A Central Role
for Microvillous Receptor Presentation
in Leukocyte Adhesion under Flow

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Summary

Leukocyte adhesion to endothelium requires specialized mechanisms for contact initiation under flow. L-selectin (CD62L), an efficient initiator of adhesion, is clustered on the tips of leukocyte microvilli. To test whether microvillous presentation is critical for contact formation ("tethering"), we transfected lymphoid cells with chimeras of L-selectin and CD44, an adhesion molecule that is excluded from microvilli. CD44 transmembrane and intracellular (TM–IC) domains targeted the L-selectin ectodomain to the planar body, whereas L-selectin TM–IC segments conferred CD44 ectodomain clustering on microvilli. Wild-type and chimeric transfectants bound similarly to anti-ectodomain MAbs in static assays, but MAb binding under flow was much more efficient in the context of microvillous presentation. Similarly, wild-type and chimeric L-selectin possessed equivalent lectin activity, but microvillous presentation dramatically enhanced contact initiation on a native ligand. These findings demonstrate a critical role for receptor topography in leukocyte adhesion and suggest a novel regulatory mechanism of leukocyte trafficking.

Introduction

A prominent feature of many highly differentiated cell types such as nerve cells or epithelial cells is the ability to compartmentalize surface membrane domains that direct nutrient transport, adhesion, and communication with the environment. This is recognizable on a light microscopic level by the cellular polarity or asymmetry brought about by dynamic interactions of cytoskeletal elements with domain-specific membrane-associated molecules. In contrast, blood-borne leukocytes appear generally spherical without obvious polarization or asymmetry. A transient shape change and polarization become apparent only after a leukocyte has become activated and adherent to the vascular lining. Thus, the surface membrane of resting leukocytes has been commonly regarded as a single functional unit covering the entire cell circumference and expressing a variety of adhesion molecules to allow binding to vascular ligands. However, on an electron microscopic level, leukocytes possess a complex surface architecture with prominent microvillus-like membrane protrusions (Beesley et al., 1979; van Ewijk, 1980), and at least some adhesion molecules are not displayed at random over the entire cell surface but are selectively targeted to or away from microvilli (Picker et al., 1991; Erlandsen et al., 1993; Berlin et al., 1995). We set out to explore whether leukocytes, like other highly specialized cell types, organize their surface in distinct functional domains (microvilli versus planar cell body) to fine-tune adhesive interactions with endothelial cells (ECs).

Leukocyte adhesion to microvascular ECs is central for both physiologic and pathologic situations involving a cellular immune response. It is viewed as a multistep process of sequential engagement of distinct receptor molecules on the leukocyte and EC surface (Butcher, 1991; Shimizu et al., 1992; Springer, 1994). This molecular cascade is reflected in vivo by initial contact and rolling of leukocytes in venules. Subsequently, rolling leukocytes encounter activating stimuli that trigger activation-dependent adhesion receptors required for firm arrest. Each link in this chain of events is essential for neutrophil function in humans; defects in either adhesion step result in a leukocyte adhesion deficiency (LAD) syndrome that is clinically characterized by severe recurrent bacterial infections due to a profound inability of neutrophils to either roll (LAD-II) or stick (LAD-I) in venules of acutely inflamed tissues (von Andrian et al., 1993a).

Neutrophil rolling in vivo is mediated by the three members of the selectin (CD62) family, termed E-, P-, and L-selectin (reviewed by Lasky, 1992), which are homologous lectin-like glycoproteins that bind sialylated carbohydrates (CHO) such as sialyl-Lewis^x (sLe^x). L-selectin (CD62L) is constitutively expressed on most blood-borne leukocytes, except a subset of memory cells. It mediates lymphocyte homing to peripheral lymph nodes (PLNs) by binding of its lectin domain to a sulfated and sialylated CHO epitope that is associated with peripheral node addressin (PNAd) glycoproteins in high endothelial venules of PLNs (Berg et al., 1991). It also is required for neutrophil trafficking in acute inflammation in vivo (Lewinsohn et al., 1987) and is a key mediator of leukocyte rolling in mesenteric venules in situ (von Andrian et al., 1991; Ley et al., 1991). Rolling via L-selectin can be mediated by its lectin domain, and,

in addition, neutrophil and monocyte (but not lymphocyte) L-selectin can present sLe^x-like CHO ligands to vascular selectins (Picker et al., 1991; von Andrian et al., 1993b). Furthermore, L-selectin on circulating neutrophils can also mediate attachment and rolling on CHO ligands presented by surface-bound neutrophils (Bargatze et al., 1994). By employing one or more of these mechanisms, L-selectin acts as a major contributor to leukocyte tethering and rolling under flow. These findings have been confirmed recently in L-selectin-deficient mice, which display profound defects in lymphocyte homing to PLNs and in neutrophil accumulation in inflamed tissues (Arbonès et al., 1994).

Initiation of cell contact under flow may be facilitated by the conspicuous surface topography of L-selectin, which is clustered on microvilli of neutrophils (Picker et al., 1991; Erlandsen et al., 1993) and lymphocytes (Hasslen et al., 1995). Consequently, it has been proposed that microvillous presentation is critical for L-selectin-mediated initiation of cell–cell contact. In contrast, β2 integrins are preferentially excluded from microvilli (Erlandsen et al., 1993) and cannot initiate selectin-independent contact under physiologic flow (Arfors et al., 1987; Lawrence and Springer, 1991; von Andrian et al., 1991), suggesting that targeting to the planar cell body may prevent ill-timed receptor engagement on blood-borne leukocytes.

Our present studies to test this hypothesis were helped by recent scanning immunoelectron microscopy investigations of a panel of surface adhesion molecules in which we found that the hyaluronate receptor CD44 on peripheral blood leukocytes had a distribution entirely opposite to that of L-selectin. We show that, in striking similarity to β2 integrins, CD44 on nonactivated cells is not clustered and is preferentially restricted to planar membrane areas covering the cell body. This offered the opportunity to engineer hybrid proteins in which functional extracellular domains of L-selectin and CD44 could be targeted to the cell body and to microvilli, respectively. Thus, our experiments allowed us to test the consequences of distinct receptor presentation patterns for the functional regulation of leukocyte adhesion under flow. We demonstrate that the surface topography of L-selectin and CD44 is independent of their extracellular components and provide evidence that microvillous presentation of adhesion molecules does indeed permit a marked increase in cellular binding under flow.

Results

Production of Transfected L1-2 Cell Lines Expressing Wild-Type and Chimeric Receptors

Stably transfected murine L1-2 pre-B cell lines were produced expressing human wild-type CD44, expressing chimeric receptors consisting of the L-selectin ectodomain linked to the transmembrane and intracellular (TM–IC) segment of CD44 (L–CD44), or expressing extracellular CD44 fused to the L-selectin TM–IC domain (CD44–L). Subcloned wild-type L-selectin transfectants described previously (Berg et al., 1992) were also utilized, and mock transfectants were employed as a control. For immunolo-
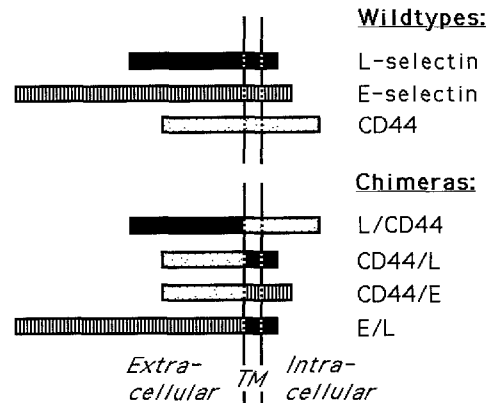

Figure 1. Schematic Representation of Transfected Wild-Type and Chimeric Receptors Used in These Studies

calization studies, L1-2 cells expressing human E-selectin (Picker et al., 1991) or chimeric receptors consisting of extracellular CD44 linked to the E-selectin TM–IC domain (CD44–E) and E-selectin joined to the L-selectin TM–IC domain (E–L) were used. A schematic representation of wild-type and chimeric molecules is shown in Figure 1.

Topographic Distribution of Transfected Wild-Type and Chimeric Receptors on L1-2 Cells

Low voltage scanning electron microscopy (LVSEM) of immunogold-labeled transfectants was carried out to determine the surface distribution of wild-type and chimeric receptors. Comparison to control cells revealed no apparent difference in the microvillous architecture of all transfectants tested in three duplicate experiments (Figure 2A). Microvilli required an intact actin-based cytoskeleton, as treatment with cytochalasin D (CD), but not dimethyl sulfoxide (DMSO), resulted in a nearly complete loss of surface projections on both wild-type and chimeric transfectants (Figure 2B).

Quantitative analysis of receptor topographies is shown in Table 1. Wild-type L-selectin and CD44–L were preferentially associated with microvilli and frequently clustered (Figure 2). In contrast, wild-type CD44 and L–CD44 were mainly found on the cell body. Clustering of wild-type CD44 was rare, while the occurrence of L–CD44 clusters was somewhat variable between cells, and the number of gold particles per cluster for the latter was smaller than on cells expressing L-selectin TM–IC domains. In contrast, L1-2 cells expressing wild-type E-selectin or CD44–E chimeras displayed receptors that were evenly distributed over the cell surface. It is unlikely that the selective expression on microvilli and clustering of antigen was due to monoclonal antibody (MAb)-induced cross-linking as the same primary and secondary antibodies were used on cells with identical ectodomains but different distribution patterns. Furthermore, although immunoreactivity tended to be lower on prefixed cells, wild-type L-selectin clustering on microvilli was identical on human neutrophils and transfected L1-2 cells when fixed either prior to or following MAb staining (Erlandsen et al., 1993; unpublished data). These findings
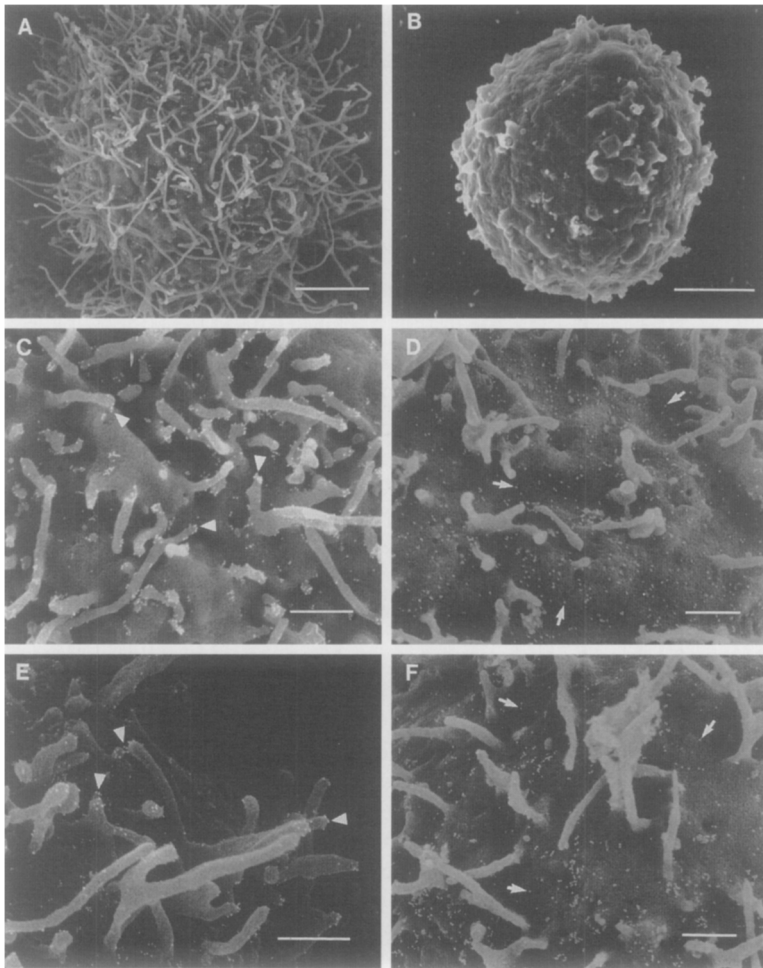
Figure 2. LVSEM Backscatter Electron Images of L1-2 Transfectants

Numerous microvilli are typically found on untreated cells (A). In contrast, CD-treated cells (B) are largely devoid of microvilli. At higher magnification, typical immunogold (the bright white dots) distribution is shown on cells expressing wild-type L-selectin (C), wild-type CD44 (D), CD44–L (E), and L–CD44 (F). Note clusters of colloidal gold on microvilli (arrowheads) and nonclustered localization on the cell body (arrows). Scale bars depict 2 μm (A and B) and 500 nm (C–F).

suggest that the TM–IC segments of L-selectin and CD44, rather than their ectodomains, control their targeting to distinct surface domains, as well as clustering.

Comparison of Native and Chimeric Receptors in Static Binding Assays

Having determined the effect of domain swapping on receptor topography, we next studied the impact of distri-

bution on cell binding to immobilized substrates. Transfectants were sorted, subcloned, and selected by flow cytometry for comparable expression levels. The staining intensity with fluorescein isothiocyanate (FITC)-conjugated MAb DREG-200 of L–CD44 transfectants was 123% ± 26% (mean ± SD) of that of wild-type L-selectin transfectants (n = 10 determinations) and that of CD44–L chimeras with anti-CD44 MAb Hermes-1 was 104% ± 17% of that

Table 1. LVSEM Analysis of Surface Antigen Topography on Transfected L1-2 Cells

Cell Line	MAb	Total Number Analyzed		Distribution (%)		Particles in Clusters (%)
		Cells	Gold Particles	Microvilli	Cell Body	
L-selectin	DREG-200	8	2052	91 ± 11	9 ± 11	73 ± 21
L–CD44	DREG-200	11	6222	5 ± 5	95 ± 5	28 ± 34
E-selectin	CL-2	3	1752	52 ± 8	48 ± 8	8 ± 6
E–L	CL-2	11	1750	83 ± 6	17 ± 6	49 ± 21
CD44	Hermes-1	8	2158	7 ± 4	93 ± 4	4 ± 3
CD44–E	Hermes-1	3	2057	47 ± 11	53 ± 11	12 ± 6
CD44–L	Hermes-1	9	824	86 ± 10	14 ± 10	71 ± 12

Colloidal gold distribution on immunolabeled cells was determined by blind analysis from electron micrographs (magnification 30,000 × –60,000 ×) using a stereoviewer. Clusters are defined as tightly packed groups of at least three particles. Data on distribution and clustering are presented as mean ± SD.
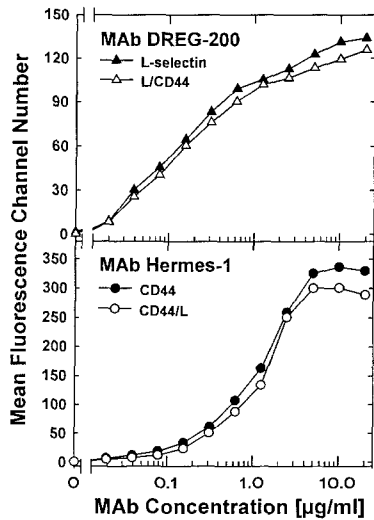
Figure 3. Equivalent Binding of Soluble MAbs to Wild-Type and Chimeric Receptors

FACS titration of MAb binding to wild-type L-selectin and L–CD44 transfectants (top) and wild-type CD44 and CD44–L transfectants (bottom). Data depict mean fluorescence channel numbers corrected for background staining of mock transfectants. MAb titration was performed in duplicates and repeated twice with similar results.

of wild-type CD44 transfectants (n = 4). Mock transfectants did not bind MAbs to human receptors and, as reported, were negative for murine L-selectin and CD44 (Berg et al., 1992). To ensure that epitope recognition by soluble MAbs (which are assumed to have equal access to the entire cell surface) was the same for wild-type and chimeric receptors, we assessed staining by serially diluted MAbs DREG-200 and Hermes-1 by flow cytometry. As shown in Figure 3, MAb titration revealed no difference in antigen binding on cells presenting wild-type or chimeric epitopes for either MAb.

Cell binding to immobilized MAbs was initially studied under static conditions. L1-2 cells were allowed to settle for 30 min on glass slides coated with bovine calf serum
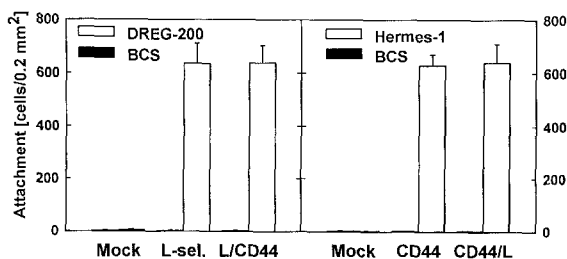

Figure 4. Static Binding of L1-2 Cells to Immobilized Antibodies

Static binding of L1-2 cells to anti-L-selectin MAb DREG-200 (left) and anti-CD44 MAb Hermes-1 (right). Cells were allowed to settle for 30 min on MAb- or BCS-coated slides. We counted six fields of view (0.24 mm² each) per well. Means ± SD of three duplicate experiments are shown. Similar results were obtained using MAbs DREG-56 and Hermes-3 to different epitopes on L-selectin and CD44, respectively (data not shown).

(BCS) or MAbs to L-selectin or CD44. Slides were washed and fixed, and bound transfectants were counted. There was no difference in binding between wild-type L-selectin and L–CD44 cells or between wild-type CD44 and CD44–L cells (Figure 4). Binding of transfectants to BCS and of control cells to MAb-coated slides was negligible. These results indicate that receptor clustering or microvillous association does not play a role in cell binding to MAbs under static conditions.

Differential Binding of Transfectants Expressing Microvillous versus Cell Body–Associated Receptors under Shear

To assess the importance of microvillous presentation under flow, we initially examined cell capturing by MAb-coated surfaces, an effect that is independent of the functional activity of transfected molecules. Captured cells were immediately arrested, did not roll, and detached rarely during 10 or 15 min of perfusion. In sharp contrast with results from static MAb binding assays, L1-2 cells presenting wild-type L-selectin on microvilli were captured much more effectively under flow than L–CD44 (cell body) transfectants (Figure 5A). This differential availability of microvillous receptors for MAb capturing increased as a function of shear. The average capturing rate of wild-type L-selectin transfectants was 1.8-fold higher than that of L–CD44 chimeras at 0.8 dynes/cm² and was 4.7 times higher at 1.6 dynes/cm². Corresponding results were obtained with capillaries coated with another anti-L-selectin MAb, DREG-56 (data not shown).

Similarly, microvillous CD44–L transfectants were captured 2.8 times more often by MAb Hermes-1 than cells presenting wild-type CD44 at 0.8 dynes/cm² (Figure 5B), and MAb Hermes-3 against a distinct epitope also captured CD44–L cells more frequently (data not shown). Cells did not bind to BCS-coated capillaries, and mock transfectants were not captured by MAb-coated capillaries.

Importance of Microvillous Presentation in L-Selectin-Dependent Contact Initiation, but Not Rolling, on a Native Ligand

We next studied the effect of topographic expression on L-selectin-mediated contact initiation and rolling on a physiologic ligand. To determine whether wild-type L-selectin and L–CD44 retained similar lectin activity, we first assessed their ability to bind FITC–polyphosphomannan ester (FITC–PPME), a specific L-selectin lectin ligand (Yednock et al., 1987). Wild-type transfectants (polyclonal cells and two subclones) and L–CD44 chimeras (polyclonal cells and three subclones), but not control cells, bound FITC–PPME in proportion to their staining with FITC–DREG-200 Fab (Figure 6). As reported previously, FITC–PPME binding was inhibitable by Ca²⁺ depletion (Yednock et al., 1987) and by anti-lectin MAb DREG-56 (Kishimoto et al., 1990). The relative ability of receptors to bind soluble CHO ligands was determined from the ratio of staining intensities with FITC–PPME:FITC–DREG-200 Fab. Using two batches of FITC–PPME with different degrees of FITC sub-
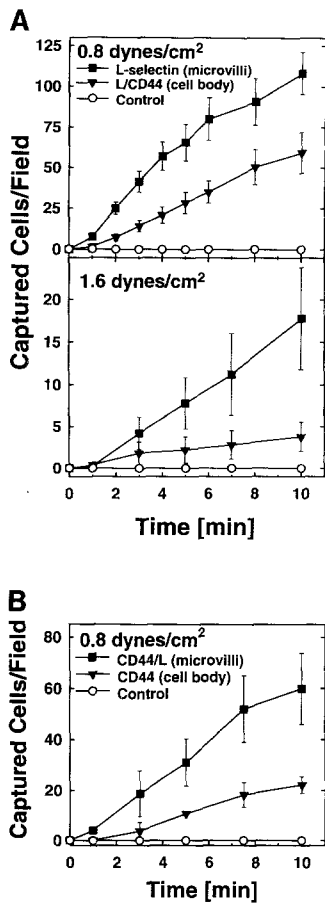
Figure 5. MAb-Mediated Capturing of Transfectants under Flow

(A) Accumulation of wild-type L-selectin and L–CD44 transfectants and control cells on immobilized MAb DREG-200 in glass capillaries; 1 × 10^6 cells/ml were perfused at 0.8 dynes/cm^2 (top) or 1.6 dynes/cm^2 (bottom). Mean ± SD of five experiments is shown.

(B) Cell capturing in anti-CD44 MAb Hermes-1 coated capillaries (shear stress, 0.8 dynes/cm^2). Capturing of mock transfectants was not detected. Mean ± SD of four experiments.
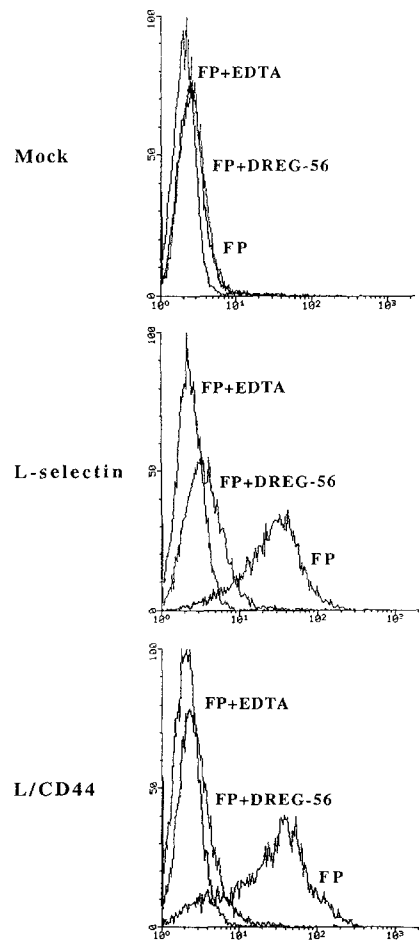
Figure 6. FACS Analysis of Wild-Type and Chimeric L-Selectin Lectin Activity

FACS histograms of mock transfectants (top) and wild-type L-selectin (middle) or L–CD44 (bottom) transfectants. Cells were incubated with FITC–PPME (FP) with or without MAb DREG-56 (20 μg/ml) or 25 mM EDTA.

stitution, this ratio averaged 0.173 (batch 1) and 0.775 (batch 2) for wild-type L-selectin and 0.177 and 0.978 for L–CD44 transfectants. Thus, soluble ligand binding by L–CD44 was as high or even slightly higher than that of wild-type receptors.

In contrast, in assays requiring lectin binding under flow to immobilized PNAd, a native L-selectin ligand, microvillous L-selectin conferred a distinct advantage over cell body chimeras (Figure 7; Table 2). As with MAb capturing, this advantage increased with shear: initial attachment of wild-type transfectants at 1.94 dynes/cm^2 was 1.9-fold higher than that of cells presenting cell body L–CD44, and this difference increased to 4.1-fold at 3.06 dynes/cm^2.

Once association with immobilized PNAd was established, the behavior of interacting cells differed from that in MAb binding assays. PNAd-coated capillaries supported slow rolling but no firm arrest as described previously for leukocyte interactions with mucosal addressin cell adhesion molecule 1 (MAdCAM-1) or PNAd in similar assays

(Berg et al., 1993; Lawrence et al., 1995). Rolling was abolished when lectins on either cell line were blocked by MAb DREG-56 (data not shown).

As an apparent consequence of differential attachment efficiencies, the rolling flux of wild-type cells was 1.5-fold higher than that of L–CD44 cells at 1.94 dynes/cm^2 and 4.1 times higher at 3.06 dynes/cm^2 (Table 2). Rolling cells were easily identified by their velocity, which was lower and often irregular when compared with the fast, smoothly moving, noninteracting cells. A similar fraction of wild-type and chimeric L-selectin transfectants displayed jerky or skipping rolling in which cells briefly appeared to detach and reattach after passing one or two cell diameters at higher velocity. Skipping cells remained within the focal plane and thus did not move away from the capillary wall, whereas cells that detached without further rolling mostly disappeared rapidly from the focal plane. The rolling flux was inversely proportional to the shear stress, and few rolling cells were observed above 3.5 dynes/cm^2.
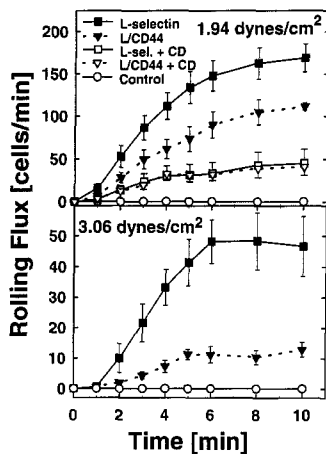
Figure 7. Rolling Flux of L1-2 Transfectants on PNAd

Rolling flux of wild-type L-selectin (microvilli) or L–CD44 (planar cell body) transfectants on immobilized PNAd at 1.94 dynes/cm² (top) or 3.06 dynes/cm² (bottom). In five experiments at 1.94 dynes/cm², microvilli were destroyed using CD. All other curves represent mean ± SEM of seven independent experiments.

The rolling flux can be influenced by several factors other than initial attachment, including the rate of cell delivery, the shear rate, the rolling velocity, and the frequency of detachment. However, the rate of cell delivery and fluid dynamics were kept constant and, as shown in Table 2, rolling velocities and fractions of skipping and detaching cells were not different between wild-type and L–CD44 cells (p > 0.05), suggesting that L-selectin topography is not important once rolling has been established. In fact, the attachment rate was the only parameter significantly distinguishing wild-type and L–CD44 cells in our flow assays. Thus, the dramatic difference in rolling of wild-type L-selectin versus L–CD44 cells was due to a higher rate of contact initiation via microvillous L-selectin.

Cytoskeletal integrity was required for optimal adhesion initiation: attachment rates and rolling flux of transfectants treated with CD, but not with DMSO alone, were significantly decreased at 1.94 dynes/cm², and very few treated cells rolled at 3.06 dynes/cm². Flow cytometry revealed no loss of L-selectin, and FITC–PPME binding was pre-

served. The reduced rolling flux may be partly explained by significantly slower rolling velocities of CD-treated cells. This may reflect a larger surface area available for close association, a larger number and altered distribution of interacting receptors on rolling cells that are devoid of microvilli, or both. Importantly, however, the differential advantage of wild-type L-selectin to initiate contact under shear was abolished by destruction of microvilli (Figure 7). In fact, there was no difference in frequency or character of attachment and rolling between CD-treated wild-type and L–CD44 cell lines (Table 2). This equivalence in cell behavior confirms the similarity in the PNAd-binding lectin activity of wild-type and chimeric receptors.

Discussion

Leukocyte interactions with ECs depend on multiple factors such as the density, functional activity, binding kinetics, and posttranslational modification of adhesion receptors and their ligands. Here we demonstrate a novel mechanism of adhesion regulation; the ultrastructural surface geography of adhesion molecules has a dramatic impact on their functional availability under flow. Using domain swapping between human L-selectin and the hematopoietic isoform of human CD44, we targeted ectodomains of both molecules to or away from microvilli on transfected murine lymphoid cells. The physiologic presentation of L-selectin on microvilli greatly facilitated leukocyte tethering on a native ligand and on anti-L-selectin MAbs. Exclusion from microvilli caused a significant and shear rate-dependent decrease in the capacity of the receptor to initiate adhesion under flow, despite normal lectin- and MAb-binding activity. Conversely, CD44 was normally expressed preferentially on the planar cell body. Its availability for capturing by immobilized MAb under flow was significantly enhanced when its ectodomain was targeted to microvilli. These data strongly suggest that the topography of adhesion receptors is a major determinant of their ability to participate in the earliest step of the adhesion cascade.

Although it was suggested as early as 1980 that interactions between leukocytes and ECs may require mechanisms associated with microvilli (van Ewijk, 1980), there

Table 2. Rolling of L1-2 Transfectants on Immobilized PNAd in Glass Capillaries

Shear Stress (dynes/cm²)	Surface Receptor	Rolling Flux (cells/min)[a]	Attachment Rate (cells/min/field)[a]	Rolling Velocity (μm/s)[b]	Skipping Fraction (%)[c]	Detaching Fraction (%)[c]
1.94	L-selectin	153.3 ± 42.5	33.6 ± 10.1	136.8 ± 28.9	22.5 ± 9	6.5 ± 4.4
1.94	L–CD44	99.9 ± 28.2[d]	17.0 ± 2.1[d]	147.7 ± 54.3	21.5 ± 11	10.4 ± 1.5
1.94	L-selectin + CD	41.4 ± 15.0	3.0 ± 2.0	70.0 ± 16.6	21.2 ± 10	6.4 ± 6.2
1.94	L–CD44 + CD	37.4 ± 17.6	3.1 ± 1.7	69.6 ± 9.9	18.6 ± 11	5.6 ± 3.9
3.06	L-selectin	47.7 ± 18.2	7.3 ± 1.4	157.2 ± 47.8	32.0 ± 10	5.5 ± 6.2
3.06	L–CD44	11.5 ± 5.3[d]	1.8 ± 0.4[d]	165.6 ± 59.4	32.5 ± 14	8.0 ± 1.6

[a] Mean ± SD of average number of attaching or rolling cells per minute during steady-state rolling.
[b] Mean ± SD of 30 consecutive cells per sample rolling across a 200 μm × 200 μm field.
[c] Mean ± SD of skipping or detaching fraction of 50 rolling cells per sample within a 400 μm × 200 μm field.
CD, cytochalasin D (5 μg/ml; 10 min at 37°C);
[d] p < 0.001 versus L-selectin (paired t test); n = 5 independent experiments in each group.

have been no attempts to investigate the functional consequences associated with a distinct geography of leukocyte surface receptors. Burns and Doerschuk noted that L-selectin was concentrated on microvilli of neutrophils (Picker et al., 1991), suggesting that this may enhance its function in initiating contact and supporting rolling. Subsequent studies confirmed the microvillous topography of L-selectin on neutrophils (Erlandsen et al., 1993) and mononuclear cells (Hasslen et al., 1995). In addition, the integrins $\alpha 4 \beta 7$ and $\alpha 4 \beta 1$, which can initiate selectin-independent lymphocyte tethering and rolling on the vascular ligands MAdCAM-1 and vascular cell adhesion molecule 1 (VCAM-1), and the newly identified P-selectin glycoprotein ligand 1 (PSGL-1), which mediates neutrophil rolling on P-selectin in vitro, are also concentrated on microvilli (Berlin et al., 1995; Moore et al., 1995; unpublished data). In conjunction with the present demonstration of the central importance of microvillous presentation, these findings suggest a general principle: receptors that act as highly reactive molecular brakes and initiate adhesion in high shear flow are presented in multivalent foci on the most distal aspects of the leukocyte surface.

When considering the role of microvilli in leukocyte adhesion, it is important to discuss venular hemodynamics that determine the frequency and degree of interactions. As a leukocyte leaves the capillary bed and travels downstream through increasingly wider vessels, these physical forces restrict contact and favor disassociation between the flowing cell and the EC. Leukocytes constitute relatively large bodies in venules with laminar flow and are thus subjected to a dispersal force that draws cells away from the vascular wall toward the geometrical axis of the vessel (Schmid-Schönbein et al., 1980; Nobis et al., 1985). Collisions with erythrocytes or disturbance of laminar flow can cause leukocyte displacement to the margin of a vessel cross section where cells may come in close proximity to ECs. However, interdigitation of large glycocalix molecules such as CD43 or CD45 may interfere with receptor–ligand interactions and may also play a role in preventing tight contact of large membrane areas due to electrostatic repulsion between negative surface charges on leukocytes (Lichtman and Weed, 1970) and ECs (Pelikan et al., 1979). In the presence of microvilli, however, the surface area making initial contact with the venular wall is predictably limited to the most peripheral aspects of the cell membrane, notably the tips of microvilli that display L-selectin, PSGL-1, or $\alpha 4$ integrins. The reduced area may decrease repulsion and provide a specialized contact-promoting environment. In addition, receptor clustering creates multivalent sticky patches, which may increase the probability and avidity of interactions involving presentation and recognition of low affinity CHO determinants.

In the present experiments, the differential effect of microvillous versus cell body presentation was a function of shear and was most dramatic in vitro at shear rates that prevail in microvessels with low to moderate flow in vivo. For anti-L-selectin MAb-mediated cell capturing, for example, microvillous and cell body epitopes yielded equivalent binding in static assays, but significant differential attach-

ment under low shear (0.8 dynes/cm^2) that increased markedly at 1.6 dynes/cm^2. Moreover, the importance of microvillous presentation was particularly striking for L-selectin interaction with its native ligand at ≥ 3 dynes/cm^2. This dependence on shear may be partly due to the fact that in our horizontal in vitro system, collisions of perfused cells with the lower capillary wall depend on gravity, which must overcome the dispersal force that draws marginated cells away from the wall. The magnitude of dispersion is proportional to the wall shear rate and, thus, causes ever more transient and less frequent contact as flow velocities and shear rates increase (which may be why little attachment is observed in most in vitro systems over 4 dynes/cm^2). Therefore, microvilli become predominant sites of initial contact at higher shear, whereas at lower shear, other aspects of the cell surface may associate with the vessel wall. In addition, the number of initial bonds required to provide the force necessary for a cell to "catch on" increases with shear (Hammer and Lauffenburger, 1987; Hammer and Apte, 1992). At low flow, the small number of L–CD44 on microvilli may suffice to establish adhesive interactions, whereas attachment at higher physiologic shear may necessitate concentration of multiple receptors on microvilli.

In vivo, additional factors such as flow disturbances and (especially) erythrocytes promote leukocyte contact with ECs (Schmid-Schönbein et al., 1980). However, postcapillary shear rates (up to ~40 dynes/cm^2) are frequently much higher than those employed in vitro (Firrell and Lipowsky, 1989), suggesting that microvillous presentation may be even more critical for contact initiation in vivo.

Following contact, rolling cells in high shear venules become flattened as they are pushed against ECs by the flowing blood (Firrell and Lipowsky, 1989). Thus, although primary adhesion of freely flowing leukocytes appears to require specialized microvillous receptors, the membrane area in contact with ECs during subsequent rolling is likely much larger and may allow participation of nonmicrovillous molecules (including CD44). Indeed, the present findings are consistent with this model, supporting a critical role for microvillous presentation in contact initiation but not in later events. Although fewer L–CD44 cells rolled, the velocity of rolling wild-type and chimeric transfectants was not different. Thus, the adhesive force acting between PNAd and wild-type or chimeric L-selectin was similar in both cases, and clustering on microvilli was not required for the rolling phase following attachment (it cannot be excluded, however, that rolling cells may rapidly alter their morphology, redistribute receptors, or both). Furthermore, similar fractions of rolling wild-type L-selectin and L–CD44 cells displayed jerking or skipping behavior. Skipping cells did not appear to loose contact with the capillary wall and thus were able to reestablish adhesive bonds even when the L-selectin ectodomain was not associated with microvilli. Such skipping may relate to uneven distribution or limited density of L-selectin ligands in vitro. Detachment rates of rolling cells were also similar for L–CD44 and wild-type L-selectin transfectants. In fact, the only behavioral parameter that distinguished the transfectants was

the rate of initial contact formation (tethering); the frequency with which freely flowing wild-type transfectants initiated contact and began to roll on PNAd was much higher, and this distinction correlated with the rolling flux.

Decreased attachment of L–CD44 cells on PNAd could also be caused by impaired function of the chimeric molecule, e.g., owing to changes in structure or in its membrane/submembrane microenvironment. However, several findings argue against this possibility. First, lectin binding to soluble FITC–PPME was not affected. Second, CD-mediated disruption of the actin-based cytoskeleton rendered cells devoid of microvilli and abolished any difference in the (reduced) frequency of attachment and rolling. Third, whether microvilli were present or not, the characteristics of rolling did not differ between wild-type and chimeric transfectants, suggesting that the lectin function of L–CD44 is not affected once it has gained access to its ligand. Finally, the importance of microvillous presentation was supported by experiments employing two molecularly independent pathways in which the static ligands were MAbs; wild-type L-selectin and CD44–L cells were captured much more frequently than cells that presented equivalent epitopes on the planar cell body. These findings do not exclude that other functions of TM–IC domains, such as cross-linking-induced signaling, were altered in chimeric molecules. However, given the rapid time course of attachment, such functions are more likely a consequence of than a prerequisite for adhesion initiation. Furthermore, the absence of differences in established rolling suggests that such functions, if indeed affected, had no effect on the parameters studied here.

Interestingly, untreated L–CD44 cells initiated rolling more efficiently than CD-treated cells lacking microvilli. This may reflect a critical contribution of the ~5%–10% L-selectin ectodomains retained on microvilli of untreated transfectants. However, CD-mediated loss of cytoskeletal anchoring through IC domain linkage of L-selectin to α-actinin (Kansas et al., 1993; Pavalko et al., 1995) and of CD44 to ankyrin (Kalomiris and Bourguignon, 1988) may play a role, and other effects of CD cannot be excluded.

In contrast with L-selectin, PSGL-1, and α4 integrins, the β2 integrins are largely restricted to the cell body of neutrophils (Erlandsen et al., 1993) and lymphocytes (Berlin et al., 1995). This distribution may be as critical as microvillous presentation in controlling leukocyte adhesion. β2 integrins cannot initiate primary adhesion under physiological flow but are specialized for secondary activation-dependent interactions (Arfors et al., 1987; Lawrence and Springer, 1991; von Andrian et al., 1992). Recruitment to the planar cell body may ensure this specialized secondary role of β2 integrins in the multistep adhesion process by precluding receptor availability at sites of initial contact under flow. In this context, the present finding that CD44 is also targeted to the cell body suggests that its physiologic role (which is not well understood; see Butcher, 1994) may also be focused on secondary events.

Not all transfected molecules display a specialized topography on L1-2 cells. Human E- and P-selectin (unpublished data) and CD44–E chimeras were found in equal

numbers in microvillous and nonmicrovillous domains, suggesting that the restricted expression of L-selectin and CD44 is highly selective. Our findings on resting leukocytes do not exclude that activation-induced events may result in substantial redistribution of these molecules, e.g., owing to cytoskeletal modifications. In fact, CD44 redistribution can occur on migrating fibroblasts (Jacobson et al., 1984), and both CD44 and L-selectin on T cells are rapidly capped upon lymphocyte activation or MAb ligation (Rosenman et al., 1993). Thus, the restricted expression of receptors within circumscribed domains on resting leukocytes may be dynamically modified by exposure to distinct stimuli, and such modifications may allow leukocytes to control intra- and extravascular interactions.

In conclusion, our studies demonstrate that the topographic distribution of L-selectin and CD44 is independent of extracellular domains. In fact, ectodomains can be targeted to or away from microvilli by linking them to the TM–IC domains of L-selectin or CD44, respectively. Furthermore, we show that receptor topography determines the ability of a molecule to initiate functional contact under flow. Clustering on microvilli favors early involvement of adhesion receptors, whereas withdrawn expression on the cell body may limit receptor engagement to specific situations in which cell–cell contact has been previously initiated by selective binding of microvillous adhesion molecules. Thus, the ability of circulating leukocytes to compartmentalize their surface geography provides cells with a means of controlling the functional availability of promiscuous surface receptors during circumscribed steps of the adhesion cascade and constitutes a novel mechanism for fine-tuning intravascular leukocyte adhesion, activation, and trafficking.

Experimental Procedures

Recombinant DNA
Full-length cDNA of human wild-type L-selectin and E-selectin had been used previously for stable transfection of L1-2 cells (Berg et al., 1992; Picker et al., 1991). A cDNA clone encoding the major hematopoietic isoform of human CD44 (Goldstein et al., 1989; Stamenkovic et al., 1989) was a gift from Dr. B. Seed. Chimeric genes were created by domain swapping using a sequence overlap extension polymerase chain reaction (PCR) protocol (Horton et al., 1989). In brief, gene fragments encoding the TM–IC domain or ectodomain of individual receptors were amplified in separate PCRs. To allow linkage of TM–IC domains from one molecule to the ectodomain of another, we designed primers so that PCR products contained a complementary extension at the TM–extracellular transition region. Amplified fragments were gel purified, mixed, and reannealed in a second PCR containing primers to 3' and 5' ends of chimeric genes. Wild-type and chimeric cDNAs were subcloned and amplified in the expression vector pMRB101, a derivative of peE6 containing the CMV promoter and Escherichia coli *gpt* gene.

Double-stranded nucleotide sequencing of wild-type L-selectin and L–CD44 revealed that both genes had a single aberration (G244→T) in their lectin domain, introducing Phe for Val in amino acid position 82. Wild-type L-selectin transfectants containing this sequence are fully functional with respect to physiologic ligand and MAb binding (Berg et al., 1991, 1992), activation-induced shedding (unpublished data), and L-selectin-mediated rolling in vivo (von Andrian et al., 1993b) and in vitro (Berg et al., 1993), suggesting that this sequence difference does not alter L-selectin function and may possibly reflect allelic diversity. No other sequence differences in wild-type L-selectin or L–CD44 were found when compared with published templates.

Antibodies

The following affinity-purified MAbs were used for these experiments: anti-human L-selectin MAbs DREG-56 and DREG-200 (both murine IgG1; Kishimoto et al., 1990) and anti-human CD44 MAbs Hermes-1 (rat IgG2a) and Hermes-3 (murine IgG2a; Jalkanen et al., 1987) were raised in our laboratory, and anti-human E-selectin MAb CL-2 (murine IgG1; Kishimoto et al., 1991) was provided by C. W. Smith. For studies involving flow cytometry or cell sorting, FITC-conjugated MAb DREG-200 (IgG or F[ab] fragments) or MAb CL-2 or MAb Hermes-1 followed by PE–goat anti-mouse or PE–mouse anti-rat F(ab)₂ fragments were used. Immunolocalization studies using electron microscopy (see below) were performed with unlabeled primary MAbs. Class-matched MAbs DREG-200 (for wild-type E-selectin and E–L transfectants), CL-2 (for transfectants expressing L-selectin ectodomains), and Mel-14 (rat IgG2a; Gallatin et al., 1983) were used as negative controls. Primary MAb labeling was followed by 10 nm colloidal gold-conjugated antibodies (Sigma) to murine and rat IgG, respectively.

Cell Lines

Murine L1-2 pre–B lymphoma cells were grown in RPMI 1640 medium containing 10% fetal calf serum and standard supplements. Cells were harvested and transfected by electroporation with pMRB101 containing inserts encoding wild-type or chimeric cDNA. Control cells were mock transfected with plasmid vector only.

Following selection, transfectants were sorted for high receptor expression on a FACStar cell sorter (Becton Dickinson) and subcloned by limited dilution. Clones with similar expression levels of L-selectin and CD44 ectodomains were chosen for functional comparison. Despite stable transfection, some subclones displayed a tendency to vary expression levels of transfected gene products. Therefore, in parallel with every experiment, expression was determined by fluorescence-activated cell sorting (FACS) analysis (see below). Experiments were excluded if differences in expression were more than 2-fold. Subclones were periodically resorted to maintain constant expression levels.

FACS Analysis

For quantitative assessment of receptor expression and cell sorting, transfectants were suspended to 1×10^7 cells/ml in HBSS and kept on ice. Primary MAb (20 µg/ml) was added for 30 min to 50 µl of cell suspension. Cells were washed twice and stained with PE-labeled second-stage serum when needed. Mock transfectants were used for determination of light scatter and fluorescence detection thresholds on a FACScan flow cytometer (Becton Dickinson).

Binding characteristics of MAbs FITC–DREG-200 and Hermes-1 to their respective epitope on transfectants from wild type chimera were determined. MAbs were serially diluted in 100 µl of HBSS plus 1% BSA, and 1×10^6 cells were added to final MAb concentrations ranging from 0.02 µg/ml to 20 µg/ml. After 30 min on ice, cells were washed, and transfectants labeled with MAb Hermes-1 were stained with excess PE–mouse anti-rat F(ab)₂ fragments. Cells were washed, fixed in ice-cold PBS plus 1% paraformaldehyde, and analyzed by FACS as above.

For assessment of lectin function, 1.5×10^6 cells expressing wild-type or chimeric L-selectin were incubated with 1:400 dilutions of two different batches of FITC–PPME (gifts from L. Stoolman and S. Rosen), a Ca^{2+}-dependent L-selectin lectin ligand (Yednock et al., 1987). Binding specificity was assessed by addition of 25 mM EDTA or 20 µg/ml MAb DREG-56 as described previously (Kishimoto et al., 1990). Mock transfectants were used to assess background staining. Aliquots of cells were labeled with FITC–DREG-200 F(ab)' fragments, and the ratio of specific staining intensities with FITC–PPME versus FITC–DREG-200 was calculated to determine the relative lectin activity as a function of receptor expression.

Disruption of Microvillous Architecture

In some experiments, transfectants (1×10^6/ml) were treated for 10 min at 37°C with 5 µg/ml CD (Sigma) to disrupt the actin-based cytoskeleton. Aliquots were prepared for LVSEM or used in flow experiments (see below). Control cells were treated with vehicle (5 µl/ml DMSO).

Ultrastructural Immunolocalization of Transfected Receptors

Transfectants were labeled on ice with primary MAb (20 µg/ml), washed, stained with colloidal gold-conjugated secondary MAb, and fixed as described previously (Erlandsen et al., 1993). Samples were assigned a code number and prepared for ultrastructural analysis of antigen distribution using a Hitachi S-900 LVSEM. Analysis was performed by counting gold particles associated with distinct surface structures on unperturbed cells using a stereoviewer for enhanced spatial resolution. Staining patterns with a majority of colloidal gold particles in localized groups of three or more were interpreted as clustering (Erlandsen et al., 1993). Investigators used blind analysis for samples in three duplicate experiments.

Static Adhesion Assays

We coated 18-well glass slides with 15 µl solution containing 100 µg/ml DREG-200, DREG-56, Hermes-1, Hermes-3, or BCS, washed the slides, and blocked them with BCS. Cells (5×10^4) in 16 µl of complete media were added to each well and allowed to settle for 30 min at 37°C. Slides were washed in HBSS and fixed by dipping in ice-cold HBSS plus 6% glutaraldehyde in HEPES buffer (pH 7.4). Bound cells were counted on an inverted microscope (Nikon). Three independent experiments were performed in duplicates or triplicates. Results are presented as mean number of cells per six fields of view (0.24 mm² each) per well.

Flow Assays

One half of glass capillaries (ID, 1.025 mm; Microcaps 100 µl, Drummond Scientific) was coated by ≥2 hr incubation at 37°C with 100 µg/ml anti-L-selectin or anti-CD44 MAbs. The entire capillary was then blocked with BCS (≥1 hr at 37°C). In some experiments, capillaries were coated instead with 1:20 diluted affinity-purified PNAd from human tonsils (Berg et al., 1991).

Using polypropylene tubing and a three-way stop cock, coated capillaries were connected to a 20 ml syringe containing culture medium and mounted on an inverted microscope (Nikon) equipped for Hofmann modulation contrast illumination. A 20× lens (Nikon) was focused on the inner surface of the capillary bottom. After 10 min equilibration with plain buffer, 12 ml or 20 ml syringes containing 1×10^6 cells/ml were fitted to the stop cock, and cells were perfused through the glass capillary using a calibrated syringe pump (model 351, Sage Instruments). The BCS-coated part of capillaries was oriented upstream of MAb- or PNAd-coated segments, and a site ~1 cm downstream of the BCS/MAb or BCS/PNAd boundary was chosen for video microscopy of cell interactions with immobilized substrate. A BCS-coated site ~2 cm upstream was used to assess unspecific interactions.

Preliminary studies showed that immobilized MAbs arrested most interacting transfectants faster than the temporal resolution of our video system (<33 ms). Captured cells were not released for at least 15 min. Transfectants presenting the L-selectin ectodomain were effectively captured at shear rates up to 160 s⁻¹ (i.e., a wall shear stress of 1.6 dynes/cm², assuming a viscosity of 0.01 P for medium at 23°C). Anti-CD44 MAbs were somewhat less efficient; cells bearing CD44 ectodomains were infrequently immobilized above 1.2 dynes/cm², but were readily arrested at lower shear. Thus, capturing assays with anti-L-selectin MAbs were performed at 0.8 dynes/cm² and 1.6 dynes/cm², whereas anti-CD44 MAbs were only used at 0.8 dynes/cm².

Video scenes were analyzed by counting captured cells during 10 min following the time when cells first appeared in the focal plane. The capturing rate was determined during the last 9 min of observation as number of arrested cells per field per minute.

Preliminary experiments had shown that PNAd does not arrest transfectants but allows attachment and slow rolling of cells up to 3.5 dynes/cm² (see also Lawrence et al., 1995). PNAd-mediated rolling in vitro resembles L1-2 cell rolling in vivo (von Andrian et al., 1993b) and is indistinguishable from L-selectin transfectant rolling on MAdCAM-1 (Berg et al., 1993). The following PNAd-mediated interactions were determined: the rolling flux, the rolling velocity, the rate of attachment, the fraction of detaching cells, and the frequency of irregularly rolling (or skipping) cells.

The Rolling Flux

The rolling flux was analyzed during 10 min following the instant when

cells first appeared in the observation window. All cells were counted that rolled across a line perpendicular to the capillary axis in the center of the field of view. Typically, the rolling flux increased steadily during the first 6 min of perfusion and reached a plateau thereafter. These kinetics were independent of the wall shear rate (at least within the range applied here) and reflect the period necessary for the system to reach equilibrium. Accordingly, all other parameters of L-selectin–PNAd interactions were determined during steady-state rolling after ≥6 min.

Rolling Velocities

Rolling velocities of 30 consecutive cells that were continuously rolling through a 200 μm × 200 μm field in the center of the field of view (which covers an area of 600 μm × 400 μm) were determined for each cell sample using an image analysis system (NIH Image). Skipping cells were not included in velocity measurements.

The Rate of Cell Attachment

The rate of cell attachment (cells per minute per field) was assessed from the number of freely flowing cells that initiated contact and began to roll within a field of view during steady-state rolling. Determination of attachment rates was facilitated by reversing the direction of the videotape during analysis.

The Fraction of Detaching Cells and the Frequency of Irregularly Rolling (Skipping) Cells

Rolling was further characterized by tracing 50 consecutive rolling cells in each sample that passed a 400 μm × 200 μm field in the center of the eyefield. From this population, the percentage of cells that detached and reattached (skipping fraction) and the fraction of cells that detached and disappeared from the focal plane and the field of view without further interaction (detaching fraction) was determined.

Acknowledgments

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