSS at a controlled physiological shear stress of 2.5 dynes/cm<sup>2</sup> using a programmable syringe pump (KD Scientific, New Hope, PA). Cells were viewed on an Axiovert 100 microscope (Zeiss, Thornwood, NY) equipped with a CCD camera (Model 300T-RC, Dage-MTI, Michigan City, IN) and viewed both as brightfield and fluorescent images. Video was recorded onto sVHS videotape, and selected sequences were digitized to TIF files using the Perception video editing package (Perception PVR-2500, Digital Processing Systems, Markham, Ontario, Canada). Video images were analyzed to yield position measurements every 1/30 s using Metamorph software (Universal Imaging Corporation, West Chester, PA). Position measurements were processed by programs written for this purpose by Dennis Kucik to

determine velocities, accelerations, and arrest durations. Counts of rolling and firmly adherent cells were determined by visual review of video sequences and confirmed by computer analysis.

## Analysis of leukocyte adhesion

### Definition of adhesion duration

Even firmly adherent cells move slightly under shear stress. Therefore, an objective definition of adhesion must be used, especially when cells are observed at high time resolution. A cell was considered firmly adhered if its velocity fell below  $5 \mu\text{s}$  for five video frames (0.16 s). This is equivalent to movement of one cell diameter in 3 s. (Note that average adhesion durations were much longer, averaging 5–25 s, as seen in Fig. 5 a). Duration of adhesion was defined as the time until the cell resumed motion at a velocity greater than  $5 \mu\text{s}$  for more than five frames. In general, the transition from arrest to rolling was not subtle, but rather was an abrupt resumption of rolling motion.

### Definition of rolling

To distinguish cells that are rolling from those flowing at the lowest hydrodynamic velocity (close to the chamber wall) without adhesive rolling interactions, a critical velocity ( $V_{\text{crit}}$ ) was calculated as described earlier (Kevill et al., 2003). Rolling was then defined as cell motion that does not meet the criteria for adhesion (above) but is below  $V_{\text{crit}}$ . *Average rolling velocity* is the mean of the instantaneous velocities (measured at 1/30-s intervals) during the period that the cell is rolling. *Rolling duration* is the total time that the cell is rolling (number of frames  $\times$  0.033 s/frame). If a cell rolled intermittently (i.e., rolling was interrupted by either free flow or firm adhesion), only the frames during which the cell met the criteria for rolling were averaged.

## Cell deformability index measurements

Images of rolling cells were observed and recorded onto videotape, and selected sequences were digitized at 30 frames/s into TIF images at a resolution of  $720 \times 486$  pixels using DPS Reality hardware and software (Leitch Technology Corporation, Florence, KY). The TIF sequences were played as movies and reviewed to identify rolling and adherent cells, and individual TIF images were selected for analysis. Using these original, sharp, still pictures, length and width of cells interacting with the endothelium were measured using Metamorph software (Universal Imaging Corporation) to an accuracy of  $\pm 0.44 \mu$ , based on the magnification of the system and assuming single-pixel resolution. Ratios were then calculated to yield cell deformability indices (Finger et al., 1996). Between 10 and 50 cells were measured for each cytochalasin D concentration.

## Scanning electron microscopy

Thermanox coverslips (Electron Microscopy Sciences, Fort Washington, PA) were incubated with  $500 \mu\text{l}$  poly-L-lysine (PLL, Sigma) for 30 min at  $37^\circ\text{C}$ , the PLL was removed, and the coverslips were washed 5 times with SEM buffer (0.1 M cacodylate buffer in water, pH 7.2, Electron Microscopy Sciences). For each coverslip,  $0.2 \times 10^6$  WEHIs were washed once with HBSS and incubated for 45 min at  $37^\circ\text{C}$ . Cells were then fixed with 2.5% electron microscopy grade glutaraldehyde (Electron Microscopy Sciences) in SEM buffer for 20 min at room temperature. They were then washed with SEM buffer 3 times, immersed in 1% osmium tetroxide in SEM buffer for 1 h at room temperature, and then washed with SEM buffer three times. Coverslips were rinsed with distilled  $\text{H}_2\text{O}$  and dehydrated at room temperature with 50%, 75%, and 95% EtOH for 4 min each, and then 3 times with 100% EtOH for 4 min each time. Cells were then dehydrated

at room temperature with 1:1 100% EtOH and hexamethyldisilazane (HMDS, Electron Microscopy Sciences) for 4 min, then twice with 100% HMDS for 4 min, and allowed to air dry overnight with a shallow covering of HMDS. Coverslips were then coated with gold and attached to an SEM stub with tape or rubber cement before SEM scanning.

## Purified substrate coating

Tissue culture dishes (35 mm) were marked with a diamond pen to outline a small area in the center. Then  $10 \mu\text{l}$  of  $20 \mu\text{g/ml}$  protein A (Sigma) in PBS was placed in the marked area and spread with the pipette tip, incubated at  $37^\circ\text{C}$  for 1 h, and then washed three times with PBS. Nonspecific binding was blocked with 2% human serum albumin (HSA, Sigma) in PBS for 2 h at  $4^\circ\text{C}$  followed by 3 washes with PBS. The marked areas were then coated with  $50 \mu\text{l}$  purified substrate ( $25 \mu\text{g/ml}$  recombinant mouse ICAM-1/Fc chimera,  $5 \mu\text{g/ml}$  recombinant mouse VCAM-1/Fc chimera,  $0.5 \mu\text{g/ml}$  recombinant mouse E-selectin/Fc chimera,  $0.5 \mu\text{g/ml}$  recombinant mouse P-selectin/Fc chimera,  $16.8 \mu\text{g/ml}$  ICAM-1/ $10 \mu\text{g/ml}$  E-selectin double-coated, or  $25 \mu\text{g/ml}$  ICAM-1/ $0.5 \mu\text{g/ml}$  E-selectin double-coated) overnight in  $4^\circ\text{C}$ . All purified coatings were obtained from R&D Systems, Minneapolis, MN.

## One-minute adhesion assay

WEHI 274.1 cells were suspended in HBSS at a concentration of  $1.25 \times 10^6/\text{ml}$ . The cells were then treated with cytochalasin D at 0, 0.3, 1, and  $3 \mu\text{g/ml}$  or 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) for 30 min at  $37^\circ\text{C}$  and washed once with HBSS. The cells were allowed to settle on the substrate-coated dishes for 1 min without flow, and then subjected to increasing shear stresses, incrementing every 7 seconds. Flow rates and durations were controlled by a PC using software written in the laboratory to operate a programmable syringe pump (KD Scientific). At the end of each interval the number of cells that remained bound was determined relative to the number of cells that had originally settled under static conditions. For each shear rate, adhesion was quantified in several fields of view.

## RESULTS

### Cytochalasin D activates adhesion of WEHI monocytes to cultured endothelium under shear flow

Mouse aortic endothelial cells were cultured as an intact monolayer in a laminar flow chamber and stimulated with  $\text{TNF-}\alpha$  ( $10 \text{ ng/ml} \times 8 \text{ h}$ ). WEHI 274.1 cells were then perfused at a physiologic shear stress of  $2.5 \text{ dynes/cm}^2$  and the number of adherent cells per minute was determined (Fig. 1 a). A low concentration of cytochalasin D activated adhesion in a dose-dependent manner, whereas at higher concentrations adhesion was low, similar to untreated controls. Peak adhesion occurred at  $0.3 \mu\text{g/ml}$ , where adhesion was increased greater than fivefold. This dose dependence is consistent with earlier studies measuring adhesion of lymphocytes to purified ICAM-1 under static conditions (Kucik et al., 1996).

### Cytochalasin D activation of adhesion is ICAM-1-dependent

To confirm the role of  $\beta_2$ -integrins, similar experiments were done using cultured aortic endothelium from ICAM-1  $-/-$

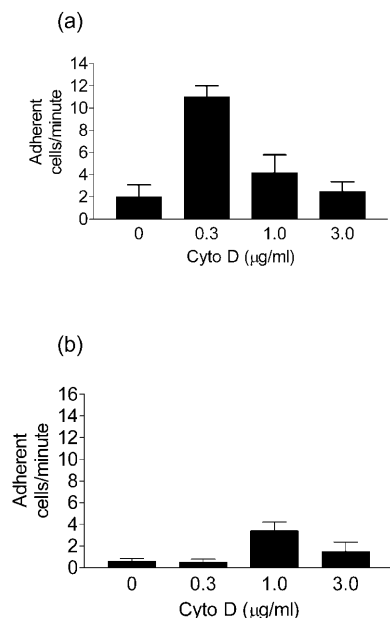


FIGURE 1 Cytochalasin D activates adhesion under flow in a dose-dependent manner. Nonrolling adhesion (arrest) of WEHI 274.1 cells on cultured primary endothelial cells from both wild-type (a) and ICAM-1  $-/-$  (b) mouse aortas was quantified. At a physiologic shear stress of 2.5 dynes/cm<sup>2</sup>, adhesion was increased fivefold on wild-type endothelium by low-dose cytochalasin D. On ICAM-1  $-/-$  endothelium, the probability of arrest was lower. Cytochalasin D had no effect on adhesion at 0.3  $\mu$ g/ml, although a small increase was seen at 1.0  $\mu$ g/ml.

mice (Fig. 1 b). On endothelium lacking ICAM-1, cytochalasin D was unable to induce the large increases in adhesion seen on wild-type endothelial cells. Although there was no effect at all at 0.3  $\mu$ g/ml cytochalasin D, a small but significant increase in adhesion was seen at 1  $\mu$ g/ml. This suggests that although ICAM-1 is important for cytochalasin D-induced activation of adhesion, other adhesion molecules may be involved as well.

### Cytochalasin D activates rolling adhesion in an ICAM-1-dependent manner

Binding of  $\beta_2$ -integrins to ICAM-1 has been shown to cooperate with selectins in stabilization of leukocyte rolling (Steeber et al., 1998, 1999; Kevil et al., 2003; Henderson et al., 2001). Therefore, we tested whether cytochalasin D could activate WEHI 274.1 rolling on cultured endothelium from both wild-type and ICAM-1  $-/-$  mice. Treatment of the WEHI cells with cytochalasin D dramatically increased the number of rolling cells in a dose-dependent manner, but only on endothelium that expressed ICAM-1 (Fig. 2).

### The concentrations of cytochalasin D that activate adhesion do not change WEHI surface morphology

To test the effect of adhesion molecule rearrangement under flow, it is important to be certain that cell morphology is not

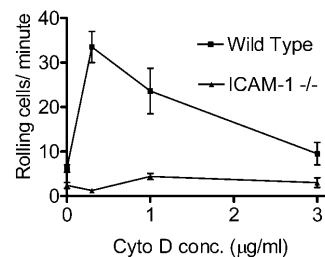


FIGURE 2 Cytochalasin D activation of rolling requires ICAM-1. Rolling adhesion on both wild-type (squares) and ICAM-1  $-/-$  (triangles) endothelium at a shear stress of 2.5 dynes/cm<sup>2</sup> was quantified. Cells were more likely to roll on endothelium that expressed ICAM-1. Moreover, on wild-type endothelium only, rolling was increased up to fourfold by low-dose cytochalasin D, with a dose dependence similar to that for firm adhesion.

altered, since this would affect the forces exerted on the cell and confound interpretation of results. In addition, loss of microvilli and other surface features could affect cell contact area. Therefore, we examined the effect of cytochalasin D on WEHI cell morphology. We found that at a concentration known to largely disrupt the cytoskeleton and inhibit adhesion in static assays (3  $\mu$ g/ml), the surface of the cell became smoother and microvilli were lost (Fig. 3 d). At the lower doses that activate adhesion, however, cell morphology was largely unaffected (Fig. 3, b and c). Thus, effects on cell adhesion are more likely due to mobilization of cytoskeleton-restricted receptors than to cell shape changes.

### Cytochalasin D does not increase deformation of WEHI 274.1 cells at a shear stress of 2.5 dynes/cm<sup>2</sup>

Cells under shear tend to become slightly elongated in the direction of flow. In neutrophils, low doses of cytochalasin D can have significant effects on cortical tension and cytoplasmic viscosity (Wakatsuki et al., 2001; Finger et al., 1996; Ting-Beall et al., 1995), which might affect cell deformability. This effect can be quantified by calculating

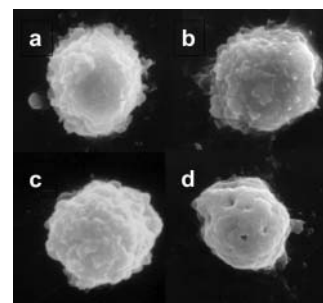


FIGURE 3 Effect of cytochalasin D on WEHI monocyte morphology. The low doses of cytochalasin D that activate adhesion (b and c) do not cause gross morphological changes, as compared to control (a). Higher doses that inhibit adhesion, however, cause loss of cell surface features (d).

a deformation index, which is the ratio of cell length to cell width (Finger et al., 1996). To quantify deformability of WEHI 274.1 cells under our flow conditions, we calculated deformability indices for rolling cells as a function of cytochalasin D concentration. Lengths and widths of cells were measured with an accuracy of  $\pm 0.44 \mu$ . We found no significant increase in deformability at the cytochalasin D concentrations and shear stress used in this study (Fig. 4).

### Cytochalasin D increases duration of both rolling and firm adhesion

To better understand the role of integrin rearrangement in cell adhesion under flow, we quantified duration of adhesion and rolling as a function of cytochalasin D treatment. We found that cytochalasin D increased the duration of both firm adhesion (Fig. 5 *a*) and rolling (Fig. 5 *b*). Interestingly, whereas the increase in firm adhesion duration peaked at 0.3  $\mu\text{g/ml}$ , increased rolling duration was sustained even at a concentration high enough (3  $\mu\text{g/ml}$ ) to disrupt cytoskeletal architecture (as seen by the morphological changes in Fig. 3), indicating that an intact cytoskeleton is less important for rolling than for firm adhesion. Adhesion and rolling durations were not determined for ICAM-1  $-/-$  endothelium due to the small number of adherent and rolling cells.

### Cytochalasin D does not affect rolling velocity

Rolling velocities for WEHI cells on wild-type endothelium were calculated by computer 30 times per second, and an average velocity for each cell was determined. Velocities were then plotted as histograms (Fig. 6) to compare the distribution of rolling velocities with and without cytochalasin D. Between 48 and 220 cell velocities were measured to produce each velocity histogram; more velocity data were available at 0.3 and 1.0  $\mu\text{g/ml}$  cytochalasin D, due to increased rolling efficiency at those doses (cf. Fig. 2). We found that, even though cytochalasin D treatment affected the number of cells rolling on wild-type endothelium,

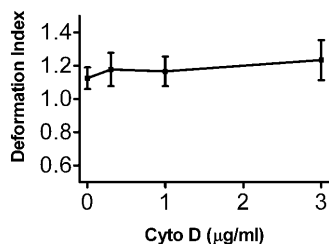


FIGURE 4 Effect of cytochalasin D on WEHI cell deformability. Cell deformability indices (ratio of cell length to cell width under conditions of shear stress) were calculated for rolling and adherent cells at the shear stress used for adhesion assays (2.5 dynes/cm<sup>2</sup>) and mean  $\pm$  SD were plotted as a function of cytochalasin D dose. No significant increase in deformability index was observed at the cytochalasin D concentrations used in this study.

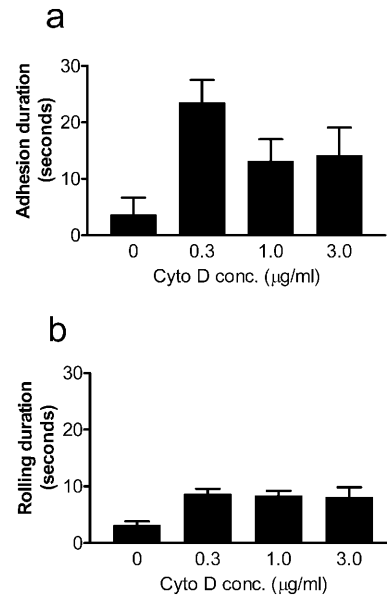


FIGURE 5 Duration of adhesion and rolling. Duration of arrest (*a*) and rolling (*b*) were quantified for WEHI monocytes on wild-type endothelium at a shear stress of 2.5 dynes/cm<sup>2</sup>. Cell trajectories were divided into segments in which the cell was rolling or stopped, as defined in Materials and Methods, to calculate these quantities. The bar graphs represent mean duration ( $\pm$  SE) of these segments for each cytochalasin D concentration. Cytochalasin D increased duration of both types of adhesion.

average velocities were not significantly different. Similarly, cytochalasin D had no effect on rolling velocities in ICAM-1  $-/-$  endothelium (data not shown).

### Cytochalasin D does not activate adhesion or rolling on purified VCAM-1 under flow conditions

The leukocyte integrin  $\alpha_4\beta_1$  mediates both rolling and firm adhesion via binding to VCAM-1. In addition, like the  $\beta_2$ -integrins, adhesion via  $\alpha_4\beta_1$  is activatable. To determine whether  $\alpha_4\beta_1$ -mediated adhesion was affected by cytochalasin D, we measured binding of WEHIs to purified VCAM-1 in the flow chamber. As with selectin substrates, cytochalasin D did not activate adhesion to purified VCAM-1 under flow (Fig. 7 *a*). When the cells were allowed to settle for 1 min and then exposed to flow (Fig. 7 *b*), cytochalasin D was still unable to increase adhesion significantly, though adhesion could be activated by PMA.

### Cytochalasin D does not activate rolling adhesion on purified endothelial selectins under flow conditions

Adhesion of leukocytes to vascular endothelium involves the simultaneous binding of several sets of adhesion molecules to their ligands. To confirm that the major effect of cytochalasin D treatment was on leukocyte integrins rather

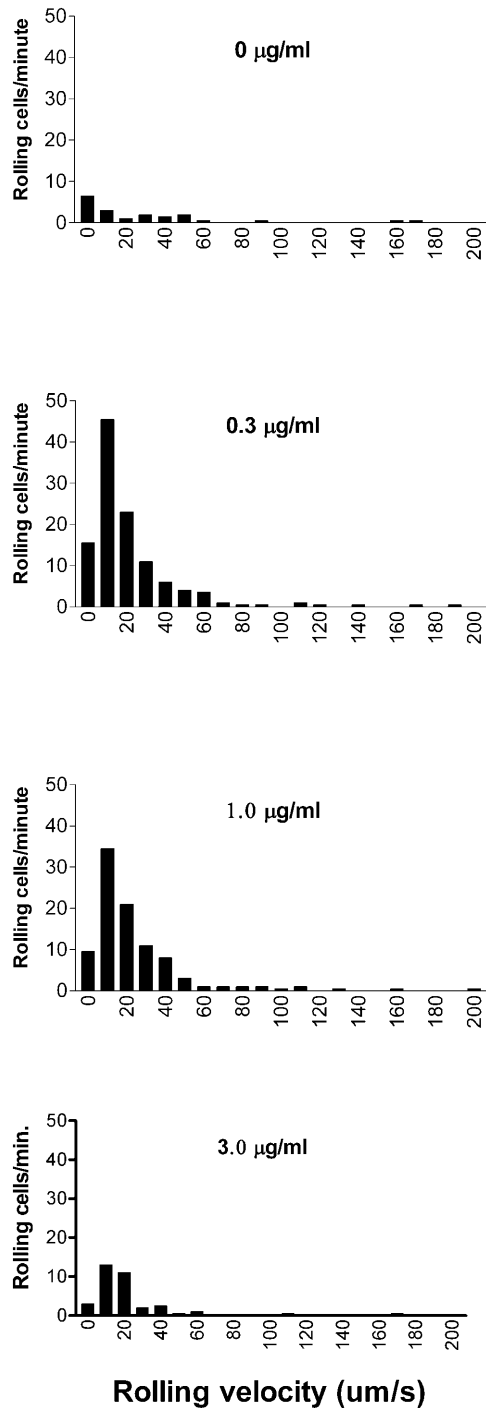


FIGURE 6 Cytochalasin D does not affect rolling velocity. Velocities of WEHI monocytes rolling on wild-type endothelium were quantified, and the distribution of average velocities was expressed as velocity histograms. The difference in bar heights among panels reflects differences in efficiency of cell capture, consistent with Fig. 2. Although cytochalasin D affected the number of rolling cells, the average velocity of those that rolled was not affected.

than on selectin ligands, we measured rolling and adhesion of WEHIs to purified endothelial selectin substrates. We found that cytochalasin D treatment had no significant effect

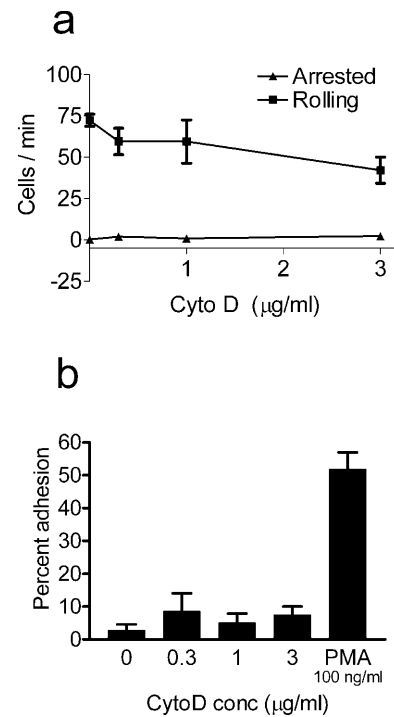


FIGURE 7 Cytochalasin D does not activate rolling or adhesion on purified VCAM-1. (a) To test for an effect of cytochalasin D on  $\alpha_4\beta_1$ -integrin avidity, rolling and firm adhesion of WEHI monocytes were quantified at a shear stress of 2.5 dynes/cm<sup>2</sup> on a substrate of the  $\alpha_4\beta_1$  ligand VCAM-1. Low dose cytochalasin D was unable to activate either type of adhesion. (b) Adhesion of WEHI monocytes to purified VCAM-1 with 1 min static incubation. WEHI cells were allowed to settle on VCAM-1 for 1 min before being subjected to a shear stress of 2.5 dynes/cm<sup>2</sup>. Cytochalasin D was unable to increase adhesion in this static assay, whereas PMA-induced adhesion was clearly demonstrable.

on the number of WEHIs rolling on or adhering to P-selectin (Fig. 8 a). On purified E-selectin, cytochalasin D treatment actually decreased the likelihood of rolling (Fig. 8 b). As expected, since the selectins are primarily rolling receptors, there was very little firm adhesion to either substrate (data not shown).

### A mixed substrate of ICAM-1 and E-selectin reconstituted the dose-dependent effect of cytochalasin D on adhesion

To confirm the role of ICAM-1 in cytochalasin D-activated adhesion, we measured adhesion of WEHI cells to purified ICAM-1. Baseline adhesion on the purified substrates differed, and seemed to be related to the ability of each substrate to capture cells from the flowing medium (Fig. 9 a). We found, however, that under flow, adhesion to purified ICAM-1 alone or on purified E-selectin alone was not increased by cytochalasin D treatment (Fig. 9 b). On a mixed substrate of both ICAM-1 and E-selectin, though, cytochalasin D was able to increase adhesion in a dose-dependent manner. This demonstrates the need for

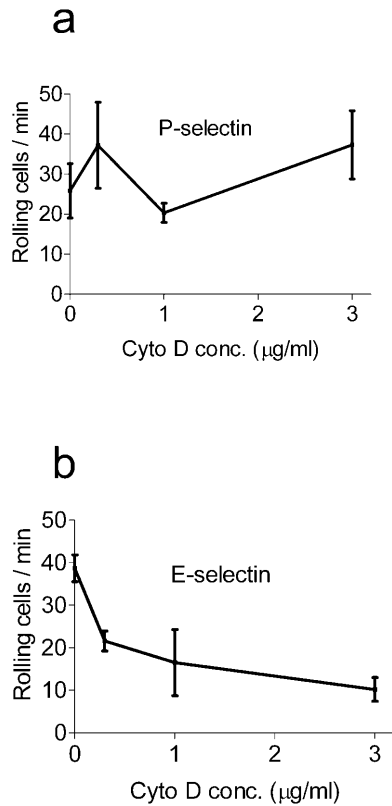


FIGURE 8 Cytochalasin D does not activate rolling on purified selectins. To test whether low-dose cytochalasin D affects avidity of selectin ligands, rolling was quantified on substrates of purified P-selectin (a) and E-selectin (b). Cytochalasin D was unable to activate selectin-mediated rolling adhesions and actually significantly decreased rolling on E-selectin.

cooperativity among adhesion receptors in activation of adhesion under flow.

## DISCUSSION

In this study, we found that treatment of WEHI 274.1 monocytic cells with low concentrations of cytochalasin D increased adhesion to vascular endothelium under flow. Both rolling and firm adhesion were increased in both frequency and duration.

It was shown previously that cytochalasin D, at low concentrations, increases  $\beta_2$ -integrin diffusion in the WEHI 274.1 cell line with little effect on other cell functions (Zhou and Li, 2000). Increased diffusion then results in increased integrin clustering (van Kooyk and Figdor, 1997; van Kooyk et al., 1999). The pro-adhesive effect of low doses of cytochalasin D on  $\beta_2$ -integrin-mediated adhesion is well established for static adhesion (van Kooyk and Figdor, 1997; Yauch et al., 1997; Stewart et al., 1998; van Kooyk et al., 1999; Zhou and Li, 2000), but had not been shown to operate under flow.

For flow studies, it was important to confirm that low-dose cytochalasin D does not alter cell morphology. This is

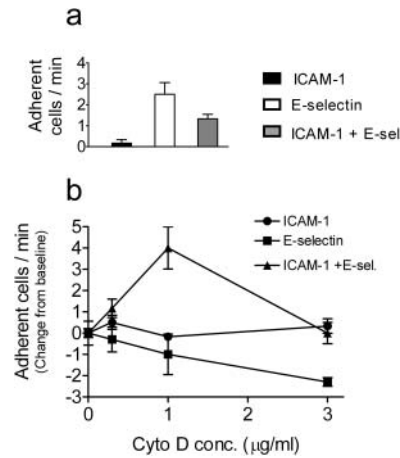


FIGURE 9 Adhesion to a mixture of purified ICAM-1 and E-selectin. WEHI monocytes were perfused at a shear stress of 2.5 dynes/cm<sup>2</sup> over substrates of purified ICAM-1, E-selectin, or a mixture of both. (a) Baseline adhesion to purified substrates without cytochalasin D treatment. Note that interaction of flowing cells with ICAM-1 alone is poor. (b) To compare activation of adhesion by cytochalasin D, baseline values are normalized to zero, and changes in adhesion are plotted as a function of cytochalasin D concentration. Although cytochalasin D failed to activate adhesion on either purified ICAM-1 or E-selectin alone, a dose-dependent effect similar to that seen with whole endothelium was observed with both adhesion molecules combined.

because alterations in cell surface features can change the profile presented to the flowing fluid, and, thus, the forces exerted on the leukocyte. We demonstrated by scanning electron microscopy that the cytochalasin D concentration that activates adhesion is lower than that needed to alter surface morphology. Fig. 3 demonstrates that at higher concentrations of cytochalasin D (3 µg/ml), microvilli collapse, making the cell surface smoother. This might increase contact area and/or decrease the forces exerted on the cell by the fluid flow. Since the pro-adhesive effect of cytochalasin D was at lower concentrations, though, where effects on surface morphology are not obvious (Fig. 3, b and c), it cannot be attributed to these cell shape changes. Note, however, that this does not rule out effects on the mechanical properties of microvilli with low-dose cytochalasin D.

It was also important, however, to examine changes in morphology under flow. If low concentrations of cytochalasin D were to significantly decrease cell stiffness, deformation of cells under shear stress might be increased. In neutrophils, cytochalasin D can have significant effects on cortical tension and cytoplasmic viscosity, even at relatively low doses (Ting-Beall et al., 1995; Wakatsuki et al., 2001; Finger et al., 1996). To determine whether decreased cortical tension affected WEHI 274.1 deformability and, thus, cell shape in the presence of shear, we used digital image processing to calculate deformation indices (Finger et al., 1996) for rolling and adherent cells as a function of cytochalasin D concentration. Using an imaging system developed for high temporal and spatial resolution (Kevill

et al., 2003), we were able to image our cells under flow at high enough resolution to reliably determine cell shape (with an accuracy of  $\pm 0.44 \mu$ ). Under the relatively low shear stress conditions used in this study ( $2.5 \text{ dynes/cm}^2$ ), there was no significant effect of cytochalasin D on cell deformability (Fig. 4). This does not rule out changes in the mechanical properties of the cells that might be detected by a more sensitive assay. It does clearly show, though, that low-dose cytochalasin D did not change the mechanical properties of the cells enough to cause them to be significantly more deformable under the conditions used for our adhesion assays.

It should be noted that effects of cytochalasin D on the physical properties of microvillous tethers between leukocytes and endothelial cells cannot be ruled out by this study. It is possible that changes in microvillus compliance might also influence adhesive interactions. At low-dose cytochalasin D, no obvious effect on the shape or number of microvilli on WEHI 274.1 cells was detected by SEM (Fig. 3). This does not, however, rule out changes in mechanical properties that might be apparent only in the presence of shear stress. These would not be detected in our system, since microvilli tethers are too small to be reliably visualized using our flow chamber. If the compliance of microvilli were increased by cytochalasin D, this could affect rolling adhesion. This would not be expected to have a large effect on  $\beta_2$ -integrin-mediated adhesion, since  $\beta_2$ -integrins are localized between microvilli, not at their tips (Erlandsen et al., 1993; Abitorabi et al., 1997). There could be an effect, however, on selectin-mediated rolling. Both L-selectin and PSGL-1 (the major ligand for P-selectin) are localized to microvillus tips (Picker et al., 1991; Moore et al., 1995). Any positive effect on PSGL-1-mediated adhesion would have to involve cooperation with other receptors, though, since cytochalasin D did not increase adhesion to purified P-selectin substrates (Fig. 8).

A key to our ability to detect the role of  $\beta_2$ -integrin diffusion in activation of adhesion under flow was the use of endothelium from ICAM-1-deficient mice. Endothelial monolayers have distinct advantages over purified substrates for understanding adhesion under flow. Although studies using purified substrates have been valuable in advancing understanding of the properties of individual receptors, they cannot truly mimic physiologic cell-cell adhesion, because, in vivo, cell adhesion involves the simultaneous interaction of multiple sets of adhesion molecules. Use of ICAM-1-deficient, gene-targeted mouse endothelial cells enabled us to detect previously unsuspected effects of  $\beta_2$ -integrin diffusion, such as enhancement of capture and rolling. This also allowed us to avoid the use of blocking antibodies, which can themselves have effects on cell activation, confounding interpretation of results.

High temporal and spatial resolution imaging (Kevil et al., 2003) made possible a detailed analysis of rolling motion to accurately quantify rolling adhesion and durations. Of

course, at high enough temporal resolution, even smooth rolling motion consists of a series of discrete steps (Smith et al., 1999; Chen and Springer, 1999). However, by using strict criteria for rolling motion (see Materials and Methods) and quantifying motion 30 times per second, it was possible to measure velocities only during rolling motion, excluding periods when the leukocytes either arrested or released from the endothelium. Quantifying motion in this way gives a truer measure of rolling velocity than lower sampling frequencies, in which nonrolling behavior is often included in the average. Thus, it could be determined, for example, that cytochalasin D increased duration of rolling with no significant effect on rolling velocity.

The ICAM-1-dependent effect of cytochalasin D on rolling (as opposed to firm adhesion) is particularly interesting. The  $\beta_2$ -integrins are classically associated with firm adhesion and cell motility, and, in some systems,  $\beta_2$ -integrins do not mediate tethering or rolling in shear flow (Lawrence and Springer, 1991; Von Andrian et al., 1991). It is becoming increasingly clear, however, that the  $\beta_2$ -integrin-ICAM-1 interaction can contribute to rolling adhesion. An isolated  $\alpha_L$  I-domain linked to a glycosphosphatidylinositol GPI anchor and expressed in baby hamster kidney cells mediated rolling better than firm adhesion, demonstrating that, under the right conditions, the integrin binding site has rolling-receptor properties (Knorr and Dustin, 1997). Similarly, when LFA-1 was transfected into K562 cells, it mediated rolling better than firm adhesion on purified ICAM-1 (Sigal et al., 2000). In the same study, however, the Jurkat T-cell line, in which regulation of  $\beta_2$ -integrins is more like that of primary cells, mediated firm adhesion rather than rolling under the same conditions.

Structural studies of the LFA-1 molecule demonstrating multiple affinity states provide clues as to how the LFA-1-ICAM-1 interaction mediates both rolling and firm adhesion, and how the form of adhesion can depend on the activation state of the cell. On a leukocyte surface,  $\beta_2$ -integrins are thought to be present in multiple affinity states, the ratio of which can change with activation (Lollo et al., 1993). Salas et al., using isolated I-domains mutationally locked in either the high-affinity ("open") or low-affinity ("closed") conformations, demonstrated that the open conformation mediates firm adhesion, whereas the closed conformation mediates rolling (Salas et al., 2002). They also showed that, when expressed in K562 cells, the wild-type  $\alpha_L\beta_2$  was mostly in the closed conformation, and these cells rolled. This makes sense in terms of the binding kinetics of these constructs. Rapid kinetics for both association and dissociation are an essential feature of rolling adhesion (Alon et al., 1995). The engineered disulfide bond that stabilizes the open conformation increases the on-rate 50-fold and decreases the off-rate 200-fold. The faster dissociation rate of the low-affinity form would be predicted to favor rolling adhesion, as was observed.

Recently, an intermediate affinity state for  $\alpha_L\beta_2$  has been defined (Shimaoka et al., 2003). The intermediate confor-



mation has a relatively fast on-rate as well as a fast off-rate, and it proves to be more effective as a rolling receptor than the low-affinity form. It is likely, then, that rolling interactions of  $\beta_2$ -integrins with ICAM-1 are mediated by low and/or intermediate conformations of the integrins.

This raises the question of whether release of integrins from cytoskeletal constraints affects affinity. The available evidence argues against this. A change in  $\beta_2$ -integrin affinity in response to cytochalasin D has not been demonstrated. Indeed, comparison of LFA-1 affinity states among K562 systems that rolled and those that adhered firmly indicated that the ability to roll depended on integrin arrangement, with no change in affinity state (Sigal et al., 2000). However, affinity measurements in that study and many others generally depend on binding of soluble ligand. This might not distinguish the intermediate from the low-affinity state, since both bind soluble ligand poorly. Therefore, it is possible that, in addition to increasing integrin diffusion, release from cytoskeletal constraints also converts integrins from low to intermediate affinity, which would also promote rolling. Given the well-known effects of cytochalasin D on  $\beta_2$ -integrin diffusion, though, and the lack of evidence that release from cytoskeletal constraints affects affinity, we feel that integrin rearrangement is the simplest interpretation of the effect of cytochalasin D on WEHI 274.1 cell adhesion.

Cytochalasin D affected not only frequency of rolling and firm adhesion, but also their duration. Adhesion of leukocytes to endothelium is not irreversible. Many cells that arrest on endothelium do so briefly, resuming rolling after a few seconds. Similarly, rolling cells often release from the endothelium after a short time to be carried away by the flow. Although cytochalasin D increased both duration of rolling and duration of firm adhesion, the dose-dependence differed. The increase in firm adhesion duration peaked at 0.3  $\mu\text{g/ml}$ , but increased rolling duration was sustained even at a concentration of cytochalasin D (3  $\mu\text{g/ml}$ ) high enough to disrupt cytoskeletal architecture (as seen in Fig. 3). The classic interpretation for the biphasic effect of cytochalasin D is that, whereas low concentrations are pro-adhesive due to their effects on integrin rearrangement, doses high enough to substantially disrupt the cytoskeleton interfere with the ability of actin to reinforce and stabilize nascent contacts (Kucik et al., 1996). Our data suggest that cytoskeletal reinforcement is less important for maintenance of rolling than for arrest.

Clearly, ICAM-1 plays an important role in adhesion between monocytes and endothelium, because far fewer cells per minute rolled and arrested on endothelium lacking ICAM-1. Not all effects of cytochalasin D could be explained by the  $\beta_2$ -integrin–ICAM-1 interaction, however. The low level of adhesion on ICAM-1  $-/-$  endothelium could still be slightly increased by pretreatment of the leukocytes with cytochalasin D (as seen in Fig. 1 *b*). One possible explanation for this is that, in the absence of ICAM-1, the  $\beta_2$ -integrins bind to other ligands. Mathematical

modeling (Bell, 1978) predicts that increased diffusion of  $\beta_2$ -integrins will increase avidity for any ligand, not just ICAM-1. Although ICAM-1 is the major  $\beta_2$ -integrin ligand found on endothelial cells, ICAM-2, another  $\alpha_1\beta_2$  ligand, is also present (Issekutz et al., 1999). In addition,  $\alpha_M\beta_2$  is known to have a number of poorly characterized ligands that may be present on activated endothelial cells (Davis, 1992). Interactions between  $\beta_2$ -integrins and any of these other ligands could account for the small amount of ICAM-1-independent adhesive effects.

Alternatively, effects of cytochalasin D on other adhesion molecules might also result in ICAM-1-independent increased adhesion. Although, so far, increased adhesiveness in response to cytochalasin D has been described only for  $\beta_2$ -integrins, we tested for effects of cytochalasin D on other adhesion molecules that might be involved in activation of leukocyte adhesion. We first tested  $\alpha_4\beta_1$  because, as with the  $\beta_2$ -integrins, leukocyte adhesion to VCAM-1 via  $\alpha_4\beta_1$  is activatable. In addition,  $\alpha_4\beta_1$  associates with the cytoskeleton in a regulated manner, clustering and binding to the cytoskeleton at sites of cell-cell contact (Sanchez-Mateos et al., 1993). To determine whether cytochalasin D affected  $\alpha_4\beta_1$  avidity, we perfused cells over a purified VCAM-1 substrate, specific for  $\alpha_4\beta_1$ -mediated adhesion. We found no increase in  $\alpha_4\beta_1$ -mediated rolling or arrest under flow in response to cytochalasin D treatment (Fig. 7 *a*). Since there was very little firm adhesion under these conditions, we also measured adhesion in a brief static assay in the same flow chamber to confirm that the substrate could mediate adhesion and that  $\alpha_4\beta_1$  was indeed activatable on our cells. Cells were allowed to settle for 1 min, and then nonadherent cells were washed away by exposure to high flow rates. Although PMA activated adhesion fourfold under these conditions, cytochalasin D had no significant effect (Fig. 7 *b*). This lack of effect of cytochalasin D on  $\alpha_4\beta_1$  function is evidence that  $\alpha_4\beta_1$  is not responsible for the ICAM-1-independent increase in firm adhesion observed on ICAM-1  $-/-$  endothelium. This is consistent with earlier studies in which  $\alpha_4\beta_1$  was transfected into Chinese hamster ovary (CHO) cells (Yauch et al., 1997). In those cells,  $\alpha_4\beta_1$ -mediated adhesion could be activated by PMA, but not by low-dose cytochalasin D. Direct measurement of  $\alpha_4\beta_1$  diffusion in that system demonstrated that cytochalasin D did not affect  $\alpha_4\beta_1$  lateral mobility.

Leukocytes have a number of ligands for endothelial selectins. If cytochalasin D were to increase diffusion of any or all of these ligands, it might activate adhesion. We reasoned that P-selectin-mediated adhesion might be affected by cytochalasin D, because the major ligand for P selectin, PSGL-1 (Sako et al., 1993; Moore et al., 1995; Snapp et al., 1998), is linked to the actin cytoskeleton in leukocytes via moesin (Snapp et al., 2002), and, thus, might undergo regulation of diffusion similar to the  $\beta_2$ -integrins. However, low-dose cytochalasin D had no significant effect on adhesion to purified P-selectin in our system (Fig. 8 *a*).

A number of cell-surface molecules serve as ligands for E-selectin, including PSGL-1 (Sako et al., 1993), and ESL-1 (Stegmaier et al., 1995). (Although L-selectin binds directly to E-selectin in humans, it is not a ligand in mice (Zollner et al., 1997)). Other E-selectin ligands may exist, since no combination of antibodies has yet been demonstrated to completely block E-selectin binding (Kansas, 2001). Therefore, it is difficult to account for all of the possible E-selectin ligands on a given leukocyte. Even without identifying the specific ligands present, however, the global effect of cytochalasin D on all E-selectin ligands can be tested functionally by measuring the dose dependence of cytochalasin D on WEHI cell rolling on a purified E-selectin substrate. We found that the low doses that activate integrin-mediated adhesion had no significant effect on firm adhesion to E-selectin (Fig. 9 b), and actually decreased rolling interactions (Fig. 8 b).

One of the most interesting findings was the requirement for cooperativity among adhesion receptors for avidity effects under flow (Fig. 9). On purified ICAM-1 alone, there was very little firm adhesion at a shear stress of 2.5 dynes/cm<sup>2</sup> (perhaps due to the inefficiency of tethering), and adhesion was not increased by cytochalasin D. Tethering was more efficient on E-selectin alone, and this resulted in some firm adhesion, but cytochalasin D had no effect. On a substrate of combined purified ICAM-1 and E-selectin, however, the pro-adhesive effect of cytochalasin D could be reconstituted. This confirms the role of ICAM-1 (and its receptors, the  $\beta_2$ -integrins) in the adhesive effects of cytochalasin D, but shows that cooperativity with another receptor is required. A simple interpretation would be that, for cytochalasin D to increase  $\beta_2$ -integrin adhesion to ICAM-1, cells must first be in contact with the endothelium, and this requires an additional receptor to mediate capture and rolling. This is consistent with previous work that suggested that E-selectin and ICAM-1 work together for optimal leukocyte adhesion. In that study, L-cells transfected with both E-selectin and ICAM-1 could support far greater adhesion, both rolling and firm, than those expressing either receptor alone (Gopalan et al., 1997).

The requirement for cooperativity between integrins and selectins may result either from physical cooperation between receptors or from receptor-mediated signaling. It has been demonstrated that efficiency of firm adhesion through  $\beta_2$ -integrins decreases as shear increases, suggesting a requirement for a collisional contact duration of at least 5 ms (Lynam et al., 1998; Neelamegham et al., 1998). Thus, interactions between E-selectin and its ligands may slow the cell and provide for increased contact duration.

E-selectin does more, however, than simply tethering the leukocyte to increase the contact time. E-selectin binding by neutrophils can activate  $\beta_2$ -integrin-mediated adhesion through mitogen-activated protein kinases (Simon et al., 2000). In this study, on a combined substrate of ICAM-1 and E-selectin, adhesion could be increased by cytochalasin D.

This suggests that signaling through E-selectin binding is not sufficient to fully activate integrin-mediated adhesion. A possible explanation is that integrin rearrangement can work synergistically with effects on affinity, with the two regulated independently. For example, signaling through E-selectin might increase  $\beta_2$ -integrin affinity, yet independent control of rearrangement might allow for further avidity increases. Future studies will be aimed at further elucidating the biochemical basis of these effects.

In summary, using low-dose cytochalasin D to mimic the increased integrin diffusion that occurs upon leukocyte activation, we have demonstrated that avidity modulation can have major effects on leukocyte adhesion to endothelium under flow. Effects involve not only firm adhesion, a well-established  $\beta_2$ -integrin function, but also capture and rolling, in which  $\beta_2$ -integrins and ICAM-1 must cooperate with selectins to exert their effects. These functional assays provide a proof of principle for regulation of leukocyte adhesion under flow by avidity regulation, and lay the groundwork for investigation of the molecular mechanisms by which integrin diffusion is controlled during adhesion in the presence of shear. Our findings not only have important implications for the mechanism of activation of adhesion, but reveal that cooperativity among sets of adhesion molecules plays a larger role than expected.

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