CELLULAR DEFORMATION MODULATES LEUKOCYTE ROLLING ON P-SELECTIN AND THE FORMATION OF PLATELET THROMBI ON ENDOTHELium IN SHEAR

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Presented to the faculty of

the School of Engineering and Applied Science

University of Virginia

In Partial Fulfillment

of the requirements for the Degree

Doctor of Philosophy in Biomedical Engineering

By

Yung Hee Park

January 2005
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ABSTRACT

The selectin family of adhesion molecules plays an important role in leukocyte recruitment to the vascular endothelium during an inflammatory response. The engagement of endothelial P-selectin with leukocyte P-selectin glycoprotein ligand (PSGL-1) is involved in the initial leukocyte tether and rolling events. Agonist stimulated platelets are also able to express P-selectin and form circulating platelet-leukocyte aggregates in vivo. In this study, PSGL-1 coated microspheres were utilized to investigate both the role of localized leukocyte deformation in modulating the kinetics of P-selectin/PSGL-1 mediated leukocyte rolling and the significance of leukocyte protrusion into the flow field from the endothelium in directing the capture of platelets.

In the first stage of the project, a cell-sized microbead system was used to analyze the force-dependent kinetics of P-selectin adhesive bonds independent of micro-mechanical properties of the neutrophil’s surface microvilli, elastic structures on which PSGL-1 is localized. Microvilli extension is hypothesized to contribute to the dynamic range of leukocyte rolling observed in vivo during inflammatory processes. To evaluate PSGL-1/P-selectin bond kinetics of microbeads and neutrophils, rolling and tethering on P-selectin coated substrates were compared in a parallel-plate flow chamber. The dissociation rates for PSGL-1 microbeads on P-selectin were briefer than those of neutrophils for any wall shear stress and increased faster with increasing flow. The microvillus length necessary to reconcile dissociation constants of PSGL-1 microbeads and neutrophils on P-selectin was 0.21 μm at 0.4 dyn/cm² and increased to 1.58 μm at 2 dyn/cm². The apparent elastic spring constant of the microvillus ranged from 2272 to 124 pN/μm at 0.4 and 2.0 dyn/cm² wall shear stress, respectively. Fixation of neutrophils to
abrogate microvillus elasticity resulted in rolling behavior similar to PSGL-1 microbeads. Scanning electron micrographs of neutrophils rolling on P-selectin confirmed the existence of micrometer-scaled tethers.

The second phase of the study looked at the possible role of leukocytes adherent to endothelium in recruiting platelets and forming thrombi. Platelets can form circulating aggregates with neutrophils or monocytes under shear conditions. To evaluate the impact of neutrophils or monocytes on thrombi formation during an inflammatory response, platelets were perfused at venular wall shear stresses ranging between 0.3–8 dyn/cm² over monocytes or neutrophils adherent on human umbilical vein endothelial cell (HUVECs) monolayers. Platelet recruitment to the endothelium via adherent leukocytes was significantly greater than platelet adhesion to the endothelium alone. Resting platelet accumulation on monocytes was inhibited by blocking either PSGL-1 or activated GPIIb/IIIa. Accumulation of activated platelets under flow was predominately mediated by platelet P-selectin interacting with leukocyte PSGL-1. Monocytes were three times more effective than neutrophils in directing platelet accumulation on HUVECs.

The final phase of the study investigated the localized fluid disturbance around adherent leukocytes and its effect on platelet accumulation. Neutrophils on cytokine-stimulated HUVECs appeared more activated than monocytes based on visual observation of ruffles and lamellipodia. Computational modeling of the local hydrodynamics around a transmigrating leukocyte reveals an approximate 6-fold deficit in the amount of shear stress reduction compared to a fully protruding leukocyte that has just initiated contact with the surface. The amount of platelet accumulation on PSGL-1
microbeads of 3-, 6-, and 10-μm in diameter matched the pattern of shear reduction indicated by the computational results.

Leukocyte morphology and deformability can have significant effects on the nature of heterogeneous cell interaction. The use of non-deformable microspheres is an effective comparative model to investigate cell deformation effects. The results suggest that microvillus extension during transient PSGL-1/P-selectin bonding may enhance the robustness of neutrophil rolling interactions. The protracted activation phenotype of monocytes in comparison to the acute neutrophil extravasation response may increase the size of localized flow stagnation zones whereby platelets preferentially accumulate on monocytes.
ACKNOWLEDGEMENTS

This work would not have been possible without the support of many other people who have guided, challenged, encouraged, and cared for me. My years here at the University of Virginia will not be thought of as a time just for academic training, but also a period for personal growth and development.

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Soli Deo Gloria
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<td>acid citrate dextrose</td>
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<tr>
<td>Dil</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine</td>
</tr>
<tr>
<td>E&lt;sub&gt;c&lt;/sub&gt;</td>
<td>interception capture efficiency</td>
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<td>ECM</td>
<td>sub-endothelial cell matrix</td>
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<td>F&lt;sub&gt;(ab')&lt;sup&gt;2&lt;/sup&gt;&lt;/sub&gt;</td>
<td>bivalent antigen binding fragment of monoclonal antibody</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>F&lt;sub&gt;g&lt;/sub&gt;</td>
<td>fibrinogen</td>
</tr>
<tr>
<td>F&lt;sub&gt;f&lt;/sub&gt;</td>
<td>force exerted on the tether</td>
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<tr>
<td>F&lt;sub&gt;s&lt;/sub&gt;</td>
<td>fluid shear force</td>
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<td>GPIIb/IIIa</td>
<td>glycoprotein IIb/IIIa (i.e. integrin αIIbβ3)</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks buffered saline solution</td>
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<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<tr>
<td>I&lt;sub&gt;cell&lt;/sub&gt;</td>
<td>mean intensity of fluorescent cell</td>
</tr>
<tr>
<td>I&lt;sub&gt;frame&lt;/sub&gt;</td>
<td>global mean intensity of frame</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
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<td>k</td>
<td>spring constant of microvillus tether</td>
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<td>stressed dissociation rate constant</td>
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<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
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<td>LFA-1</td>
<td>lymphocyte function-associated antigen-1</td>
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<td>lever arm</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>OG</td>
<td>octylglucopyranoside</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>prostaglandin I&lt;sub&gt;2&lt;/sub&gt; (i.e. prostacyclin)</td>
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<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
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<tr>
<td>Q</td>
<td>flow rate</td>
</tr>
<tr>
<td>R</td>
<td>radius of bead/cell</td>
</tr>
<tr>
<td>Re</td>
<td>Reynolds number</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDF</td>
<td>stromal derived factor</td>
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<td>standard error of mean</td>
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<td>TRAP</td>
<td>thrombin receptor activating peptide</td>
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<td>T&lt;sub&gt;s&lt;/sub&gt;</td>
<td>shear torque</td>
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<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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<td>VLA-4</td>
<td>very late antigen-4</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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<td>Symbol</td>
<td>Definition</td>
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</tr>
<tr>
<td>( \delta )</td>
<td>gap width between particle and flat surface</td>
</tr>
<tr>
<td>( \gamma_w )</td>
<td>wall Shear rate</td>
</tr>
<tr>
<td>( \rho_f )</td>
<td>fluid density</td>
</tr>
<tr>
<td>( \nu_f )</td>
<td>fluid kinematic viscosity</td>
</tr>
<tr>
<td>( \theta )</td>
<td>angle between tether and lower wall</td>
</tr>
<tr>
<td>( \theta_p )</td>
<td>exclusion angle for platelet binding</td>
</tr>
<tr>
<td>( \eta )</td>
<td>uniaxial viscosity of microvillus tether</td>
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<td>( \tau_w )</td>
<td>wall shear stress</td>
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Chapter 1

Introduction

1.1 Background

Blood circulation is necessary to deliver oxygen and essential nutrients to tissues and to remove carbon dioxide along with other waste products of metabolism. The viscous flow of fluid along a surface such as the blood vessel wall will impose a tangentially directed force along the surface. This force is called shear stress, $\tau$, which may be expressed as the frictional force per unit surface area.

The role of shear stress on cells of the vascular system has many important implications in physiology. Flow in the vascular system is for the most part laminar, but time-dependent flow patterns can develop in regions of complex geometry (Buchanan et al., 2003; Wootton and Ku, 1999). Wall shear stresses range from 1-6 dyn/cm$^2$ in veins. Arteries have a broader wall shear stress range of 10-70 dyn/cm$^2$, while coronary stents and complex plaques have wall shear stresses that can range from 70 to over 100 dyn/cm$^2$ (Kroll et al., 1996; Malek et al., 1999). The development of atherosclerotic lesions is primarily localized to regions of fluid recirculation. Endothelial cells in a shear field will remodel from a cobblestone appearance and elongate in the direction of flow. Endothelial gene transcription of cytokines and leukocyte adhesion molecules can be induced through shear stress regulation, possibly due to mechanotransduction through cytoskeletal filaments (Dai et al., 2004; Gimbrone et al., 1997; Helmke and Davies, 2002; Resnick and Gimbrone, 1995; Wang et al., 1993).
Figure 1.1 Cells present in the vascular system. Erythrocytes, leukocytes, and platelets circulate in the bloodstream of a vessel lumen. The single cell monolayer of endothelial cells protects the circulating blood from pro-coagulant proteins present in the sub-endothelial matrix.

Leukocyte rolling on inflamed venous endothelium is regulated by the continual association and dissociation of adhesion receptors. The selectin family of adhesion receptors are the primary molecules responsible for the rolling process under fluid shear forces. At higher shear, leukocyte rolling can be seen progressing at a higher average velocity. The mechanism of selectin bond breakage is a function of the amount of force that is loaded on the bond(Bell, 1978; Chen and Springer, 2001). Certain selectin bond association events have also been shown to be affected by shear. Little or no leukocyte interactions can be seen through L-selectin binding at low shear but multiple events are observed at higher shear (Finger et al., 1996; Lawrence et al., 1997).
The differential involvement of adhesion receptors in platelet interactions with sub-endothelial cell matrix (ECM) proteins upon endothelial damage/denudation is regulated by the presence of shear. Platelet deposition on ECM at high shear is primarily regulated by GPIb-IX-V interactions with von Willebrand factor (vWF), but low shear platelet attachment is governed by GPIIb/IIIa anchorage to fibrin/fibrinogen (Ruggeri, 1994; Savage et al., 1998; Savage et al., 1996).

The vascular system presents a wide range of fluid shear stresses that circulating cells may experience. The interaction of cells in the circulation through adhesive protein bonds is specifically regulated by the shear regime of cell associations and the molecular entities involved. Cellular deformation on the microstructure and global scale could decrease the likelihood of bond dissociation in fluid flow. Leukocyte microvilli elongation under increasing shear stress may allow selectin bond interactions to last longer. The localized formation of secondary flow regions around adherent leukocytes may promote platelet attachment and accumulation on endothelial surfaces.

1.2 Specific Aims

Chapter 2 examines the preservation of leukocyte, specifically neutrophil, rolling at increasing fluid shear stresses. In order to understand the influence of cell surface features in the initial phase of selectin-mediated leukocyte capture and rolling, a ligand-coated microbead analysis method is presented. The selectin bond dissociation rate kinetics are analyzed using first order principles of chemical reactions. The influences of increasing shear force and cell structure deformation are broken down into mechanical free body diagrams to solve the equilibrium force loading equations. Microbeads can be
functionalized with various adhesion molecules for bond characterization studies. The lack of deformability along with the absence of surface features of these particles offers an optimal comparative tool for leukocyte tethering/rolling analysis. Rolling kinetic analysis of paraformaldehyde-fixed neutrophils was also compared, and structural material properties of the leukocyte microvilli were extracted. This chapter presents much of the work I published in 2002 (Park et al., 2002).

Chapters 3 and 4 are primarily investigating the role of platelet capture and recruitment to the endothelial surface by leukocytes. The P-selectin/PSGL-1 interaction involved in leukocyte rolling on endothelium during acute inflammatory responses could also be exploited by activated platelets, which express P-selectin. The role of leukocytes in the participation of venous thrombosis has not been fully investigated. During tissue inflammation, leukocytes are recruited from circulation to the endothelial surface. These leukocytes will transiently interact with the endothelial surface and some may become firmly adherent to the intact vascular lumen. The association and dissociation rates of receptors presented on the surface of an activated platelet may favor binding with endothelial-adherent leukocytes as opposed to direct platelet interactions with the endothelium.

Chapter 4 examines the fluid dynamics around an adherent leukocyte that may promote platelet attachment and accumulation. Neutrophils and monocytes both present receptors available for platelet attachment. The activation state of the two leukocyte subtypes interacting with cytokine-stimulated endothelial cells differs, with neutrophils displaying more of a spread and asymmetric appearance than monocytes. Fluid profiles of different cell projection heights were examined using computational fluid dynamics.
Chapter 1 ~ Introduction

Experimental analysis of cell protrusion in platelet capture from flow was performed using ligand-coated microbeads of standardized sizes.

Chapter 5 summarizes the significance of this work and future directions for research in specific scientific areas or utilizing the tools developed in these studies.
Chapter 2

Microvilli Extension Stabilizes Leukocyte Rolling on P-Selectin

2.1 Introduction

During an inflammatory response leukocytes are recruited to the vascular endothelium through a series of sequential adhesive events that consists of tethering, rolling, and firm adhesion (Fig. 2.1). The first two steps typically require engagement of selectin receptors, a family of structurally related, membrane anchored C-type lectins that bind to carbohydrate epitopes on opposing membranes (Somers et al., 2000). Due to the favorable binding kinetics of selectins, circulating leukocytes are able to form bonds with the endothelium under flow conditions, resulting in leukocyte rolling along the vessel wall. The selectin family of receptors consists of three members, L-, P-, and E-selectin, all specialized for mediating rolling interactions between platelets, leukocytes, and endothelium. P-selectin in particular is necessary for the recruitment of neutrophils in response to the release of mediators of acute inflammation such as thrombin or histamine. The storage of P-selectin pre-formed in endothelial cell secretory granules permits rapid upregulation P-selectin on the endothelium, resulting in the accumulation of rolling neutrophils. Rolling of neutrophils on P-selectin expressed surfaces is mediated by a membrane anchored glycoprotein that has mucin like characteristics, named P-selectin Glycoprotein Ligand-1 (PSGL-1) (Moore et al., 1995; Norman et al., 1995).

PSGL-1 has an unusual surface distribution on neutrophils in that it is found concentrated on the tips of microvilli that extend from the cell body (Moore et al., 1995). Two other adhesion receptors, L-selectin and Very Late Antigen-4 (VLA-4), are also
localized on the microvilli tips of leukocytes (Berlin et al., 1995; Picker et al., 1991). The location of PSGL-1 on microvilli tips (which extend 0.2 to 0.3 microns from the leukocyte surface) may enhance association with P-selectin on apposing cell surfaces, as has been suggested for L-selectin and VLA-4 with their respective ligands (Berlin et al., 1995; Picker et al., 1991; von Andrian et al., 1995). In contrast to the surface distribution of selectins, the β2 integrins on neutrophils are only found on the planar regions of the membrane between the microvilli, a location that may limit interactions with endothelial cell ligands during rolling (Bruehl et al., 1996; Erlandsen et al., 1993).

Attachment of leukocytes to the vessel wall likely depends on fast binding kinetics of the selectins relative to the β1 and β2 integrins that mediate firm adhesion (Lawrence and Springer, 1991). Association \((k_{on} \sim 4.4 \times 10^6 \text{ M}^{-1} \text{ second}^{-1})\) and dissociation \((k_{off} \approx 1.4 \text{ seconds}^{-1})\) constants of PSGL-1/P-selectin bonds have been determined in the fluid phase (Mehta et al., 1998), but these parameters appear to be significantly altered when the bond is mechanically stressed by biologically relevant forces (Alon et al., 1995a; Fritz et al., 1998; Kaplanski et al., 1993; Pierres et al., 1995). From analysis of neutrophils rolling on P-selectin substrates, un-stressed bond dissociation constants \((k_{off}^{u})\) have been estimated to range between 0.9 - 2.4 seconds\(^{-1}\), increasing exponentially up to approximately 100 pN/bond (Alon et al., 1995b; Smith et al., 1999).

As shear forces are increased, leukocyte rolling velocities on P-selectin typically increase in a non-linear fashion, such that a plateau in velocity is evident at physiological flowrates (Atherton and Born, 1973; Lawrence and Springer, 1993). In contrast, physical models of bond dissociation predict an exponential decrease in selectin bond lifetime with force (Bell, 1978). As predicted by such models, P-selectin bond lifetimes decrease...
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exponentially with force in the 0 to 100 pN/bond range (Smith et al., 1999). However, above an estimated 100 pN/adhesive bond cluster, neutrophil P-selectin dissociation constants increase only linearly with additional force (Smith et al., 1999). Since both cellular $k_{off}$ and rolling velocity plateau with increasing force, it may be hypothesized that mechanisms exist at the cellular level to control rolling.

Since PSGL-1 bonds are localized to microvilli that may stretch (Shao et al., 1998), it is possible that the geometrical relationship between the anchorage point and the rolling neutrophil may change significantly under stress. Indeed, individual microvilli on neutrophils stretch into surprisingly long tethers if the bond at the anchorage point lasts long enough (Schmidtke and Diamond, 2000), suggesting that microvilli may elongate at forces a neutrophil might encounter on the blood vessel wall. Additionally, the contribution of cellular deformation to the stability of neutrophil rolling may also become significant at in vivo wall shear stresses if the contact area available for receptor ligation increases (Cao et al., 1998; Firrell and Lipowsky, 1989; Lei et al., 1999). Both microvillus stretching and increases in leukocyte-endothelium membrane contact area may therefore contribute to the ability of leukocytes to roll at in vivo flow rates.

In this study, I compared selectin dissociation kinetics of microbead binding to a selectin surface with that of neutrophils to examine the hypothesis that neutrophil microvillus elongation may regulate P-selectin mediated rolling and partially explain the stability of rolling at in vivo flow rates. Purified native PSGL-1 molecules immobilized on non-deformable microbeads allowed the measurement of P-selectin interactions in the absence of cellular factors such as cellular deformation and complexities of surface topography such as microvilli as has been used previously to characterize the PSGL-1
binding pocket (Goetz et al., 1997; Rodgers et al., 2000). Comparison of the dynamics of PSGL-1 microbeads with neutrophils suggested the plateau in $k_{\text{off}}$ at higher wall shear stresses was a consequence of microvilli extension that depended on the level of fluid shear stress. Additionally, the plateau in P-selectin $k_{\text{off}}$ above 1 dyn/cm$^2$ wall shear stress was eliminated on neutrophils by reducing deformability through glutaraldehyde fixation. Both high-resolution video microscopy and scanning electron microscopy of rolling neutrophils indicate the existence of microvilli tethers on the scale of 1 to 4 μm under dynamic flow conditions. These observations suggested that local membrane deformation dramatically modified PSGL-1/P-selectin bond dissociation constants and neutrophil rolling dynamics.

2.2 Experimental Methods

Antibodies and Protein Substrates

KPL1, a P-selectin and L-selectin blocking monoclonal antibody (mAb) to PSGL-1, was developed as previously described (Snapp et al., 1998). The mAb against LFA-1, TS1/22, was purified from hybridoma supernatant as described (Sanchez-Madrid et al., 1982). E-selectin blocking mAb BB11 was a gift from R. Lobb (Biogen, Inc., Cambridge, MA). FITC-conjugated goat, anti-mouse secondary antibody was purchased from Pharmingen (San Diego, CA). Human P-selectin was purified from platelet lysates as described previously (Lawrence et al., 1997). PSGL-1 was purified from harvested HL-60 cells homogenized in 20 mM Tris, 140 mM NaCl, 0.025% sodium azide (TSA buffer) with 5 mM EDTA, and 1% Triton X-100 in the presence of protease inhibitors 10 μM leupeptin and 0.1 U/ml aprotinin adjusted to pH 8.0. The cell lysate was passed
twice over a column of CNBr-activated Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) coupled to KPL1 (1.3 mg/ml). The column was washed (30 column volumes) with TSA adjusted to pH 7.6 and containing 1% octylglucopyranoside (OG; Sigma, St. Louis, MO). PSGL-1 was eluted from the column using TSA, pH 12.0, with 1% OG, and neutralized with approximately 10% (V/V) 1 M Tris, pH 4.0, 1% OG. PSGL-1 purification was verified by Western Blot analysis using KPL1.

*Neutrophil Isolation*

Neutrophils were isolated from human whole blood with a one-step separation consisting of 94% Mono-Poly Resolving Medium (MPRM, ICN Biochemicals, Aurora, OH) and 6% sterile water (Taylor et al., 1996). After isolation, the neutrophils were suspended in Hank's Balanced Salt Solution (HBSS) without calcium and magnesium, that was supplemented with 10 mM HEPES, pH 7.4, and 0.1% human serum albumin (HSA), and placed on ice. For flow assays, the neutrophils were taken as needed from this reserve and washed into HBSS with 2mM CaCl₂, 10 mM HEPES, pH 7.4, and 0.1% HSA at room temperature.

*Preparation of Microbeads*

Polystyrene microbeads (9.76 ± 0.86 μm) were purchased from Polysciences, Inc. (Warrington, PA). For adsorption of PSGL-1, the microbeads (5×10⁷ beads/ml) were washed twice with alternating steps of water and ethanol, followed by incubation with PSGL-1 in 0.1 M borate buffer, pH 8.5 for four hours at room temperature under end-to-end rotation. To facilitate adsorption, PSGL-1 (solubilized in 1% OG) was diluted to
well below the critical micelle concentration of 0.7% in buffer, and was then added to the bead suspension. It is likely that PSGL-1 is adsorbed to the polystyrene microbead through its hydrophobic transmembrane domain which would be the thermodynamically favored anchorage point. PSGL-1, like many cell surface proteins, is highly glycosylated and the hydrophilicity of the active domains have significantly lower chemical potentials for interaction. Adsorption of glycoproteins that are fused to Fc domains or have their transmembrane domains removed by recombinant DNA technology will often adsorb well to plastic, but appear to require consistently much higher concentrations (10 to 100-fold) to achieve equivalent site densities. While the exact orientation of the adhesive glycoprotein on the surface of either the wall of the flow chamber or on the microbead is unknown, the adsorbed receptor retains functional activity similar to that observed for PSGL-1 or P-selectin anchored on cellular membranes that is blockable by the appropriate antibodies. The large size of domains of adhesive glycoproteins in general may contribute to maintaining their functional activity after adsorption onto a surface, particularly if other, non-functional domains provide anchorage. Anchorage of P-selectin or E-selectin to plastic may actually be superior to anchorage in transfected cell lines that may lack critical cytoskeletal components required to keep the adhesive receptor from being extracted. After washing with HBSS, the microbeads were incubated in 0.5% Tween-20 solution for 30 minutes with agitation to block the remaining surface against nonspecific adhesion. The microbeads were stored in HBSS containing 0.5% Tween-20. Prior to use with the laminar flow chamber, the microbeads were resuspended in assay media (HBSS, 10 mM HEPES, pH 7.4, and 1 mM CaCl₂) at a concentration of $5 \times 10^5$ beads/ml.
Additionally, PSGL-1 was covalently coupled to the microbead surface using carbodiimide chemistry. Briefly, 10 μm diameter carboxylate-modified polystyrene microbeads (Polysciences, Warrington, PA) were washed in alternating stages of carbonate (pH 9.6) and phosphate buffer (pH 4.0) following the manufacturer’s instructions. The washing phase was followed by incubation of the microbeads in a 2% carbodiimide solution in phosphate buffer for 3 hours at room temperature on a rotary shaker. The microbeads were then washed repeatedly with borate buffer and then were incubated in a 1 μg/ml concentration of PSGL-1 in borate buffer overnight at room temperature with end-to-end rotational mixing. The PSGL-1 microbeads were then blocked with a Tween-20 solution as described above. In experiments to remove sialic acid residues, PSGL-1 microbeads were incubated with 0.1 U/ml neuraminidase (Calbiochem, La Jolla, CA) for 1 hour at room temperature. Microbeads were then washed twice with HBSS and stored in assay media before use.

**Laminar Flow Assay**

Polystyrene slides were cut from bacteriological Petri dishes (Falcon 1058) and the diluted adhesion molecules were applied to the plates and allowed to adsorb for 2 hours at room temperature. The slides were then blocked for nonspecific adhesion with 0.5% Tween-20 in HBSS for 1 hour at room temperature. The site densities of the adhesion molecules used as a substrate were determined by radioimmunoassay. The P-selectin site densities used were 100 sites/μm² (1:200 dilution) and 25 sites/μm² (1:650 dilution). The wall shear stress of the parallel plate flow chamber (GlycoTech, Rockville, MD) was regulated by the gasket thickness, which was 250 μm thick with a channel width of 5 mm.
and 20 mm long (Fig. 2.2). The wall shear rate and shear stress is a function of the flow rate, fluid viscosity and chamber dimensions:

$$\tau_w = \nu f \gamma_w = \nu f \frac{d\nu}{dy} \bigg|_{y=0} = \frac{6\nu f Q}{wh^2}$$ (2-1)

where $\tau_w$ is the wall shear stress, $\gamma_w$ is the shear rate, $\nu f$ is the fluid viscosity, $u$ is the fluid velocity in the x direction, $Q$ is the flow rate, $w$ is the width of the chamber, and $h$ is the height of the chamber. Cell suspensions were drawn into the chamber at room temperature using a syringe pump (Harvard Apparatus, South Natick, MA). The chamber was mounted over an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Garden City, NY) at 20x magnification. High-resolution differential interference contrast microscopy of neutrophils rolling on P-selectin was obtained using a Kodak MotionCorder Analyzer SR-500 (Eastman Kodak, Motion Analysis Division, San Diego, CA). Neutrophil and microbead motion was viewed at a frame rate of 240 frames per second (fps) with this system. Standard video frame rate of 30 fps was used in all other assays unless indicated.

**Flow Cytometry**

Bead or neutrophil preparations ($1 \times 10^6$ particles/ml) were incubated in 2 μg/ml of primary mAb in HBSS containing 1% HSA for 30 minutes. The microbeads or neutrophils were washed three times and then incubated for 30 minutes with fluorescein isothiocyanate (FITC)-conjugated, goat anti-mouse secondary antibody specific for the Fc region of the primary mAb. Samples were washed and resuspended in 400 μl of 0.5%
paraformaldehyde. Fluorescence was detected with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using incorporated CellQuest software.

**Scanning Electron Microscopy**

Neutrophils were perfused over 200 sites/µm² P-selectin at 2 dyn/cm² wall shear stress for 5 minutes followed by the perfusion of 1% glutaraldehyde. The remaining adherent neutrophils were further fixed in 2.5% glutaraldehyde followed by incubation in 1% osmium tetroxide. With the assistance of Jan Redick at the University of Virginia Center for Advanced Microscopy, the fixed cells were dehydrated through a series of graded ethanol solutions and critical point dried in liquid CO₂. The slides were placed on aluminum stubs with silver paste, coated with gold-palladium, and observed in a JSM-6400 scanning electron microscope (JEOL, Japan).

**Data Analysis**

Images from the VCR playback were digitized on an AG-5 frame acquisition card (Scion Corp., Frederick, MD) installed in a G3 Macintosh (Apple, Inc., Cupertino, CA) with NIH Image v.1.62 (Bethesda, MD). Cell position and velocity measurements of individual particles were acquired using an automated tracking program coded in MATLAB 5 (Cheezum et al., 2001), which uses a sum-of-absolute-difference algorithm to identify the cell in consecutive image frames generated through NIH Image 1.62. Velocity tracings and pause time distribution data was collected using a CCD camera (Vicon Industries, Melville, NY). In order to resolve microbead and neutrophil motion from high-speed video images (240 frames/second), an interpolation routine was
incorporated into the tracking program that allowed sub-image pixel resolution of changes in position. To determine the lifetime of a PSGL-1/P-selectin tether, the time a cell remained bound was determined by counting the number of image frames in which it remained stationary. Cell flux measurements were obtained by individually counting the number of microbeads that transiently attached at least once and firmly adhered in a given area for about 1 minute. Microbeads were considered firmly adherent if they remained bound for >20 seconds.

Geometrical parameters of the system (Fig. 2.3) and hydrodynamic force equations (Goldman et al., 1967) were used to quantitate the specific bond lengths necessary to support rolling with certain dissociation rates. The force and momentum balance equations were:

\[ F_s = F_b \cos \theta \]  
\[ T_s + R \cdot F_s = F_b \cdot l \cdot \sin \theta \]

where \( F_s \) is the shear force, \( F_b \) is the tensile bond force, \( \theta \) is the angle between the tether and lower wall, \( T_s \) is the shear torque, \( R \) is the particle radius, and \( l \) is the lever arm.

The torque and force equations used were as follows:

\[ T_s = 4 \pi \nu \gamma R^3 \left( T_{y}^{\ast} \right) \]  
\[ F_s = 6 \pi \nu \gamma R \delta \gamma \left( F_{x}^{\ast} \right) \]

where \( \nu \) is the fluid viscosity, \( \gamma \) is the shear rate, \( \delta \) is the gap width of the particle centroid to the surface, \( T_{y}^{\ast} \) is the normalized non-dimensional shear-induced torque, and \( F_{x}^{\ast} \) is the normalized non-dimensional shear-induced force. The limiting solution where \( \delta \rightarrow 0 \), \( F_{x}^{\ast} = 0.944 \) and \( T_{y}^{\ast} = 1.7 \) (Goldman et al., 1967).
Figure 2.1  The leukocyte adhesion cascade during inflammation. Leukocyte recruitment is a multi-step process starting with capture and rolling mediated by selectins, firm adhesion mediated by integrins, and migration also mediated by integrins. (Adapted from Jung, et al., 1998.)

Figure 2.2  Parallel plate flow chamber apparatus. The parallel plate flow chamber is composed of a polycarbonate block with an inlet and outlet sandwiched by vacuum with a gasket and slide. The gap in the gasket is 20 mm long by 5 mm wide by 250 μm high. This allows the perfusion of cells over a cellular or molecular substrate that has been immobilized on the slide.
Figure 2.3 Mechanical forces on spherical particles near planar surfaces resulting from fluid shear flow. (A) Initial tether formation of a leukocyte with a P-selectin surface will originate through PSGL-1 bonds present on the microvillus tips. (B) Free body diagram of an interacting cell/bead illustrates the resultant bond force governed by the force (Eq. 2-1) and momentum balance equations (Eq. 2-2).
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The dissociation rate constant, $k_{\text{off}}$, was obtained from the slope of the natural log of cumulative pause events in relation to the pause durations for each wall shear stress. The extreme outliers comprising the longest 10% of events were excluded from analysis to minimize the possible influence of multivalent interactions. The neutrophil $k_{\text{off}}$ value was matched to the curve derived from PSGL-1 microbead interactions with P-selectin describing the bond lifetime versus force relationship to determine the absolute force acting on the ligand-microvillus complex. The force estimated from the bond dissociation constant was inserted in equation (2-5) to determine the correct bond length.

$$F_b = \frac{T_p + R \cdot F_s}{\ell \cdot \sin \theta} = \frac{T_p + R \cdot F_s}{\sqrt{(R + b)^2 - R^2 \left(\frac{R}{R + b}\right)}}$$

(2-5)

with the microbead, the parameters $\ell$, $\theta$, $b$ (the microvillus length), and $R$ are set by the geometry, so the flowrate can be directly related to the force on the bond, $F_b$. Comparing PSGL-1 bead $k_{\text{off}}$ to the observed neutrophil $k_{\text{off}}$ then allows the recalculation of the actual $F_s$ on the bond, and the tether arm length is solved for directly. Values of $R$ for the neutrophil were 4.25 $\mu$m and for the microbead $R$ was 4.9 $\mu$m.

The relationship between the loading force and the dissociation rate constant of the P-selectin/PSGL-1 bond was evaluated using the Bell model (Bell, 1978; Smith et al., 1999).

$$k_{\text{eff}} = k_{\text{off}}^\circ \exp \left(\frac{\sigma F_b}{k_B T}\right)$$

(2-6)

where $k_{\text{off}}^\circ$ is the unstressed dissociation rate constant, $\sigma$ is the bond reactive compliance, $k_B$ is the Boltzmann constant, and $T$ is the absolute temperature. Dissociation constants
were fitted to the Bell model with a nonlinear, least-squares fitting routine using the Levenberg-Marquardt algorithm (IgorPro v4.0; WaveMetrics, Lake Oswego, OR).

2.3 Results

2.3.1 PSGL-1 Presentation on Microspheres

PSGL-1-coated microbeads were developed to explore the bond dissociation kinetics of leukocyte rolling on P-selectin in isolation from cellular factors. PSGL-1 levels on the 10 µm beads were assessed through flow cytometry for two different concentrations of PSGL-1: a 1:100 dilution (0.01 µg/ml) and 1:25 dilution (0.04 µg/ml) of the protein in detergent-free buffer. Flow cytometry using monoclonal antibody KPL1 (Snapp et al., 1998) confirmed the adsorption of PSGL-1 on the microbeads (Fig. 2.4 A-C). A monoclonal antibody to the I-domain of the β2 integrin LFA-1, TS1/22 (Knorr and Dustin, 1997), did not recognize the PSGL-1-coated microbeads (Fig. 2.4 D). Flow cytometry demonstrated comparable levels of PSGL-1 expression on human neutrophils and the 1:100 adsorbed PSGL-1 beads (Fig. 2.4 E and F). Based on a comparison to neutrophils with similar mean fluorescence values and 30,000 PSGL-1 receptors/neutrophil (Moore et al., 1992), the 1:100 dilution of PSGL-1 was estimated to adsorb onto the polystyrene microbeads at a site density of approximately 95 sites/µm².

To match the PSGL-1 surface density of the microbeads with that of neutrophils as closely as possible, the 1:100 concentration of adsorbed PSGL-1 was used in the reported experiments. Binding of KPL-1 antibody to PSGL-1 microbeads and other surfaces suggested an intact antigen epitope still existed subsequent to immobilization (Ramos et al., 1998; Snapp et al., 1998).
Figure 2.4 Flow cytometry results displaying presence of PSGL-1 on the microbeads. (A-C) Varying concentrations of PSGL-1 were adsorbed onto 10-μm microbeads. The microbeads were then incubated with mAb KPL-1, specific for PSGL-1, followed by an FITC-conjugated goat anti-mouse secondary antibody. (D) TS1/22, antibody to LFA-1, isotype control experiment on the PSGL-1 coated microbeads. (E) KPL-1 binding to PSGL-1 on human neutrophils. (F) Isotype control experiment for neutrophils with E-selectin mAb BB11.
2.3.2 Specificity of PSGL-1 Interactions with Immobilized P-selectin

PSGL-1 microbead flux was quantified to assess the tethering frequency to purified P-selectin under shear and to establish the specificity of interaction. Rolling has been previously demonstrated using both a N-terminal 19 amino acid sequence of PSGL-1 or sialyl Lewisx glycoconjugates immobilized on a microbead surface (Brunk et al., 1996; Brunk and Hammer, 1997; Goetz et al., 1997; Rodgers et al., 2000). We used surface immobilized purified P-selectin as the substrate rather than CHO cells transfected with P-selectin (Goetz et al., 1997; Ramachandran et al., 1999) to minimize potential effects of membrane deformation of cellular substrates.

PSGL-1 microbeads bound to purified P-selectin coated substrates in flow and were detected by videomicroscopy (Fig 2.5). Most PSGL-1 microbeads formed transient interactions lasting less than 2 seconds before continuing on to form successive adhesive events. Transient interactions appeared to be similar in qualitative terms to neutrophil rolling on sparsely coated selectin substrates. Non-specific, firm adhesion events were infrequently observed. On average, less than 0.3% of the microbeads contacting the surface formed firm adhesions as defined by pauses lasting longer than 20 seconds. The firm adhesion events were EDTA-insensitive and tended to not occur after several minutes of flow.

In order to determine the role of sialic acid residues of PSGL-1 in P-selectin recognition independent of cellular membrane carbohydrate, the PSGL-1 beads were treated with 0.1 U/ml neuraminidase (Fig 2.6). Enzymatic removal of sialic acid of PSGL-1 on microbeads eliminated (>98%) interactions with the P-selectin substrate, suggesting that tyrosine sulfation of PSGL-1 is by itself insufficient to mediate microbead
rolling at forces associated with 0.7 dyn/cm² wall shear stress (estimated 340 pN/bond for a 92 nm bond length and lever arm of 0.95 μm [Eq. 2-5]). Interactions between desialylated PSGL-1 with P-selectin appear to be detectable at lower forces as recently reported (Rodgers et al., 2001). The PSGL-1 microbead rolling was inhibited by KPL1, a monoclonal antibody to PSGL-1, confirming receptor specificity. The presence of 5 mM EDTA in the media completely abolished adhesion by chelating calcium cations.

PSGL-1 or P-selectin receptor detachment for the polymer surface may be a possible mechanism of cell dissociation during rolling interactions. To address this concern, 4 μg/ml purified PSGL-1 was coupled to carboxylate microbeads through carbodiimide chemistry. Microbeads thus prepared supported rolling with pause times similar, if slightly briefer, than microbeads with adsorbed PSGL-1 (Table 2.1). Adsorption of PSGL-1 appeared to have created an adequate anchorage for micro bead rolling at the shear forces tested.

<table>
<thead>
<tr>
<th>Shear Stress (dyn/cm²)</th>
<th>Covalently Coupled</th>
<th>Physically Adsorbed</th>
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<tr>
<td></td>
<td>k&lt;sub&gt;off&lt;/sub&gt; (1/s)</td>
<td>R²</td>
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<tr>
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<td>6.2</td>
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</tr>
<tr>
<td>1</td>
<td>13</td>
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Table 2.1 Comparison of PSGL-1 attachment with bead dissociation rate. k<sub>off</sub> values obtained for 4 μg/ml PSGL-1 microspheres adhering to P-selectin at 100 sites/μm²

2.3.3 Rolling Comparisons of PSGL-1 Microbeads and Neutrophils

A representative PSGL-1 microbead velocity profile indicated that rolling on P-selectin consisted of distinct start and stop motions (Fig. 2.7 A). Between pauses, the microbeads traveled at the free-stream velocity. At the same wall shear stress,
neutrophils displayed a pattern of longer pause events interspersed with smaller steps and lower velocities between pauses (Fig. 2.7 B). Comparison of Figures 2.7A and 2.7B demonstrates the striking difference in rolling dynamics between beads and neutrophils despite both having similar PSGL-1 surface densities.

To explore the role of microvillus extension and microvilli presentation of PSGL-1 as the source of the significant differences between the rolling dynamics of microbeads and neutrophils, glutaraldehyde-fixed neutrophils were compared to the PSGL-1 microbeads. Fixation alters the viscoelastic properties of the neutrophil by chemically cross-linking the free amino groups on various proteins (including the cytoskeleton) with each other, effectively “freezing” the neutrophil into a significantly more rigid and less deformable shape (sufficient to allow thin sectioning for electron microscopy). The fixation process preserves antigenicity of PSGL-1 and L-selectin binding epitopes and does not rearrange their surface distribution (Hasslen et al., 1996). The possibility that glutaraldehyde fixation may alter selectin bio-recognition and related molecular properties, such as the receptor’s bond compliance cannot be ruled out at this time. The fixed neutrophils had dynamic adhesion properties much closer to those of the coated microbeads than to the untreated neutrophils (Fig. 2.7 C). In particular, they possessed the same distinctive stop and go activity as the ligand-coated microbeads, clearly approaching critical velocity between pauses (Fig. 2.8 A). Pause durations of fixed neutrophils were intermediate between the microbeads and untreated neutrophils at 0.5 dyn/cm² wall shear stress.

The behavior of the PSGL-1 microbeads and the fixed and non-fixed neutrophil populations were further contrasted by determining their average rolling velocities. The
PSGL-1 microbeads, with surface densities approximating those of neutrophils, rolled faster than the fixed or untreated neutrophils despite significantly higher P-selectin substrate densities used to insure a comparable number of interactions of microbeads (Fig. 2.8B). Neutrophils traveled only 7.4% of the speed of the PSGL-1 beads while fixed neutrophils were found to roll at 31.3% of the velocity of the microbeads.

2.3.4 Pause Time Distributions Reveal Kinetic Properties of Selectin Bonds

The role of bond lifetimes in the observed rolling dynamics of the microbeads was investigated in order to isolate the PSGL-1/P-selectin binding properties from the contributions due to neutrophil microvilli deformability (Shao et al., 1998; Smith et al., 1999). Transient interactions between PSGL-1 microbeads and the P-selectin substrate averaged approximately 0.076 seconds in duration at a wall shear stress of 0.5 dyn/cm² (Fig. 2.9) while the average of neutrophil pauses was much longer, at 0.317 seconds. Glutaraldehyde-fixed neutrophils were observed to exhibit an intermediate mean pause lifetime of 0.215 seconds.

Selectin bond dissociation can be described through first order reaction kinetics when the interactions are dominated by single bond events, or bond clusters that behave functionally like single bonds. The dissociation constants ($k_{off}$) of neutrophil and microbead PSGL-1 from P-selectin, calculated from pause time distributions, increased with increasing wall shear stress (Fig. 2.9), consistent with previous observations of all three selectins (Smith et al., 1999). The $k_{off}$ values for the PSGL-1 microbead system on P-selectin ranged from 3.1 to 14.7 seconds⁻¹ at wall shear stresses of 0.11 to 0.5 dyn/cm² (Fig. 2.9 A). The dissociation rate constants of the PSGL-1 beads were significantly faster than those of neutrophils when matched at the same wall shear stress (Fig. 2.9 B).
On P-selectin, neutrophil dissociation constants ranged from 3.6 to 10.2 seconds\(^{-1}\) as the wall shear stress increased from 0.4 to 2.0 dyn/cm\(^2\).

The force on a bond for the rigid bead can be calculated from the relationship: \(F_b = F_s / \cos \theta\), where \(\theta\) is evaluated for a bond length of 92 nm (Li et al., 1996; Ushiyama et al., 1993), \(F_s\) represents the shear force from flow, and \(F_b\) represents the resultant force on the bond (Fig. 2.10). Nonlinear fitting of the microbead dissociation kinetic constants to the Bell model (Eq. 2-6) produced a bond separation distance (\(\sigma\)) (also described as reactive bond compliance) of 0.37 Å and an unstressed dissociation constant (\(k_{off}\)) of 1.5 seconds\(^{-1}\) which agreed closely with previous observations (Alon et al., 1995b; Smith et al., 1999). The nonlinear fit of the Bell equation to the \(k_{off}\) data of the microbeads was better (\(\chi^2 = 1.19\)) than the fit of data from a similar analysis of rolling neutrophils (\(\chi^2 = 6.7\)) (Smith et al., 1999), suggesting that PSGL-1/P-selectin interactions mediated through microbeads fit the Bell model better than in a cellular context.

In contrast to observations with PSGL-1 microbeads, the \(k_{off}\) values of neutrophils tethering on P-selectin exhibited a plateau effect as the wall shear stress was increased up to and above 1 dyn/cm\(^2\), as previously observed (Smith et al., 1999). There was no apparent plateau in dissociation constants observed with the PSGL-1 microbeads at estimated forces of the same or greater magnitude (Fig. 2.9 D). It is possible that at higher flow rates a plateau in PSGL-1 microbead \(k_{off}\) might exist; however, the low frequency of microbead tethering and the brevity of rolling interactions precluded reliable statistical sampling. Fixed neutrophils appear to dissociate with relatively similar off-rates as resting neutrophils at low wall shear stresses (0.4 – 0.7 dyn/cm\(^2\)), but their dissociation constants increased exponentially with wall shear stress (Fig. 2.9 C and D).
Figure 2.5 PSGL-1 bead interactions with P-selectin substrate. Video image averaging over a 1 second duration illustrates the different interaction times of the adsorbed PSGL-1 beads flowing at a wall shear stress of 0.7 dyn/cm² over 90 sites/μm² P-selectin substrate. Green circles represent the start position and the red circles represent the final position at the end of 1 second for each bead. Cell flux interactions of the adsorbed PSGL-1 beads.
Figure 2.6 Microbead flux interactions of PSGL-1 beads at 0.7 dyn/cm² wall shear stress over 90 sites/µm² P-selectin substrate. The flux interactions were assessed in a 256,000 µm² observable field of view. The blue bars represent transiently adhered microbeads while the red bars represent beads that were firmly adherent for more than 20 seconds. Microbeads treated with neuraminidase (N’dase) or KPL-1 were shown to inhibit attachment to the substrate. Addition of EDTA was shown to eliminate transient interactions. Microbeads immobilized with P-selectin mAb G1 (10 µg/ml) were able to interact with the P-selectin plate. Error bars represent one standard deviation.
Figure 2.7 Instantaneous velocity profiles of cell and microbead rolling at 0.5 dyn/cm² wall shear stress. Free-flow velocity near the plate surface was approximately 120 μm/second. (A) PSGL-1 microbead rolling over 90 sites/μm² P-selectin. (B) Neutrophil rolling over 12 sites/μm² P-selectin. (C) Fixed neutrophil rolling over 12 sites/μm² P-selectin. Dashed line indicates the hydrodynamic velocity for each respective particle system with a gap width of 100 nm between the particle and the surface.
Figure 2.8 Translocation of PSGL-1 microbeads, fixed neutrophils, and untreated neutrophils. Average velocity of particles between pause events at 0.5 dyn/cm² wall shear stress (A). Average rolling velocities at 0.5 dyn/cm² wall shear stress (B). Error bars represent one standard deviation.
Figure 2.9  Pause time distributions used to calculate the bond dissociation constants of the PSGL-1 microbeads interacting with the P-selectin substrate. The dissociation constant values, $k_{off}$, correspond to the negative slope of the logarithmic plots of the cumulative distributions. Shown are the cumulative distributions of pause times that indicate the existence of an exponential distribution of pause time durations versus number of interactions. Dissociation constant ($k_{off}$) values were calculated for the (A) PSGL-1 microbeads, (B) untreated neutrophils, and (C) fixed neutrophils. The extreme outliers comprising the longest 10% of events were excluded from the linear regression. (D) Relationship between the off-rate of PSGL-1 and P-selectin relative to the wall shear stress. Data from the PSGL-1 microbeads rolling on 90 sites/μm² P-selectin, along with that of the fixed neutrophils and untreated neutrophils rolling on 12 sites/μm² P-selectin.
Figure 2.10  Effect of lever arm elongation on increasing interaction time under shear flow. PSGL-1 microbead dissociation rates were recalculated with longer lever arm lengths at different wall shear stresses (A). Experimental data was analyzed using longer lever arm lengths (B). Green line indicates the experimentally measured data. Lever arm of 3.06 μm is an initial lever arm estimated for neutrophil rolling (Alon et al., 1995). Increasing the tether/lever arm length of the bond interaction inhibits bead dissociation at higher wall shear stress.
2.3.5 Microvillus Extension Lowers Resultant Force on the Bond

Defining a lever arm length \((l)\) allows the calculation of force exerted on the receptor-ligand bond \((F_c)\) under various wall shear stresses when a cell or microbead is anchored to the substrate. Without the lever arm value, it is impossible to estimate the actual force acting on a bond holding a sphere in static equilibrium in shear. Figure 2.3 shows a graphic representation of the PSGL-1 bead binding to the P-selectin plate under flow with the relevant applied forces in static equilibrium, i.e., all forces are balanced. The lever arm calculated for a microbead (radius = 4.9 \(\mu m\)) was determined to be a maximum of 0.95 \(\mu m\) with a bond angle of 79° based on the geometrical constraints of a sphere in contact with a flat surface (Kuo and Lauffenburger, 1993) and 92 nm for the length of the P-selectin/PSGL-1 bond (Li et al., 1996; Ushiyama et al., 1993). The combination of the bond and microvillus is referred to as the tether length. Therefore, the bonds formed between PSGL-1 microbeads and P-selectin at 1 dyn/cm\(^2\) wall shear stress would be subjected to 480 pN of force. In contrast, the lever arm of a neutrophil was first estimated to be a constant 3.06 \(\mu m\) based on flow reversal measurements (Alon et al., 1997), resulting in a force per bond estimate of 124.4 pN at 1 dyn/cm\(^2\) wall shear stress. Despite the limitations of flow reversal measurements (dead times and unsteady flow velocities on the order of several P-selectin bond lifetimes), the initial estimates of neutrophil tether arm length informed by knowledge of static microvilli lengths were reasonable starting points.

At a given fluid shearing force, a longer lever arm requires the bond to support less force in order to balance the torque generated by the shear flow acting on the leukocyte or cell. As \(l\) increases, \(\theta\) decreases which in turn reduces the force on the bond.
Chapter 2 ~ Microvilli Extension Stabilizes Leukocyte Rolling

\( F_b = F_c / \cos \theta \) as \( \cos \theta \) increases. The decrease in \( F_b \) resulting from a longer lever arm will decrease the selectin dissociation constant as predicted by the Bell model (Fig 2.10 A). Consequently, a lever arm length such as estimated for a neutrophil will necessitate a higher wall shear stress to achieve the same dissociation kinetics as observed for the PSGL-1 microbeads (Fig. 2.10 B). A doubling of lever arm length in the flow rates analyzed in this study would be estimated to reduce P-selectin \( k_{off} \) by as much as four-fold at 0.5 dyn/cm\(^2\) wall shear stress, likely having an even more significant effect on rolling velocity and new bond formation rates at higher forces such as those found \textit{in vivo}.

Hypothesizing that the differences in the off-rate constants between PSGL-1 microbeads and neutrophils were due to the extension of microvilli, the corresponding microvillus length was calculated at each wall shear stress in order to superimpose the neutrophil dissociation constants onto that of the PSGL-1 microbeads when \( k_{off} \) is plotted against \( F_b \) rather than wall shear stress (Fig. 2.11). Equating the dissociation constant of the PSGL-1 microbead to those of neutrophils allowed determination of the force acting on the microvillus by using the characteristic bond lifetime of PSGL-1/P-selectin as a direct measure of the force on the bond. The lever arm was then recalculated on the neutrophil and thus the microvillus length at a given wall shear stress.

A comparison of PSGL-1 microbeads with the fixed neutrophils (Fig. 2.11) indicated the average microvillus length of the fixed neutrophils was 0.25 ± 0.07 µm throughout the range of shear forces investigated and did not increase with flow. The microvillus length on fixed neutrophils appeared to be constant, a conclusion supported by the close match of the \( k_{off} \) to that of the PSGL-1 microbeads at equivalent forces. The
estimate of microvillus length of 0.25 microns scaled closely to values from electron
microscope measurements of 0.3 microns for resting neutrophils (Bruehl et al., 1996;
Hasslen et al., 1996; Shao et al., 1998),

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Table 2.2 Microvillus lengths and associated spring constants for non-fixed neutrophils. The spring constant was calculated by dividing the force on a single tether/bond anchorage point (pN) by the change in neutrophil microvillus length (µm) that was determined by comparing neutrophil and microbead k_{off} constants. Microvillus extension is modeled as Hookean spring, so that \( F = k \times L \), with the spring constant \( k \) in units of (pN/µm) and the length \( L \) in units of (µm), i.e., the extension of the microvillus. Strain calculated by dividing \( \Delta l \) by the resting microvillus length.

By mapping the unfixed (resting) neutrophil data onto that of the rigid beads and fixed neutrophils, the neutrophil microvillus length was calculated to range from 0.17 µm at 0.4 dyn/cm² wall shear stress up to 1.82 µm at 2 dyn/cm² (Table 2.2). A spring constant was then calculated from the relationship between the \( F_b \) at rupture and the change in length of the microvillus, which increased non-linearly with force. For extensions estimated to be on the order of a 0.1 µm, a length that represents 30% strain (large compared to strains typically observed in non-biologic material deformation), the spring appeared to be very stiff. As force was increased, it appeared that it became progressively easier to extend the microvillus. At a wall shear stress of 2 dyn/cm² and
over 200 pN at the anchorage point, the microvillus spring constant appeared to have fallen almost 30-fold in stiffness, approaching more closely values measured by micropipette aspiration (Shao et al., 1998). Varying average microvilli lengths from a resting value of approximately 0.17 μm to more than 1.8 μm completely reconciled the $k_{\text{eff}}$ versus $F_b$ relationship obtained with beads and unfixed neutrophils (Fig. 2.11 and Table 2.2). Similarly, the assumption of a noncompliant 0.25 μm microvilli on fixed neutrophils completely reconciles the $k_{\text{eff}}$ versus $F_b$ relationship obtained with PSGL-1 microbeads.

2.3.6 High-Speed Video Analysis of Microvillus Viscoelasticity

Using high-temporal resolution video microscopy and sub-pixel image interpolation, time-dependent microvillus extension during rolling was estimated from the deceleration of the neutrophil as it formed a bond with P-selectin (Fig. 2.12 A). By tracking the motion of the neutrophil immediately following tethering, the “creeping” displacement of the neutrophil centroid could be measured using high-speed video (4 ms per image frame). Motion of neutrophils following a P-selectin bond formation event and before bond dissociation was typically less than a micron and considerably less than the distance between selectin mediated pauses at the low site densities examined. It was hypothesized that the submicron displacement was related to or a consequence of stretching of the anchored microvillus following a bond formation event. The time-displacement curve created by deceleration of the tethered neutrophil allowed the estimation of both the viscous and elastic components of the microvillus.

Neutrophil skipping motions on P-selectin were obtained with a temporal resolution of 240 frames/second. Five representative trajectories showing a bond release
event followed by a discrete bond formation event were used to generate a displacement (stretch) versus time curve. Microvillus stretching slows the deceleration of the neutrophil, as suggested by the much steeper deceleration curve of a PSGL-1 microbead under the same flow conditions (Fig. 2.12 A). The time duration between motion of a PSGL-1 microbead in flow and a detectable pause on the substrate was one camera frame rate or 4 milliseconds. There was no detectable pivot motion or stretching for PSGL-1 microbeads that formed tethers on P-selectin at the limit of the computerized image interpolation algorithm. For the last time point before neutrophil detachment, the lifetime of the PSGL-1/P-selectin bond corresponded to a 42% bond breakage probability. Immediately following tethering, the “creeping” displacement of the neutrophil centroid measured using high-speed video was less than 4% of the hydrodynamic velocity. It was hypothesized that the submicron displacement of the neutrophil’s centroid was a consequence of stretching of the anchored microvillus following a bond formation event.

The mechanical behavior of the microvillus was modeled using a Voigt viscoelastic model (Fig. 2.13 A). In the Voigt model, a viscous element dampens the temporal response of the spring to changing forces, but does not affect the final displacement in response to a given force. Consequently, at long times, as shown in Figure 2.13 B, displacement as predicted by the Voigt model reached a maximum. Changes in the estimated force on the bond for a representative microvillus extension (Fig. 2.13 B) are concentrated in the first 15% of the median lifetime of the tether. As the tether linkage (bond plus stretched microvillus) lengthened, the resultant force changed progressively less for each unit of length extension. Long duration (>1 second) of creeping-like motions (Schmidtke and Diamond, 2000; Shao et al., 1998) were not
observed in any of the PSGL-1 microbead interactions with P-selectin but could be easily observed when neutrophils rolled on P-selectin.

Under 2.0 dyn/cm² wall shear stress, or 209 pN maximum force on the selectin bond, measurements of the microvillus spring constant ranged from 133.5 – 228.1 pN/μm and the uniaxial viscosity varied from 2.66 – 5.28 Pa·s·μm (Table 2.3). An average spring constant obtained from the five tethers was 170 ± 40 pN/μm, while the average uniaxial viscosity was 3.9 ± 1.1 Pa·s·μm for 209 pN of tensile force. The spring constant estimated by comparison of neutrophils to microbead dissociation constants (koff) was 152 pN/μm, a very close match of essentially independent approaches to the determination of the microvilli elastic properties at brief extensions characteristic of neutrophil rolling interactions. Visualized PSGL-1 tether extensions of microvilli under high magnification were also plotted; they matched at the short time points very well with the microvillus extension determined from neutrophil displacement subsequent to tether formation. At longer times (> 0.1 seconds), the elastic structure of the microvillus appears to break down, and the extension appears to be determined largely by the viscous properties of the cytoplasm filling the tether, as shown by the steady extension of the tether with time at constant force (Schmidtke and Diamond, 2000). The presence of microvillus tethers modulating neutrophil rolling could be observed in the scanning electron micrographs (SEM), as shown by a representative image in Figure 2.14. The neutrophil exterior maintains its characteristic ruffled appearance during rolling on P-selectin, even with the microvilli trailing the cell body. A few of the adherent cells displayed two or three tethers. The presence of these multiple tethers is believed to be a result of the high P-selectin site density (200 sites/μm²) of the substrate. This site density was chosen
because the approximately half of the neutrophils rolling on the substrate had a tendency to detach upon the entry of glutaraldehyde into the flow chamber and with lower site densities too few neutrophils would have remained bound for reliable analysis.

Microbead and neutrophil rolling experiments were conducted under conditions of lower site densities to ensure a high proportion of single or quantal bond interactions. A majority of the extensions examined under SEM were observed to be single or double tethers ranging from 1-3 μm. This measurement correlates very well with the dynamic analysis of microvilli extension at 2 dyn/cm². The calculated estimate of 1.58 μm and the visual surveillance of tethers 1-3 μm in length are consistent in scale. This further strengthens the conclusion that microvilli extension is a fundamental component of neutrophil rolling.

<table>
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Table 2.3 Viscoelastic measurements of microvilli tethers. Displacement data from transiently tethered neutrophils at 2 dyn/cm² wall shear stress were analyzed using a Voigt model of structural deformation.
Figure 2.11 Off-rate of the PSGL-1/P-selectin bond as a function of the force exerted on the linkage. Comparison of the $k_{\text{off}}$ of neutrophils, glutaraldehyde-fixed neutrophils, and PSGL-1 coated microbeads. Data from the ligand coated microbeads were fitted to Bell’s equation (green line). The unstressed dissociation constant ($k_{\text{off}}^{0}$) was 1.83 seconds$^{-1}$ with a bond compliance (σ) of 0.36 Å. The arrows indicate the direction of the force change necessary to equate the molecular dissociation properties of PSGL-1 on the beads with PSGL-1 expressed on neutrophils. Initial lever arm length predictions for the untreated neutrophils (0.25 μm) appear underestimated at higher ranges. Fixed neutrophils were plotted with the same data from the PSGL-1 microbeads and microvilli lengths at each force measurement were calculated to extract an average microvillus length of 0.25 μm (Inset).
Figure 2.12 Measurement of dynamic microvilli extension directly from velocity profile. High-speed resolution of PSGL-1 microbead tethering (A) and neutrophil attachment (B) at 1.0 dyn/cm² on P-selectin. High temporal resolution of 240 frames/second (4 msec/frame) can discern the minuscule motions of the neutrophil after an attachment has formed with the substrate (C).
Figure 2.13  Viscoelastic measurements of tether elongation during neutrophil rolling.  Schematic of the Voigt viscoelastic model and the governing equations (A).  Neutrophil displacement data (®, ◇, +, △) after initial attachment under 2.0 dyn/cm² wall shear stress. The predicted force loaded on the selectin bond (×) during the elongation process was calculated for the cell labeled + (B).
Figure 2.14 Surface topography of rolling neutrophils on 200 sites/μm² P-selectin. Neutrophils were subjected to 2 dyn/cm² wall shear stress in assay media prior to infusion of 2.5% glutaraldehyde at the same flow rate. (A) Low magnification images of two adjacent rolling neutrophils showing a singlet and doublet tether restraining the cells. (B) Low magnification and (C) high magnification image of a tethered neutrophil. The arrow shows a possible tether just beginning to form. Bars, 1 μm.
2.4 Discussion

Comparison of the dynamics of rolling neutrophils with PSGL-1 microbeads suggested that the elasticity of structures such as microvilli that display PSGL-1 significantly lowered the stresses on PSGL-1 bonds with P-selectin during rolling. The ability of microvilli to elongate under force appeared to be much greater than the elasticity of the PSGL-1/P-selectin molecular linkage (Alon et al., 1997; Howard, 2001) and may therefore serve to stabilize neutrophil rolling interactions with vascular endothelium in vivo. PSGL-1, like L-selectin, is localized on the tips of microvilli (Bruehl et al., 1996; Moore et al., 1995) or more precisely, on microridges formed by the neutrophil’s excess membrane area (Schmid-Schonbein et al., 1980). Presentation of PSGL-1 on the tips of leukocyte or neutrophil microvilli has been hypothesized to facilitate capture or tethering under flow conditions, presumably by concentrating binding sites on the point first in contact with an apposing cell surface (Moore et al., 1995; Picker et al., 1991; von Andrian et al., 1995). In addition it has been recently hypothesized that the position of PSGL-1 on microvilli tips may facilitate neutrophil rolling on P-selectin, particularly if microvilli can stretch under physiologically relevant forces (Schmidtke and Diamond, 2000; Shao et al., 1998).

The effect of force on non-covalent selectin bond lifetimes has been quantified by analysis of the time-varying motions of leukocytes as they roll in shear (Alon et al., 1995b; Kaplanski et al., 1993; Smith et al., 1999). In parallel, studies of leukocyte rolling using microbead systems have demonstrated that the physical chemistry of selectins imparts the ability to support rolling interactions in the absence of cellular factors (Goetz et al., 1997; Rodgers et al., 2000; Rodgers et al., 2001). A significant advantage of the
rigid microbead system exploited in this study is that bond formation is limited to a small area within reach of the adhesive receptors on the respective surfaces of the bead and substrate. For instance, the force on an selectin anchorage point is constrained to a minimum 480 pN per dyn/cm² wall shear stress by the length of the stressed PSGL-1/P-selectin bond (Fritz et al., 1998; Li et al., 1996; Ushiyama et al., 1993). Unlike the case with a microbead, however, the force on a neutrophil’s PSGL-1/P-selectin bond cluster additionally depends on the microvillus length and any other structure that contributes to the tether arm. Analysis of tethered neutrophil motion during rapid flow reversal has previously suggested microvilli are approximately one micron in length, a value 3-fold greater than estimates based on electron micrographs (Alon et al., 1997).

In contrast to the model of a rigid microvillus, recent micropipette measurements indicate that neutrophil microvilli might be elastic enough to extend in response to forces which neutrophils might encounter in vivo (Shao et al., 1998). Pulling of neutrophil membrane tethers by micropipette aspiration suggests that under a stress of 34 pN or less, microvilli exhibit a spring constant of 43 pN/μm (Shao et al., 1998). With forces in this range the microvillus eventually reaches an extension of approximately 1 μm, resulting in a lower force on the bond due to the movement of the anchorage point relative to the neutrophil during rolling. A transition regime above 34 pN and below 61 pN also exists which both elastic behavior and membrane “tether” formation can be observed. At stresses above 61 pN, most microvilli convert to tethers (Shao et al., 1998) and extend steadily with time. Membrane tethers pulled from microvilli may appear therefore to have substantially different viscoelastic properties depending on the force applied and duration of pulling.
Chapter 2 ~ Microvilli Extension Stabilizes Leukocyte Rolling

The ability of microvilli to stretch and form membrane tethers as neutrophils roll on immobilized platelets and P-selectin substrates has been directly observed by high-resolution microscopy (Schmidtke and Diamond, 2000) and takes place at forces considerably below what is necessary to significantly deform a rolling neutrophil (Cao et al., 1998; Firrell and Lipowsky, 1989; Lei et al., 1999). Consequently, in this study I focused my comparison of microbeads and neutrophils at forces where the influence of microvilli elongation on rolling dynamics would be predicted to be most apparent. Critical to the comparison of rolling dynamics of microbeads and neutrophils was the assumption that PSGL-1 bonds with P-selectin shared the same mechanical properties in the two systems. In support of this assumption, even the N-terminal 19 amino acid truncated form of PSGL-1 embodies sufficient structure to support microbead rolling on P-selectin (Goetz et al., 1997; Rodgers et al., 2000). The significantly longer native form of PSGL-1 used in this study is likely to be even less affected by the surface properties of the microbead. Additionally, purified PSGL-1 immobilized on surfaces has also been shown to support L-selectin dependent neutrophil rolling (Ramos et al., 1998; Walcheck et al., 1996), supporting the hypothesis that purified and cellular PSGL-1 have similar functionality. Therefore, with the assumption that PSGL-1 receptors on microbeads and neutrophils share equivalent molecular mechanics, it became possible to exploit the stochastic properties of P-selectin bonds as a reporter for the loading force on the bond.

A second major assumption underlying the comparison of PSGL-1 microbead rolling dynamics to those of neutrophils is that the same numbers of PSGL-1/P-selectin bond clusters are being compared in the two systems to determine the dissociation constant. If the bond clusters mediating microbead and neutrophil tethering were
significantly different in number or size then the conclusion that microvillus extension
imparts greater dynamic stability on a rolling neutrophil would be subject to uncertainty.
The effect of multiple bonds has been recently examined in the special case of the
influence of dimerization on PSGL-1 and P-selectin interactions (Ramachandran et al.,
2001). When dimeric P-selectin is compared to monomeric P-selectin, the apparent \( k_{off} \)
of neutrophils is lowered, presumably because at least two bonds are supporting the load.
As a result, the entire force versus dissociation constant curve is flattened and the bond’s
apparent reactive compliance is lowered. By contrast, the dissociation constants of
neutrophils rolling on P-selectin shift from an exponential dependence on force to an
almost linear dependence at 1 dyn/cm\(^2\) wall shear stress (Smith et al., 1999). In order for
such a shift to be a consequence of a shear induced increase in bond number, the effect
would have to be extremely abrupt, almost as if a switch were flipped and the neutrophil
interactions became mediated by multiple rather than single bonds. An alternative
explanation is that the shift to a linear \( k_{off} \) increase may indeed reflect the effect of
microvilli extension. At this time it is clear that further modeling analysis, perhaps using
adhesive dynamics simulations (King and Hammer, 2001), will be necessary to fully
explore the consequences of the potentially complex force distributions that might exist
during multibond rolling interactions.

While it is difficult at this time to resolve the question of whether multiple or
single bonds are controlling PSGL-1 microbead and neutrophil rolling, experimental
evidence offered some support for the validity of the quantal bond cluster hypothesis.
The strongest evidence was the apparent absence of small bond breakage events leading
to steps during the adhesive interactions of both microbeads and neutrophils while
tethering (or skipping) on P-selectin. In particular, PSGL-1 microbeads decelerated within an average of 4 milliseconds from hydrodynamic velocity and became stationary until release from the surface. In order to escape detection, the P-selectin bond with PSGL-1 would have to be briefer by almost an order of magnitude than previously supposed (Alon et al., 1995b; Smith et al., 1999; Tees et al., 2001) and be closer to a sub-millisecond lifetime as recently proposed for L-selectin interactions with PSGL-1 (Evans et al., 2001). In the case of neutrophils, motion subsequent to tethering was very slow and appeared to be dominated by viscoelastic extension of a putative microvillus structure. Neutrophil steps between adjacent bonds stressed in series would have to be smaller than 20 nm and considerably briefer than 4 milliseconds to escape detection. A further point to be weighed in the question of possible differences in the number of bonds being compared between microbeads and neutrophils is that $k_{off}$ estimates matched most closely at low forces where the effect of microvilli stretching would be expected to be least significant.

Consistent with the estimates derived from the PSGL-1 microbead and neutrophil bond cluster lifetime comparisons, scanning electron microscopy of neutrophils rolling on P-selectin indicated that microvillus tether lengths of 1 to 3 microns were frequent at 2 dyn/cm$^2$ wall shear stress as predicted by the bond lifetime analysis. While the apparent stiffness of the microvillus spring constant decreased 30-fold between 0.4 and 2.0 dyn/cm$^2$ wall shear stress, its lowest value was still at least four-fold higher (stiffer) than that estimated using monoclonal antibody anchored tethers (Shao et al., 1998). One explanation for the discrepancy between the two sets of observations may be the significant difference in the time-scale of the measurements. It is important to note that
only a few of the neutrophil pauses analyzed in this study lasted as long as the shortest-lived membrane tether pulled by micropipette aspiration. Over the broad range of pulling times examined in this study and that of Shao and co-workers (1998), microvillus mechanical stiffness might vary significantly as cytoskeletal elements disassemble or otherwise rearrange under the applied loads (Fig. 2.15). For instance, the long membrane tethers occasionally formed by neutrophils rolling on P-selectin extended steadily, much like those detected during micropipette pulling experiments.

Another important consideration in the interpretation of the PSGL-1 microbead data is the potential contribution of cell body deformation to increasing the neutrophil contact area with a P-selectin expressing surface. The moment the neutrophil is anchored by a bond it experiences a force perpendicular to the plane of the substrate from the fluid shear induced torque. If the neutrophil were flattened to any significant degree its contact area with the substrate would be expected to increase, enhancing the likelihood of additional bond formation (Lawrence et al., 1997). In fact, blood flow in vivo can visibly deform a rolling neutrophil into a more hydrodynamic profile, which likely increases the contact area with the vascular endothelium (Firrell and Lipowsky, 1989). In vitro flow chamber studies indicate that an increase from 4 to 20 dyn/cm² wall shear stress can compress the height of an HL-60 myeloid leukemic cell by as much as two microns and double the contact area with the substrate (Lei et al., 1999). Under the flow conditions (<2 dyn/cm² wall shear stress) at which I examined neutrophils, the reduction in height would be calculated to be less than 0.2 μm, significantly constraining the potential for contact area increase and bond formation at the low site densities examined. At this time, however, the effect of neutrophil flattening as a possible explanation for the differences
in behavior compared to microbeads and fixed-neutrophils in this study cannot be rigorously excluded.

The highly elastic nature of microvilli in conjunction with whole cell deformation would appear to have a significant influence on the strength of neutrophil rolling interactions *in vivo*, particularly when coupled with the effects of force-sensitive adhesive bonds. Mathematical modeling efforts have suggested that selectin bonds must be very close to 'ideal' bonds, in that their lifetimes must be nearly force independent to support rolling interactions over the wide range of fluid shear forces that exist *in vivo* (Chang et al., 2000; Hammer and Apte, 1992). However, direct measures of selectin bond lifetimes under stress (Alon et al., 1995b; Smith et al., 1999) indicate that breakage increases more rapidly with force, i.e. selectins appear to be 'slip' bonds (in the Bell model, the bond separation distance ($\sigma$) is greater than 0) rather than 'ideal' ($\sigma = 0$) to use the terminology of Dembo and colleagues (Dembo et al., 1988). Recent studies have suggested the presence of 'catch' bond ($\sigma < 0$) behavior in P-selectin/PSGL-1 and L-selectin/PSGL-1 interactions (Evans et al., 2004; Yago et al., 2004). The ability of catch bonds to form more stable attachments at higher loading forces may cooperate with the force dampening mechanism of microvilli elongation to reduce the apparent dissociation rate of selectin bonds.

Microvillus extension, by shielding the selectin bond from the torque it would experience if anchored on a non-extensible surface, could contribute to creating an ideal bond, or more properly, an 'ideal linkage' that would facilitate rolling in shear over a wide range of fluid forces. Both the positioning of PSGL-1 on extensible microvilli and the unique physical chemistry of its bond with P-selectin may both therefore contribute
the complex mechanical and molecular process that control leukocyte trafficking and homing to inflammatory sites. Neutrophil microvillus extension subsequent to selectin tether formation may therefore have important physiological implications on neutrophil recruitment \textit{in vivo} through the modulation of selectin bond lifetimes.

\textbf{Figure 2.15} Cytoskeletal reorganization within an extended microvillus tether. The cytoskeletal filaments within an unstressed microvillus would be numerous. The tether structure may start to exclude cytoskeletal filaments under high tensile force as the microvillus elongates, resulting in a less rigid object susceptible to further deformation.
Chapter 3

Leukocyte Recruitment of Platelets to the Endothelium

3.1 Introduction

Platelet adhesion to damaged vascular endothelium is one of the first steps in hemostasis and thrombosis (Barstad et al., 1994; Baumgartner, 1973; Bonnefoy et al., 2000; Mosesson, 1992; Turitto and Baumgartner, 1979). The subendothelial matrix is known to be a highly adhesive substrate for platelet recruitment and activation through the involvement of collagen, fibronectin, fibrinogen, and/or vWF (Badimon et al., 1988; Ruggeri, 1994; Savage and Ruggeri, 1991). Platelet attachment has also been shown to occur on intact endothelium in vivo under flow, given the prerequisite of an activated or dysfunctional endothelial phenotype such as atherosclerotic lesions or inflamed endothelium (Frenette et al., 1995; Huo et al., 2003). Under these pathological conditions, individual platelets can be observed rolling events directly on endothelium.

Normally intact endothelium is inherently anti-thrombogenic to flowing blood due to its ability to neutralize coagulation pathways and prevent platelet activation. In particular, endothelial cell production of prostacyclin and nitric oxide limits platelet aggregation, while cell-surface expression of heparan sulfate and thrombomodulin act to inhibit thrombin activity (Bevilacqua et al., 1985; Frangos et al., 1985; Loscalzo, 2001; Muluk et al., 1998). Consequently, the capacity for platelet accumulation on intact endothelial surfaces appears to be limited compared to injured vascular regions with exposed underlying extracellular matrix. Denudation of vascular endothelium, such as
through FeCl₃ treatment, can induce rapid thrombus formation and subsequent vessel occlusion within minutes (Ni et al., 2000).

Endothelial activation can transform the vasculature to a more thrombogenic state by decreasing blood flow and initiating the synthesis of tissue factor and type-1 plasminogen activator inhibitor (PAI-1). Localized tissue inflammation also causes an up-regulation of various adhesion receptors on endothelial cells for leukocytes, including E- and P-selectin, ICAM-1, and VCAM-1. The endothelial-expressed adhesion molecules are involved in regulating the multi-step cascade transition from initial leukocyte tethering to slow rolling and arrest (Lawrence et al., 1990; Lawrence and Springer, 1991; Ley et al., 1991). In inflamed venules, P-selectin is rapidly mobilized to the endothelial surface after secretion from Weibel-Palade bodies but its expression levels decrease after a few hours while E-selectin expression is more prolonged (Bevilacqua et al., 1987; Jones et al., 1996; Jung and Ley, 1997). ICAM-1 is up-regulated on the surface of endothelial cells upon cytokine-activation, and is the main ligand for leukocyte β2 integrins LFA-1 and Mac-1 (Ding et al., 1999; DiVietro et al., 2001; Petruzzelli et al., 1998; Seo et al., 2001). Cellular activation and longer leukocyte-endothelial interaction times are favorable for integrin binding, resulting in leukocyte arrest and transmigration. Clusters of rolling and adherent leukocytes can accumulate in such large numbers in vivo on cytokine-stimulated venules that up to 80% of the observed venular surface is covered with interacting leukocytes (Kunkel et al., 1997). Few studies have looked into the role of rolling or arrested leukocytes in recruiting platelets from flow. A recent investigation has shown a correlation between leukocyte adhesion and platelet localization to the venular surface after ischemia/reperfusion-induced injury (Russell et al., 2003).
Circulating platelet-leukocyte aggregates are considered to be clinical prognostic markers for acute coronary syndromes, such as myocardial infarction and unstable angina (Entman and Ballantyne, 1996; Kupatt et al., 2000; Sarma et al., 2002). Platelet activation is a prerequisite condition for the formation of platelet-leukocyte complexes, however the mechanism by which activation occurs in vivo remains unknown. Platelet activation will expose P-selectin and activate integrin GPIIb/IIIa on the platelet surface to enable interactions with leukocyte adhesion receptors (Fig. 3.1). The formation of platelet-leukocyte complexes in vivo has recently been reported to amplify the formation of atherosclerotic lesions by recruiting monocytes to the lesion, normally non-interactive with flowing leukocytes (Huo et al., 2003). The heterotypic aggregation of platelets with leukocytes is augmented under shear conditions and is primarily mediated by platelet binding to leukocyte PSGL-1 and Mac-1 (Konstantopoulos et al., 1998; Rinder et al., 1991b). Activated platelets forming heterotypic aggregates with leukocytes in vitro display a preference for monocyte attachment (Rinder et al., 1991b; Sarma et al., 2002).

Low venous shear stresses that promote leukocyte-endothelial rolling via selectin and integrin pathways could likewise contribute towards platelet-leukocyte interactions (Fig. 3.2). The probability of cellular and receptor associations increase at lower flow rates as determined through adhesive dynamic simulations of interactions taking place between 1 - 22 s⁻¹ (Chang and Hammer, 1999). Receptor bonds that form during leukocyte rolling are more stable at lower wall shear stresses due to the lower amount of tensile force loaded on the bond (Bell, 1978; Park et al., 2002; Puri et al., 1997; Smith et al., 1999). In vitro and in vivo studies have also demonstrated that low blood flow promotes a margination effect at which the cellular components separate from a
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homogenized state to create a high hematocrit core (Bloch, 1962; Fahraeus, 1958; Goldsmith and Spain, 1984; Schmid-Schonbein et al., 1980). This results in an increased concentration of leukocytes and platelets flowing near the periphery of the endothelial wall. The excess of near-wall cellular species could potentially increase the frequency of interactions among the three cell types, possibly leading to large thrombi formation.

The defined role of the endothelium in mediating the formation of venular thrombus under flow remains poorly understood. Under inflammatory conditions, the rolling and adherent leukocytes could present a surface of high affinity platelet adhesion ligands on the endothelium such as PSGL-1 and Mac-1. The endothelial-adherent leukocytes may provide a more favorable binding surface for the recruitment of circulating activated platelets than the endothelium alone. Leukocyte PSGL-1 binding to endothelial P-selectin is a high affinity interaction (Kansas, 1996; Mehta et al., 1998; Springer and Lasky, 1991; Ushiyama et al., 1993), and the same mechanism may be exploited for activated platelet recruitment to the endothelium by leukocyte bridging.

I hypothesize that these adherent leukocytes promote platelet recruitment and aggregate growth on the cytokine-stimulated endothelium in a model of inflammation. The accumulation of platelets around adherent monocytes or neutrophils was investigated at a range of venous flow rates in a parallel plate flow chamber. To determine whether adherent leukocytes may be potential sites for developing venous thrombi, the following issues were addressed: 1) whether arrested leukocytes significantly recruit more platelets to the endothelial surface than the endothelium alone; 2) determine whether there is a leukocyte subtype preference for platelet recruitment from flow; and 3) the molecular-recognition and fluid dynamic constraints for the development of substrate-anchored
multi-cellular aggregates. The results indicate that endothelium-adherent leukocytes are able to directly accumulate large numbers of platelets to the endothelial surface, potentially amplifying the thrombogenic state of intact venous thrombosis. Adherent monocytes appear more receptive for platelet recruitment from flow than neutrophils.

3.2 Methods

Antibodies and reagents

Human P-selectin (Lawrence et al., 1997), PSGL-1 derived from HL-60 cells (Park et al., 2002), and human ICAM-1 were purified as described previously (Lawrence et al., 1995). Human E-selectin was purified from Chinese Hamster Ovary (CHO) cell lysates transfected with wild-type human E-selectin cDNA as previously described (Lawrence et al., 1997; Lobb et al., 1991). KPL1, a functional blocking monoclonal antibody to P-selectin glycoprotein ligand 1 (PSGL-1), was kindly provided by K.R. Snapp (Northwestern Univ., Evanston, IL). β2 integrin monoclonal antibody TS1/18 was purified from a hybridoma cell line (Sanchez-Madrid et al., 1982). Anti-E-selectin mAb BB11 was a gift from R. Lobb (Biogen, Inc., Cambridge, MA). Anti-Mac-1 mAb 2LPM19c (Dako Cytomation, Carpinteria, CA), anti-VLA-4 HP2/1 (Immunotech, Marseille, France), anti-P-selectin G1 (Ansell, Bayport, MA), anti-GPIb SZ2 (Biodesign, Saco, ME), and anti-GPIIb/IIIa F(ab')2 fragment c7E3 (Centocor, PA) were purchased. Pac-1, a FITC-labeled mAb against the activated-conformation of GPIIb/IIIa (GPIIb/IIIa*), was purchased from BD Biosciences (San Diego, CA). Goat anti-human IgG (Biodesign, Saco, ME) and FITC-labeled secondary F(ab')2 fragment (Zymed, S. San Francisco, CA) were purchased.
Figure 3.1 Translocation of platelet glycoproteins during activation. Upon agonist stimulation, platelet α granules secrete P-selectin to the membrane surface, GPIIb/IIIa affinity and expression levels increase, and GPIb/IX/V complex is internalized.

Figure 3.2 Receptor ligation pathways for attachment among heterogeneous cell populations.
Platelet isolation

Blood obtained from healthy volunteers was drawn through an antecubital vein with a 21-gauge needle into a syringe anti-coagulated for a 1:9 dilution of acid-citrate-dextrose (ACD; 85 mM tri-sodium citrate, 67 mM glucose, 42 mM citric acid), added 95% air/5% CO₂ gas, and centrifuged for 7 minutes at 310 g. Platelet rich plasma (PRP) was collected in 1:20 v/v ACD, supplemented to 2 U/ml apyrase, 80 nM prostaglandin I₂ (PGI₂), and 2.8 μM indomethacin (Gear, 1982), and subsequently labeled with 1 μM calcein AM (Molecular Probes, Eugene, OR) for 20 minutes in the dark at room temperature. PRP was centrifuged for 20 minutes at 550 g and the supernatant was discarded. The platelet pellet was resuspended with the addition of 1/2 PRP volume of ACD, 80 nM prostaglandin I₂ (PGI₂), and 2.8 μM indomethacin. All platelet isolation reagents were purchased from Sigma (St. Louis, MO). Platelet wash step was repeated and platelets were resuspended in Hanks balanced salt solution without calcium and magnesium, supplemented with 10 mM HEPES and adjusted to pH 7.4 (HBSS). Isolated platelet suspensions were kept at 37°C to minimize platelet activation. In some experiments, 8 μM thrombin receptor activating peptide (TRAP, Sigma) was used to activate the platelets in suspension for 3 minutes.

Leukocyte isolation

Monocytes were obtained from human peripheral blood by first separating into a mononuclear cell population by layering human whole blood over Polymorph Prep Isolation Media (Accurate Chemicals, Westbury, NY). Monocytes were isolated using
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Mid MACS monocyte isolation kit (Miltenyi Biotec, Auburn, CA). Neutrophils were isolated by obtaining the polymorphonuclear cell layer from the same density gradient solution used to obtain the monocytes. To allow for fluorescence visualization, isolated monocytes/neutrophils were labeled with 20 μM 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) for 20 minutes. After isolation, the leukocytes were suspended in HBSS with 1% HSA. Prior to experimentation, 2 mM CaCl₂ and 2 mM MgCl₂ were supplemented to the cell suspension.

Flow Cytometry of Leukocytes and Platelets

Isolated monocytes and neutrophils were incubated with 150 μg/ml goat anti-human Ig to block Fc receptor uptake of mAbs. Cell suspensions were added to individual vials of anti-PSGL-1 (KPL1), anti-L-selectin (LAM1-116), anti-Mac-1 (2LPM19c), anti-LFA-1 (TS1/22), anti-VLA-4 (HP2/1), and an isotype control mAb (BB11). Isolated leukocytes were incubated with the mAbs for 20 minutes in staining buffer (2% FBS in PBS) at 4°C, washed twice, and incubated with FITC-labeled secondary F(ab')₂ for 15 minutes. Cell wash was performed twice and resuspended in staining buffer containing 2% paraformaldehyde.

Isolated platelets were incubated with anti-P-selectin (G1) and anti-GPIIb/IIIa* (Pac-1) for 20 minutes at room temperature and washed twice. In some experiments, 8 μM TRAP was also added during antibody incubation. FITC-labeled secondary F(ab')₂ was incubated with the anti-P-selectin platelet samples for 15 minutes at room temperature and washed twice. mAb BB11 was used as an isotype control.
Relative fluorescence values for expression level comparison were obtained by calculating the fluorescence ratio. The mean fluorescence intensity of positively gated cells was divided by the total fluorescence intensity of the isotype control.

**Preparation of adhesive substrates**

2 μg/ml E-selectin and 0.75 μg/ml ICAM-1 were co-immobilized over polystyrene plates for 3 hours in a humid environment. The protein slides were blocked against non-specific adhesion by incubation with 0.5% Tween-20. With the assistance from Andrew Pryor and Brett Blackman, human umbilical vein endothelial cells (HUVEC) were harvested from normal term cords. Isolated HUVECs were cultured in T-75 flasks (Nalge Nunc, Rochester, NY) with Medium 199 (Gibco, Carlsbad, CA) containing 20% heat-inactivated fetal bovine serum (Gemini Bio-products, Woodland, CA), 25 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), and 100 U/ml penicillin - 100 μg/ml streptomycin (Gibco). 100 μg/ml heparin (Sigma) and 50 μg/ml endothelial cell growth supplement (ECGS; Biomedical Technologies, Stoughton, MA) were added to suppress smooth muscle cell proliferation and increase HUVEC growth rate, respectively. The cells were maintained in an incubator set at 37 °C with 5% CO₂ gas, and growth media was replenished every 2-3 days. Once the HUVECs became confluent, the monolayer was washed with PBS buffer and split by incubating with 0.05% trypsin – 0.53 mM EDTA (Gibco) for 2 minutes at 37 °C. HUVECs were passaged at a 1:4 or 1:5 splitting ratio. Endothelial cells of up to passage 6 were plated until confluence on glass slides or 35 mm Petri dishes pre-coated with 0.1% gelatin (Sigma). HUVECs were stimulated
with 5 ng/ml recombinant IL-1β (R&D Systems, Minneapolis, MN) for 4 hours prior to experimentation.

Endothelial cell matrix substrates were prepared by detaching confluent HUVEC monolayers from non-gelatinized dishes. Endothelial cells were cultured 3-4 more days after the monolayers reached confluency. To detach HUVECs, cells were incubated with 10 mM EDTA for 30 minutes at room temperature and repeatedly washed with PBS until endothelial cells could not be detected in the Petri dish. 100 μg/ml human fibrinogen (Sigma) or von Willebrand factor (vWF; Calbiochem, San Diego, CA) were adsorbed to polystyrene surfaces for 3 hours and blocked with 0.5% Tween-20.

**Laminar flow assays**

The slides were attached to a parallel plate flow chamber (GlycoTech, Gaithersburg, MD) and the chamber was mounted over an inverted differential interference contrast microscope equipped with epifluorescent illumination (TE2000; Nikon, Melville, NY) using a 100-W HBO mercury lamp source coupled to a bandpass fluorescein and rhodamine filter set (Chroma Technology, Brattleboro, VT). A 12-bit cooled-CCD camera (SensiCam QE; Cooke Corp., Auburn Hills, MI) capable of differentiating 4096 intensity levels was used for image digitization. Fluorescent and brightfield images were directly captured by a G3 Macintosh with IPLab v3.5.5 (Scanalytics, Fairfax, VA) for observation of fluorophore-labeled monocytes and/or platelets interacting with surface substrate. The wall shear stress in the parallel plate flow chamber is controlled by the dimensions of the gasket and the fluid flow regulated with a syringe pump (Harvard Apparatus, Holliston, MA). A water bath was used to maintain cell suspensions and
assay media at 37°C for the duration of experimentation. Monocytes or neutrophils
resuspended to 0.3×10⁶/ml were initially perfused over the HUVEC or P-selectin/ICAM-
1 substrate for 5 minutes at 1 dyn/cm² wall shear stress. Due to monocyte detachment at
higher shear stresses, monocyte suspension concentration was increased to 0.5×10⁶/ml for
platelet flow experiments performed at 6 and 8 dyn/cm² wall shear stress. Fibrinogen
was added to the platelet suspension to obtain 100 µg/ml, and the cation concentration
was adjusted to 2 mM/L CaCl₂ and 2 mM/L MgCl₂ prior to experimentation. A solution
of assay media was followed for 1 minute at 1 dyn/cm² wall shear stress. Platelets were
resuspended at 30×10⁶/ml and were perfused into the flow chamber at the indicated wall
shear stresses for 20 minutes.

Dynamic platelet rolling observations were obtained by connecting the
microscope to a CCD camera equipped with a GenIIsys image intensifier (DAGE-MTI,
Michigan City, ID). The images were recorded using a SVHS recorder (Panasonic,
Secaucus, NJ) and were digitized from tape using an AG-5 frame acquisition card and
Scion Image v1.63 (Scion Corp., Fredrick, MD).

Image Quantitation and Data Analysis

Seven sequential non-overlapping images of HUVEC plates containing fluorescently
labeled platelets were acquired at 20× magnification with the camera exposure time set at
200 ms. Each image was subtracted against background and analyzed with NIH ImageJ
http://rsb.info.nih.gov/ij/ (Rasband, 1997-2004). In each group, ten individual platelets
were selected and the mean of their fluorescent intensity was used to obtain a standard
platelet fluorescence value. Individual platelets or platelet aggregates were outlined and
their respective intensity values were divided by the standard platelet fluorescence to obtain platelet counts. Fluorescent intensities of individual platelets or platelet clusters were obtained (Fig 3.3). No significant changes in average platelet intensity were detected for platelet aggregates of up to 5 platelets. Platelet aggregates of larger numbers could not be calibrated due to limitations in discerning actual platelet numbers. Decreases in fluorescence intensity per platelet for very large aggregates (> 10 platelets) cannot be ruled out. The resulting uncertainty would create an underestimation of the actual number of platelets present in an aggregate. The total area of one optical field at 20× corresponded to 0.138 mm².

3.3 Results

3.3.1 Platelet Interactions with Endothelial Surfaces

Platelet interactions directly with endothelial monolayers were assessed after 10 minutes of platelet perfusion. The firm adhesion of unstimulated platelets to IL-1β activated endothelium was virtually non-existent (Fig. 3.4 A). Platelets stimulated with 8 μM TRAP displayed a slight enhancement of platelet attachment onto HUVEC monolayers. TRAP will cause platelet degranulation resulting in P-selectin surface expression and activation of GPIIb/IIIa. Unlike thrombin, TRAP will not induce the proteolytic cleavage of fibrinogen into fibrin, and the formation of fibrin strands.

Extracellular matrix proteins were much better substrates for platelet arrest under flow. Non-activated platelets were able to attach firmly onto the sub-endothelial matrix in significantly more numbers (P<0.05) than activated platelet binding to endothelial cells at wall shear stresses less than or equal to 1 dyn/cm² (Fig. 3.4 B). Considerable platelet
binding could be detected on immobilized fibrinogen at wall shear stresses less than 1
dyn/cm² but dropped off quickly with increasing shear. Platelet arrest was not detectable
on vWf at any of the shear rates tested, however the number of transient interactions (<5
s) increased when wall shear stress was greater than 1 dyn/cm² (Fig. 3.4 C).

3.3.2 Leukocyte Bridging Facilitates Platelet Sequestration to the Endothelium

Visualization of activated platelet binding directly onto cytokine-stimulated
endothelial cells after 10 minutes of flow showed some patterns of platelet string
formation, possibly indicating secondary capture (Eriksson et al., 2001) (Fig. 3.5 B).
Monocytes that were rolling on or firmly adherent to activated endothelium were able to
recruit platelets to the endothelial surface in greater quantities than the endothelium alone
(Fig. 3.5 C). Platelet aggregate formation was preferential to the downstream face of the
adherent monocytes. Platelets interacting with these monocytes were seen to attach to the
surface of the monocyte and roll in a pattern similar to selectin-mediated leukocyte-
endothelial interactions (Fig. 3.6 A). Once adherent to the monocyte surface, platelet
rolling would occur through a series of pauses and ratcheting motions. The initial
tethering events of platelets to the monocyte surface are predominately located on the
upstream face of the leukocyte (Fig. 3.6 B). Platelet rolling on the adherent leukocyte
predominately followed a circumferential path with rare events of platelet translocation
directly over the peak of the leukocyte. Platelets skipping over the attached monocytes
had two outcomes: detachment and return to the flowing platelet suspension, or rolling to
the downstream-end of the adherent monocyte before coming to arrest. The duration of
platelet rolling before detachment into the fluid flow ranged from 0.8 – 7.5 seconds at 1
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dyn/cm². 98% of the non-stimulated platelets observed on the endothelial surface were localized within a half-cell diameter of the adherent monocytes.

The trajectories of flowing platelets interacting on the surface of the monocytes converge as the platelets traverse from the upstream-facing side of the adherent leukocyte and roll to the downstream end. In contrast with TRAP-activated platelets which are able to form attachments with the stationary monocyte, non-interacting particles that are perfused past an adherent leukocyte do not display any asymmetry in flow streaklines (Fig. 3.7).

Surface-adherent monocytes were able to attract platelets in amounts greater than or equal to the amount of activated platelets binding directly to endothelial surfaces (Fig. 3.8 A). Since platelets were almost exclusively localized around the adherent monocytes, the amount of platelet deposition onto the HUVEC monolayer was a strong function of the number of monocytes present. Platelet accumulation was normalized to the number of monocytes present in each field of view, which correlated to a platelet aggregate size measurement. Larger aggregates of 6.78 ± 1.16 platelets were able to form at the low wall shear stress of 0.3 dyn/cm² compared to higher shear (6-8 dyn/cm²), which averaged 2 or less platelets per aggregate (Fig. 3.8 B). Per monocyte, 48% less platelets attached at 4 dyn/cm² than at 0.3 dyn/cm² wall shear stress. Platelet accumulation per monocyte was significantly higher (P<0.05) at 0.3 dyn/cm² compared to the higher wall shear stress (≥4 dyn/cm²). Platelets that were maximally activated with TRAP did not display a modulation in monocyte capture of platelets in relation to shear stress until wall shear stress was increased to 6 dyn/cm² and higher.
Figure 3.3 Fluorescence calibration of labeled platelets. The fluorescence intensity of single platelets, doublets, triplets, quadruplets, and quintuplets were measured (A). Accurate platelet numbers were difficult to discriminate for larger platelet aggregates. Fluorescence intensity was normalized per platelet (B). Data values represent mean ± SD.
Figure 3.4 Direct platelet attachment to endothelial cells or adhesive protein surfaces. Unstimulated platelets or platelets activated with 8 μM TRAP were perfused over IL-1β stimulated HUVECs for 10 minutes (A). Deposition of unstimulated platelets on sub-endothelial matrix or 100 μg/ml fibrinogen substrates after 10 minutes of flow (B). Due to the transient nature of platelet tethering to immobilized vWF (100 μg/ml), the number of individual platelet attachment events was assessed over 30 second intervals (C). Data represent means ± SE of 3 experiments.
Figure 3.5 Platelet accumulation on endothelium at 1 dyn/cm² requires adherent leukocytes. Monocytes were labeled with membrane dye DiI (red) and platelets were loaded with Calcein AM (green). Representative areas for each condition were obtained using Nomarski and fluorescent imaging. TRAP-activated platelets were perfused over non-stimulated HUVECs (A). Suspensions of TRAP-activated platelets alone (B) or with monocytes (C) were perfused over IL-1β activated HUVEC for 20 minutes. Bar, 50 μm.
Figure 3.6 Platelet rolling on an adherent monocyte. A series of epifluorescent images of a fluorescently-labeled platelet interacting with a monocyte attached to an E-selectin/ICAM-1 co-immobilized surface (A). Time point of 0 seconds shows the initial capture of the platelet from flow until its release at 3.6 seconds. Seven individual platelet rolling trajectories around the adherent monocyte (dashed lines) were plotted (B). Platelet attachment (o) and detachment (×) centroid locations were noted for each trajectory. Bar, 10 μm.
Figure 3.7 Streaklines around a surface-adherent neutrophil. Two representative high-speed video microscopy images at 250 frames per second show the streaklines produced by particles that do not interact with the surface-bound leukocyte. Phase-contrast images were superimposed over each other using ImageJ. 1.87±0.05 μm polystyrene beads were perfused at 2 dyn/cm² wall shear stress. Bar, 10 μm.
Figure 3.8 Shear stress modulation of platelet recruitment to adherent monocytes. Non-stimulated platelets and platelets activated with 8 µM TRAP were perfused over monocytes pre-adherent on IL-1β stimulated HUVECs (A). Platelet aggregate size was assessed individually on a per monocyte basis (B). Data labels in panel (A) refer to the mean monocyte counts per field of view in each condition. Data values represent means ± SE of 3 experiments.
3.3.3. Monocyte Cluster Recruitment of Platelets from Flow

Monocyte recruitment to the endothelium under flow displayed patterns of cluster formation, likely due to leukocyte secondary capture mechanisms (Eriksson et al., 2001; Lim et al., 1998). Platelet attachment to isolated adherent monocytes or monocyte clusters were evaluated to determine if leukocyte cooperativity in platelet recruitment was taking place (Fig 3.9). Monocytes were considered clustered or adjacent to one another if the cell was less than a cell diameter (8 μm) away from another cell. At 1 dyn/cm$^2$ wall shear stress, monocyte clusters recruited more TRAP-activated platelets than solitary monocytes adherent on endothelial cells (Fig 3.9 B). Normalizing the amount of platelet accumulation to the number of monocytes associated with each platelet thrombus resulted in similar values regardless of whether monocytes formed clusters or not (Fig 3.9 C). Monocyte proximity on the endothelium did not appear to influence the effectiveness of an adjacent monocyte in recruiting platelets from flow.

3.3.4 Flow Cytometry of Leukocyte and Platelet Adhesion Receptors

Quantitation of adhesion receptor presentation on monocytes, neutrophils, and platelets was performed using flow cytometry (Fig. 3.10). The mean fluorescence intensity (MFI) of the positive-gated cells was ratioed against the total background fluorescence produced by the isotype control labeled cells (Fig. 3.11). Monocytes and neutrophils express similar levels of PSGL-1 (20.3 ± 5.1 vs. 20.7 ± 7.1, respectively) and L-selectin (17.0 ± 3.9 vs. 42.0 ± 11.1, respectively). Integrin expression levels were consistently higher on monocytes compared to neutrophils. Monocyte expression of the β2-integrins Mac-1 and LFA-1 were 73.4 ± 19.5 and 42.2 ± 18.1, respectively. Neutrophil fluorescence values for Mac-1 and LFA-1 were 17.5 ± 4.6 and 9.3 ± 1.2,
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respectively. The MFI for P-selectin expression on resting platelets was $37.0 \pm 15.5$ and increases to $136.5 \pm 56.5$ with $8 \mu M$ TRAP activation. The presence of activated GPIIb/IIIa as detected by Pac-1 mAb on resting platelets was low ($14.5 \pm 4.7$ MFI) but increases substantially after TRAP activation ($236.9 \pm 71.9$ MFI).

3.3.4 Receptor Dependency for Attachment at Different Flow Rates

To investigate the shear-sensitive mechanism for platelet accumulation on monocytes, adhesion receptors on both platelets and monocytes were blocked with antibodies at successive shear rates. The antibody blocking experiments were performed on monocytes pre-adherent on E-selectin and ICAM-1 co-immobilized on plastic plates. PSGL-1 blocking mAb (KPL-1) eliminated platelet accumulation at $1 \text{ dyn/cm}^2$ and higher (Fig. 3.12). At $0.3 \text{ dyn/cm}^2$, KPL-1 was not as effective but was still able to decrease platelet accumulation by $54\%$ ($5.3 \pm 0.66$ to $2.45 \pm 0.2$ platelets/monocyte). 2LPM19c blocking of the Mac-1 $\alpha$ subunit that mediates fibrinogen attachment (Diamond and Springer, 1993) was equally effective at $0.3 \text{ dyn/cm}^2$ in reducing platelet aggregate size ($5.3 \pm 0.66$ to $2.64 \pm 0.4$ platelets/monocyte). While KPL-1 was able to abolish platelet accumulation at $1 \text{ dyn/cm}^2$ and $4 \text{ dyn/cm}^2$ wall shear stress, Mac-1 inhibition resulted in a $50\%$ decrease at $1 \text{ dyn/cm}^2$ ($3.49 \pm 0.46$ to $1.76 \pm 0.26$ platelets/monocyte) and had no effect at $4 \text{ dyn/cm}^2$. The GPIIb/IIIa blocking Fab fragment c7E3 was able to block platelet aggregation around monocytes at all the fluid shear rates tested with the non-stimulated platelets. Blocking the $\beta2$ integrin subunit with TS1/18 did not decrease the aggregate sizes formed from resting platelet accumulation around adherent monocytes.

Platelets activated with TRAP (Fig 3.12 D and E) did not show a decrease in accumulation on monocytes at any shear rate when incubated with 2LPM19c. Blocking
PSGL-1 was as effective in suppressing thrombi growth by the activated platelets as with
the non-stimulated platelets. At 0.3 dyn/cm², similar aggregate sizes were found with
PSGL-1 inhibition on non-stimulated and activated platelets after 20 minutes of flow
(2.45 ± 0.2 vs. 2.39 ± 1.24 platelets/monocyte). At 1 dyn/cm² and higher, KPL-1
abolished accumulation of activated platelets. c7E3 did not reduce the amount of
activated platelet accumulation. Similar results were found for activated platelets flowing
at 4 dyn/cm² wall shear stress as with 1 dyn/cm². The same relationship of platelet
accumulation with flow shown in Figure 3.5 C using HUVEC monolayers as a substrate
for monocyte attachment can also be seen with monocytes adherent on the immobilized
E-selectin/ICAM-1 mimetic endothelial surface.

3.3.5 Monocytes Provide Better Substrate for Platelet Capture than Neutrophils

In the presence of a shear force, isolated neutrophils have shown an ability to
form aggregates with platelets which have not been exogenously stimulated. The ability
of surface-adherent neutrophils to capture platelets was compared to what was observed
with monocytes. The ability of neutrophils in capturing activated platelets from flow was
three-fold less than observed with monocytes (Fig. 3.13 A). Activated platelet
recruitment by neutrophils also did not display a shear-sensitivity between 0.3-4 dyn/cm².
Fluorescence visualization of the platelet-leukocyte aggregates on the HUVEC
monolayer revealed a difference leukocyte shape appearance. After a maximum
leukocyte-endothelial interaction time of 15 minutes and 10 minutes of platelet flow,
monocytes still appear to retain their original spherical shape (Fig. 3.13 B). However,
several of the adherent neutrophils were observed to be asymmetrical with pseudopodial
structures branching out, indicating an activated state (Fig. 3.13 C).
Figure 3.9 Monocyte proximity influence on platelet capture from flow. Monocyte clusters (red) and isolated adherent cells capture activated platelets (green) at 1 dyn/cm² wall shear stress (A). Platelet attachment to adherent solitary monocytes, doublets, or triplets were quantified (B). Platelet accumulation was normalized to the number of monocytes associated with the platelet thrombi (C). Data values represent mean ± SE. Bar, 50 μm.
Figure 3.10 Flow cytometric analysis of leukocyte receptor expression levels. Adhesion receptor expression on isolated neutrophils (A-F) and monocytes (G-L) were assessed. E-selectin mAb, BB11, was used as an isotype negative control and gate setting for neutrophils (A) and monocytes (G). PSGL-1 (B & H), L-selectin (C & I), Mac-1 (D & J), LFA-1 (E & K), and VLA-4 (F & L) were incubated with the neutrophils and monocytes, respectively. Data values in each panel represent the percentage of cells passing through the positive gate.
Figure 3.11 Comparison of neutrophil and monocyte receptor expression. Mean fluorescence intensity measurements for each mAb reporter were normalized against the total background fluorescence reported by an isotype control mAb (E-selectin mAb, BB11). Data values represent means ± SE of 3 experiments.
Figure 3.12 Receptor determinants for platelet accumulation on monocytes. Before platelet infusion, the adherent monocytes were incubated for 2 minutes with the following mAbs: 2LPM9c (anti-Mac1 α, 5 μg/ml), KPL-1 (anti-PSGL1, 5 μg/ml), and TS1/18 (anti-β2, 5 μg/ml). 10 μg/ml c7E3 (anti-GPⅡb/Ⅲa) was incubated with the platelet suspension and co-infused into the flow chamber. Resting platelet suspensions were perfused over adherent monocytes at 0.3 (A), 1 (B), and 4 dyn/cm² (C). TRAP-activated platelets were similarly perfused over adherent monocytes at 0.3 (D) and 1 dyn/cm² (E). Note difference in scales between panels A-C and D-F. Data represent means ± SE of 3-5 experiments.

Figure 3.13 Comparison of capture efficiency of monocytes versus neutrophils. Total TRAP-stimulated platelet deposition (A) and accumulation per leukocyte (B) was determined after 10 minutes of platelet flow over arrested monocytes/neutrophils. Calcein-AM (green) labeled platelet attachment around Dil (red) labeled monocytes (C) or neutrophils (D). Values in panel A and B represent means ± SE of 3-4 experiments. Data labels in panel A represent mean monocyte/neutrophil counts per field of view in each condition. Bar, 10 μm.
3.4 Discussion

The accumulation of platelets on the vascular endothelium is likely an initial step in the development of venous thrombosis. Platelet recruitment on exposed subendothelial matrix under flow is a rapid and substantial response. Due to the non-thrombogenic nature of intact endothelium, formation of large surface-anchored platelet aggregates is normally inhibited. It is not well understood what mechanisms trigger platelet adhesion to the endothelium during an inflammatory stimulus. The data suggest that leukocytes, specifically monocytes, interacting with the endothelium during an inflammatory response may act as potential sites for the initiation of thrombosis. The accumulation, and ultimately dislodgement, of large platelet aggregates from the vascular surface increases the probability for the incidence of pulmonary embolism (Kakkar et al., 1969; Lip et al., 2002; Ni et al., 2003) and may be catalyzed by monocyte adhesion to the endothelium.

Direct observation of platelet attachment to surface-adherent leukocytes under flow revealed several interesting findings. Firmly arrested monocytes are able to recruit resting platelet suspensions to the endothelium in greater amounts than can be found even from activated platelet binding directly to endothelium. Endothelial cells alone have some ability to form attachments with platelets, mainly through fibrinogen-mediated bridging of platelet GPIIb/IIIa and endothelial ICAM-1 (Bombeli et al., 1998). In addition, endothelial P-selectin and vWF have also been demonstrated to mediate platelet interactions to the vascular surface (Andre et al., 2000; Frenette et al., 1995). However, the interactions of platelets directly with endothelium under flow are generally transient in nature and appear resistant to the formation of large surface-bound aggregates.
Endothelial cells present glycosaminoglycans such as the heparan sulfate (Munoz and Linhardt, 2004). The anti-coagulative properties of heparin and heparan sulfate are due to its high affinity for the protease inhibitor antithrombin. In addition, endothelial cells also synthesize PGI$_2$, ADPase, and nitric oxide to inhibit platelet activation and aggregation (Bombeli et al., 1997).

Cytokine-activated endothelial cells may synthesize tissue factor and suppress the activity of membrane-associated thrombomodulin (Bevilacqua et al., 1984; Morrissey, 1993; Verheul et al., 2000), which together promote the production of thrombin on the endothelial surface. Localized thrombin activity on the endothelial surface induces the proteolytic cleavage of fibrinogen into fibrin, resulting in fibrin deposition on the endothelium (Kirchhofer et al., 1994; Verheul et al., 2000). Despite the possibility for platelets to capture onto endothelial-immobilized fibrin, monocytes present on the endothelial surface significantly enhance platelet recruitment from flow relative to the endothelium alone (Fig. 3.5). It is likely that the adherent leukocytes present a more favorable surface for platelet capture from flow than the endothelial layer.

The dissociation constant ($K_d$) for activated GPIIb/IIIa binding to fibrinogen is approximately 300 nM (Du et al., 1991; Marguerie and Plow, 1981), very similar to PSGL-1 affinity for P-selectin (Mehta et al., 1998; Ushiyama et al., 1993). Fibrinogen bound to activated GPIIb/IIIa is a very stable interaction, indicative of slow dissociation rate constant ($k_{off}$). The $k_{off}$ for GPIIb/IIIa interactions with immobilized fibrinogen is $\sim$4-5$x10^3$ s$^{-1}$ (Huber et al., 1995), much slower than P-selectin dissociation from PSGL-1 ($k_{off} = 1.4$ s$^{-1}$). In contrast, selectin interactions with their respective ligands are characterized by rapid dissociation and association rates (Kansas, 1996; Springer and
Leukocyte capture to the endothelium is predominately due to the ability of selectin molecules to form rapid attachments under flow. The slower association rate ($k_{on}$) for activated GPIIb/IIIa binding to fibrinogen may favor platelet interactions through P-selectin binding to available PSGL-1 molecules.

The contribution of receptor-ligand pairs in mediating platelet accumulation around arresting leukocytes dramatically differs depending on shear rate and platelet activation state. Non-stimulated platelets display a shear-sensitive response to accumulation around leukocytes that requires GPIIb/IIIa at all shear rates examined. Individually, blocking P-selectin or Mac-1 appears to have a role in decreasing but not eradicating platelet adhesion at 0.3 dyn/cm² wall shear stress. Shear appears to increase the contribution of the P-selectin/PSGL-1 bond at higher wall shear stresses where Mac-1 is not a factor in platelet accumulation. It is likely that the interaction time of platelet rolling at the higher shear rates precludes the leukocyte integrin from becoming involved due to its lower binding affinity compared to selectin bonds.

The ability of GPIIb/IIIa antibody blockade to diminish leukocyte interactions with un-activated but not with activated platelets is consistent with previous observations (Rinder et al., 1991a). In contrast, activated platelet deposition around adherent leukocytes is wholly governed by the P-selectin/PSGL-1 bond at all the shear rates examined (Larsen et al., 1989). The shear-insensitivity of activated platelet accumulation on adherent monocytes initially appear conflicting with the well-characterized force dependency of P-selectin bond lifetimes (Alon et al., 1995b; Park et al., 2002; Smith et al., 1999). An activated platelet can display over 350 P-selectin molecules/μm² on its surface (McEver and Martin, 1984). This high site density coupled with the fast
association rate kinetics of selectin-ligand coupling creates an optimal situation for the formation of multiple bonds. It appears that even with a 100-fold increase in GPIIb/IIIa affinity upon activation (Du et al., 1991; Marguerie and Plow, 1981), its ability to mediate platelet accumulation is secondary to the role of P-selectin. Once these multiple P-selectin bonds firmly bind the platelet to the adherent leukocyte and other platelets, ligation of activated GPIIb/IIIa integrins may be favored. It has been shown previously that the involvement of Mac-1 is a prerequisite for firm adhesion of leukocytes to activated platelet monolayers (Diacovo et al., 1996; Ostrovsky et al., 1998). However, the discrepancy seen in Mac-1 bond participation could be due to fundamental differences in interaction dynamics of leukocyte-platelet versus platelet-leukocyte binding. Under the same flow conditions, the larger size of a traversing leukocyte compared to a platelet will impose approximately a 16-fold greater translational shear force (Goldman et al., 1967). The divergence in tensile bond force between the two interaction schemes is even greater. Under a single bond assumption, leukocyte rolling on a flat adhesive surface will generate an estimated 30-fold greater bond force than platelet rolling (Doggett et al., 2002; Park et al., 2002).

The equivalent number of PSGL-1 adhesion receptors on both neutrophils and monocytes would be suggestive of a similar capacity to recruit platelets. Visualization of platelet attachment to fluorescent leukocytes qualitatively shows the differential response of monocyte and neutrophil activation on IL-1β stimulated HUVEC monolayers. Monocytes tightly bound to the apical surface of unstimulated endothelial cells can be seen round without any pseudopodial extensions after 45 minutes of incubation (Muller and Weigl, 1992). As a leukocyte proceeds to transmigrate through the endothelial layer...
via cytokine-stimulation or chemotactic gradients, less of its cellular body becomes exposed to the vascular flow (Luscinskas et al., 1991; Luu et al., 2000; Shaw et al., 2001). This can restrain platelet accumulation in a few ways. I show that platelet capture and rolling is predominately initiated on the upstream end of the adherent leukocyte (Fig. 3.6). The activated leukocyte would provide more of a streamlined profile and less surface for the flowing platelets to impinge on for the initiation of leukocyte-platelet tethering bonds (Fig. 3.14). The activated/transmigrating leukocyte would also present less adhesion receptors available for the platelet to form bond associations. The disturbance in fluid shear on the downstream end of the adherent leukocyte, while minute on a macro-scale level, could have significant impact on a small particle the size of a platelet. As the platelet tethers, rolls, and comes to rest downstream of the leukocyte, the protruding body may be able to shield the platelet from fluidic forces necessary to promote detachment.

A model for the initiation of venous thrombosis elicited by leukocytes arrested on cytokine-stimulated endothelium is presented here. The initial platelet-leukocyte-endothelium complex could potentially form even larger heterotypic aggregates, which may eventually occlude the vessel or embolize (Fig. 3.15). Activated platelets injected into circulation are rapidly sequestered by neutrophils and monocytes for up to three hours, and enable monocytes to interact with atherosclerotic lesions (Huo et al., 2003). This study did not examine the role of pre-formed circulating platelet-leukocyte aggregates in the progression of venous thrombosis. The sequence of platelet or endothelial stimulation and range of wall shear stress may play a large role in determining the involvement of circulating platelet-leukocyte complexes.
Figure 3.14 Schematic of fluid flow differences resulting from leukocyte protrusion. Activated leukocytes on adhesive surfaces will gradually have less of the cell body exposed to fluid flow (A). Leukocyte activation may reduce the collision events of flowing platelets, resulting in less accumulation (B).

Figure 3.15 Progression of thrombosis at the venular surface during inflammation. The continual passage of circulating leukocytes and activated platelets over the initial aggregate may lead to thrombi growth.
Chapter 4

Cell-Localized Flow Disruption Promotes Platelet Deposition

4.1 Introduction

The flow of blood in the circulatory system produces both tangential and normal forces along the vascular wall. The range of shear stress within the vascular network varies greatly depending on location and pathological condition. Wall shear stresses range from 1-6 dyn/cm² in veins, arteries have a broader range of 10-70 dyn/cm², while coronary stents and complex plaques have wall shear stresses that can range from 70 to over 100 dyn/cm² due to their complex geometries (Kroll et al., 1996; Malek et al., 1999). Platelets and leukocytes present within the circulating blood express adhesion molecules which allow them to interact with the vascular endothelium upon injury. Leukocyte interactions with endothelial surfaces has been shown to be highly sensitive to fluid shear (Bjerknes et al., 1986; Lawrence et al., 1987; Ley et al., 1989; Lipowsky et al., 1988). Their initial tether event and rolling processes are predominately controlled by selectin adhesion receptors (Lawrence and Springer, 1991; Ley et al., 1995; Ley and Gaehtgens, 1991; Rosen and Bertozzi, 1994). Selectin bonds appear to be characterized by relatively fast association rates and dissociation rates which increase with bond-loading force (Alon et al., 1995b; Fritz et al., 1998; Rinko et al., 2004; Smith et al., 1999). The kinetics of the selectin receptor interactions, in conjunction with cell microvilli and cell body deformation, are able to modulate leukocyte rolling in shear flow (Firrell and Lipowsky, 1989; Park et al., 2002; Smith et al., 2002).
Platelets express adhesion receptors that allow interactions at both low and high shear forces. The leucine-rich sialoglycoprotein complex GPIb-IX-V mediates transient attachments of flowing platelets to von Willebrand factor (vWF) on exposed subendothelial matrix and endothelial cells (Andre et al., 2000; Cranmer et al., 1999; Cruz et al., 2000; Moroi et al., 1997). Platelet interactions on vWF display a shear threshold phenomenon similar to leukocyte L-selectin interactions (Alon et al., 1998; Greenberg et al., 2000; Lawrence et al., 1997). Dense platelet tethering and attachment events can be detected at high shear on von Willebrand factor (vWF). In contrast, platelets show limited ability to form attachments at low shear (Cranmer et al., 1999; Doggett et al., 2002; Savage et al., 1996). While ineffective at high shear, the interaction of platelet integrin GPIIb/IIa (αIIbβ3) with fibrin/fibrinogen present on the subendothelial matrix surface can significantly increase platelet accumulation at low shear stresses (Goto et al., 1998; Ruggeri et al., 1999; Savage et al., 1996).

High flow rates combined with geometric asymmetries are responsible for the generation of large recirculation zones near arterial bifurcations. The location of disturbed flow patterns strongly correlate with vessel wall intimal thickening and atherosclerotic lesion development (Malek et al., 1999; Rosfors et al., 1998; Ross, 1993; Stewart-Phillips and Lough, 1991). Polymeric casts arterial branches or back step flow models used to recreate atherosclerotic-prone regions have demonstrated that monocyte interactions occur more frequently in regions of secondary flow (Buchanan et al., 2003; Malinauskas et al., 1995; Wootton and Ku, 1999). Wall shear stresses in the secondary flow regions can vary between -5 and 5 dyn/cm² (Dai et al., 2004; Malek et al., 1999; Wootton and Ku, 1999), ideal low shear ranges for the engagement of endothelial ligands.
with monocyte selectins.

Low flow and recirculation zones are also present in the venous circulatory system. Secondary flow regimes localized around the valve cusp pockets of large veins help initiate the development of venous thrombi (Libertiny and Galland, 2001; Raju et al., 2000; Schaub et al., 1984; Severyn et al., 2004). Activated platelets can accumulate in these low flow regions and aggregate with each other (Bell et al., 1989; Skilbeck et al., 2004). Platelets interacting with adherent monocytes on human umbilical vein endothelial cells (HUVEC) show larger amounts of accumulation than platelet accumulation on HUVECs alone. Visual observation of the leukocytes suggests that neutrophils may be more activated with a more asymmetric appearance than monocytes on cytokine-stimulated HUVECs as seen in Chapter 3. In this study, I compare monocyte and neutrophil activation and transmigration rates on stimulated HUVECs, and the effect of changes in leukocyte apical protrusion profile on platelet accumulation. PSGL-1 microspheres of three different sizes were used to standardize “cell” projection height. Computational fluid dynamics of the downstream region of leukocytes at various transmigration stages was used to analyzed the presence of secondary flow regions of single cell projections. Areas of reduced shear produced downstream of the adherent cells/beads offer an explanation for the differential platelet accumulation amounts seen on monocytes versus neutrophils.
4.2 Experimental Methods

Antibodies and reagents

Human P-selectin (Lawrence et al., 1997) and PSGL-1 derived from HL-60 cells (Park et al., 2002) were purified as described previously. KPL1, a functional blocking monoclonal antibody to P-selectin glycoprotein ligand 1 (PSGL-1), was kindly provided by K.R. Snapp (Northwestern Univ., Evanston, IL). Anti-E-selectin mAb BB11 was a gift from R. Lobb (Biogen, Inc., Cambridge, MA). FITC-labeled secondary F(ab')2 fragment (Zymed, S. San Fransisco, CA) was purchased.

Platelet isolation

Blood obtained from healthy volunteers was drawn through an antecubital vein with a 21-gauge needle into a syringe anti-coagulated for a 1:9 dilution of acid-citrate-dextrose (ACD; 85 mM tri-sodium citrate, 67 mM glucose, 42 mM citric acid), added 95%air/5% CO₂ gas, and centrifuged for 7 minutes at 310 g. Platelet rich plasma (PRP) was collected in 1:20 v/v ACD, supplemented to 2 U/ml apyrase, 80 nM prostaglandin I₂ (PGL₂), and 2.8 μM indomethacin (Gear, 1982), and subsequently labeled with 1 μM calcein AM (Molecular Probes, Eugene, OR) for 20 minutes in the dark at room temperature. PRP was centrifuged for 20 minutes at 550 g and the supernatant was discarded. The platelet pellet was resuspended with the addition of 1/2 PRP volume of ACD, 80 nM prostaglandin I₂ (PGL₂), and 2.8 μM indomethacin. Platelet wash step was repeated and platelets were resuspended in Hanks balanced salt solution without calcium and magnesium, supplemented with 10 mM HEPES and adjusted to pH 7.4 (HBSS). Isolated platelet suspensions were kept at 37°C to minimize platelet activation. In some
experiments, 8 μM thrombin receptor activating peptide (TRAP) was used to activate the platelets in suspension for 3 minutes. All platelet isolation reagents were purchased from Sigma (St. Louis, MO).

Leukocyte isolation

Monocytes were obtained from human peripheral blood by first separating into a mononuclear cell population using Polymorph Prep Isolation Media (Accurate Chemicals, Westbury, NY). Monocytes were isolated using MidiMACS monocyte isolation kit (Miltenyi Biotec, Auburn, CA). Neutrophils were isolated by obtaining the polymorphonuclear cell layer from the same density gradient solution used to obtain the monocytes. To allow for fluorescence visualization, isolated monocytes/neutrophils were labeled with 20 μM 1,1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) for 20 minutes. After isolation, the leukocytes were suspended in HBSS with 1% HSA. Prior to experimentation, 2 mM CaCl₂ and 2 mM MgCl₂ were supplemented to the cell suspension.

Preparation of adhesive substrates

Isolated HUVECs were cultured in T-75 flasks (Nalge Nunc, Rochester, NY) with Medium 199 (Gibco, Carlsbad, CA) containing 20% heat-inactivated fetal bovine serum (Gemini Bio-products, Woodland, CA), 25 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin - 100 μg/ml streptomycin (Gibco), 100 μg/ml heparin (Sigma), and 50 μg/ml endothelial cell growth supplement (ECGS; Biomedical Technologies, Stoughton, MA). The cells were maintained in an incubator set at 37 °C.
with 5% CO₂ gas, and growth media was replenished every 2-3 days. Once the HUVECs became confluent, the monolayer was washed with PBS buffer and split by incubating with 0.05% trypsin – 0.53 mM EDTA (Gibco) for 2 minutes at 37 °C. HUVECs were passaged at a 1:4 or 1:5 splitting ratio. Endothelial cells of up to passage 6 were plated until confluence on glass slides or 35 mm Petri dishes pre-coated with 0.1% gelatin (Sigma). HUVECs were stimulated with 5 ng/ml recombinant IL-1β (R&D Systems, Minneapolis, MN) for 4 hours prior to experimentation.

Octylglucopyranoside-solubilized PSGL-1 (1 μg/ml) was immobilized to polystyrene plates for 3 hours in a humid environment at room temperature. The protein slides were blocked against non-specific adhesion by incubation with 0.5% Tween-20. PSGL-1 microbeads were prepared as previously described (Park et al., 2002). 3-μm (3.01 ± 0.11 μm), 6-μm (5.61 ± 0.40 μm), and 10-μm (10.50 ± 1.00 μm) polystyrene microbeads (Polysciences, Warrington, PA) were washed and incubated with varying concentrations of purified PSGL-1 for at least 4 hours. The protein microbeads were incubated with 0.5% Tween-20 to block non-specific adhesion and were used within two days. For PSGL-1 microbead arrest, a high concentration of P-selectin (5 μg/ml) was immobilized onto polystyrene plates for 3 hours and subsequently blocked with 0.5% Tween-20 as described previously.
Figure 4.3 Calibration of PSGL-1 occupancy on microspheres. The adsorption of 0.5 – 4 μg/ml PSGL-1 onto the microsphere surface was examined with flow cytometry. Dashed red line indicates the PSGL-1 bead expression level used in the experimental platelet recruitment flow assays.

Flow Cytometry of PSGL-1 microbeads

PSGL-1 microbeads were incubated with anti-PSGL-1 (KPL-1) or an isotype control mAb (BB11) for 15 minutes in staining buffer (2% FBS in PBS) at room temperature. The beads were washed twice and incubated with FITC-labeled secondary F(ab')2 for 15 minutes at room temperature. Bead wash was performed twice and resuspended in staining buffer containing 2% paraformaldehyde. Relative fluorescence values for expression level comparison were obtained by calculating the fluorescence ratio. The mean fluorescence intensity of positively gated beads was divided by the total
fluorescence intensity of the isotype control. For experimental assays, the PSGL-1 concentrations used to obtain equivalent ligand presentation were 1.5 μg/ml, 1.0 μg/ml, and 1.0 μg/ml for the 3, 6, and 10 μm beads, respectively (Fig. 4.1). PSGL-1 site density at this concentration was approximately 380/μm² based on corresponding fluorescence intensity reported by neutrophils (Moore et al., 1992; Park et al., 2002).

Laminar flow assays

The slides were attached to a parallel plate flow chamber (GlycoTech, Gaithersburg, MD) and the chamber was mounted over an inverted differential interference contrast microscope equipped with epifluorescent illumination (TE2000; Nikon, Melville, NY) using a 100-W HBO mercury lamp source coupled to a bandpass fluorescein and rhodamine filter set (Chroma Technology, Brattleboro, VT). A 12-bit cooled-CCD camera (SensiCam QE; Cooke Corp., Auburn Hills, MI) capable of differentiating 4096 intensity levels was used for image digitization. Fluorescent and brightfield images were directly captured by a G3 Macintosh with IPLab v3.5.5 (Scanalytics, Fairfax, VA) for observation of fluorophore-labeled monocytes and/or platelets interacting with surface substrate. The wall shear stress in the parallel plate flow chamber is controlled by the dimensions of the gasket and the fluid flow regulated with a syringe pump (Harvard Apparatus, Holliston, MA). A water bath was used to maintain cell suspensions and assay media at 37°C for the duration of experimentation. Monocytes or neutrophils resuspended to 0.5×10⁶/ml were perfused over stimulated HUVEC monolayers for 5 minutes, followed by assay media up to the indicated time. 1% paraformaldehyde was
perfused into the flow chamber for 5 minutes to fixate the leukocytes present on the HUVEC monolayer.

PSGL-1 microbeads resuspended to $0.5 \times 10^6$/ml were initially perfused over the P-selectin substrate for 5 minutes at 0.5 dyn/cm$^2$ wall shear stress. Due to detachment of 10-µm PSGL-1 microbeads at higher shear stresses, bead concentration was increased to $1.0 \times 10^6$/ml for platelet flow experiments performed at 1 and 2 dyn/cm$^2$ wall shear stress. Assay media flow was continued for 1 minute to remove non-adherent PSGL-1 beads from the flow chamber. Platelets were resuspended at $30 \times 10^6$/ml and were perfused into the flow chamber at the indicated wall shear stresses for 20 minutes.

**Imaging and Quantitation**

Three dimensional images of fluorescently labeled leukocytes were acquired using a Bio-Rad MicroRadiance confocal scanner attached to a Nikon TE-300 inverted microscope with a 40x Plan Fluor objective. The z-axis step motor was set at 0.5 µm intervals. Differences in the refractive indices between the immersion medium and the sample's mountant can cause aberrations along the observed Z-axis (Errington et al., 1997). Z-axis scaling was calibrated using 6-µm carboxyl YG fluorescent microspheres (Polysciences).

All image processing was done with NIH ImageJ (Rasband, 1997-2004). Images were run through a 3x3 median bandpass filter to remove shot noise while preserving edge detection. The images were further subjected to a high pass filter (size = 1.7 pixels (0.5 µm)) to suppress image intensity variations not associated with linear features then subsequently smoothed out with a Gaussian filter.
Figure 4.2 Intensity threshold methodology for labeled adherent leukocytes. The image frame with the highest global mean intensity was chosen out of the stack to generate the cell outline mask. Mean cell intensities ($\overline{I}_{cell}$) within each outline were compiled and the average value was used as a lower threshold level of cell detection. Bar, 50 μm.
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For each image stack, the image frame with the largest global mean intensity 
($\bar{I}_{frame}$) was used to create an outline mask (Fig. 4.2). The upper threshold level was
adjusted until the red mask ($\bar{I} = 0$) completely covered the black background. Average
intensity measurements of the labeled cells ($\bar{I}_{cell}$) were measured inside the threshold
mask. Threshold level of the image stack was readjusted so that $\bar{I}_{cell}$ was equivalent to
the minimum threshold level. As the cell moves out of the focal plane, less of the red
region inside each cell will be visible. The apical and basal position of each adherent
cell was determined by counting the frames where the red mask is present in each cell.

Seven sequential non-overlapping images of fluorescently labeled platelet
attachment to microbeads were acquired at 20× magnification with the camera exposure
time set at 200 ms. Each image was subtracted against background and analyzed with
ImageJ. In each group, ten individual platelets were selected and the mean of their
fluorescent intensity was used to obtain a standard platelet fluorescence value. Individual
platelets or platelet aggregates were outlined and their respective intensity values were
divided by the standard platelet fluorescence to obtain platelet counts. The total area of
one optical field at 20× corresponded to 0.138 mm².

Available PSGL-1 microbead surface area for platelet attachment was calculated
as follows:

$$SA = 4\pi R^2 \left(1 - \frac{2\theta_p}{360}\right)$$

(4-1)

where $\theta_p$ is the exclusion angle and $R$ is the microbead radius (Fig. 4.3). $\theta_p$ for 3-, 6-, and
10-µm microspheres were calculated to be 70.53°, 48.19°, and 36.87°, respectively. The
resulting surface area accessible for platelet adhesion was 17.20 \( \mu \text{m}^2 \), 82.82 \( \mu \text{m}^2 \), and 249.81 \( \mu \text{m}^2 \) for 3-, 6-, and 10-\( \mu \text{m} \) microspheres, respectively.

**Figure 4.3** PSGL-1 bead surface area available for platelet attachment. An exclusion height of one platelet radius (1 \( \mu \text{m} \)) was used to calculate the exclusion angle for platelet binding (\( \theta_p \)). The surface area of the exclusion zone (orange) was subtracted from the overall surface area of the bead.

The efficiency of secondary capture mechanisms of platelet adhesion to PSGL-1 coated microbeads was approximated by particle interception theory neglecting lubrication effects as well as surface attraction (Spielman and Goren, 1970; Yao et al., 1971). Interception capture efficiency of a collecting cell in axisymmetric flow for a non-diffusing particle is the ratio of mass flow rate of particles following streamlines divided by the mass flow rate excluded by the collecting cell:

\[
E_c = \frac{2\pi CR_c A \Psi}{\pi R_c^2 C_o U} = \frac{3\pi C_o U R_p^2}{2 R_c^2 C_o U} = \frac{3}{2} \left( \frac{R_p}{R_c} \right)^2
\]  

(4-2)

where \( R_p \) is the particle radius, \( R_c \) is the radius of collecting cell, \( \Psi \) is the velocity field stream function, and \( C_o \) is the bulk suspension concentration. The small spherical particle
follows an undisturbed fluid streamline near the larger collecting sphere until the particle
and collector touch, and the particle is retained by adhesion (Probstein, 1994). This
model assumes that the collecting sphere is suspended in fluid flow, away from surface
boundaries. The resulting non-dimensional factor for capture efficiency is solely
dependent on the dimensions of the flowing particle and the non-motile collecting cell.

*Leukocyte Transmigration*

3-μm pore size 12-well polyester Transwell plates (Corning Costar, Acton, MA) were
coated with 0.1% gelatin for HUVEC attachment. 80,000 HUVECs in 400 μl were
inserted into each well and were cultured 1-2 days until confluent. The lower
compartment of the Transwell dishes contained 1.5 ml of the HUVEC growth medium
during culture and in the experiments. HUVEC were stimulated with 5 ng/ml
recombinant IL-1β for 4 hours prior to experimentation. The medium in the lower
compartment was replaced with pre-warmed HUVEC medium with varying low
concentrations of IL-8 (0, 0.1, and 1 ng/ml). Dil-labeled monocytes/neutrophils (0.3×10⁶
cells/ml) were layered over the HUVEC monolayer and placed back into the incubator.
The number of leukocytes that transmigrated into the bottom well was assessed using an
epifluorescent microscope and obtaining the average number of labeled cells in five fields
of view for each fraction.

*Finite Element Analysis*

Computational fluid dynamic analysis of shear stresses and velocity patterns near
leukocyte interfacial surfaces was conducted using the finite element software package
FEMLAB 3.0 (Comsol, Burlington, MA) running on a 1.8 GHz Pentium 4 personal computer with 1.3 GB of RAM (International Business Machines, White Plains, NY).

The flow chamber dimensions used in the model were 0.2×2 mm, similar to the experimental flow chamber. Five 8 μm-diameter circular projections representing adherent leukocytes were positioned 0.2 mm apart on the same line (y=0). The height of exposure for each circle increased in 1-μm increments ranged from 4 μm (semi-circle) to 8 μm (full circle). Assumed buffer media viscosity (1.0×10^{-3} kg m^{-1}s^{-1}) and density (9.98×10^{2} kg m^{-3}) values at 20°C were used in the equations for incompressible Navier-Stokes fluid flow:

\[ \rho_f \frac{\partial \vec{u}}{\partial t} - \nabla \cdot \nabla \vec{u} + \rho_f (\vec{u} \cdot \nabla) \vec{u} + \nabla P = \vec{F} \]  \hspace{1cm} (4-3)

\[ \nabla \cdot \vec{u} = 0 \]  \hspace{1cm} (4-4)

where \( \rho_f \) is the fluid density, \( \nu_f \) is the fluid kinematic viscosity, \( \vec{u} \) is the 2-dimensional (2-D) velocity field in the x- and y- direction, \( P \) is the pressure, and \( \vec{F} \) is the volume force field (i.e. gravity). Flow was assumed to be steady (\( \frac{\partial \vec{u}}{\partial t} = 0 \)) and external forces were considered negligible (\( \vec{F} = 0 \)). No slip (\( \vec{u} = 0 \)) boundary conditions were set at the fluid interfacial surfaces with the wall and cell borders. Outflow pressure was set for free flow.

The inlet velocity was calculated from the 2-D Poiseuille equation of flow:

\[ u(y) = \frac{\Delta P}{2 \nu_f L} \left[ \frac{h}{2} \right]^2 - y^2 \right] = \frac{4 u_{\text{max}}}{h} \frac{y}{(1 - \frac{y}{h})} \]  \hspace{1cm} (4-5)

where \( \Delta P \) is the pressure drop across the chamber, \( L \) is the length of the chamber, \( h \) is the chamber height, and \( u_{\text{max}} \) is the maximum fluid velocity in the x-direction. Maximum
inlet velocities \( (u_{\text{max}}) \) were set between 0.0025 – 0.075 m/s, which corresponded to wall shear stresses of 0.5 – 15 dyn/cm². Shear rates and shear stresses were computed:

\[
\tau_w = \nu_f \gamma_w = \nu_f \frac{du}{dy}
\]  

(4-6)

Stokes regime flow was verified by calculating the Reynolds number:

\[
Re = \frac{L_c \bar{u}}{\nu_f}
\]  

(4-7)

where \( L_c \) is the characteristic length (channel height = 200 \( \mu \)m) and \( \bar{u} \) is the average flow velocity. The Reynolds number for the highest flow rate simulated \( (u_{\text{max}} = 0.075 \) m/s) was 0.0002. A predefined mesh size within the software package was set to 'Extra Fine'.

The mesh for the fluid domain was refined to a 10 nm average size, 50 \( \mu \)m upstream and downstream of each solid circular projection to resolve possible flow irregularities that may occur (Fig. 4.4). A grid of 9700 elements was generated to solve the 2-D fluid flow solutions, requiring about 140 CPU seconds.

Shape dimension profiles of HL-60 cell rolling on P-selectin at 2 dyn/cm² and 15 dyn/cm² wall shear stress were obtained from images shown in Dong, D and Lei, XX (2000). Ellipsoid outlines of the shear-deformed cells were generated and measured in ImageJ, and the dimensions were inserted in the finite element model. At 2 dyn/cm² wall shear stress, the rolling HL-60 cell ellipsoid profile has a major axis of 6.8 \( \mu \)m, minor axis of 6.13 \( \mu \)m, a rotational offset of -20°, and a centroid height elevation of 2.546 \( \mu \)m.

For 15 dyn/cm² wall shear stress, the rolling HL-60 cell profile has a major axis of 8.754 \( \mu \)m, minor axis of 6.67 \( \mu \)m, a rotational offset of -40°, and a centroid height elevation of 0.99 \( \mu \)m (Fig. 4.5).
Figure 4.4 Finite element grid mesh of fluid region inside the parallel plate flow chamber. 8-μm diamter circular projections with varying height increments (1 μm) were used to represent varying stages of leukocyte transmigration.
Figure 4.5 Adherent cell deformation under shear flow. Side-view flow chamber video micrographs show HL-60 cell rolling on 375 sites/μm² P-selectin at 2 dyn/cm² (A) and 15 dyn/cm² (C) wall shear stress. The geometric parameters of the shear-deformed cells were incorporated into the finite element model (B & D). Side-view flow chamber images were obtained from Dong, C and Lei, X (2000).
4.3 Results

4.3.1 Adherent Leukocyte Asymmetry on Stimulated Endothelium

The surface morphology of leukocytes adherent on IL-1β stimulated HUVECs was assessed using laser-scanning confocal microscopy (Fig. 4.6). Neutrophils perfused over stimulated HUVECs for 15 minutes at 2 dyn/cm² wall shear stress displayed a spread appearance near the endothelial surface. Lamellipodial extensions were observed protruding out from the main cell body. In contrast to the observed activated state of neutrophils, monocytes appeared to retain much of their spheroid shape after 15 minutes of flow. The average height of monocyte adherent on cytokine-stimulated HUVEC was 8.12 μm with a standard deviation of 1.07 μm. Neutrophils adherent on HUVEC monolayers had significantly less height protrusion (P<0.05). Average neutrophil height on stimulated endothelium was 6.5 ± 1.15 μm as determined through confocal image analysis.

4.3.2 More Rapid Transmigration of Neutrophils versus Monocytes

I proceeded to examine differences in monocyte and neutrophil adhesion and transmigration patterns on endothelial monolayers. The isolated leukocyte populations were layered over confluent HUVECs cultured on Transwell plates. Over a 6-hour period, neutrophils transmigrated across the endothelial layer into the lower well of the Transwell dish in more numbers than monocytes (Fig. 4.7 A). Neutrophils also transmigrate across the cytokine-stimulated endothelial monolayer at a faster rate than monocytes. After 30 minutes, neutrophils have already begun to appear and increase in numbers at later time points (Fig. 4.7 B). It is not until two hours after leukocyte addition that appreciable numbers of monocytes are seen. After 3 hours, significantly more
neutrophils than monocytes have transmigrated across the stimulated HUVEC monolayer by an average of 83.08 ± 4.70 and 26.24 ± 3.78, respectively. Neutrophils were able to transmigrate across cytokine-stimulated endothelium alone in similar amounts compared with the presence of a low IL-8 gradient (0.1 ng/ml). IL-1β stimulation of endothelial cells does not appear to be a prerequisite for monocyte transmigration in the static assay. Without HUVECs present, monocyte and neutrophil populations can already be seen after 15 minutes in the lower well containing 1 ng/ml IL-8. More neutrophils appear at the early onset but after three hours, the numbers of monocytes and neutrophils that have transmigrated are similar in the absence of endothelial cells.

4.3.3 Platelet Attachment on PSGL-1 Microbeads Determined by Bead Size

To directly examine the particle protrusion assistance of platelet capture, microbeads of different sizes were coated with PSGL-1. A concentrated P-selectin surface was used to immobilize PSGL-1 microbeads on the planar surface. TRAP-activated platelets were perfused over PSGL-1 presented on flat plates or surface-bound PSGL-1 microbeads at a wall shear stress between 0.3 - 2 dyn/cm², and the amount of platelet accumulation was assessed after 10 minutes of platelet flow (Fig. 4.8 and 4.9 A). Observations in each field of view indicated that platelet attachment correlated with the number of PSGL-1 microbeads present on the surface (Fig. 4.9 B). Firmly attached platelets were observed all around the bead but were more prevalent downstream of each bead. Platelet interactions (tethering or firm arrest) with the P-selectin substrate were not detected. Platelet attachment to each of the adhesive substrates was normalized to the PSGL-1 surface area available for platelet binding (Fig. 4.9 C).
Capture efficiency of immobilized PSGL-1 beads for flowing platelets appears to be affected by both bead size and flow rate. At 0.3 dyn/cm², the smaller 3-μm beads were more effective in platelet capture than the 6- and 10-μm beads (89,535/mm² vs. 53,757/mm² and 27,467/mm², respectively). At 2 dyn/cm², the 3-μm beads became less efficient in platelet capture than the 6- and 10-μm beads (10,936/mm² vs. 26,417/mm² and 24,061/mm², respectively). Theoretical estimations of platelet attachment efficiency were normalized to the values at 0.5 dyn/cm² for the 10-μm bead. The particle interception estimation method predicts a 5-fold high platelet per surface area value than observed with the 3-μm beads at 0.5 dyn/cm² wall shear stress, and a 1.5-fold higher amount for the 6-μm beads at 0.5 dyn/cm². The flat PSGL-1 substrates were not as efficient in platelet capture as the immobilized PSGL-1 beads.

The amount of platelet accumulation around the PSGL-1 microbeads was proportional to the bead size (Fig. 4.10). At 0.3 dyn/cm² wall shear stress, activated platelets accumulated on the 3-μm PSGL-1 beads at 1.54 ± 0.1 platelets per bead which increased to 4.45 ± 0.49 for the 6-μm bead and up to 6.86 ± 0.73 platelets per bead for the 10-μm bead. Increasing wall shear stress from 0.3 to 2 dyn/cm² had a larger effect in attenuating platelet accumulation for the 3-μm bead than the 6-μm bead (87.8% vs. 50.8%, respectively). The 10-μm PSGL-1 beads appear to effectively shield platelet aggregate formation from increases in flow.
Figure 4.6 Visualization of fluorescently-labeled leukocytes adherent on stimulated HUVEC monolayers after 15 minutes of flow at 2 dyn/cm² wall shear stress. Maximum intensity projection of the confocal image stack was performed to obtain the three-dimensional image. Surface X-Y view of neutrophils (A) and monocytes (C) arrested over endothelium. Image projection at a rotational offset of 70 degrees about the x-axis for neutrophils (B) and monocytes (D). Bar, 20 μm.
Figure 4.7 Monocyte and neutrophil transmigration across HUVEC monolayer. Monocytes (blue bars) or neutrophils (red bars) were layered over non-stimulated HUVECs (0) or HUVECs stimulated with IL-1β the lower well containing 0 ng/ml (M0/N0), 0.1 ng/ml (M1/N1), or 1 ng/ml (M2/N2) IL-8. Monocyte or neutrophil transmigration in the absence of HUVECs was also compared (M'0/N'0). Data values represent means ± SE of 3 experiments.
Figure 4.8 Brightfield and fluorescent images of platelet attachment to PSGL-1 surfaces. Fluorescently labeled platelets (green) were observed on PSGL-1 planar surfaces (A), 3-μm (B), 6-μm (C), and 10-μm (D) PSGL-1 coated microspheres. Bar, 50 μm.
Figure 4.9 Shear stress modulation of platelet recruitment to immobilized PSGL-1 surfaces. Schematic of PSGL-1 orientation on the surfaces examined (A). TRAP-activated platelets were perfused over a flat surface of PSGL-1 or PSGL-1 microbeads of varying sizes (B). Platelet accumulation counts were normalized to the available PSGL-1 surface area and theoretical prediction of particle capture was estimated from Eq.4.2 (C). Data labels in panel (B) refer to the mean PSGL-1 microbead counts per field of view in each condition. Data values represents means ± SE of 3 experiments.
Figure 4.10 Platelet recruitment by PSGL-1 microbeads. TRAP-activated platelets were perfused over surface-adherent PSGL-1 microbeads at different wall shear stresses. Data represent means ± SE of 3 experiments.
4.3.4 Flow Perturbation Downstream of Cellular Protrusions

The significance of leukocyte activation and resulting morphological changes could affect the fluid flow around the adherent leukocyte. The antibody blocking experiment performed in the previous chapter verified that leukocyte capture of activated platelet is primarily mediated by the P-selectin/PSGL-1 bond (Fig. 3.11). Since the formation and dissociation of selectin bonds are dependent on the fluid shear force, alterations in this parameter can significantly influence the amount of platelet capture and accumulation. The variation in fluid dynamics around adherent leukocytes of different shapes was examined using a finite element model.

Laminar fluid flow at 8 dyn/cm² wall shear stress over a 8 μm-diameter circular object show large regions of low (< 4 dyn/cm²) or negative shear stress (Fig. 4.11 A). Evaluating the shear stress profiles along horizontal cross-sections across the circular protrusion depict the steep transitions proximal to the cell (Fig. 4.11 B). Negative shear stress values indicate stress in the opposite direction of flow and the presence of small recirculation zones. Arbitrarily choosing 50% of wall shear stress as a low shear parameter permitted comparison of fluid disturbance in relation to the circular body exposure to fluid flow. The length along the wall surface of reduced shear stress produced downstream by a semi-circle 4 μm in height is 5.2 μm while a 8 μm diameter leukocyte just initiating contact would provide a low fluid stress field 17 μm downstream. The maximum area of reduced shear stress produced by fluid disruption increases with cell surface exposure (Fig. 4.12). The amount of low shear produced by an 8 μm-diameter circular body protruding to a maximal height of 4 μm is almost a magnitude lower than the area produced when the full circle is allowed to disrupt flow.
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Figure 4.11 Shear stress perturbation effects from leukocyte protrusion. The finite element model predicts low shear stress regions proximal to the adherent leukocyte at 8 dyn/cm² wall shear stress (A). Cross-sections of the fluid region proximal to the adherent leukocyte depict the variations in shear stress that can occur around a stationary leukocyte (B). Heights in legend of panel B correspond to the red cross-sectional lines shown in panel A.
Figure 4.12 Regions of reduced shear increases with cell protrusion. A criteria of 50% wall shear stress as a low shear cutoff demonstrates that an increase of leukocyte exposure to fluid flow will promote more of a reduction of shear stress. The volume of fluid experiencing reduced flow around an adherent leukocyte with all of its body protruding out to the fluid flow is a magnitude greater than a leukocyte that has partially transmigrated and has half of its body exposed to the fluid shear forces.
Figure 4.13 Area of disturbed flow downstream of projection preserved at high shear. Schematic of the relative flow disturbance produced by a 4 μm and a 7 μm circular projection (A). The red zone under the dashed line denotes the reduced shear area. A threshold of 1 dyn/cm² for reduced shear was evaluated downstream of a 7 μm (black bar) or a 4 μm (grey bar) protrusion (B).
Figure 4.14 Computational analysis of shear stress asymmetry around shear-deformed cell adherent on the P-selectin surface. Shear stress profiles of horizontal cross-sections around an HL-60 cell adherent at wall shear stresses of 2 dyn/cm² (A) and 15 dyn/cm² (B) upstream and downstream along the x-axis. Red dashed line indicates the criteria level for reduced shear (50% wall shear stress). Blue dashed line indicates an absolute criteria level for reduced shear of 1 dyn/cm².
4.3.5 Low-Shear Regions Downstream of Deformed Rolling Cell

The steady state evaluation of flow disturbance generated downstream of a myeloid leukemic HL-60 cell rolling on P-selectin was analyzed. Visualization of cell deformations occurring with increasing shear is seen with side-view flow chamber images of HL-60 cell rolling on P-selectin (Dong and Lei, 2000), and the observed cellular profiles were used in the computational fluid simulation (Fig. 4.4). Larger areas of flow reduction can be seen downstream of the shear-deformed cell than upstream (Fig. 4.14). Table 4.1 shows the reduced shear areas utilizing both threshold criteria for 2 dyn/cm² and 15 dyn/cm² wall shear stress downstream of the asymmetric shear-deformed cell. For a wall shear stress of 15 dyn/cm², increasing the reduced shear level threshold from 1 dyn/cm² to 2 dyn/cm² increased the area from 9.19 μm² to 13.6 μm², and a 4 dyn/cm² threshold criteria generated a reduced shear area of 23.02 μm². The areas of low shear are likely zones for preferential platelet accumulation.

<table>
<thead>
<tr>
<th>Wall Shear Stress</th>
<th>Cell Height</th>
<th>50% Reduced Shear Area</th>
<th>Shear Area &lt; 1 dyn/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 dyn/cm²</td>
<td>8.81 μm</td>
<td>49.2 μm²</td>
<td>49.2 μm²</td>
</tr>
<tr>
<td>15 dyn/cm²</td>
<td>8.54 μm</td>
<td>48.85 μm²</td>
<td>9.19 μm²</td>
</tr>
</tbody>
</table>

Table 4.1 Simulation results of fluid region downstream of deformable cells.

4.4 Discussion

Areas of low or recirculatory blood flow are potential sites for the development of venous thrombosis (Libertiny and Galland, 2001; Raju et al., 2000; Severyn et al., 2004).

In this chapter, I have shown that areas of low shear that form immediately downstream
of adherent cells expressing PSGL-1 correlate with the accumulation of activated platelets. I have shown previously that platelets form larger thrombi downstream of adherent monocytes than neutrophils, even though both leukocyte subtypes present equivalent levels of PSGL-1 on their surface. Confocal microscopy demonstrates that monocytes present a more spherical and taller structure on cytokine-stimulated endothelial cells than neutrophils. 3-, 6-, and 10-μm PSGL-1 microspheres were used to standardize cell projection height and observed larger platelet thrombi formation downstream of the larger beads. Computational fluid dynamics of the region surrounding the surface-adherent bead verify the presence of reduced shear regions proximal to the bead-wall interface and the maintenance of the low shear zone at high wall shear stresses.

Stimulated endothelial cells will immediately secrete P-selectin to the membrane surface (McEver et al., 1989), promote the rapid induction of E-selectin and VCAM-1 mRNA transcripts (Bevilacqua et al., 1987; Chan et al., 2001; Cybulsky et al., 1991; Shimizu et al., 1991), and upregulate ICAM-1 expression (Lusczinskas et al., 1991; Smith et al., 1990; Staunton et al., 1988). In addition to leukocyte adhesion molecule presentation, the endothelium can act as a source of cytokines for leukocyte activation such as IL-1, IL-8, GM-CSF, and MCP-1 (Carr et al., 1996; Furie and Randolph, 1995; Gerszten et al., 1999; Griffin et al., 1990; Middleton et al., 1997). The potency of endothelial-derived cytokines on leukocyte activation is dependent on cell expression of the respective chemokines receptors (Campbell and Butcher, 2000; Johnston and Butcher, 2002; Seo et al., 2001; Zlotnik and Yoshie, 2000). The discrepancy in appearance of activation for monocytes versus neutrophils on stimulated HUVECs may be due to the
differential secretion of leukocyte activators by the endothelium (Luster, 1998; Tanaka et al., 1993).

The extravasation rate of neutrophils is higher than that of monocytes after intradermal injection of Escherichia coli, peaking after 2 hours versus 3-4 hours, respectively (Issekutz and Movat, 1980; Issekutz et al., 1981). Induction of inflammation through cytokine-stimulation \textit{in vivo} results in a similar early onset of neutrophil extravasation, followed by emigration of monocytes and lymphocytes a few hours later (Jung et al., 1996; Xie et al., 1997). As a leukocyte proceeds to transmigrate through the endothelial layer via cytokine-stimulation or chemotactic gradients, less of its cellular body is exposed to the vascular flow (Luscinskas et al., 1991; Luu et al., 2000; Shaw et al., 2001). This can restrain platelet accumulation in several ways. The activated leukocyte would provide more of a streamlined profile and less surface for the flowing platelets to impinge on for the initiation of leukocyte-platelet tethering bonds. The activated/transmigrating leukocyte would also present fewer adhesion receptors available for platelet capture or tethering. The disturbance in fluid shear on the downstream end of the adherent leukocyte, while minute on a macro-scale level, can have significant impact on a small particle the size of a platelet. As the platelet tethers, rolls, and comes to rest downstream of the leukocyte (Fig. 3.6), the protruding body is able to shield the platelet from fluid forces necessary to promote detachment.

In order to isolate the contribution of cell size and protrusion from the wall towards enhancing platelet thrombi formation, immobilized PSGL-1 microbeads were utilized as reactive sites for platelet adhesion under flow. Platelet attachments could be detected all around the periphery of the immobilized PSGL-1 bead, in contrast to the
nearly exclusive downstream accumulation of platelets observed on leukocytes as seen in
Chapter 3. Platelet interactions with PSGL-1 beads displayed less rolling and more firm
adhesion events than leukocytes once platelet – bead contact was initiated. Insufficient
non-specific blocking could be a contributing factor to the large proportion of immediate
firm adhesion events. Bare beads with the same blocking protocol will not interact with
immobilized protein substrates (Park et al., 2002), however interaction of beads with
cells/tissues may incorporate more non-specific van der Waals forces that were not
significant in polystyrene bead and synthetic coated surfaces (Florin et al., 1994;
Leckband et al., 1994). Another possibility may be due to an increase in ligand activity
associated with presentation on a polymer matrix. Conformational changes or clustering
of fibrinogen upon surface immobilization have been implicated for the observation of
resting platelet interactions with adsorbed fibrinogen substrates (Luscher and Weber,
1993; Zaidi et al., 1996).

The effectiveness of platelet binding increases as the PSGL-1 bead size decreases
for wall shear stresses ≤1 dyn/cm² as predicted by interception theory (Spielman and
Goren, 1970; Yao et al., 1971). The dramatic decrease in platelet capture by the 3-μm
beads, and to a lesser extent for the 6-μm beads, at higher shear is most likely due to
mass transport limitations near the planar surface on which the PSGL-1 beads are
anchored. The interception theory is appropriate for axisymmetric flows around the
collecting cell. At low flow around large surface-adherent cells, the mass flux of
platelets will be a closer approximation to the axisymmetric flow modeled. However, the
model breaks down at higher flow and smaller beads probably due to shear gradient
generation and wall exclusion of platelets. A more appropriate model which takes into
account the interference of the wall surface in particulate attachment by a collecting cell may need to be considered.

Activated platelet interactions with PSGL-1 through P-selectin are shear sensitive and form longer attachments at lower shear (Alon et al., 1995b; Konstantopoulos et al., 1998; Schmidtke and Diamond, 2000). Arbitrarily choosing a threshold of shear stress less than 1 dyn/cm² for platelet firm arrest, computational fluid dynamics demonstrates that a half-circle representation of a transmigrating leukocyte with height protrusion of 4 μm would only have enough of a reduced shear area for two platelets (3.14 μm²/platelet) whereas an attached leukocyte with 7 μm protruding out would enable 9 platelets to accumulate in the downstream area for wall shear stress of 2 dyn/cm². It is difficult to extrapolate from the 2-D finite element data the actual number of platelets that could reside in a volume of reduced shear downstream of an adherent bead/cell. Translating the geometry of a 2-D circular projection into a three-dimensional (3-D) analysis produces an infinitely long cylinder. The fluid reduction downstream of a cylinder would be different from the reduction produced by a spherical object. The 2-D computational model offers a relative look at the influence cell protrusion may have in decreasing shear downstream of the object. With more computing resources and investigational time, a more appropriate 3-D model of fluid flow around the surface-adherent cell/bead could be constructed.

The platelet accumulation patterns seen around the surface-adherent microbeads resembled the downstream platelet deposition shown with monocytes arrested on HUVEC. Although the bead model cannot separately isolate and analyze the three features outlined as influencing platelet deposition, the relationship of amounts of platelet
aggregation per bead and fluid shear stress attenuation with respect to bead/cell height are very similar. Analysis of cell profiles obtained from HL-60 cell rolling on P-selectin (Dong and Lei, 2000) show asymmetries in the amount of low shear present downstream versus upstream of the cell. These asymmetries developed during flow may account for the increased downstream preference of platelets accumulation on leukocytes compared to PSGL-1 beads. It appears that fluid disturbance downstream may be the main factor which regulates platelet accumulation around an adherent leukocyte, provided that the platelets are able to interact and roll on the surface of the adherent leukocyte.
Chapter 5

Concluding Remarks

5.1 Summary

In the vascular system, cell-cell interactions under fluid shear are important in normal physiology and in pathologic situations. During an inflammatory response, leukocyte arrest on the endothelium and extravasation are required for normal immune function. Leukocyte rolling, primarily through selectins, is a prerequisite for arrest via engagement of leukocyte integrins to endothelial ligands (Bullard et al., 1996; Frenette et al., 1996; Jung et al., 1998). Susceptibility to recurrent bacterial infections and impaired wound healing are common symptoms in people with inherited defects in the β2-integrins Mac-1 or LFA-1 (Anderson and Springer, 1987). The recruitment and attachment of bone marrow-derived endothelial progenitor cells to sites for re-endothelialization or angiogenesis may also require the involvement of selectins and integrins (Urbich and Dimmeler, 2004). The presentation of activated platelets on the surface of circulating leukocytes or tumor cells enhances endothelial interactions at high shear (Bastida et al., 1989; Huo et al., 2003). The specific molecular interactions that vascular cells may employ for cell adhesion are closely intertwined with the local fluid shear stress environment.

Cell shape changes can affect the local fluid environment around an adherent cell and on the interaction force of the cell with the adhesive substrate (Firrell and Lipowsky, 1989; Hyman, 1972; Smith et al., 2002). In order to circumvent leukocyte deformation issues, I designed ligand-coated microbeads to assist my analysis of cell interactions
occurring in fluid shear flow. The lack of deformability and narrow size distributions associated with the microbead system enable me to account for cell shape variations.

The significant contributions of this study are:

1. Microvilli present on the surface of circulating leukocytes prolong interaction times of selectin-mediated bonds under shear flow. Bonds associated with long tethers will experience less loading force than shorter tethers. Microvilli also act as force dissipating structures by elongating with increasing tensile force. The material properties obtained from microvilli tethers formed under fluid shear force show that the nature of elongation is viscoelastic.

2. Platelet accumulation on endothelium at venular shear rates is enhanced in the presence of adherent leukocytes. Platelets roll on the arrested leukocyte and accumulate on the downstream side of immobilized leukocytes. Platelet interaction mechanisms with adherent leukocytes are dependent on the amount of fluid shear and level of platelet activation. Activated platelet accumulation amounts are greater on endothelium-adherent monocytes than adherent neutrophils.

3. Neutrophils on cytokine-stimulated endothelium are more spread-out and with less height projection into fluid flow than monocytes. The amount of activated platelet accumulation downstream of immobilized PSGL-1 microbeads is proportional to the microbead size. The downstream areas of adherent leukocytes could generate regions of secondary flows and provide an ideal fluid shear environment for platelet accumulation.
5.2 Future Directions

Interesting new avenues of research can be pursued from the experimental observations and scientific tools developed in this study. Design of a more effective drug delivery carrier is of an interest to many in academia and industry. Bioconjugation of particles with adhesion molecules can increase the effectiveness of localized delivery, however the fluid shear stress present in blood circulation remains a large barrier that needs to be overcome. Decreasing the force experienced by the adhesion molecules present on the particles could increase the interaction duration of the bound particle. A variety of chemical and biological functionalization strategies could be employed to increase the tether length for adhesion (Fig. 5.1 A).

Poly(ethylene glycol) molecules are widely available through commercial sources and come with many different chemical modifications for easy conjugation. Proteoglycan complexes could be utilized as ‘stiffer’ molecular linkages for enhanced adhesion molecule presentation away from the particle body. Titin polypeptides possess immunoglobulin regions that can bind with each other and dissociate under force, resulting in an elongated the molecule. Particles may be conjugated with single-stranded DNA of various sequences. Customizable tether lengths could be hybridized upon inclusion of complementary DNA sequences that are functionalized at one end with an adhesion protein. Other strategies include modifying the carrier itself to display tethers for enhanced capture and retention (Fig 5.1 B) (Rychak, 2004).

Immobilized ligand-coated beads can be used to study multi-cell aggregation, such as platelets, or single cell adhesive interactions. Fluorescent nano-beads may be functionalized with adhesion receptor ligands before immobilization to a planar surface.
(Fig. 5.2 A). Verification of location and dispersity could be detected using high-resolution fluorescence microscopy or atomic force microscopy (AFM). Depending on the type of adhesion molecule present on the nano-bead, biological applications include regulation of adhesion receptor site density, affinity versus avidity differentiation of bond formation, or spatial control of endothelial cell focal adhesion complexes (Fig. 5.2 B).

This study may also address some technical issues that may arise in optimization with tissue engineering and/or bioprocess manufacturing. Fluid flow is necessary to deliver oxygen and essential nutrients to the cells and remove carbon dioxide and other waste from the cells. Greater fluid flow over surface immobilized cells may be necessary for cells with high metabolic activity or to enhance product yield. Shielding cells or cell aggregates from high shear is important to prevent cells cell detachment (Fig. 5.3). Engineering strategies could be implemented to balance effective fluid delivery with protection against destructive shear stresses.
Figure 5.1 Tether elongation strategies to increase lever arm length. Adhesion receptor modification with poly(ethylene glycol) (PEG), proteoglycan (PG), titin, or single stranded DNA may be used to couple with drug delivery particles. Increasing the tether length may decrease the loading force on the adhesion receptor and increase interaction time of the drug delivery particle (A). Microvilli-like lipid shell ruffles can be created on gas-filled microbubbles (B) (Rychak, JJ, 2004).
Figure 5.2 Functionalized nanobead immobilization on planar surfaces. Fluorescent beads may be visualized on a surface to determine specific locations of immobilization (A). Nanobeads coated with adhesion receptors may be used to tightly control cell ligand site densities or adhesion receptor clustering (B).
Figure 5.3  Shear stress protection of cellular aggregates in tissue engineering or biocatalysis applications. Cellular aggregates directly cultured on a flat substrate are directly exposed to the fluid shear stress (A). Insertion of a barrier upstream of the cellular aggregate may possibly limit disruption of the aggregate (B). Cells cultured in micro-channel troughs experience less exposure to direct shear (C).
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