Nano-to-Microscale Mechanical Switches and Fuses Mediate Adhesive Contacts between Leukocytes and the Endothelium

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INTRODUCTION

As a vital part of the inflammatory response, the leukocyte adhesion cascade involves the capture of white blood cells at the vascular endothelium, a progressively slowing, “rolling” motion of cells along the vessel walls, firm cell adhesion at target sites, and eventually, the transmigration of activated cells into the inflamed tissue1 for an instructive overview see also http://bme.virginia.edu/ley/). Studies aiming to reveal the biophysical mechanisms underlying these processes face the difficult challenge of covering a large range of relevant scales: nanoscale interactions between adhesive molecules, local deformations of subcellular structures under point loads, and whole-cell activation and motion. Yet multiscale approaches also offer particularly rewarding insights, for example, by providing an understanding of single-molecule interactions within their natural, larger-scale biological context and, conversely, by tracing macroscopically observed cellular phenomena directly to their molecular origins.

The present work focuses on nano-to-microscale regulatory aspects that mediate the initial stages of the adhesion cascade. Early events, such as the capture of leukocytes and their initial rolling exploration at the endothelium, occur usually too fast to be regulated biologically, i.e., through the expression of proteins. We thus expected that most mechanisms governing initial leukocyte adhesion under a broad range of blood-flow conditions had to be “preprogrammed” into molecular-to-subcellular structures and that they had to be primarily mechanical. Accordingly, our experimental approach was to study in vitro the nano-to-micromechanical response of individual leukocytes that were loaded at a point with a rapidly increasing force.

The adhesion bond between P-selectin (expressed in vivo on the endothelium and on activated platelets), and its ligand PSGL-1 (resident in the leukocyte membrane) is a key player in the initial adhesion stages.4,5 We exploited this bond to form point attachments between polymorphonuclear leukocytes (PMNs) and functionalized microspheres that had been coated with P-selectin. To distinguish the dynamic properties of an individual P-selectin:PSGL-1 bond from mechanisms governed by other cellular structures, we also tested this bond on its own using isolated PSGL-1 that had been immobilized on a second batch of microspheres.

Both types of interaction, i.e., P-selectin:PSGL-1 as well as P-selectin:PMN, were characterized by dynamic force spectroscopy6,7 over exceptionally wide ranges of force-loading rates. Complementary test surfaces were first brought into soft, feedback-controlled contact to allow for the formation of bonds between immobilized, reactive molecules. Then, while moving the surfaces apart at preset velocities, we recorded the force experienced by attachments between them. Piconewton forces were reported by the biomembrane force probe,8,9 a prototypical biophysical tool that is uniquely suited to study the dynamics of biologically relevant, ultraweak interactions.

This approach enabled us to inspect in detail two possible candidates for principal regulatory mechanisms in early leukocyte adhesion. One is a serial molecular attachment consisting of (i) the extracellular P-selectin:PSGL-1 bond and (ii) a putative, weak biochemical link that anchors the intracellular tail domain of the transmembrane PSGL-1 to the cortical cytostructure.10 The other is a hierarchy of rheological cell responses to point loads: from slow viscoelastic deformation of the whole cell, and a soft-elastic displacement of the cell cortex, to the extrusion of thin membrane tubes (“tethers”) that can easily grow—at quasi-constant pulling forces—to several micrometers in length. The formation of tethers from fluid membranes under point loads is a ubiquitous phenomenon, as evidenced by a large body of work on lipid vesicles11–14 and on various types of biological cells.15–18 A number of pioneering studies also investigated the mechanical properties of PMN tethers19–21 and their importance in leukocyte rolling.22,23

Perhaps even more interesting than these regulatory mechanisms by themselves is the intriguing interplay between them. For example, the lifetime of weak attachments under stress depends critically on the history of force application. In the present case where the leukocyte itself links adhesive molecules to rigid surfaces, any subcellular soft structures will act to dampen the sudden impact of force and thus modulate the force history experienced by the actual adhesion bonds. Therefore, the lifetime, or strength, of adhesive attachments is directly affected by the rheology of cell deformation under point loads. On the other hand, the extrusion of tethers from PMNs appears to commence only
after the cytoskeletal anchor of PSGL-1 has been released. This demonstrates how the failure of a nanoscale attachment may trigger the crossover between distinct regimes of the rheological response of the leukocyte, e.g., from soft-elastic displacement of the cell cortex to tether formation.

The present paper summarizes our recent results on a number of such mechanical, regulatory aspects of PMN adhesion that contribute to the stabilization of leukocyte rolling velocities within a narrow range despite wide variations in shear stresses. Technical and methodological details of our approach have been published before. Therefore, we will include here only the basic principles of experimental operation inasmuch as they are required to understand the interpretation of our results.

BACKGROUND

Dynamic Force Spectroscopy - A Generic Approach to Characterize Weak Interactions. Single-molecule force measurements have been performed with a variety of sensitive force-probe instruments. The required piconewton force resolution can be achieved, to varying degree, by the atomic force microscope (AFM), optical tweezers, the biomembrane force probe (BFP), magnetic pullers (often also called “magnetic tweezers”), and other force microscopes that have used specialized force transducers such as glass fibers or flow in a micropipet. The two most common protocols of force application using these instruments are force clamp and force ramp. Force clamps step up the applied force and then hold it constant at the desired value while recording distance changes that signify molecular events such as bond rupture or intramolecular transitions. In addition to changes in length, the quantity most interesting to assess molecular kinetics is simply the time that goes by until a transition occurs. On the other hand, force ramps increase the applied force gradually, preferably in a linear fashion. Since the applied force-loading rate is known, time and force are interchangeable (until a molecular transition occurs). It has become customary to record the transition force in this mode.

Since molecular-scale transitions are strongly mediated by the random impulses imparted by the thermal environment, lifetimes and transition forces are stochastic quantities. A large number of events need to be recorded to establish the most likely lifetime (at a given holding force) or transition force (at a given force-loading rate). Perhaps one of the most important fundamental insights gained from the interpretation of single-molecule force measurements is that the most likely transition force depends on the applied loading rate, just as the lifetime depends on the holding force. Therefore, the strength of a molecular interaction cannot be characterized by a single value of force. Any statement about the (most likely) rupture force of a weak molecular bond has to indicate, at the very least, the rate of force loading under which the force was measured. A more complete characterization of the molecular interaction must be based on measurements performed at a spectrum of loading rates (or holding forces). Dynamic force spectroscopy is a generic approach for the quantification of biomolecular interactions on the basis of such experimental spectra. It is independent of the particular force-probe instrument and has, indeed, been successfully used in the interpretation of data obtained with the BFP, AFM, optical tweezers, flow chambers, and other dynamic force techniques.

We start from the conceptual view of an energy landscape that governs the interaction between biomolecules (including intramolecular configurational transitions), as has been common in chemical kinetics since Arrhenius and Eyring. The simplest possible energy landscape for the formation/ release of a bond between two molecules is depicted schematically in Figure 1. It maps out the interaction energy along the optimum pathway of dissociation (the “reaction coordinate”). This path leads from a deep minimum—the bound state—over a single, sharp energy barrier to the free, unbound state. The rates of the unbinding (“forward”) and rebinding (“reverse”) transitions are given by (for notation see Figure 1)

\[ k_{\text{off}} = A_{\text{off}} \exp \left( - \frac{E_0 + E_b}{k_B T} \right), \quad k_{\text{on}} = A_{\text{on}} \exp \left( - \frac{E_b}{k_B T} \right) \]  

In solution, Eyring’s prescription for the empirical attempt frequencies (prefactors to the exponentials) has to be augmented by the Brownian damping due to the liquid environment. Kramers’ theory has provided us with a physical basis for the interpretation of these prefactors in terms of the local diffusivity and of the shape of the energy landscape local to the minimum and the barrier.

Here, we are interested in the role of force, which primarily affects the exponentials. The main effect of adding the mechanical potential of a force probe to the interaction energy is a tilt in the energy landscape, pulling down the barrier as shown in Figure 2. (Small shifts in the positions of the minimum and the barrier can be neglected when dealing with deep, narrow minima and sharp barriers. We denote the fixed position of the barrier, or of the “transition state”, relative to the bound state by \( x_{\text{ts}} \).) In the simplest case
of applying a force $f$ that is roughly constant along the reaction coordinate, the reduction in barrier height, $-f\kappa_{\text{un}}$, is the dominating contribution to the change in the off-rate (eq 1):

$$k_{\text{off}}(f) = A_{\text{off}} \exp\left(-\frac{E_0 + E_{\text{un}} - f\kappa_{\text{un}}}{k_B T}\right)$$

(2)

Denoting the spontaneous off-rate $k_{\text{off},0} \equiv k_{\text{off}}(0)$, this is rewritten as

$$k_{\text{off}}(f) = k_{\text{off},0} \exp\left(\frac{f\kappa_{\text{un}}}{k_B T}\right) = k_{\text{off},0} \exp(ff_{\beta})$$

(3)

where the second equality introduces the characteristic force scale for rate exponentiation defined as $f_{\beta} \equiv k_B T/\kappa_{\text{un}}$. First proposed by Bell,$^{29}$ eq 3 clearly illustrates how force exponentially increases the rate of dissociation.

How can we make the connection between these general considerations and experimental force-ramp measurements that give us a distribution of transition forces? Reinterpreting traditional kinetic rate equations as master equations describing a Markov process, we may write for the present simple case

$$\frac{dS_0}{df} = -k_{\text{off}}S_0 + k_{\text{on}}(1 - S_0)$$

(4)

where $S_0$ is the normalized likelihood for occupancy of the bound state. Most force-probe experiments ramp the force too quickly, or use a clamping force too high, to allow rebinding. These experiments are thus performed far from equilibrium, causing the second term in eq 4 to vanish.

The solution of the remainder of eq 4 is nontrivial because $k_{\text{off}}$ depends on force (cf. eq 3), which in turn depends on time. Considering linear ramps in force and denoting the loading rate $r_f \equiv f/t$, we find

$$\frac{dS_0}{df} = -k_{\text{off},0}S_0 \frac{k_{\text{off},0}}{r_f} \exp(ff_{\beta})$$

(5)

The location of the peak in a measured distribution of transition forces can easily be calculated from eq 5 if one recognizes that the measured transition will occur most frequently at the force where the survival likelihood decreases most rapidly with force. Setting the derivative of eq 5 with respect to force to zero, and reinserting the original equation into the resulting right-hand side, we obtain for the most frequent transition force

$$f^* = f_{\beta} \ln\left(\frac{r_f/f_{\beta}}{k_{\text{off},0}}\right)$$

(6)

This hallmark result of DFS appeared first in 1997.$^6$ It predicts a logarithmic dependence of the most frequent transition force $f^*$ on the loading rate $r_f$ for the simple case shown in Figure 2. It also clearly illustrates that two parameters are needed to characterize even the simplest biomolecular interaction: the generalized interaction strength given by the force scale $f_{\beta}$ and the apparent unstressed off-rate (or inverse lifetime) $k_{\text{off},0}$.

If a sufficiently large number of transition forces have been collected at each loading rate, the comparison of the measured histograms of forces with theoretical predictions can significantly increase the confidence in data interpretation. The probability density $\rho(f)$ for the transition to occur at the force $f$ is simply the negative rate of change of the survival likelihood of the ground state, $\rho(f) = -dS_0/df$. The now-required solution of eq 5 is straightforward and leads to the Evans distribution$^7$ of transition forces that can be written as

$$\rho(f) = C_S s \exp(-s) \quad \text{where} \quad s \equiv \exp\left(\frac{f-f^*}{f_{\beta}}\right)$$

(7)

with $f^*$ given by eq 6. (The scaling prefactor $C_S \equiv \exp(f_{\beta}k_{\text{off},0}/r_f)_{f_{\beta}}$ has units of 1/force.) Whenever possible, this universal distribution should be matched to experimental transition-force histograms as a self-consistency test for the results.

The above treatment outlines the general approach to the quantitative interpretation of molecular force-probe experiments. For simplicity, we have considered only the simplest case where a single, sharp energy barrier impedes the unbinding of a weak molecular bond. A surprising amount of experimental data was found to be in excellent agreement with the predicted logarithmic dependence of the transition force on loading rate, eq 6. On the other hand, for molecular interactions with possible direct involvement in the regulation of biological processes one might expect a more complex behavior.

**Biomembrane Force Probe — A Unique Instrument to Study the Dynamics of Ultraweak Interactions.** Figure 3 illustrates the biomembrane force probe (BFP)$^8$ as used in the present P-selectin:PMN tests. Originally designed and applied to measure the strength of single molecular bonds over an exceptional dynamic range (5–6 orders of magnitude in force-loading rate, with a damping factor as small as $\sim 0.0005$ PNs/nm),$^7$ the figure demonstrates how the BFP is also easily adopted for experiments probing the softness of linkers on various scales, including macromolecules, subcellular structures, or even whole cells.
On the left side of both parts of Figure 3, a stationary glass micropipet holds a partially aspirated, preswollen red blood cell to which a small glass bead—the probe tip—was glued chemically. The pressurized red cell is basically an empty membrane bag that behaves like an ideal Hookean spring when deformed along the axis of the rotationally symmetric assembly. A major advantage of the BFP over other piconewton force transducers is that it provides the experimentalist with a handle on its spring constant $k$. Not only is $k$ known fairly accurately from membrane mechanics, it can even be adjusted over a considerable range ($\sim 0.1 - 5 \text{ pN/nm}$) by changing the suction pressure in the pipet that holds the red blood cell. Given by

$$k \approx 2\pi \frac{\alpha}{\ln[4R_0^2/(R_pR_c)]}$$

(8)

the BFP spring constant depends on the membrane tension $\alpha$ set by the aspiration pressure $\Delta p$ through

$$\alpha = \frac{1}{2} \frac{R_0 P_p}{R_0 - R_p} \Delta p$$

(9)

and on three geometric parameters: the radius $R_0$ of the spherical portion of the aspirated cell (measured in the absence of an axial force), the radius $R_p$ of the micropipet, and the radius $R_c$ of the contact disk between red cell and probe-tip bead. A recent experimental study assessing the accuracy of eq 8 gave a sound confirmation of the validity of this equation.30

Multiplying $k$, by the deflection of the force transducer (red blood cell) gives force. The transducer deflection is determined from the displacement of the dark diffraction pattern at the rear of the probe tip. Optical microscopy (Carl Zeiss, Inc., Thornwood, NY) combined with fast video imaging (SensiCam, Cooke Corp., Auburn Hills, MI) allows us to track this diffraction pattern with a resolution of 5–10 nm at frame rates up to $\sim 1500$ frames per second (fps). The 50-fold increase in temporal resolution over the conventional video framing speed (30 fps) is critical for detection of events over a range of 5–6 orders of magnitude in force-loading rate. Monitoring the force in real time at $\sim 0.6$-ms intervals has also enabled us to implement a fast feedback algorithm to control, for example, the impingement force upon touch of a test object to the transducer. Dedicated software allows us to set the approach speed, the feedback-controlled (negative) impingement force, duration of touch, and rate of force loading as needed in each experiment. At high pulling speeds the force reported by the BFP needs to be corrected for viscous damping. The damping coefficient of the (parallel) viscous component of the BFP transducer is easily determined from its exponentially decaying recoil after detachment from any test surface.27

The probe tip is decorated with the receptor (here: P-selectin) for an adhesive molecule (here: PSGL-1) that is immobilized on a test surface. Using a second micropipet to hold and translate the test surface gives our setup great versatility. For example, the test surface can be the chemically modified surface of another glass bead, as in our P-selectin:PSGL-1 tests. Alternatively, as in the case shown in Figure 3, the membrane of an intact neutrophil can be used as the test surface, thus allowing us to study membrane-based adhesion molecules in their natural environment. Translating this test surface into contact with the force probe and subsequently retracting it at the desired rate of force-loading allows us to determine the dynamic adhesion strength and, if needed, the softness of linkers. Translation with nanometer positioning accuracy is achieved by a closed-loop piezo actuator (Polytec PI, Inc., Auburn, MA).

A typical measurement runs for up to 500 test cycles, after which a new BFP (fresh red cell, probe bead, and test surface) is assembled before proceeding with the experiment. When probing dynamic bond strength, up to $\sim 500$ bond-failure forces are usually collected at each loading rate, ideally with an attachment frequency of $\sim 10\%$ of the total number of touches. This low bond frequency provides high confidence that the recorded attachments are mostly single bonds. (For example, assuming Poisson-distributed attachment events, the confidence for bonds to be single is 95% at an attachment frequency of 10%).

RESULTS

Figure 4 presents an overview of two different BFP experiments that we performed to test the P-selectin:PSGL-1 bond (Figure 4A) and the P-selectin:PMN interaction (Figure 4B). Force-time curves recorded during typical test cycles are shown at the bottom. Negative forces mark the feedback-controlled compression of the BFP spring as observed after the test surface had been translated into contact with the probe bead. Subsequently, the test surface was retracted at a preset “nominal” force-loading rate $rf \equiv k v_{pull}$ defined as the product of the BFP spring constant $k$ and the piezo pulling speed $v_{pull}$. This retraction created an increasing force load on any adhesive attachments that had formed during the contact between the two reactive surfaces. The true loading rate experienced by all serial linkages during this stage is the local slope $rf \equiv df/dt$ of the measured force-time curve. Final detachment of the BFP from the test surface was observed as the recoil of the BFP spring to zero force.

For bead-vs-bead tests such as in Figure 4A, the slope $rf$ of the linear force rise is roughly the same as the nominal loading rate $rf$. The maximum force immediately before detachment was recorded as the rupture force of an individual bond, tested at the given loading rate. Collecting a large number of P-selectin-PSGL-1 rupture forces over a wide range of loading rates and plotting the most frequent rupture forces as a function of the logarithm of the loading rate provided us with the dynamic force spectrum of this adhesion bond (see the next subsection).

The example curves in Figure 4B show intriguing force histories recorded in P-selectin:PMN tests at various nominal force-loading rates $rf$. Dotted lines indicate the behavior that one would expect for a rigid linkage where $rf = rf$. The measured forces rise considerably slower, which reflects linker softness—in this case, the softness of the PMN. At all loading rates, the typical force-time behavior of sufficiently long-lived attachments revealed two distinct regimes of PMN softness. The first was an initial elastic deformation of the cell cortex by $\sim 200 - 500$ nm, evident in the linear force rise up to the crossover force labeled $f_0$. In the second regime beyond $f_0$, the rheological response was a continually growing cell extension (up to several micrometers) at quasi-constant force, resembling plastic flow. At this stage,
nanoscale membrane tubes ("tethers") were extruded from the neutrophil. The tethers were generally invisible, apart from an occasional funnel-shaped protrusion at the cellular tether base. As illustrated in Figure 5, we interpret the abrupt transition from elastic to plastic-like behavior at the force \( f_X \) as the failure of a molecular linkage that anchors the intracellular tail domain of PSGL-1 to the cortical cytoskeleton of the leukocyte.\(^{10,25}\) To avoid speculation, we will call this attachment in the following the "cytoskeletal anchor of PSGL-1", without attempting to identify the exact nature of the possibly complex linkage, nor where exactly it may rupture under force.

Presuming the existence of this cytoskeletal anchor of PSGL-1 in normal neutrophils, the formation of an extracellular P-selectin:PSGL-1 adhesion bond will initially establish a membrane-spanning attachment between P-selectin and the PMN cortex, with PSGL-1 acting as a transmembrane "bridge". Such a serial bond is an excellent candidate for a "preprogrammed" molecular structure with functional importance in the mechanoregulation of leukocyte adhesion. Clearly, the P-selectin-mediated PMN attachment to the endothelium will terminate if the extracellular P-selectin:PSGL-1 bond fails. The force history experienced by this bond, however, is dramatically different depending on whether PSGL-1 is anchored to the cytoskeleton or not, which in turn augments the lifetime of the extracellular adhesion bond and thus the duration of the contact between the PMN and the endothelium. Our experimental approach has allowed us not only to characterize both constituents of this serial bond separately but also to quantify the directly related two-step rheological cell response to point loads. The results are summarized in the following subsections.

The P-Selectin:PSGL-1 Catch Bond — A Mechanochemical Molecular Switch that Prevents Leukocyte Arrest in Slow Flow. A peculiar shear-threshold behavior\(^{31-33}\) of rolling neutrophils or test particles has frequently been observed in flow-chamber experiments. Below a certain shear rate, the lifetimes of transient attachments of neutrophils to selectin-coated substrates did unexpectedly not increase further. Instead, the attachments appeared to become weaker, despite the reduced shear stresses that the cells were subjected to. Recent findings showed that this behavior can be traced directly to the molecular interaction between selectins and PSGL-1.\(^{24,34,35}\)

Figure 6 presents the dynamic force spectrum of the P-selectin:PSGL-1 bond that we measured with the setup shown in Figure 4A.\(^{24}\) Focusing first on the filled squares, a clear logarithmic dependence between rupture force and loading rate is seen at \( \sim 300 \text{ pN/s} \), in close agreement with the prediction of eq 6. For this range of loading rates, the interaction is characterized by a force scale \( f_0 \approx 18 \text{ pN} \) and an apparent unstressed off-rate of \( k_{off} \approx 0.37 \text{ s}^{-1} \). However, when bonds were loaded by force ramps with rates below \( \sim 300 \text{ pN/s} \), the most frequent rupture force dropped to near zero (filled upside-down triangles), in agreement with the observed shear-threshold behavior of rolling neutrophils. On the other hand, when the same slow
loading rates were applied after a quick initial step in force (to 20–30 pN within 5–6 ms), the most frequent rupture force (empty squares) followed the same spectrum that had been found at high loading rates using the continuous force ramp. Clearly, the initial force jump “locked” the interaction in the bound state. Interestingly, Dembo et al. anticipated this behavior as early as 1988, calling bonds of this type “catch-slip bonds”.36

These observations, and a number of additional experimental results,24 led us to the conclusion that the dissociation of the P-selectin:PSGL-1 bond can occur along two distinct unbinding pathways. A conceptual view of the envisioned energy landscape underlying this behavior is shown in Figure 7. An important detail is the existence of two ground states. In the absence of force, or if the bond is loaded very slowly, the dissociation follows the “easy-out” pathway (1) across a small energy barrier. This pathway is little affected by force; hence it was sketched perpendicular to the direction of force application. On the other hand, the initially high energy barrier impeding pathway (2) as well as the second ground state are lowered by force. This gradually “empties” the ground state of path (1) and thus prevents dissociation along that pathway—as long as the force increases fast enough. Eventually, pathway (1) shuts down completely, and the situation in the (2)-direction becomes the simple, one-dimensional case discussed above in the background section on DFS.

This example demonstrates how single molecules can act as mechanochemical switches. In the present case, the strength of an important cellular adhesion bond is toggled between low and high depending on the force-loading rate. The resulting dramatic weakening of transient adhesion bonds between leukocytes and the endothelium under low shear stress is believed to prevent clogging in blood vessels where flow is slow.

The Cytoskeletal Anchor of PSGL-1 Exhibits the Signature of a Weak Chemical Bond. Using the setup shown in Figure 4B, we also collected the values of the crossover forces $f_X$ obtained over a 200-fold range of (average) measured loading rates into histograms.25 These histograms were well described by the probability distribution eq 7, and the dynamic force spectrum of the most likely crossover forces (shown in Figure 8) revealed a linear dependence of the transition force on the logarithm of the loading rate, as predicted by eq 6. This good agreement between the measured crossover forces and the theoretically predicted behavior of a single, weak chemical bond is a strong indication that the transition at $f_X$ reflects the failure of a single linkage, i.e., the weakest link in the possibly complex molecular structure that anchors PSGL-1 to the cytoskeletal cortex of PMNs. The force scale that best characterizes this interaction was found to be $f_{bi} \approx 17$ pN, with the apparent unstressed off-rate in the range of $k_{off} \approx 0.7–1.7$ s$^{-1}$.

A Two-Step Rheological Cell Response Dramatically Decreases the Load on the Adhesion Bond. To quantify the observed two-step rheological response of PMNs to point loads,25,26 we have adopted the following phenomenological description of the measured force-time curves (such as shown in Figures 4B and 9). A straight line was matched to the linear force rise in the initial elastic regime (cf. Figure 9). Its slope is the measured loading rate $r_f$ in this regime. The spring constant $k_i$ for this elastic deformation of the PMN’s cytoskeletal cortex is easily calculated as

$$k_i = k_i \frac{1}{r_f^{0}/r_f - 1}$$

recognizing that the PMN is in series with the force transducer. The subsequent approach to an apparent force

Figure 6. Most frequent rupture force (highest peak in individual histograms) as a function of the loading rate for the P-selectin:PSGL-1 bond.24 Filled symbols (squares and triangles) were obtained by loading single attachments with a steady ramp of force. Empty squares were obtained using a different force protocol that consisted of an initial fast step to 20–30 pN, followed by a linear ramp at the indicated loading rate.

Figure 7. Conceptualized energy landscape with two separate unbinding pathways. A landscape of this form is thought to govern the P-selectin:PSGL-1 interaction.

Figure 8. Most frequent transition force for release of the cytoskeletal anchor of PSGL-1 as function of the average loading rate.25
The apparent slight increase of the plateau force was almost always well described by an exponential fit of the form (cf. Figure 9)

\[ f(t) = f_\infty - (f_\infty - f_1) \exp \left( -\frac{t - t_1}{\tau} \right) \]  

with the plateau force \( f_\infty \) and the characteristic time \( \tau \) as empirical fitting parameters. (Another fitting parameter, \( f_1 = f(t_1) \), is the force at the first data point of a suitably chosen fitting range.)

Figures 10 and 11 summarize the main results of this analysis. Figure 10 shows the spring constant \( k_i \) (cf. eq 10) for the initial soft-elastic response of the PMN cortex to a point load as function of the PMN extension rate. The semilog plot reveals a value of \( k_i \approx 0.2-0.25 \) pN/nm that is roughly constant over a large range of rates, as expected for ideal elastic behavior. (The apparent slight increase of the \( k_i \)-values at larger rates indicates a slow viscous component, most likely due to deformation of the whole cell.) This initial elastic regime acts to soften the impact experienced by both constituents of a serial P-selectin:PSGL-1:cytostructure attachment, i.e., the P-selectin:PSGL-1 adhesion bond as well as the cytoskeletal anchor of PSGL-1. A similar, additional effect can be expected in vivo from the softness of the vessel walls.

The crossover to viscoelastic behavior at \( f_0 \) (Figures 4B and 9) causes a substantially greater reduction in the stress experienced by the P-selectin:PSGL-1 bond. In this regime, the continuing growth of the total cell extension is accompanied by an exponential-like force approach to a rate-dependent plateau \( f_\infty \). Characteristic for tether extrusion, this apparently Maxwellian rheological response commences only after the release of the cytoskeletal anchor of PSGL-1. A plot of the tethering-plateau force as a function of the pulling speed is shown in Figure 11, where we have also included the result of pioneering studies of leukocyte-tether mechanics by Hochmuth and co-workers.20 The data obtained with the biomembrane force probe reveals clearly that tether flow is not Newtonian. Instead, tether extrusion at higher rates is greatly facilitated by a shear-thinning behavior of cell material as it flows onto the tether. Obtained from a log-log plot of the BFP data, the power law

\[ \frac{f_\infty}{[\text{pN}]} \approx 60 \left( \frac{v_{\text{pull}}}{[\mu\text{m}/\text{s}]} \right)^{0.25} \]  

was the best match to the dependence of the tethering force on the full range of measured pulling speeds. We would like to emphasize that the previously published original data19-22 are in near-perfect agreement with our tethering forces in the respective ranges of pulling speeds. However, being able to pull tethers over an exceptional, almost 1000-fold range of speeds leads us to the reinterpretation of the leukocyte-tethering dynamics in terms of eq 12. Additional support for this shear-thinning behavior is provided by our measurements of the characteristic times \( \tau \) for the exponential-like approach to a constant plateau force (cf. eq 11). The dependence of the characteristic time on pulling speed was found to follow the inverse power law

\[ \frac{\tau}{[\text{s}]} \approx 0.3 \left( \frac{v_{\text{pull}}}{[\mu\text{m}/\text{s}]} \right)^{-0.75} \]  

Mechanoregulation of Transient Leukocyte Attachments. Together, the above results allow us to predict the typical scenario for a transient PMN attachment to immobilized P-selectin. As mentioned earlier, an intact extra-
Figure 12. Combined dynamic force spectra of the two constituents of the serial transmembrane linkage. Filled symbols (squares and triangles) mark the catch-bond response of the extracellular P-selectin:PSGL-1 adhesion bond (cf. Figure 6). Open circles are the most frequent failure forces of the cytoskeletal anchor of PSGL-1 (cf. Figure 8). The sketch at the top illustrates the dramatically different behavior depending on which part of the serial linkage fails first under a rising force load (taken here as the drag exerted by flow as denoted by the arrow). At low rates (left of the vertical dashed line), the extracellular P-selectin:PSGL-1 bond breaks first, leading to PMN detachment from immobilized P-selectin. At rates above a threshold of ~300 pN/s, the cytoskeletal anchor of PSGL-1 is the weaker link in the serial attachment. Its failure enables tether extrusion, which fundamentally changes the force history experienced by the still-intact P-selectin:PSGL-1 bond. The situation now resembles plastic-like flow, i.e., a quasi-constant-force displacement at fixed speed. This results in a significant increase of the mean survival time of the P-selectin:PSGL-1 bond, or equivalently, of the PMN attachment. In other words, the release of the cytoskeletal anchor of PSGL-1 and the subsequent tether flow represent a mechanical fuse that prevents premature cell detachment from immobilized P-selectin. Interestingly, the force-plateau levels ($f_{\infty}$) in Figure 11) are not linearly proportional to the tether-extrusion speed. Instead, a shear-thinning tether flow causes an additional reduction in the force load exerted on any surviving P-selectin:PSGL-1 bonds at high extrusion speeds. In vivo, the resulting prolonged lifetime of PMN attachments, in combination with the extrusion of membrane tethers, will enable PMNs to move slowly downstream of the blood flow for some time. The shear flow will, at the same time, act to push the tethered PMN against the vessel wall. This gives the cell the opportunity to form new adhesive bonds with the endothelium before the current P-selectin:PSGL-1 bond eventually fails.

CONCLUSIONS

We used the biomembrane force probe to test the adhesive strengths of attachments between surface-bound P-selectin and either intact neutrophils or immobilized PSGL-1. Combining single-molecule with single-cell experiments has enabled us to paint a comprehensive picture of intriguing nano-to-micromechanical processes that play key roles in the regulation of the initial stages of leukocyte adhesion. In addition to the dependence on the bloodstream’s shear rate and on the surface densities of adhesion sites, we have shown that the duration of endothelial contacts of a leukocyte (via P-selectin) is significantly affected by (i) the mechanical switch that is preprogrammed into the structure of the P-selectin:PSGL-1 complex, and that selects one of two unbinding pathways depending on the loading rate, (ii) the dynamic strength of the intracellular linkage that anchors PSGL-1 to the cortical cytoskeleton, (iii) the relative strengths of the above two constituents of the serial transmembrane linkage P-selectin:PSGL-1: cortical cytoskeleton, (iv) the rheological response of soft linkers supporting the attachment, i.e., the crossover from an initial elastic extension of the PMN cortex to viscoelastic, constant-force tether flow, and (v) the additional reduction in bond load at high rates due to the shear-thinning behavior of cell material as it flows onto a tether. Together, these mechanisms not only prevent permanent cell arrest at sites where blood flow is low but also enable...
leukocytes to patrol vessel walls largely unaffected by variations in the bloodstream’s flow rate in regions where this rate is above the shear threshold.

Finally, it is worthwhile to note that our force tests against intact neutrophils represent the prototype of a novel, non-invasive experimental technique to study intracellular mechanics of single cells. The technique relies on membrane-embedded macromolecules (such as PSGL-1) that act as transmembrane “handles”. Forming specific extracellular attachments between such molecular handles and an ultra-sensitive force probe, we are able to inspect nano-to-microscale mechanical features inside cells, for example, the intracellular anchoring strength of adhesion receptors, or the cohesive strength of the actin cytoskeleton.

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