A Differential Role for Cell Shape in Neutrophil Tethering and Rolling on Endothelial Selectins Under Flow[1]

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We investigated the role of neutrophil microvilli in interactions with E-selectin and P-selectin in hydrodynamic shear flow by disruption with cytochalasin B, hypotonic swelling, and chilling. Cytochalasin B only marginally reduced microvilli numbers (from 30 ± 6 to 16 ± 6 per cell perimeter, $p < 0.005$) as shown by electron microscopy, completely disrupted tethering in shear flow to E-selectin and P-selectin, increased the strength of rolling adhesions on E-selectin and P-selectin, and increased cell deformability in shear flow with a likely increase in the area of cell:substrate contact. Hypoosmotic swelling markedly reduced microvilli number (to 6 ± 5 per perimeter, $p < 0.005$), almost completely inhibited tethering on E- and P-selectin, and increased the strength of rolling adhesions on P-selectin but not on E-selectin. Chilling almost completely abolished microvilli (to 3 ± 3 per perimeter, $p < 0.005$), but pseudopod-like structures were present, and had little effect on tethering in flow. Immunogold labeling of L-selectin, which is normally clustered on tips of microvilli, showed that in the absence of microvilli it remained in small clusters. Our studies show that alterations in cell morphology and viscoelasticity can have opposing effects on tethering and rolling, showing that they are independently regulatable. Furthermore, our results suggest that the association of molecules that mediate rolling with microvilli tips may be important not just to enhance presentation, but for other functions such as to promote resistance to extraction from the membrane or cooperative interactions among clustered receptors. *The Journal of Immunology,* **1996, 157: 5085–5096.**

Neutrophil localization to sites of inflammation is a multistep process requiring the interaction of leukocytes with endothelium (1). Neutrophils are first observed to roll along the walls of postcapillary venules, which brings them into close proximity with the endothelium where arrest and emigration through the endothelial lining and into the tissues can occur. Rolling interactions through selectins are required for subsequent arrest of the rolling cell and the development of firm adhesion through integrins that precedes diapedesis (2–4). The three members of the selectin glycoprotein family are characterized by an N-terminal C-type lectin domain; an epidermal growth factor domain; a variable number of short consensus repeats; and transmembrane and cytoplasmic domains (1, 5). E-selectin (CD62E) is expressed on endothelial cells following exposure to TNF, IL-1, or LPS and requires de novo protein synthesis (6). P-selectin (CD62P) is stored preformed in α and dense granules of resting platelets as well as in endothelial Weibel-Palade bodies, and is rapidly redistributed to the cell surface following stimulation with various inflammatory cytokines (7). L-selectin (CD62L) is constitutively expressed on circulating neutrophils, monocytes, and most lymphocytes (8), but is shed upon cellular activation (9, 10).

The selectins recognize sialylated carbohydrate structures related to sialyl Lewis × (5). L- and P-selectin recognize carbohydrate structures on heavily *O*-linked mucin-like molecules. P-selectin recognizes the *O*-glycoprotease-sensitive mucin P-selectin glycoprotein ligand-1 (PSGL-1) (11, 12). L-selectin binds to peripheral node addressin (PNAd)[3] in lymph node high endothelial venules (HEV) (13, 14), which is composed of a mixture of mucins that include GlyCAM-1 (15) and CD34 (16). L-selectin is unique among molecules known to support rolling in that it requires shear stress above a threshold in order for cell adhesion to occur (17). E-selectin recognizes carbohydrate ligands presented on both glycoproteins and glycolipids (18–20). On neutrophils, L-selectin is a sialyl Lewis ×-bearing glycoprotein that presents or is associated with a carbohydrate ligand for E-selectin (21–23).

The surface of the resting neutrophil contains numerous protuberances of the plasma membrane that may be characterized as stubby microvilli (24). The overall surface area of membrane is in large excess of the minimum required to contain the cell's volume (25). The microfilamentous structure of actin and associated proteins determine the cell shape in human leukocytes, which is altered when the leukocyte is activated (26). In contrast to the much longer microvilli on many epithelial cells, which serve to increase surface area and adsorptive capacity, the function of microvilli on circulating leukocytes is not fully described. L-selectin has been shown to cluster on neutrophil microvilli and surface foldings, and is only minimally present on the flat portions of the cell membrane, raising the hypothesis that this localization may facilitate cell interactions through selectins (21, 27–29). Deletion of the L-selectin cytoplasmic domain and cytochalasin B treatment of intact cells has been shown to decrease rolling in vivo and to decrease attachment to HEV in vitro (30). Redistribution of L-selectin to the body of the cell through domain swaps with nonmicrovillus protein has

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[3] Abbreviations used in this paper: PNAd, peripheral node addressin; HEV, high endothelial venules; RVD, regulatory volume decrease; TEM, transmission electron microscopy.

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shown a decrease in attachment rate and rolling flux in vitro (31). PSGL-1 has also been shown to localize to leukocyte microvilli (12), but the role of leukocyte microvilli in rolling on the endothelial selectins (E- and P-selectin) has not been described.

Selectin-mediated rolling of neutrophils has been demonstrated for L-, E-, and P- selectins in vitro (2, 14, 32), where the functional components of rolling can be dissected and studied individually. Tethering is the initial interaction wherein a cell attaches to the endothelial substrate. It is measured as the frequency with which leukocytes in hydrodynamic flow adhere to the vessel wall, and is appreciated by a dramatic slowing in velocity as the leukocyte moving at the hydrodynamic velocity tethers and begins rolling. The strength of rolling adhesions can be measured by resistance to detachment by increasing shear flow, which increases as selectin density increases, and by rolling velocity, which decreases as selectin density increases (2, 19, 32).

In this study, we have tested whether microvilli are important in neutrophil interactions with E- and P-selectin. We show that cytochalasin B and hypoosmotic swelling partially and largely decrease microvilli number, respectively, dramatically decrease tethering efficiency to E-selectin and P-selectin, and by contrast do not decrease the strength of rolling adhesions on E-selectin and actually increase the strength of rolling adhesions on P-selectin. Chilling almost completely disrupted microvilli, but did not decrease tethering efficiency. Using L-selectin as a marker for a microvillus-associated membrane protein, we show that such treatments do not affect expression or clustering. Our results demonstrate the independent regulation of tethering and rolling by cellular properties, and a more complex association between clustering on microvilli and selectin-mediated functions than previously appreciated.

Materials and Methods

Antibodies and proteins

L-selectin mAb DREG 200 (33), P-selectin mAb WAPS 12.2 (34), and E-selectin mAbs BB11 (35) and CL-3 (36) were used as purified Abs. CD18 mAb TS1/18 (37) was used as ascites. Purified P-selectin was a generous gift of Rodger McEver (38). E-selectin was affinity purified from transmembranal E-selectin-transfected CHO cells by modification of the procedure previously described (35). Briefly, a Triton X-100 lysate was passed over an anti-E-selectin mAb (BB11) sepharose column, eluted with 0.1 M glycine, 1% *N*-octyl β-D-glucopyranoside, pH 3.0, and neutralized. Recombinant soluble E-selectin used in deformation assays was a generous gift of Dr. Roy Lobb, Biogen, Cambridge MA (35).

Preparation of planar bilayers

E- and P-selectin were incorporated in phosphatidylcholine liposomes by *N*-octyl β-D-glucopyranoside dialysis (2). Glass microscope slides were cleaned by soaking in a sulfuric/chromic acid cleaning solution for 30 min, washed extensively with water, boiled for 30 min in 1:7 dilution of Linbro 7× detergent, and washed in deionized water for at least 24 h. A solution of E- or P-selectin liposomes (20 μl) was spotted on the cleaned slide, overlaid with a clean 12-mm diameter coverslip, and incubated for 20 min at room temperature. The slides were then blocked with 1% HSA in HBSS, 10 mM HEPES, pH 7.4, for at least 30 min. Bilayers were never exposed to air.

Determination of site densities

E- and P-selectin bilayers were prepared in glass test tubes in the same manner as for the glass slides. The bilayers were washed with PBS and blocked with 1% BSA, and 1 mg/ml human IgG in PBS. Saturation binding of ^{125}I-labeled CL-3 and WAPS 12.2 was used to measure site densities for E- and P-selectin, respectively, as reported in each figure legend (2).

Isolation of neutrophils

Neutrophils were isolated from citrate anti-coagulated blood by dextran sedimentation and Ficoll-Hypaque density separation (39). The remaining erythrocytes were lysed in deionized H_2O at 4°C. The purified neutrophils were washed twice and stored in HBSS, 0.25% HSA, 1 mM EDTA, 10 mM HEPES, pH 7.4, on ice. Cells were allowed to warm fully to room temperature before use.

Cytochalasin B treatment

PMN were suspended in HBSS, 0.25% HSA, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4, at 5×10^5/ml. Cytochalasin B (Calbiochem, San Diego, CA) was added to 2, 20, or 100 μM from a 25 mg/ml stock in DMSO. The cells were incubated for 10 min at room temperature and immediately used in tethering or detachment assays in the presence of cytochalasin B throughout.

Cell swelling

PMN were resuspended in a solution of 80 mM NaCl, 60 mM KCl, 2 mM $CaCl_2$, 0.25% HSA, 10 mM HEPES, pH 7.4, and diluted to the indicated osmolality with 2 mM $CaCl_2$, 0.25% HSA, 10 mM HEPES, pH 7.4, at 5×10^5 cells/ml. Osmolality was confirmed by freezing point depression (Precision Systems, Inc., Natick, MA). Cells were assayed for attachment or detachment within 5 min in the same medium.

Cold treatment

PMN were isolated as above and stored in HBSS, 0.25% HSA, 1 mM EDTA, 10 mM HEPES, pH 7.4, on ice for at least 1 h before use, and assayed in the cold as described in the legend of Figure 12.

Light microscopy/cell size measurements

PMN were resuspended in media of indicated osmolality and viewed with Hoffman optics on a Nikon Diaphot 300 inverted microscope (Nikon, Inc., Garden City, NY) as images were captured using a Scion LG-3 frame-grabber in a Macintosh PowerPC 7100 and analyzed with the National Institutes of Health Image analysis program. The focal plane was adjusted until cells appeared to have a single dark spherical ring around the circumference of the cell. Cell diameters were measured orthogonally, then averaged.

Laminar flow assays

The glass-supported bilayers were assembled in a parallel plate flow apparatus (2). Flow rates were controlled with a syringe pump. Cell interaction with the planar bilayer was observed on an inverted phase-contrast microscope (Diaphot-TMD), and recorded on videotape for later analysis. Infused cells were suspended at 5×10^5 cells/ml in HBSS, 0.25% HSA, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4, plus indicated treatment agents, in hypotonic media, or in chilled medium as indicated. All assays for a given experiment were performed on a single field of view of the bilayer.

Tethering assays

PMN were infused over the planar bilayer at shear stresses from 1.8 to 3.6 dyn/cm^2 for 1 to 3 min. The number of cells was counted that tethered, as defined by a sudden drop in velocity below that of the hydrodynamic velocity as rolling was initiated. Only primary interactions of cells with the selectin-containing bilayer were scored. Cells that first bumped into a previously tethered cell, or that tethered within five cell diameters directly downstream of a previously tethered cell(s), were not counted.

Detachment assays

PMN were allowed to tether under low-flow conditions (≤0.35 dyn/cm^2) and the medium was changed to the identical medium free of cells. Shear stress was increased incrementally every 10 s to a maximum of 35 dyn/cm^2. The percentage of cells remaining at the end of each flow rate was assessed and nonspecific attachment at the end of each experiment was determined by perfusing HBSS, 0.25% HSA, 5 mM EDTA, and 10 mM HEPES, pH 7.4. Routinely, 100% of remaining cells detached with EDTA.

Rolling velocity determination

Rolling velocities were determined from videotapes of detachment assays by monitoring cell positions for 5 to 10 s. For each cell treatment and shear stress, rolling velocities of 15 to 50 cells were measured.

Cell deformation measurement

Recombinant-soluble E-selectin (0.89 μg/ml in sodium carbonate buffer, pH 9.3) was adsorbed on plastic petri dishes as described (32). The plates were blocked with 1% HSA in PBS for 1 h, washed with 1% Tween-20 in PBS for 1 min, washed extensively in PBS, and assembled in the flow chamber. PMN were allowed to settle under static conditions for 30 to 60 s,

and then flow was initiated at 0.73 dyn/cm^2 and increased in increments to 35 dyn/cm^2. Images were analyzed with the National Institutes of Health Image analysis program on a Macintosh PowerPC 7100 computer for cell diameters parallel and perpendicular to the direction of flow. The diameter parallel to flow divided by the diameter perpendicular to flow was defined as the deformation index.

Electron microscopy/pre-embedding immunocytochemistry

After the various treatments described above, cells were immediately fixed in 2% p-formaldehyde, 0.05% glutaraldehyde in 0.1 M PBS, pH 7.4, for 30 min at 22°C or in hypotonic medium as indicated below. Neutrophils were then washed three times in HBSS, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4, and incubated with DREG-200 (2 mg/ml) at a 1:200 dilution in DMEM, 1% albumin for 30 min at 22°C. They were washed as described above, and then incubated in rabbit anti-mouse Ig (Zymed; 1 mg/ml) in a 1/100 dilution for 30 min at 22°C, and washed again three times. Subsequently the cells were incubated with 5-nm gold particles conjugated to protein A (University of Utrecht, The Netherlands) at a 1 to 50 dilution for 45 min at 22°C. After washing, they were refixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, plus 1% sucrose for 30 min at 22°C. Subsequently the cells were concentrated into small pellets by centrifugation, post-fixed with OsO_4, stained with aqueous uranyl acetate, embedded in Epon, sectioned, and stained with uranyl acetate and lead citrate. Swollen cells were fixed in 0.5% glutaraldehyde in hypoosmotic medium for morphometric analysis alone.

For morphometric determinations, photomicrographs of sectioned neutrophils were traced using a SummaSketch Plus (Summagraphics Corp., Fairfield, CT) digital drawing tablet interfaced with a computer. The traces were analyzed using Bioquant System IV morphometry software (R & M Biometrics, Inc., Nashville, TN). Measurements were made only on those sections that contained sections of the Golgi complex or multiple lobes of the nucleus. The length of plasma membrane and area of the sectioned cells were obtained simultaneously by tracing the perimeter of the sectioned cells, and the diameter was determined by averaging the length and width. Length measurements were taken by measuring the longest axis that passed through the center of the cell and width measurements by measuring the longest axis perpendicular to the length axis. The axes measured spanned the sectioned cells between the flat portions of the plasma membrane and did not include microvilli. The average of three tracings was reported for each data point.

Pointed projections of the plasma membrane extending at least 50 nm from what would otherwise be a smooth continuous contour of the cell surface were considered a microvillus (40). Microvillus length was measured from the midpoint of the base (determined as the line that would exist if the plasma membrane did not extend from the cell surface as a microvillus) to the midpoint of the tip. Microvillus tip width was measured perpendicular to the microvillus axis at a point 75 to 90% of the length out from its origin at the cell body. Data are representative of two to four experiments for each experimental condition with neutrophils from at least two separate donors.

Adhesion to HUVEC

HUVEC were harvested and cultured in monolayers as described (41). Confluent endothelial monolayers (passage 2) were stimulated with 100 U/ml TNF-α (Genzyme, Boston, MA) for 4 h, washed, and assembled in the flow apparatus. PMN were preincubated with TS1/18 CD18 mAb (1:400 dilution of ascites) before assay for tethering efficiency and shear resistance in the same manner as for selectin bilayers.

Statistics

Data are presented as mean ± SD or SEM as indicated. Two-tailed Student's *t* tests were performed with Microsoft Excel. Statistical significance is noted as follows: $*p < 0.05$; $**p < 0.01$; and $***p < 0.005$.

Results

Cytochalasin B decreases the number of microvilli on neutrophils

To investigate the role of cell surface topology in cell adhesion, we sought to test the morphologic and functional effects of cytoskeletal disruption with cytochalasin B. Transmission electron microscopy (TEM) of resting human neutrophils reveals numerous microvilli extending from the cell surface (Fig. 1*A*). On average there are 30 ± 6 microvilli per section at a mean frequency of 0.8 microvilli per micron of plasma membrane (Table I), which is similar to the value reported elsewhere (29). The average microvillus measures 270 ± 200 nm in length. Immunogold labeling for L-selectin confirms previous studies (21, 27) showing that L-selectin is preferentially localized to microvilli (Fig. 1*B*), and is almost entirely absent from the interceding regions of plasma membrane.

Cytochalasin B causes a 47% reduction in the number of microvilli per PMN section ($p < 0.005$, Fig. 1*C*, Table I), but does not change the mean length of the microvilli ($p > 0.1$). The average width of microvilli near the tip was 190 ± 25 nm. The sum of the widths of the tips of microvilli can be used as an approximation of the length of plasma membrane in contact with the bilayer during rolling. This value was 3.04 ± 0.98 μm per section for cytochalasin B-treated cells, compared with 3.70 ± 0.70 μm per section for control cells ($p < 0.005$). Cytochalasin B treatment also appears to polarize the cells by generating long segments of smooth plasma membrane without microvilli while preserving short segments of plasma membrane with microvilli appearing like those on control cells (compare Fig. 1, *C* and *A*). A 14% increase in average PMN diameter was also observed ($p < 0.005$, Table I). The length of the plasma membrane and the area of the sectioned cell, however, remained nearly unchanged (Table I). Reduction in the number of microvilli was accompanied by an altered distribution of L-selectin on the body of the cell. L-selectin remained in clusters both on the cell body and on the few persisting microvilli. Neutrophils remain unactivated following isolation and cytochalasin B treatment as assayed by maintenance of L-selectin expression, little CD18 up-regulation, and absence of changes in gross cell morphology (data not shown), all of which are indicators of cellular activation (1, 26, 42). Similarly, cytochalasin B had no effect on expression of CD63, a marker for primary (azurophilic) granule fusion with the cell membrane (43) as measured by FACS (data not shown).

Cytochalasin B treatment of neutrophils results in decreased tethering in hydrodynamic flow, but increases the strength of subsequent rolling adhesions

To determine the role of microvilli in tethering, rolling velocity, and resistance to detachment in shear flow, neutrophil interactions with E- and P-selectin were studied in a transparent parallel-wall flow apparatus in which a selectin-containing lipid bilayer formed the floor of the chamber. Neutrophil suspensions were infused at a flow rate controlled by a syringe pump attached to the outlet. We measured the number of cells that tethered at a wall shear stress of 1.8 to 3.6 dyn/cm^2, which is within the range of wall shear stresses (1 to 30 dyn/cm^2) found in post-capillary venules in vivo. Tethering is the initial adhesive interaction between a cell in hydrodynamic flow and the selectin-containing vessel wall, and is measurable as a dramatic reduction in the velocity of a cell from its hydrodynamic velocity to a rolling velocity. Treatment of neutrophils with cytochalasin B inhibited tethering to E-selectin under flow by 89 to 95% ($p < 0.005$, Fig. 2*A*).

The strength of rolling adhesions was determined by measuring resistance to detachment. In separate experiments neutrophils were allowed to tether to the E-selectin-containing bilayer at a wall shear stress of ≤0.35 dyn/cm^2. At these subphysiologic shear stresses, agents that affected cell shape had no effect on tethering rate (data not shown). Subsequently, the shear stress was increased and the percentage of cells that remained adherent after 10 s at each shear stress was determined (Fig. 2*B*). When allowed to tether at these low shear stresses, cytochalasin B-treated neutrophils were able to sustain rolling interactions with the E-selectin bilayer as shear stress was increased even into the upper physiologic range. The resistance to detachment of cytochalasin B-treated

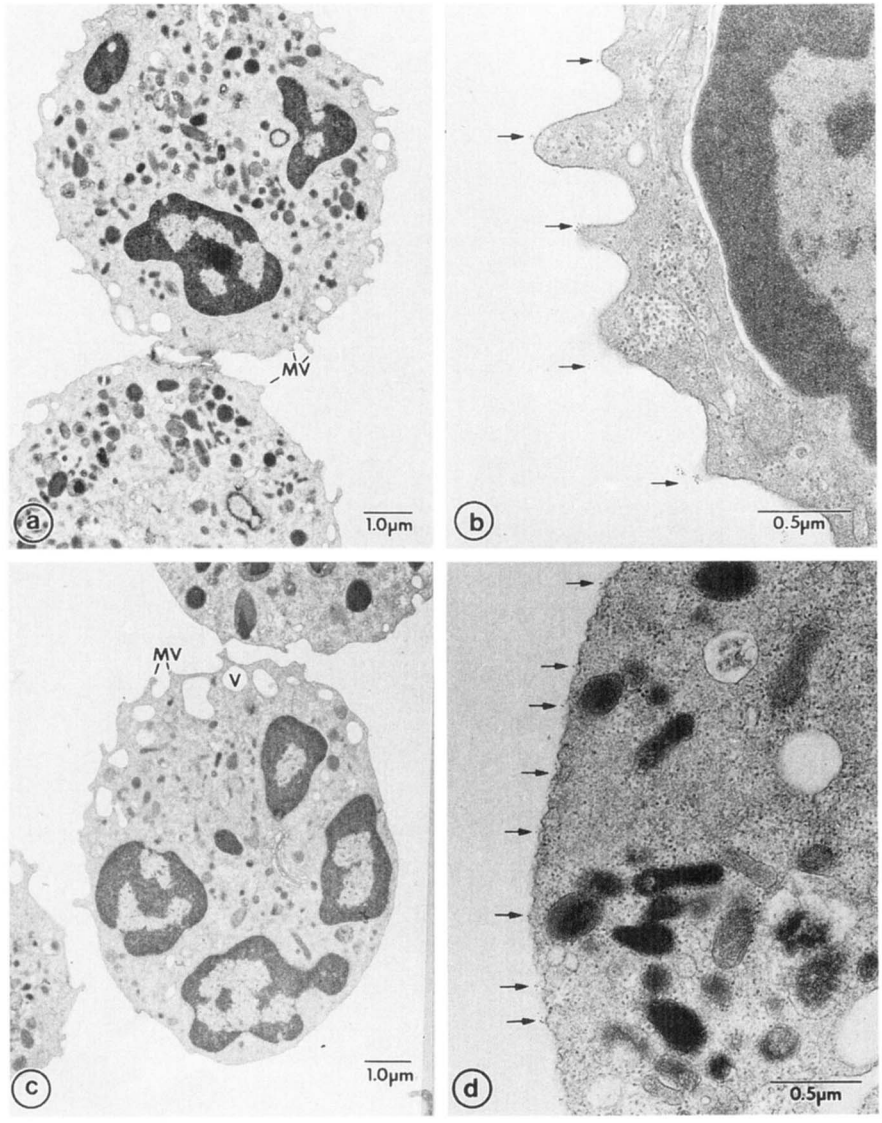

FIGURE 1. Electron microscopy and immunolocalization of L-selectin on control and cytochalasin B-treated neutrophils. *A*, Numerous microvilli (MV) are present on untreated resting human neutrophils. *B*, At higher magnification, immunogold labeling illustrates the preferred localization of L-selectin to the tips of the microvilli on untreated neutrophils (arrows). *C*, Treatment of PMN with 20 μM of cytochalasin B for 10 min at 22°C reduces the number of microvilli, generates long segments of flat plasma membrane, and polarizes the cell. Several microvilli and vacuoles (V) can be seen in the *upper left* portion of the section (9 to 2 o'clock), and a marked decrease in the number of microvilli can be seen in the *lower right* (2 to 9 o'clock). *D*, Gold particles (arrows) can be seen still existing in groups on cytochalasin B-treated neutrophils but are randomly distributed along a flat region of the plasma membrane. Cells were fixed in 2% paraformaldehyde, 0.05% glutaraldehyde, and 0.1 M sodium phosphate, pH 7.4, before immunogold labeling. Magnification: *A*, ×9,000; *B*, ×39,000; *C*, ×9,000; *D*, ×39,000.

Table I. *Morphologic analysis of electron photomicrographs of control, cytochalasin B-treated, hypotonically swollen and cold-exposed neutrophils* [a]

Parameter	Control	Cytochalasin B	Hypotonic	4°C
Microvilli per section	30 ± 6	16 ± 6	6 ± 5	3 ± 3
Length of microvillus (nm)	270 ± 200	280 ± 150	180 ± 90	620 ± 430
Width of microvillus tip (nm)	120 ± 71	190 ± 25	160 ± 26	230 ± 94
Sum of microvillus tip widths per section (μm)	3.7 ± 0.7	3.0 ± 1.0	1.0 ± 0.8	0.6 ± 0.6
Length of PMN plasma membrane per section (μm)	39 ± 4	35 ± 3	35 ± 2	28 ± 10
PMN section diameter (μm)	7 ± 1	8 ± 0	9 ± 1	6 ± 2
PMN section area (μm^2)	47 ± 7	51 ± 6	71 ± 7	39 ± 15

[a] Photomicrographs of sectioned PMN were traced with a drawing tablet interfaced with a computer as described in *Materials and Methods*. Twenty-five neutrophil micrographs were sampled from each experiment and 758, 406, 153, and 73 microvilli were surveyed from the control, cytochalasin B (20 μM) treated, hypotonically swollen, and cold-treated PMN, respectively. Despite inclusion of criteria for selection of sections that bisect cells near their center (see *Methods and Materials*), sections will rarely intersect the center, and thus average values for sections will be lower than for a section through the center. Values are mean ± SD.

cells was significantly greater than that of control cells at these higher shears.

Similar experiments were conducted on neutrophil interactions with P-selectin incorporated into lipid bilayers. Cytochalasin B treatment dramatically decreased PMN tethering to P-selectin under flow. A 97% reduction in tethering at a wall stress of 1.8 dyn/cm^2 was seen with 20 μM of cytochalasin B treatment ($p <$ 0.01, Fig. 3*A*). Resistance to detachment was assayed after allowing PMN to attach at low wall shear stress. Control and cytochalasin B-treated cells both demonstrated rolling interactions. Cytochalasin B-treated cells showed a dramatically increased resistance to detachment over control cells throughout the range of shears tested (Fig. 3*B*), similar to results on E-selectin. Thus, cytochalasin B treatment strengthened rolling adhesions.

We examined the effect of cytochalasin B on deformability of cells in shear flow. Treated cells remained grossly round in stasis,

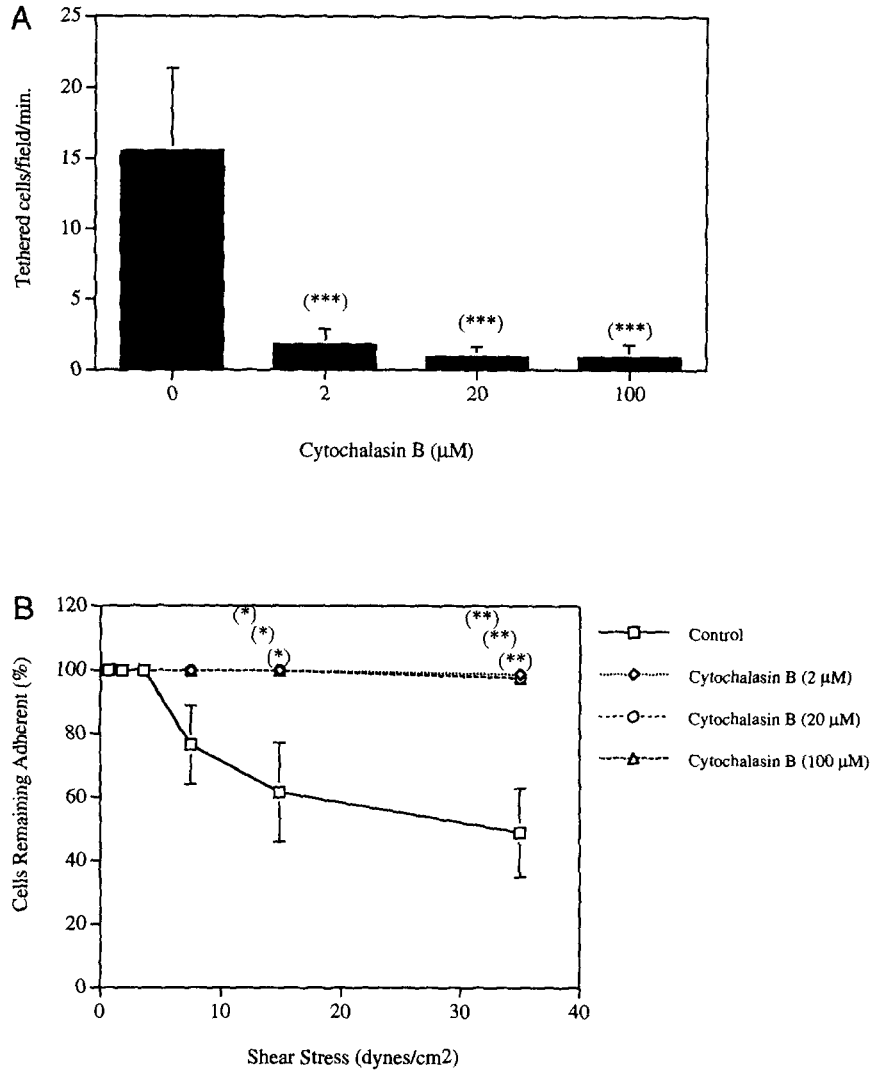

FIGURE 2. Tethering and resistance to detachment of cytochalasin B-treated neutrophils on E-selectin. Tethering and detachment of cytochalasin B-treated cells were assessed on phospholipid bilayers with E-selectin at 200 sites/μm^2. Neutrophils were treated with varying concentrations of cytochalasin B for 10 min at 22°C. Tethering and shear resistance were assayed in this same treatment medium. *A*, Tethering in flow of cytochalasin B-treated or control PMN at a wall shear stress of 3.6 dyn/cm^2. Data are mean tethers per 4 × field per min ± SD, $n \approx 3$ to 7. *B*, Control or treated cells were infused at low flow rate (≤a wall shear stress of 0.35 dyn/cm^2) and allowed to tether to the bilayer. The medium was switched to a cellfree solution identical to the medium in which the cells were treated. The flow rate was increased incrementally every 10 s and the percentage of cells remaining at the end of 10 s at each flow rate was determined (±SEM, $n = 3$–5).

and while rolling and tethering at low wall shear stresses, as shown by light microscopy. However, at wall shear stresses ≥14 dyn/cm^2, cytochalasin B-treated but not control cells visibly deformed into a teardrop shape, with the point of the drop at the upstream end of the cell. By determining the ratio of the cell diameters parallel and perpendicular to the direction of flow, we obtained an index of cell deformation. At wall shear stresses above 2 dyn/cm^2, cytochalasin B-treated cells increasingly deformed, whereas control cells and hypotonically swollen cells tended to maintain their shape (Fig. 4). Deformation is related to cortical and cytoplasmic elasticity, and since the cytoplasm must flow to maintain a teardrop shape for a rolling cell, to cytoplasmic viscosity (44, 45). The increased deformation of cytochalasin B-treated cells in shear flow may support increased shear resistance by allowing an increased contact area between the cell and the selectin-containing bilayer. These data on the effects of cytochalasin B suggest that leukocyte microvilli are required for efficient tethering to endothelial selectins, but are not required for subsequent rolling.

Tethering of hypotonically swollen neutrophils

To further test the hypothesis that microvilli are important for tethering of cells in flow, but are not required for rolling, neutrophils were swollen in hypotonic media to stretch the plasma membrane and smooth the surface. Leukocytes suspended in hypotonic media swell as predicted by osmotic theory; however, following this increase in cell size, leukocytes normally undergo regulatory volume decrease (RVD) and return to normal size (46). The major mechanism of RVD is K^+ channel opening allowing efflux of K^+ accompanied by the passive efflux of freely diffusible Cl^- (46). By using an extracellular medium containing a product of K^+ and Cl^- concentrations equal to the product of intracellular K^+ and Cl^- concentrations, RVD is predicted to be inhibited (46, 47). We tested whether this would minimize RVD over the time course of our experiments (5–10 min). Neutrophils visibly swelled from a diameter of 8.1 μm in 295 mOsm (iso-osmotic) medium to 9.0 μm in 105 mOsm medium and to 10.0 μm in 75 mOsm medium. These sizes were stable longer than the 10-min period used in experiments (Fig. 5). Flow cytometry showed that hypotonic swelling did not affect the level of L-selectin or CD18 expression and that there was only a slight increase in CD63 expression, indicating partial primary granule fusion (data not shown).

Hypotonically swollen neutrophils were fixed in hypotonic medium, immunogold labeled for L-selectin, and analyzed by TEM (Fig. 6*A*). The average number of microvilli per section was reduced by 80%, from 30 ± 6 to 6 ± 5 ($p < 0.005$) and, unlike the cytochalasin B-treated PMN, the mean length of the persistent microvilli on swollen cells was reduced 33% from 270 ± 200 nm to 180 ± 90 nm ($p < 0.005$, Table I). The width of microvilli near

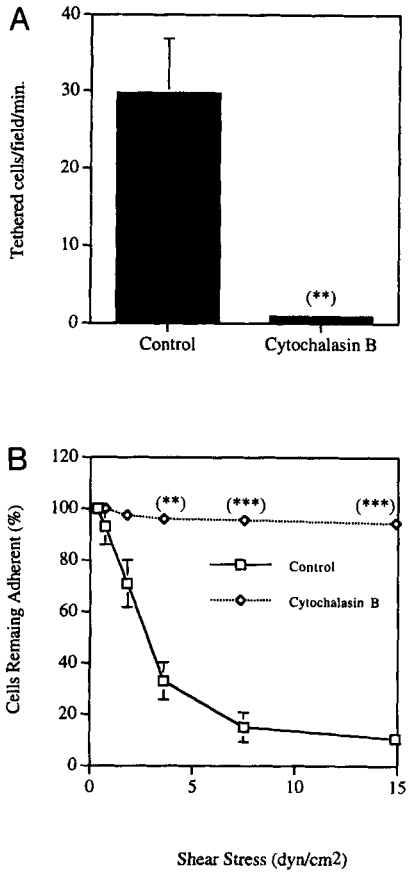

FIGURE 3. Tethering and resistance to detachment of cytochalasin B-treated neutrophils on P-selectin. Control neutrophils or neutrophils treated with 20 μM of cytochalasin B for 10 min at 22°C were tested for tethering and resistance to detachment on phospholipid bilayers with P-selectin (90 sites/μm^2) as described in the Figure 2 legend. *A*, Tethering in flow at 3.6 dyn/cm^2 (tethers per min per 4 × field ± SD, n = 3). *B*, Resistance to detachment. Cells were tethered at a wall shear stress of ≤0.35 dyn/cm^2, the flow rate was increased every 10 s, and cells remaining bound were determined (mean ± SEM, n = 4–6).

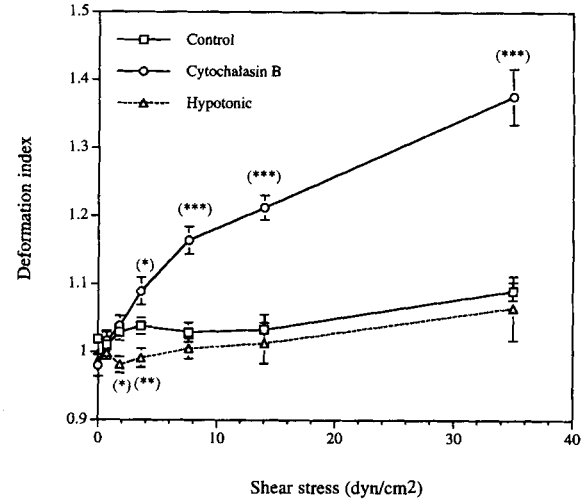

FIGURE 4. Deformation of rolling PMN in shear flow. Control neutrophils or neutrophils treated with 20 μM of cytochalasin B for 10 min at 22°C, or swollen in 75 mOsm media were allowed to settle onto E-selectin adsorbed to plastic. The cells were then subjected to increasing shear stress. The ratio of diameters parallel and perpendicular to the direction of flow for each of 10 to 50 cells in each treatment group at each of the indicated shear stresses was determined by computer-assisted image analysis. The deformation index is the diameter parallel to flow divided by the diameter perpendicular to flow. Data are mean ± SEM.

the tip was 160 ± 26 nm, and the sum of the tip widths was 1.0 ± 0.8 μm per section, a reduction of 73% compared with control neutrophils ($p < 0.005$). Despite the more pronounced effect on microvillus frequency and length compared with cytochalasin B treatment, the flat regions of the plasma membrane of the swollen cells were noticeably less smooth compared with the cytochalasin-treated cells (compare Figs. 1*C* and 6*A*). Similar to cytochalasin B-treated PMN, L-selectin on swollen cells was present on flat regions of the plasma membrane and remained associated in clusters (Fig. 6*B*). Swelling increased the average section diameter and area (Table I). Other morphologic alterations induced by hypotonic swelling were dispersion of the nuclear heterochromatin (Fig. 6*A*), dilation of the Golgi cisternae (data not shown), and generation of cytoplasmic vacuoles. Some cells also appeared hypogranular.

Hypotonically swollen PMN were assessed for tethering, rolling velocity, and resistance to detachment on bilayers containing E-selectin. Tethering at 1.8 dyn/cm^2 was inhibited by 81 and 94% by hypotonic swelling in 105 and 75 mOsm medium, respectively ($p < 0.05$, Fig. 7*A*). There was no significant difference in resistance to detachment in shear between cells assayed in 295 mOsm, 105 mOsm, and 75 mOsm media (Fig. 7*B*). Thus, control and swollen cells were functionally different in their tethering efficiency and not in strength of rolling adhesions.

Similar experiments were conducted to examine interaction of hypotonically swollen cells with P-selectin in shear flow. Hypoosmotic swelling completely abolished tethering to P-selectin at 3.6 dyn/cm^2 ($p < 0.005$, Fig. 8*A*). Swollen rolling cells on P-selectin were more resistant to detachment than control cells (Fig. 8*B*).

Examination of rolling velocities confirmed differential effects of hypoosmotic swelling on the behavior of rolling adhesions on E-selectin and P-selectin. As shear stress was increased, PMN rolling velocity on both P-selectin and E-selectin increased (Fig. 9). The rolling velocity of PMN on E-selectin was little affected by swelling (Fig. 9*A*), correlating with little effect on resistance to detachment (Fig. 7*B*). By contrast, hypotonic swelling resulted in a marked decrease in rolling velocity on P-selectin (Fig. 9*B*), correlating with the substantially greater resistance to detachment of swollen cells (Fig. 9*B*).

The parallel discrepancy between tethering ability and subsequent rolling of cytochalasin B-treated and hypotonically swollen neutrophils provides confirmatory evidence that microvilli are required for tethering but not rolling.

Tethering and rolling of control, cytochalasin B-treated, and hypotonically swollen neutrophils on HUVEC monolayers

To test these observations in a more physiologic system, HUVEC monolayers were stimulated with TNF-α to up-regulate E-selectin (48). Tethering and shear resistance were measured following cytochalasin B treatment and hypotonic cell swelling. Cytochalasin B and hypotonic swelling decreased tethering at 2.7 dyn/cm^2 by 85 and 95%, respectively ($p < 0.005$ for both, Fig. 10*A*). Shear resistance was not affected by these treatments (Fig. 10*B*). Similar findings were found for neutrophil tethering and shear resistance on P-selectin expressing platelet layers (not shown). These data confirm our initial observations on purified molecules.

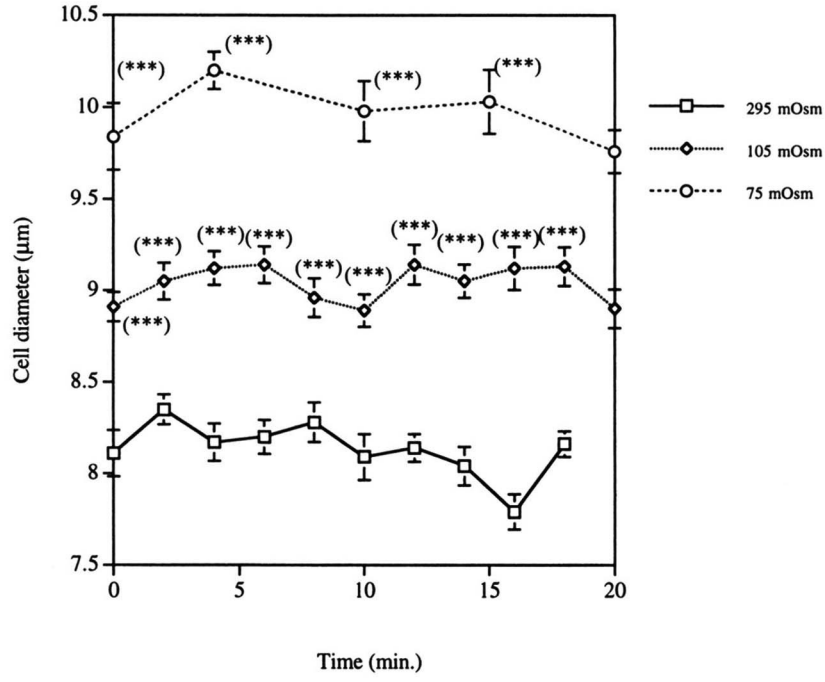

FIGURE 5. Sizes of hypotonically swollen neutrophils. PMN were resuspended in 295 mOsm, 105 mOsm, or 75 mOsm medium and every 2 to 5 min were allowed to settle onto plastic petri dishes as described in Figure 4. Images were captured directly for computer-assisted measurement of cell diameters. Data are mean diameter (± SEM) of 20 to 50 cells.

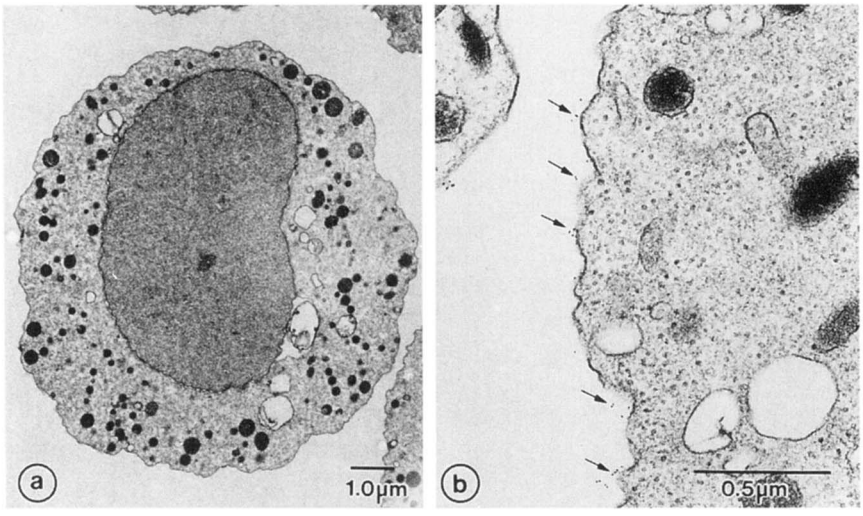

FIGURE 6. Electron microscopy and immunolocalization of L-selectin on hypotonically swollen neutrophils. Neutrophils swollen in hypotonic medium (75 mOsm) for 5 min at 22°C were fixed in the same hypotonic medium with 0.5% glutaraldehyde (*A*) or in 2% paraformaldehyde, 0.05% glutaraldehyde in phosphate buffer (*B*). *A*, Note the marked diminution of microvilli and the change in nuclear heterochromatin (compare to Figs. 1, *A* and *C*). *B*, Gold particles are found in groups and appear randomly distributed along the plasma membrane, which presents no well-formed microvilli. Magnification: *A*, ×9,000; *B*, ×39,000.

Tethering and rolling behavior of neutrophils exposed to cold

Cold has previously been shown by immunofluorescence to reduce the number and lengths of microvilli on lymphocytes (49). To further confirm the role of leukocyte microvilli in tethering to endothelial selectins, we tested the effect of chilling on cell adhesion. Treatment of neutrophils at 4°C for 60 min before fixation resulted in dramatic changes in gross morphology and surface microstructure. Electron micrographs revealed changes in the overall shape of the cells from round to ovoid and irregular, and a 90% reduction in the number of microvilli per section (Table I). There was a concomitant generation of pseudopod-like extensions of the cytoplasm (Fig. 11*A*) and blebbing of the plasma membrane (Fig. 11*B*). These morphologic features were absent in control cells. Cold-exposed PMN presented only 3 ± 3 microvilli per section compared with 30 ± 6 microvilli on control cells ($p < 0.005$), and the average length of microvilli on these cells increased from 270 ± 200 nm to 620 ± 430 nm ($p < 0.005$, Table I). The tip width of microvilli on cold-exposed PMN was increased to 230 ± 94 nm compared with 120 ± 71 nm on control cells ($p < 0.005$). The sum of the microvillus tip widths on cold-exposed PMN was 0.6 ± 0.6 μm, a reduction of 83% compared with control cells ($p < 0.005$). Pseudopod-like structures on cold-treated neutrophils were defined as broad irregular projections on the cytoplasm containing little or no organelles (Fig. 11*A*). Some of these cytoplasmic extensions were smooth along their leading edge while others were serrated, and many were gold labeled. The average cold-exposed PMN section presented 2.1 ± 1.6 pseudopod-like structures averaging 890 ± 560 nm in length. These structures were highly irregular in shape and ranged from 100 to 2870 nm in width near the tip, with an average of 860 ± 600 nm. The sum of the tip widths of the pseudopod-like structures averaged 2.3 ± 1.8 μm per section. Ring-like structures of the plasma membrane (Fig. 11*B*, inset) were seen both projecting from the cell surface and disconnected from the main cell body and were gold labeled. There were 0.6 ± 0.7 ring-like structures on or associated with each section. The length of plasma membrane per section including these rings was 29 ± 11 μm, considerably less than the length of control cell

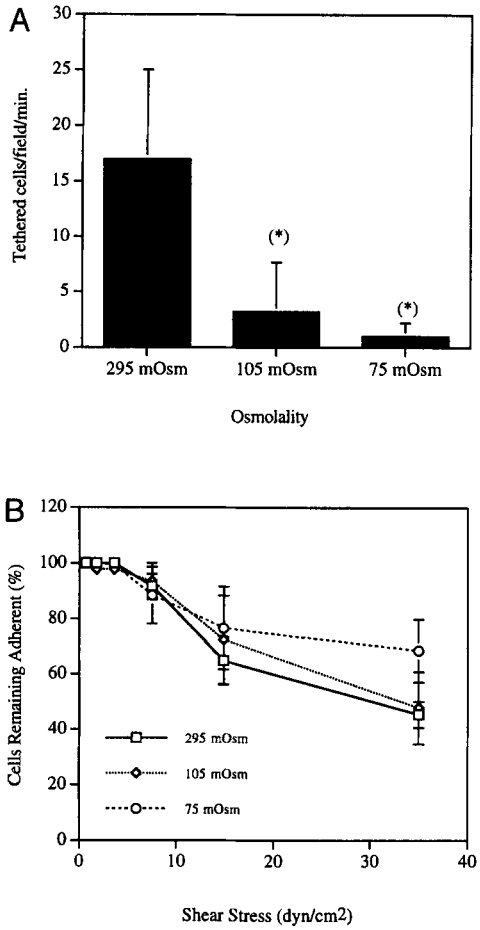

FIGURE 7. Tethering and resistance to detachment of hypotonically swollen neutrophils on E-selectin. *A*, Tethering in flow at a wall shear stress of 1.8 dyn/cm^2. *B*, Resistance to detachment. Neutrophils were incubated in balanced potassium medium of the indicated osmolarity for 1 to 5 min before measuring tethering or resistance to detachment in the same medium on phospholipid bilayers containing 400 sites of E-selectin/μm^2 as described in the Figure 2 legend. Data are mean ± SD, n = 3 to 4 (*A*) or mean ± SEM, n = 3 (*B*).

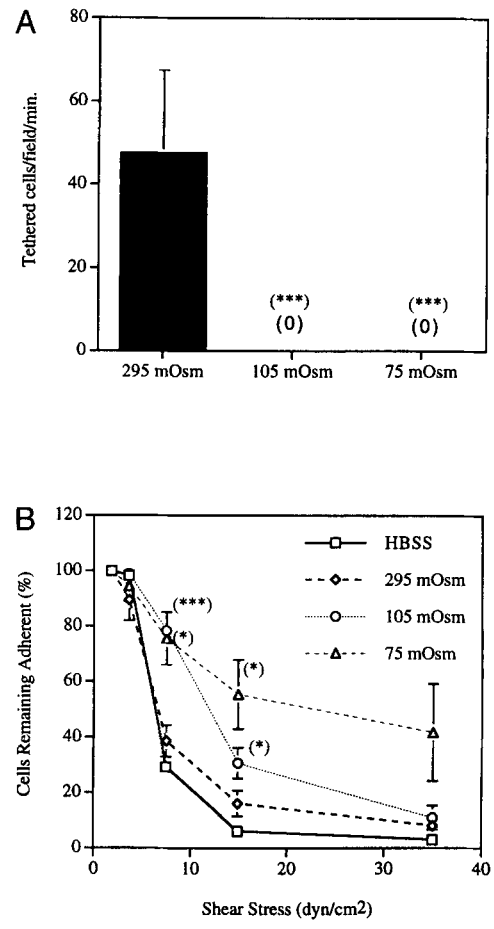

FIGURE 8. Tethering and resistance to detachment of hypotonically swollen neutrophils on P-selectin. *A*, Tethering in flow at a wall shear stress of 3.6 dyn/cm^2. *B*, Resistance to detachment. Neutrophils were incubated in balanced potassium medium of the indicated osmolarity for 1 to 5 min before measuring tethering or resistance to detachment in the same medium on phospholipid bilayers containing 90 sites of P-selectin/μm^2 as described in the Figure 2 legend. Data are mean ± SD, n = 3–5 (*A*) or mean ± SEM, n = 4–6 (*B*).

plasma membrane. This may reflect plasma membrane blebbing and the loss of the ring structures in the supernatant during washing and labeling procedures.

We hypothesized that chilled neutrophils that lack microvilli would behave similarly to cells treated in other manners to reduce microvilli. Neutrophils exposed to cold for 1 h were examined for tethering to E-selectin and P-selectin in an ice bath and compared with control cells held at 22°C and tethered at 22°C (Fig. 12). Despite greatly reduced numbers of microvilli, cold-treated PMN were unaffected in tethering efficiency on E-selectin (Fig. 12*A*) and P-selectin (Fig. 12*B*). As a positive control, treatment with cytochalasin B greatly reduced tethering on the same substrates. Cold-exposed neutrophils had decreased shear resistance and increased rolling velocity (data not shown), but these measures are likely modified by changes in cell membrane stiffness and intrinsic kinetic parameters. Tethering at reduced temperature provides contradictory results on the absolute necessity of leukocyte microvilli for efficient tethering and suggests that the function of these structures is more complex than solely increasing the accessibility of selectin ligands.

Discussion

The neutrophil surface has significant fine-scale structure that cannot be seen by ordinary light microscopy. These protrusions and the cytoskeletal elements that form them have been implicated in such cellular processes as shape change, diapedesis, chemotaxis, secretion, and phagocytosis (26, 50, 51). However, the specific functions of such microvillous structures on circulating leukocytes have not yet been fully described.

The role of microvilli in leukocyte adhesion is only recently being appreciated. Three different classes of adhesion molecules that mediate rolling (L-selectin (21, 27–29), PSGL-1 (12), and the integrin $\alpha 4\beta 7$ (52)) are localized to microvilli. The common microvillous localization suggests that this presentation should play an important role in tethering and rolling interactions. The tips of these microvilli are in a highly accessible position such that they are the first portion of plasma membrane to interact with the endothelium as a neutrophil comes into close contact with the vessel wall before tethering and rolling.

Of the molecules involved in rolling, the functional importance of expression on microvilli has thus far only been investigated for

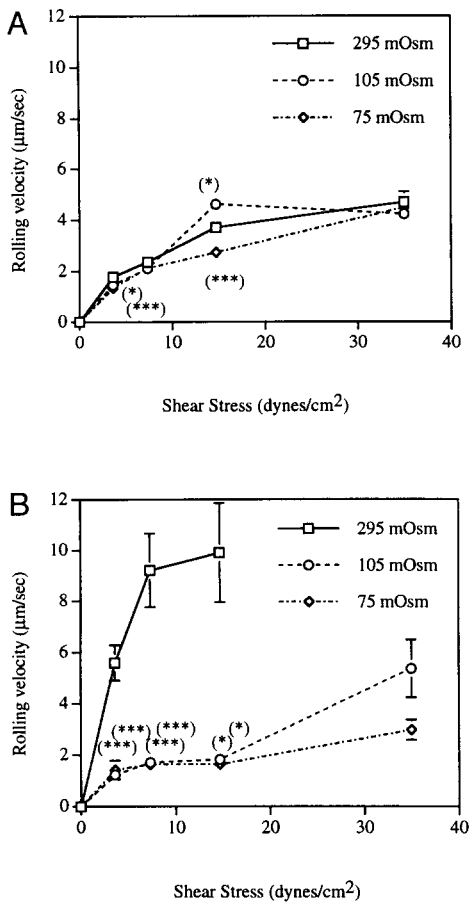

FIGURE 9. Rolling velocities of swollen PMN on E-selectin and P-selectin. *A*, Rolling velocities on E-selectin at 400 sites/μm^2. *B*, Rolling velocities on P-selectin at 90 sites/μm^2. Velocity was calculated from the distance traveled for 5 to 10 s of rolling for 15 to 50 cells at each flow rate. Data are mean ± SEM.

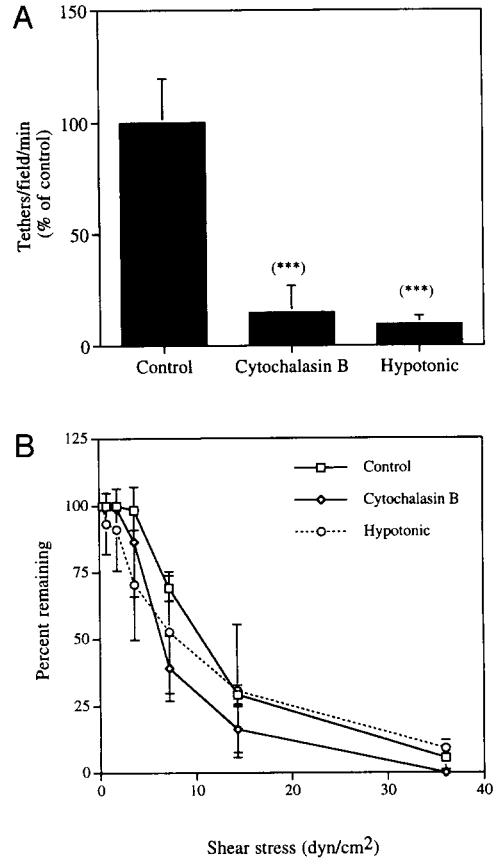

FIGURE 10. Tethering and shear resistance of cytochalasin B-treated and hypotonically swollen neutrophils on HUVEC. Neutrophil tethering at 2.7 dyn/cm^2 (*A*) and shear resistance (*B*) on TNF-α stimulated HUVEC monolayers were assayed as in Figures 2 and 7. Data are mean ± SD; n = 3–8 (*A*), 3–4 (*B*).

L-selectin. CD44 is excluded from microvilli and domain substitution with the cytoplasmic domain of CD44 has been used to exclude chimeric molecules with the extracellular domain of L-selectin from microvilli (31). In this state, L-selectin maintains ligand binding activity, but in shear flow has a decreased attachment rate and rolling flux on its ligand PNAd. Cytochalasin B treatment and L-selectin cytoplasmic domain deletion result in reduced rolling flux in vivo and decreased binding to HEV in vitro (30). Cytoplasmic domain deletions result in a lack of binding of L-selectin to the cytoskeletal protein α-actinin, but microvillous localization is maintained (53). These studies suggest that while localization of L-selectin to microvilli seems to be important for function in hydrodynamic flow, microvillous presentation is not sufficient to confer cell binding, even when ligand recognition is intact. These studies do not address the role of microvilli in the function of the endothelial selectins, and also fail to discriminate between leukocyte tethering and subsequent rolling, which are two separable components of these initial adhesive events. Although enhancement of rolling by receptor concentration on microvilli has been assumed in previous studies to confer greater accessibility for presentation to ligands on vessel walls, other factors may also be important, including greater resistance to extraction from the membrane by the hydrodynamic drag forces that are exerted on the receptor and ligand. These forces are greater than those required to extract hydrophobic anchors from the membrane (54). In the current investigation, we have focused on the role of cell surface topology in neutrophil adhesion to E- and P-selectin. We have quantitatively characterized how cytochalasin B, hypoosmotic swelling, and chilling affect cell morphology, and how these treatments affect tethering and rolling behavior.

Although leukocyte tethering is prerequisite for rolling, we were able to experimentally distinguish between these two measures of selectin-dependent adhesive functions that are important for the initiation and maintenance of rolling in vivo. We found that cytochalasin B markedly decreased neutrophil tethering to both P-selectin and E-selectin, but actually increased the shear resistance of rolling cells. This underscores the distinction between tethering and rolling and demonstrates their independent regulation by cellular characteristics. Cytochalasin B only partially decreased the number of microvilli and sum of tip width per section, by 47% and 18%, respectively. This incompleteness is in agreement with the greater susceptibility of cytoplasmic than cortical actin filaments to disruption by cytochalasin B (S. Tsukita, personal communication). Hypotonic swelling of neutrophils resulted in substantially greater obliteration of microvilli and decrease in the sum of microvillous tip widths per section than cytochalasin B, and also dramatically decreased tethering on P-selectin and E-selectin. Swelling stabilized rolling on E-selectin slightly and markedly stabilized rolling on P-selectin. These results were confirmed by rolling velocity measurements; greater shear resistance correlated well with slower rolling.

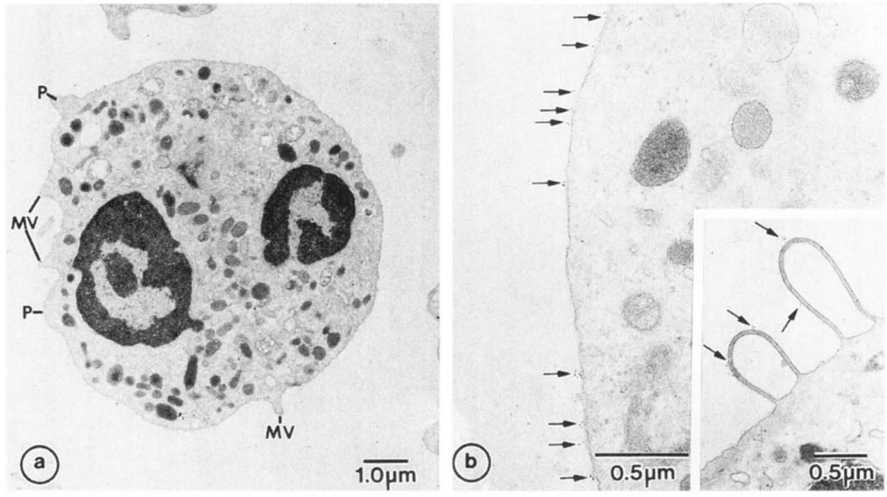

FIGURE 11. Electron microscopy and immunolocalization of L-selectin on cold-exposed neutrophils. *A*, TEM of immunogold-labeled neutrophil incubated at 4°C for 60 min before fixation. *A*, Whole cell section. Note the change in surface microstructure relative to the control section shown in Figure 1*A*. The section shown here has only three microvilli (MV) and presents three pseudopod-like structures (P), which are absent in control cells. Note that despite these changes, the composition and appearance of the cytoplasm with respect to granules and nuclear morphology (condensed peripheral heterochromatin) is not changed relative to control cells (compare to Fig. 1*A*). *B*, High magnification view of the plasma membrane. Note the random distribution of grouped gold particles (arrows), and the smoothness of the plasma membrane. *Inset*, Rings of plasma membrane blebbing from the cell surface. Note the presence of grouped gold particles (arrows). Neutrophils were incubated at 4°C in HBSS, 0.25% HSA, 1 mM EDTA, 10 mM HEPES, pH 7.4, for 60 min before fixation in 2% paraformaldehyde, 0.05% glutaraldehyde, 0.1 M sodium phosphate, pH 7.4, and then immunogold labeled for L-selectin. Magnification: *A*, ×8,900; *B*, ×41,000; *Inset*, ×21,000.

Our finding that cytochalasin B-treated cells retain substantial microvilli yet fail to tether suggests that microvilli are not sufficient for tethering. These findings with E-selectin and P-selectin correlate with the finding that L-selectin cytoplasmic deletion mutants remain on microvilli yet are ineffective in cell adhesion assays (30). Thus, presentation on microvilli, while possibly important for enhancing the probability of contact with ligand in shear flow (31), may be an incomplete explanation for the finding that receptors that mediate rolling are concentrated on tips of microvilli. Microvilli are points of attachment of actin bundles to the plasma membrane, and concentration of receptors on microvilli may reflect associations with the cytoskeleton that enable these receptors to resist extraction from the membrane by the substantial force exerted on the receptor and ligand by the hydrodynamic drag force acting on the tethered cell in shear flow. Partial disruption of the actin cytoskeleton by cytochalasin B, as appreciated here by the substantial increase in cell deformability in shear flow, may render receptors more susceptible to extraction.

Cytochalasin B and hypoosmotic swelling dramatically decreased tethering but enhanced the stability of some rolling adhesions. Not only do our results show that these functions are regulated independently, they also show that changes in characteristics that include cell shape, elasticity, and viscosity have opposing effects on tethering and the stability of rolling. Both tethering and the maintenance of rolling adhesions appear to be physiologically important for leukocyte accumulation at inflammatory sites in vivo. There appears to be a trade-off between optimum tethering and rolling. The fine structure and shape of the neutrophil cell surface, which is largely affected by actin polymerization (26), cortical and cytoplasmic elasticity, and viscosity of neutrophils, may be evolutionarily adjusted to balance the need for both efficient tethering and rolling. Several factors may contribute to the greater shear resistance of cytochalasin B-treated neutrophils. The microvilli are somewhat fewer in number and would be expected to bend more readily when positioned between the neutrophil and the substrate, allowing greater contact between the body of the cell and the substrate. The greater elasticity of the cell is predicted to make a substantial contribution to increased shear resistance by enabling a greater area of contact with the selectin-bearing surface. This is because cortical tension that keeps a cell rounded and lowers its elasticity resists the formation of a large area of cell-substrate contact (45).

Biomechanical factors are predicted to make small contributions to the decrease in tethering efficiency of hypoosmotically swollen cytochalasin B and cold-treated cells. The greater surface area of swollen cells increases the force on the cell by the square of the ratio of cell diameters (55), or approximately $10.0^2/8.1^2 = 1.52$-fold higher than the value for control cells. Another factor is the lever arm, the distance between the tether point and the projection of the center of the neutrophil on the substrate. The lever arm reflects the local geometry and determines the ratio between the hydrodynamic force on the cell and the force on the selectin:ligand bond at the tether point. Longer lever arms moderate the force on the bond. Disruption of microvilli is predicted to decrease the lever arm distance.[4] In preliminary experiments this value was reduced from 3.65 μm to 2.89 μm and 2.71 μm for control, cytochalasin B-treated, and chilled neutrophils, respectively, and thereby increased the force on the bond only 1.16- to 1.22-fold (E. Finger, S. Chen, and T. Springer, unpublished observations). The lever-arm for hypotonically swollen neutrophils was not amenable to measurement by these same assays, but would be predicted to be shorter than for control cells. Shortening of the effective lever arm and increasing the drag force together combine to create a greater disruptive force on the selectin bond. However, it should be noted that swelling did not decrease the strength of rolling adhesions, despite the increased force on the cells.

Cell swelling illustrated some differences between E-selectin and P-selectin. Cell swelling resulted in increased shear resistance and decreased rolling velocity on P-selectin with little change on

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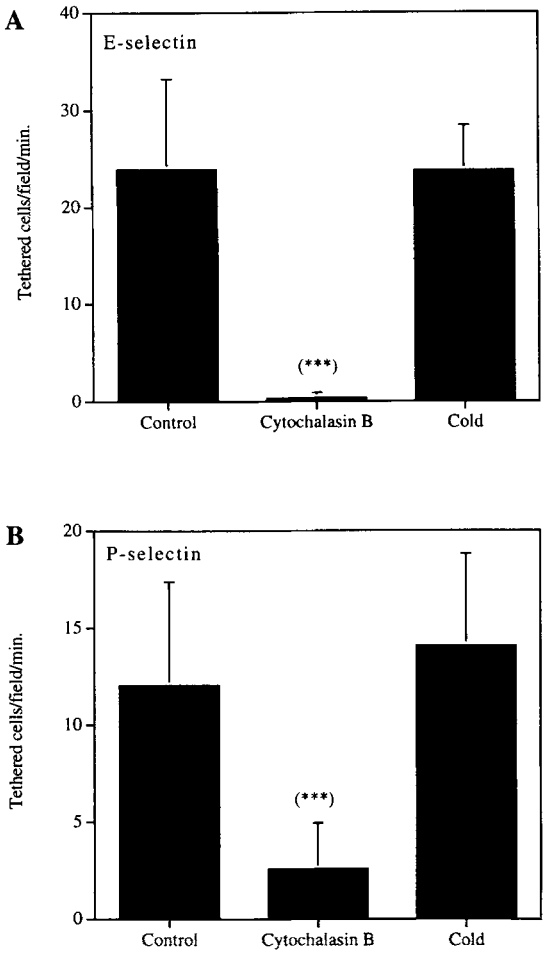

FIGURE 12. Tethering of cold-exposed neutrophils to E-selectin and P-selectin. *A*, Tethering to 200 sites of E-selectin/μm^2 at 3.6 dyn/cm^2. *B*, Tethering to 90 sites of P-selectin/μm^2 at 2.7 dyn/cm^2. For cold treatment, neutrophils were stored on ice for ≥1 h. The parallel plate flow chamber was placed in a petri dish ice bath on the microscope and temperature equilibrated by perfusion with ice-cold PBS at a high flow rate before initiation of the experiment by perfusion with cold-treated neutrophils. Control measurements at 22°C on cells held at 22°C were carried out in the same flow chamber before cold treatment. Cells treated with cytochalasin B were also examined in the same chamber. All measurements were on the same field of view. Data are mean ± SD, $n = 4-6$.

E-selectin. PMN interaction with P-selectin requires glycoprotein ligands (18, 38), of which only one high affinity P-selectin ligand has been identified on neutrophils (11). Several glycoproteins and glycolipids have been shown to support E-selectin binding (18, 20, 21, 56). It is likely that the whole of the cell surface displays potential ligands for E-selectin. While the microvilli-associated molecules may direct tethering on E-selectin, it is possible that the other ligands are more evenly distributed, and support rolling. Redistribution of E-selectin ligands to the body of the cell may not enhance rolling since ligands are already present on the cell body. On the other hand, PSGL-1 is present on microvilli, and after the cell membrane is swollen, will be present on the body of the cell where more area is available for contact with the surface. This may allow more P-selectin:PSGL-1 bonds to form, or it may shorten the distance between successive bonds. Selectin ligands such as PSGL-1 that are on microvilli may have an increased probability of initiating an adhesive interaction with the substrate. On the other hand, excluding such molecules from the body of the cell may lower their ability to support rolling.

The lack of effect of chilling on neutrophil tethering to E- and P-selectin despite an almost complete loss of microvilli is provocative, although conclusions must be tempered by our findings that pseudopod-like structures were present that presented an equivalent sum of tip widths per section. Pseudopod-like structures, however, were much fewer in number and wider than microvilli, and our findings argue that microvilli per se are not required for tethering. In our studies, exposure of PMN to cold temperatures resulted in marked decrease in the number of microvilli as assayed by TEM. This is supported by early results using fluorescence microscopy to quantify microvilli (49). Other groups have shown persistence of microvilli in neutrophils and other leukocytes that had been stored in cold temperatures (27, 28, 52); however, these studies were performed with scanning electron microscopy in which the cells are dehydrated before observation. Although dehydration decreases cell volume, it does not affect surface area. Actin bundles that are attached to the membrane at microvilli appear to provide a skeleton over which the membrane is draped during dehydration, giving rise to microvillous processes that are much longer than those observed here by TEM.

Although we did not observe microvilli after chilling, actin bundles may nonetheless have remained associated with the membrane at points where selectin ligands or L-selectin were concentrated, and this may have been an important contributor to the maintenance of tethering. Increased bond lifetime or decreased effect of force on bond lifetime (57) at lower temperature might increase tethering efficiency, and we cannot rule out the possibility that this could at least partially compensate for a loss in microvilli.

Our results with chilled cells, cytochalasin B-treated cells, and swollen cells point out the complex relationship between expression of adhesion receptors on microvilli, the efficiency with which they support tethering of a cell in shear flow, and the strength with which they support rolling of a cell in shear flow. We have shown that tethering and rolling are not only operationally separable experimentally, but are also opposingly regulatable by cellular characteristics such as shape and viscoelasticity.

Leukocyte microvilli are not required for efficient rolling interactions, but disruption of microvilli with two of three cell morphology-altering treatments resulted in near complete inhibition of tethering under shear flow in the physiologic range. The effects of these three cell treatments on neutrophil tethering may be ascribed to a combination of changes in accessibility of leukocyte receptors, alteration in the biomechanical translation of drag force to bond force, alteration in resistance of receptors to extraction from the membrane and cytoskeleton, and alteration in cell viscoelasticity. There appears to be a trade-off between optimization of tethering efficiency and the strength of rolling adhesions, and the biomechanical properties of leukocytes may be evolutionarily adapted to achieve a balance that permits both. Further study will be required to achieve a detailed understanding of these properties.

Note added in proof: Subsequent TEM experiments using cryosectioned as well as epon embedded samples have shown that PMN lose microvilli during chilling and retain these structures at 22°C regardless of whether the fixation medium is at room temperature or cold. These findings confirm that loss of microvilli at low temperature is not a fixation artifact.

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