Direct Observation of Membrane Tethers Formed during Neutrophil Attachment to Platelets or P-selectin under Physiological Flow

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Abstract. Adhesion and subsequent aggregation between neutrophils and platelets is dependent upon the initial binding of P-selectin on activated platelets to P-selectin glycoprotein ligand 1 (PSGL-1) on the microvilli of neutrophils. High speed, high resolution videomicroscopy of flowing neutrophils interacting with spread platelets demonstrated that thin membrane tethers were pulled from neutrophils in 32 ± 4% of the interactions. After capture by spread platelets, neutrophil membrane tethers (length of 5.9 ± 4.1 μm, $n = 63$) were pulled at an average rate of 6–40 μm/s as the wall shear rate was increased from 100–250 s^{-1}. The average tether lifetime decreased significantly ($P < 0.001$) from 630 to 133 ms as the shear rate was increased from 100 s^{-1} ($F_{bond} = 86$ pN) to 250 s^{-1} ($F_{bond} = 172$ pN), which is consistent with P-selectin/PSGL-1 bond dynamics under stress. Tether formation was blocked by antibodies against P-selectin or PSGL-1, but not by anti-CD18 antibodies. During neutrophil rolling on P-selectin at 150 s^{-1}, thin membrane tethers were also pulled from the neutrophils. The characteristic jerking motion of the neutrophil coexisted with tether growth (8.9 ± 8.8 μm long), whereas tether breakage (average lifetime of 3.79 ± 3.32 s) caused an acute jump in the rolling velocity, proving multiple bonding in the cell surface and the tether surface contact area. Extremely long membrane tethers (>40 μm) were sometimes pulled, which detached in a flow-dependent mechanism of microparticle formation. Membrane tethers were also formed when neutrophils were perfused over platelet monolayers. These results are the first visualization of the often hypothesized tethers that shield the P-selectin/PSGL-1 bond from force loading to regulate neutrophil rolling during inflammation and thrombosis.

Key words: thrombosis • P-selectin • PSGL-1 • hemodynamics • tether

Introduction

Adhesion and aggregation of platelets and neutrophils to each other and to the blood vessel wall are critical events in inflammation and thrombosis, and are relevant to patients with stroke, acute myocardial infarction, extracorporeal circulation, and coronary angioplasty (Rinder et al., 1992; Kostantopoulos et al., 1995; Mickelson et al., 1996; Neumann et al., 1997; Hagberg et al., 1998). The initial bonding event between neutrophils and platelets is mediated by the P-selectin present on the surface of activated platelets (~12,000 molecules/cell) with P-selectin glycoprotein ligand 1 (PSGL-1;[1] ~10^4 molecules/cell) located on the tips of neutrophil microvilli (Moore et al., 1995; Bruehl et al., 1997). Under hemodynamic conditions, the rolling and arrest of neutrophils on spread platelets or activated endothelium involves the transition from P-selectin–mediated tethering to more stable β_2-integrin bonding (Lawrence and Springer, 1991; Buttrum et al., 1993; Diacovo et al., 1996; Yeo et al., 1994; Weber and Springer, 1997).

The mechanics and kinetics of the P-selectin/PSGL-1 bond that regulate the adhesive dynamics have been studied using several different experimental techniques. Parallel-plate flow assays have employed flowing neutrophils over monolayers of adherent platelets (Hamburger and McEver, 1990; Yeo et al., 1994; Lalor and Nash, 1995; Diacovo et al., 1996), sparse islands of platelets (Bahara and Nash, 1998), and P-selectin–coated surfaces (Lawrence and Springer, 1991; Alon et al., 1995). The P-selectin/PSGL-1 bond displays a modest increase in dissociation when force loaded, with an unstressed off rate $k^0_{off} = 1$–2.4 s^{-1} and a stressed off rate of 3.5–6.3 s^{-1} at a wall shear stress of 1 dyne/cm^2 (bond loading of 110 pN; Alon et al.,

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[1]*Abbreviations used in this paper:* DIC, differential interference microscopy; PSGL-1, P-selectin glycoprotein ligand 1.

1995; Smith et al., 1999). Plasmon resonance measurements of the affinity and kinetics of the bond between soluble monomeric human P-selectin and immobilized PSGL-1 from human neutrophils yielded values of $k^0_{on} = 4.4 \times 10^6\ M^{-1}\ s^{-1}$, $k^0_{off} = 1.4\ s^{-1}$, and $K_d = 320$ nM (Mehta et al., 1998). In contrast, atomic force microscopy measurements of single complexes between a PSGL-1/IgG chimera immobilized on the tip of an atomic force microscopy cantilever with a surface coated with P-selectin/IgG chimera (Fritz et al., 1998) displayed higher avidity (K_d = 60 nM) than the native P-selectin/PSGL-1 bond (K_d = 320 nM), and resulted in off rates from 0.02 s^{-1} under zero force up to 15 s^{-1} under applied forces.

Proposed mechanisms of neutrophil detachment during rolling and adhesion under fluid shear forces involve the following: (1) the dissociation of the receptor bond (Alon et al., 1995); (2) the extraction of membrane receptors (Evans et al., 1991; Shao and Hochmuth, 1999); and/or (3) the failure of the membrane. Of these mechanisms, force-induced bond dissociation is the favored model of cell detachment. Microvilli rheology may also have a role during neutrophil rolling (Shao et al., 1998; Chen and Springer, 1999). Shao and Hochmuth (1996) using micropipette manipulation and antibody-coated beads demonstrated that microvilli on neutrophils, under a pulling force, can be extended (*microvillus extension*, 1 μm) or can form long thin membrane cylinders (*tether formation*, up to 20 μm) depending on the magnitude of the force (Shao et al., 1998). Although these measurements were made under nonphysiological conditions, it is predicted that tether formation could also occur as a result of a bond between PSGL-1 on the neutrophil microvilli and platelet (or endothelial) P-selectin under physiological hemodynamic conditions. Similarly, recent work by Chen and Springer (1999) supports the role for microvilli extension during rolling adhesion by demonstrating neutrophil recoil upon sudden flow deceleration, while Alon et al. (1997) detected a moment arm upon flow reversal; both observations are suggestive of a tether structure.

In this study, we investigated the initial adhesive interactions between neutrophils and platelets using high speed, high resolution differential interference contrast (DIC) videomicroscopy. Under physiological flow conditions, we found that when flowing neutrophils collided with immobilized platelets or rolled on P-selectin–coated surfaces, membrane tethers were pulled from the neutrophil surface. These results are the first reported direct visualization of such tethers forming under venous flow conditions, and may have significant implications in the understanding of bond loading and neutrophil rolling dynamics.

Materials and Methods

Antibodies and Reagents

Human P-selectin, purified from outdated platelet lysates (Lawrence et al., 1997), was provided by Dr. Michael B. Lawrence (University of Virginia, Charlottesville, VA). Function-blocking anti–PSGL-1 mAb PL1, anti-CD18 mAb IB4, and anti–P-selectin mAb G1 were purchased from Ancell, whereas human serum albumin (HSA) was obtained from Golden West Biologicals.

Neutrophil Isolation

Venous human blood for neutrophil isolation was obtained from healthy donors by venipuncture and anticoagulated with EDTA (nine parts blood to one part K_3EDTA). Neutrophils were isolated by centrifugation over neutrophil isolation medium (Cardinal Associates). After isolation, neutrophils were resuspended in either a Mg^{2+}-free Hepes-buffered Tyrode's solution, pH 7.35, containing BSA (3.5 mg/ml), 137 mM NaCl, 2.7 mM KCl, 3.8 mM NaH_2PO_4, 3.8 mM Hepes, 2 mM $CaCl_2$, and 5.5 mM glucose or in Mg^{2+}-free Hank's balanced salt solution (HBSS; Gibco Laboratories) supplemented with 2% HSA and 2 mM $CaCl_2$, pH 7.4. Immediately after suspension in buffer, cells were counted electronically by a Coulter counter (model Z2) and used in the flow experiments at 10^5 cells/ml. All subsequent procedures were performed at room temperature.

Microcapillary Flow Chambers

Flow chambers were constructed with rectangular glass capillaries (microcapillary; VitroCom) with a cross-section of 0.2 × 2.0 mm and a wall thickness of 0.15 mm. Microcapillaries were cleaned by overnight incubation in 20% nitric acid, followed by extensive washing with deionized water. The microcapillaries were rinsed with 100% dry ethanol and dried under argon. The ends of the clean microcapillary (length = 7 cm) were inserted into silastic tubing (I.D. 1.47 mm × OD 1.96 mm) and a hermetic seal between the glass capillary and the silastic tubing was established by covering the joint with adhesive lined, heat shrinkable tubing (3M Electronics).

Fibrinogen and P-selectin–coated Surfaces

To promote platelet adhesion, the clean microcapillary flow chambers were incubated with a human fibrinogen solution (100 μg/ml; Enzyme Research Labs) for 1 h at room temperature. After incubation, each flow chamber was washed of excess fibrinogen solution by perfusing Ca^{2+}- and Mg^{2+}-free HBSS containing HSA for at least 20 min. Human platelets were sparsely adsorbed on the fibrinogen before binding studies with neutrophils. In some experiments, monolayers of platelets were prepared by perfusing washed platelets into the chamber and allowing them to settle for at least 30 min. P-selectin–coated surfaces were prepared by incubating clean microcapillary flow chambers with affinity-purified human platelet P-selectin (~1 μg/ml final concentration) in Ca^{2+}- and Mg^{2+}-free HBSS for at least 3 h at room temperature. The flow chamber was washed by perfusing Ca^{2+}- and Mg^{2+}-free HBSS containing 2% HSA through the chamber for at least 30 min, to remove any excess or unbound protein. For site density determinations, clean microcapillary chambers were incubated with the dilute P-selectin solution for 3 h, followed by washing/blocking with the 2% HSA/HBSS for 30 min. The glass microcapillaries were incubated with a solution of ^{125}I-labeled anti–P-selectin antibody clone G1 (3.95×10^6 cpm/μg; Ancell) and diluted in 1% BSA/PBS. After antibody incubation, the tubes were washed with PBS, crushed, and counted in triplicate with a gamma counter.

Cell Treatments

For blocking P-selectin on the surface of platelets, flow chambers coated with platelets were filled with mAb G1 at 20 μg/ml and incubated at room temperature for 20 min before perfusion of neutrophils. To block bonding via PSGL-1 or β_2-integrins, neutrophils were incubated with either 20 μg/ml mAb PL1 (anti–PSGL-1) or 20 μg/ml mAb IB4 (anti-CD18) in 2% HSA/HBSS medium containing 2 mM Ca^{2+} for 30 min before perfusion. The neutrophil suspension was diluted in a 3–10-fold volume of HSA/HBSS medium and immediately perfused into the flow chamber. Energy-depleted neutrophils were prepared by incubating isolated neutrophils with 30 mM sodium azide for 30 min at room temperature before perfusion.

High Resolution, High Speed Imaging under Flow

The flow chamber was mounted on a Zeiss Axiovert 135 microscope. DIC microscopy was conducted with a 63× (NA 1.40) oil immersion objective lens (Plan-Apochromate) and an oil immersion condenser (NA 1.4). During perfusion, neutrophils were withdrawn from a reservoir through the flow chamber at controlled flow rates by a syringe pump (Harvard Apparatus). The wall shear stress imposed on the surface was calculated from the equation $\tau_w = (6Q\mu)/(B^2W)$, which is the solution of the Navier-Stokes equation for laminar flow of a Newtonian liquid between parallel plates, where τ_w represents the wall shear stress (dynes/cm^2), Q represents

the flow rate (cm^3/s), μ represents the viscosity (0.01 Poise at room temperature), B represents the total plate separation (0.02 cm), and W represents the width (0.2 cm). The wall shear rate, γ_w (s^{-1}), was calculated as $\gamma_w = 6Q/(B^2W)$. Distortions in the flow field, because of the sidewalls, were minimal for the 1:10 aspect ratio of the gap separation to the chamber width and for the imaging of events only in the middle of the chamber away from the sidewalls. Digital images were captured with a high speed digital camera (MotionCorder Analyzer; Eastman Kodak Co.) at imaging rates of 30–240 frames per second (fps). Images from the high speed camera were played back at slow motion (5 fps) or frame-by-frame and recorded on videotape. Control of the videotape recorder was performed using LabVIEW and IMAQ Vision Software (National Instruments) with serial communication to a Pentium-based workstation, while images were digitized via an on-board capture card (model PCI-IMAQ 1408; National Instruments). During video playback, some images were contrast enhanced with background subtraction and grey scale stretching using an Argus 20 Image Processor (Hamamatsu Corp.).

Analysis of Neutrophil–Platelet Interactions

When unstimulated neutrophils were perfused over spread platelet singlets, three general types of interaction occurred: (type 1) membrane tether formation during neutrophil translation downstream without rotation (see Fig. 1 a); (type 2) neutrophil rolling over the platelet without observable membrane tether formation and with an observed neutrophil rotation; or (type 3) pausing without an observable membrane tether formation or rolling. Type 1 interactions were analyzed by determination of cell center positions in successive video frames from the digitized images using Scion Image software. The position of the cell center was determined within one pixel accuracy (± 0.09 μm/pixel) at each instant. During type 1 events, these tethers were extremely thin and short-lived, and often crossed the focal plane of the DIC image. Thus, the growing tethers were sometimes difficult to completely visualize in the image plane because manual refocusing was not possible in real-time during the adhesive events. However, when the microscope was readjusted to

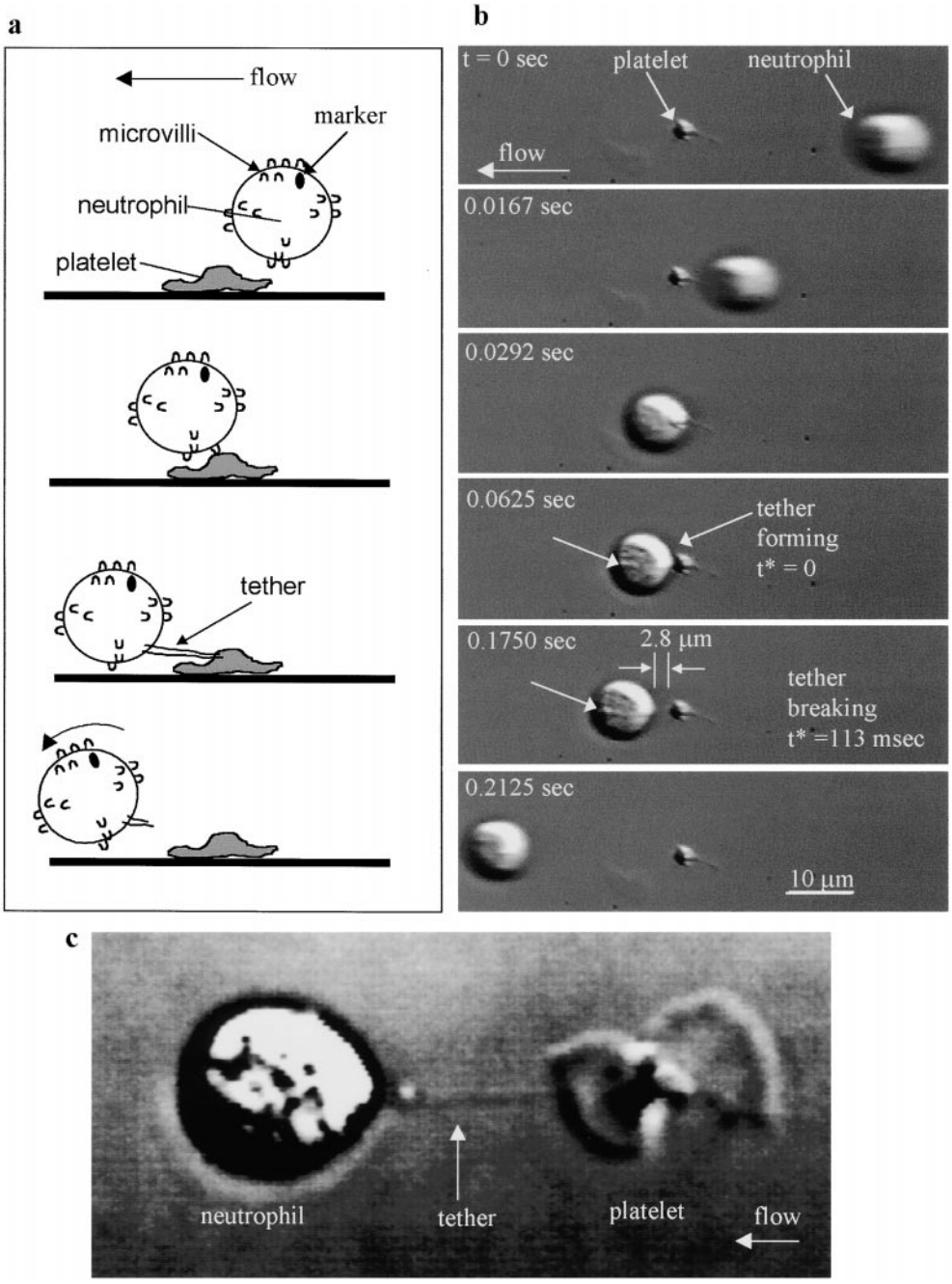


Figure 1. Neutrophil tethering to platelets under flow. (a) Schematic of a flowing neutrophil colliding with a spread platelet and forming an elongated membrane tether. (b) Formation of a neutrophil–platelet tether at $\gamma_w = 200\ s^{-1}$. Digitized images taken from a video sequence of a neutrophil approaching an adherent platelet (t = 0–0.0167 s), capture (t = 0.0292 s), growth of tether (t = 0.0625–0.1750 s), and release (t = 0.2125 s). The arrows on the left in the frames at t = 0.0625 and t = 0.1750 point to a bright surface feature on the neutrophil, demonstrating that the cell is translating and not rotating as the tether grows. Video capture rate, 240 fps (time resolution = 4.5 ms). (c) A single membrane tether was observed between a teardrop-shaped neutrophil and an adherent platelet (flow from right to left). Bar, 10 μm.

the correct focal plane, tethers could be directly and definitively visualized.

Results

Tethering of Neutrophils to Spread Platelets

Neutrophils were perfused over surfaces sparsely coated with platelets at wall shear rates of 50–300 s^{-1} to determine whether, under physiological flow conditions, the initial adhesive interactions between free-flowing neutrophils and immobilized platelets can lead to the formation of elongated membrane tethers. During perfusion, elongated membrane tethers were pulled from neutrophils after capture by spread platelets (Fig 1 a). The process of tether formation caused a slight teardrop-shaped deformation of the neutrophil (Fig. 1, b and c). In real-time, the tethering process was easily distinguished from a rolling or pausing neutrophil by identification of surface features on the neutrophil that did not rotate as the neutrophil translated downstream from the platelet at a velocity considerably slower than the flow velocity (Fig. 1 b). Fig. 1 b shows a free-flowing neutrophil traveling at 1,285 μm/s (wall shear rate = 200 s^{-1}) being captured by an adherent platelet, forming a 2.8-μm-long tether, and then releasing after 113 ms. Fig. 1 c shows an example of an extremely thin tether formed between a neutrophil and a platelet.

For the neutrophils that formed tethers, determination of the cell center position with time demonstrated the rapid approach velocity of a neutrophil in a streamline above the platelet, the extremely rapid deceleration during capture by the platelet, tether extension, and the acute release of the neutrophil into a streamline closer to the surface (Fig. 2). In Fig. 2 a, the cellular motion of the collision event imaged in Fig. 1 b demonstrated that the upstream velocity of neutrophil was uniform before the adhesive event, whereas the downstream velocity after detachment was markedly slower but almost instantaneously obtained after release. Fig. 2 b shows the distance traveled by the neutrophil as it approached the spread platelet, as a membrane tether grew, and finally as the tether detached from the stationary platelet, and the neutrophil translated downstream (with observable rotation of surface features). The slopes of the lines drawn in Fig. 2 b represent the average velocity of each of these stages, and they demonstrate the distinct transitions between each phase of approach, tether growth, and release.

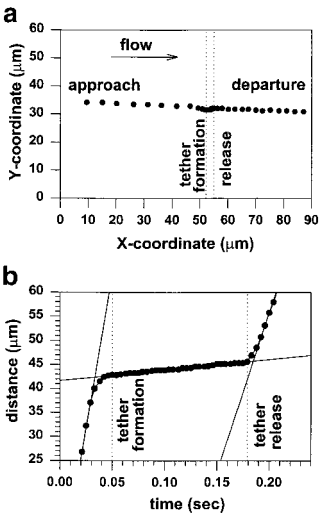


Figure 2. Motion of a free-flowing neutrophil that tethers to a stationary platelet. (A) The x-y position of the cell centroid of the neutrophil shown in Fig. 1 b, before, during, and after the neutrophil has formed a membrane tether with the adherent platelet. (b) The distance traveled with time by the neutrophil relative to its entrance point. The slopes of the solid lines represent the average velocity for each stage: the approach velocity, tether growth velocity, and the release velocity.

Effect of Wall Shear Rate on Neutrophil–Platelet Membrane Tether Growth

To gain insight into the strength and the rate of growth of the tethers formed between neutrophils and platelets, the influence of wall shear stress on tether growth rate, length, and duration was studied. Wall shear rates ranging from 50 to 300 s^{-1} (τ_w = 0.5–3.0 dyne/cm^2) were investigated by varying the flow rate of the perfusion solution through the chamber. The average tether growth rate increased significantly ($P < 0.001$) from 6 to 40 μm/s as the wall shear rate was increased from 100 to 250 s^{-1} (Fig. 3 a), whereas the average time of tether duration decreased significantly ($P < 0.001$) from 630 to 133 ms with an increasing wall shear rate (Fig. 3 b). Although the average tether length appeared to go through a maximum at 200 s^{-1} (Fig. 3 c), these values were not statistically different with the overall average tether length being 5.9 ± 4.1 μm (range 1–25 μm, n = 63 tethers). At wall shear rates below 50 s^{-1} and above 300 s^{-1}, transient tethers were not detected.

For neutrophil collisions with spread platelets involving tether formation, there is a movement of neutrophil positions with gap separations that are about a micron away from the surface to positions with gap separations <0.3 μm (Fig. 4). Fig. 4 a is a plot of the average free-flowing neutrophil velocity before and after tethering, with the stationary platelet as a function of wall shear rate. As expected, both the approach velocity and release velocity increased with an increasing wall shear rate. By using the measured approach and release velocities, we calculated the distance between the neutrophil (effective hydrodynamic radius, R = 4.25 μm) and the wall using the theoret-

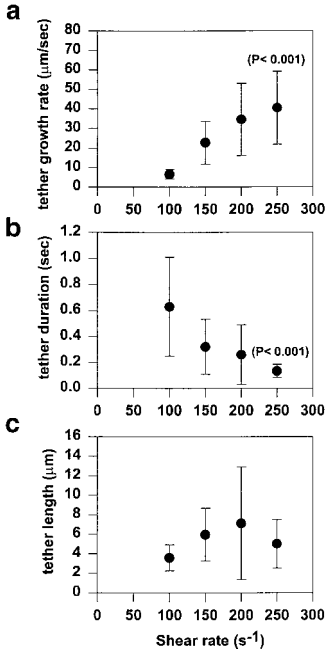


Figure 3. Effect of wall shear rate on (a) tether growth rate, (b) tether duration, and (c) tether length. Data points are the average ± SD of n measurements at each shear rate (γ_w = 100 s^{-1}, n = 9; γ_w = 150 s^{-1}, n = 21; γ_w = 200 s^{-1}, n = 23; and γ_w = 250 s^{-1}, n = 10).

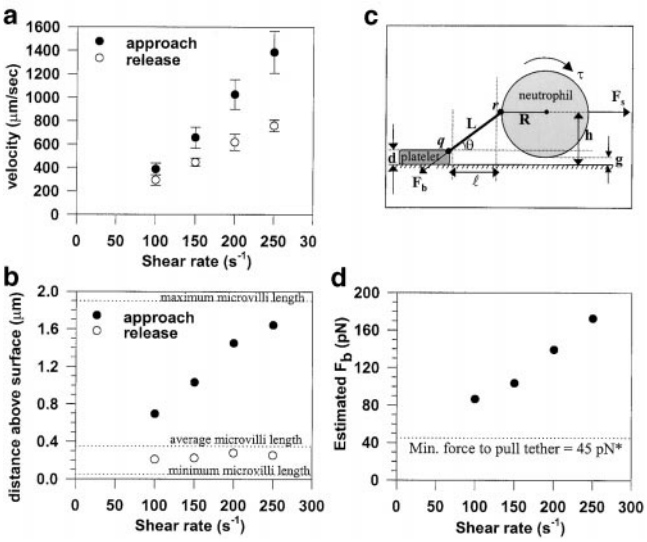


Figure 4. Velocity, height, and force parameters of free-flowing and tethered neutrophils. (a) Average velocity of free-flowing neutrophils before and after tethering to adherent platelets as a function of wall shear rate. (b) Calculated gap distance between the neutrophil and the wall before and after tethering to a spread platelet. (c) Force balance on tethered neutrophil. L, the length of the tether; F_s, shear force on the cell; F_b, force on the tether bond; R, radius of the cell; g, gap distance between neutrophil and wall; h, distance of the cell centroid from wall; and ℓ, the measured average projection of the tether length. (d) Estimated force on tether bond as a function of wall shear rate. The relationship between wall shear stress and force on the cell was estimated from Goldman's equation ($F_s = 6\pi\mu Rh\gamma_w C$), where μ, viscosity; R, cell radius; h, distance from the center of the cell to the wall; γ_w, wall shear rate; and C, a numerical factor that depends on h/R (Goldman et al., 1967). From geometry, $F_s = F_b \cos(\theta)$. F_b represents the case where the tether is bonded between point *q* on the platelet and point *r* on the neutrophil (i.e., $\theta = \tan^{-1}(h-d/h)$), where ℓ is the measured tether length. Asterisk denotes the minimum force to pull a tether was obtained from Shao et al. (1998).

ical results of Goldman et al. (1967). Fig. 4 b is a plot of the neutrophil distance from the surface with shear rate. The gap distance of the neutrophil above the wall after tether release was relatively constant at ~0.2–0.3 μm. The gap distance of the approaching neutrophils captured by adherent platelets increased modestly from 0.7 to 1.7 μm, with an increasing shear rate from 100 to 250 s^{-1}, an observation that may relate to the frequency of surface encounter of the longest neutrophil microvilli or to very subtle lift forces that may exist (particle Reynolds number, ~0.02).

To estimate the force on the tether between the neutrophil and platelet, a force balance was performed (Fig. 4, c and d). The relationship between wall shear stress and force on the cell was estimated from Goldman's equation ($F_s = 6\pi\mu Rh\gamma_w C$), where μ represents the viscosity, *R* represents the cell radius, h represents the distance from the center of the cell to the wall, γ_w represents the wall shear rate, and C represents a numerical factor that depends on *h*/*R*. The relationship between the force on the cell and the force on the tethered bond was based upon a force balance $F_b\cos\theta = F_s$. The force on the tether bond can be estimated by visualizing the projected length of the tether (ℓ) and making the following assumptions that: (1) the tether began at the edge of the platelet (point *q*); (2) the gap distance between the wall and the neutrophil was the height of the neutrophil after release (g ~0.3 μm); and (3) the tether originated from the neutrophil at point *r*. Values for d and h were estimated from the height determined from the neutrophil's approach and release velocities using the method of Goldman et al. (1967). Since the ratio of the tether growth velocity (v_{tether}) to the cellular free flowing velocity (v_{flow}) is typically below 1:20, the assumption of mechanical equilibrium is sufficiently accurate since the shear force is overestimated by <5% for a correction factor of $1-v_{tether}/v_{flow} \sim 0.95$. As the first available estimate of the P-selectin/PSGL-1 bond strength where both receptors reside in their natural cellular membranes, the force on the bond increased from 86 to 172 pN as the shear rate increased from 100 to 250 s^{-1} (Fig. 4 d).

Tether Formation Frequency and Antibody Blocking Experiments

The percentage of initial adhesive events between flowing neutrophils and immobilized platelets, which resulted in the formation of elongated membrane tethers, as compared with transient pausing or rolling is shown in Fig 5. At a shear rate of 200 s^{-1}, 43 out of 139 collisions observed between free-flowing neutrophils and immobilized platelets resulted in a membrane tether (*n* = 3 experiments with individual donors). To determine if the observed tether formation was due to P-selectin/PSGL-1 bonding, we studied platelet–neutrophil interactions in the presence of function-blocking mAbs to P-selectin and PSGL-1. Incubating adherent platelets with mAb G1, which binds to an epitope in the lectin domain of P-selectin and blocks binding of P-selectin to leukocytes (Hamburger and McEver, 1990) and PSGL-1 (Moore et al., 1995), completely blocked tether formation (Fig. 5). Similarly, incubation of neutrophils with the mAb PL1, which binds to the NH_2-terminal region of PSGL-1 and blocks P-selectin binding to this region (Moore et al., 1995), also resulted in the total inhibition of tether formation (Fig. 5). In contrast, incubation of neutrophils with either mAb IB4

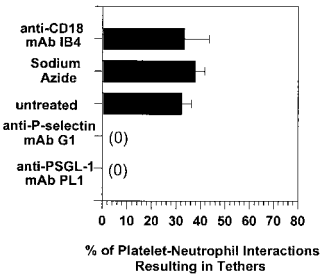
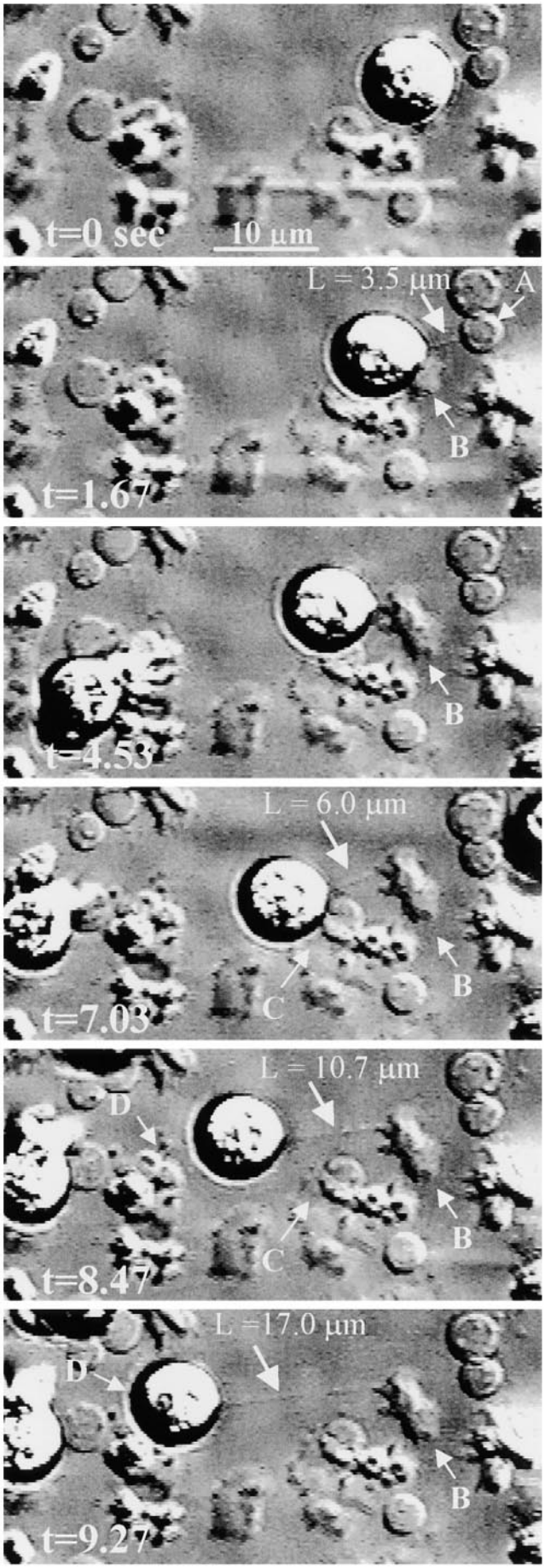


Figure 5. Role of the P-selectin/PSGL-1 bond in neutrophil tether formation with adherent platelets. Neutrophils were perfused over adherent platelets at 200 s^{-1} in the absence (*n* = 3 experiments with individual donors) or the presence of mAbs, and the percentage of neutrophil–platelet collisions that resulted in membrane tether formation was determined. Incubation of platelets with anti–P-selectin antibody (G1), or neutrophils with anti–PSGL-1 (PL1) resulted in a complete inhibition of tether formation (*n* = 2 experiments for each mAb). In contrast, the incubation of neutrophils with anti-CD18 antibody (IB4) (*n* = 3 experiments) or with the metabolic inhibitor sodium azide did not prevent tether formation (*n* = 3 experiments).



against CD18 or with 30 mM sodium azide (NaN_3) had no effect on tether formation (Fig. 5).

Tether Formation on Platelet Monolayers

To study whether observable membrane tethers were formed between neutrophils and monolayers of platelets, neutrophils were perfused over a subconfluent monolayer of immobilized platelets at a wall shear rate of (150 s^{-1}). When islands of platelets were present, neutrophils rolled across them. However, when there were gaps in the platelet surface coverage, neutrophils often formed membrane tethers as they translated from one island to the next. Fig. 6 shows a neutrophil that has been rolling on platelets and subsequently forms a series of membrane tethers as it translates between the gaps between upstream and downstream islands of platelets.

Neutrophil Rolling on Purified P-selectin–coated Surfaces

To demonstrate that the tether membrane originates from the neutrophil (not the platelet), and that P-selectin/PSGL-1 interactions supports tether formation, we investigated whether membrane tethers were formed when neutrophils rolled on P-selectin–coated surfaces. Tethering in this system was easier to visualize by DIC microscopy since the tethers formed in close proximity to the glass surface of the flow chamber. When neutrophils were perfused over P-selectin–coated surfaces ($\sim$10 sites/μm^2, $n = 3$) at a wall shear rate of 150 s^{-1}, they rolled over the surface and formed thin membrane tethers similar to those observed with platelets (Fig. 7 a). The neutrophil displayed a jerky motion of stops and starts as it rolled on the P-selectin surface concomitant with the growth of a membrane tether bonded to the surface. On purified P-selectin, distinct pauses in the neutrophil motion were detected (Fig. 7 c), the majority of which were $<$1 s, which is consistent with the known lifetime of the P-selectin/PSGL-1 bond. In many recordings, the breaking of the tether occurred at the terminal site of attachment with the surface, and the tether rapidly snapped back to the neutrophil (Fig. 7 a, t = 3.967 s). At the instant that the tether was released from the surface, the neutrophil experienced a sudden rotation forward that was driven by the shear forces (Fig. 7, b and c). To test whether β_2-integrins were involved in the formation of these long-lived tethers, neutrophils were treated with mAb IB4 against CD18. Blockade of CD18 did not alter the ability of neutrophils to form tethers

Figure 6. Neutrophil tether formation between islands of platelets. A neutrophil formed a series of membrane tethers as it translated between gaps in upstream and downstream islands of platelets. As the neutrophil translated from platelet island A to island B, a membrane tether was pulled (t = 0–1.03 s). The neutrophil remained relatively stationary on platelet island B while tethered at island A (t = 1.03–1.73 s). When the tether broke, the neutrophil rolled over and paused on island B (t = 1.73–4.53 s). The neutrophil formed a new tether as it translated away from island B to island C (t = 4.53–4.83 s). The neutrophil paused on island C (t = 4.83–7.73 s) before it released and translated downstream to island D (t = 7.73 to 9.1 s). The tether finally broke at t = 10.13 s.

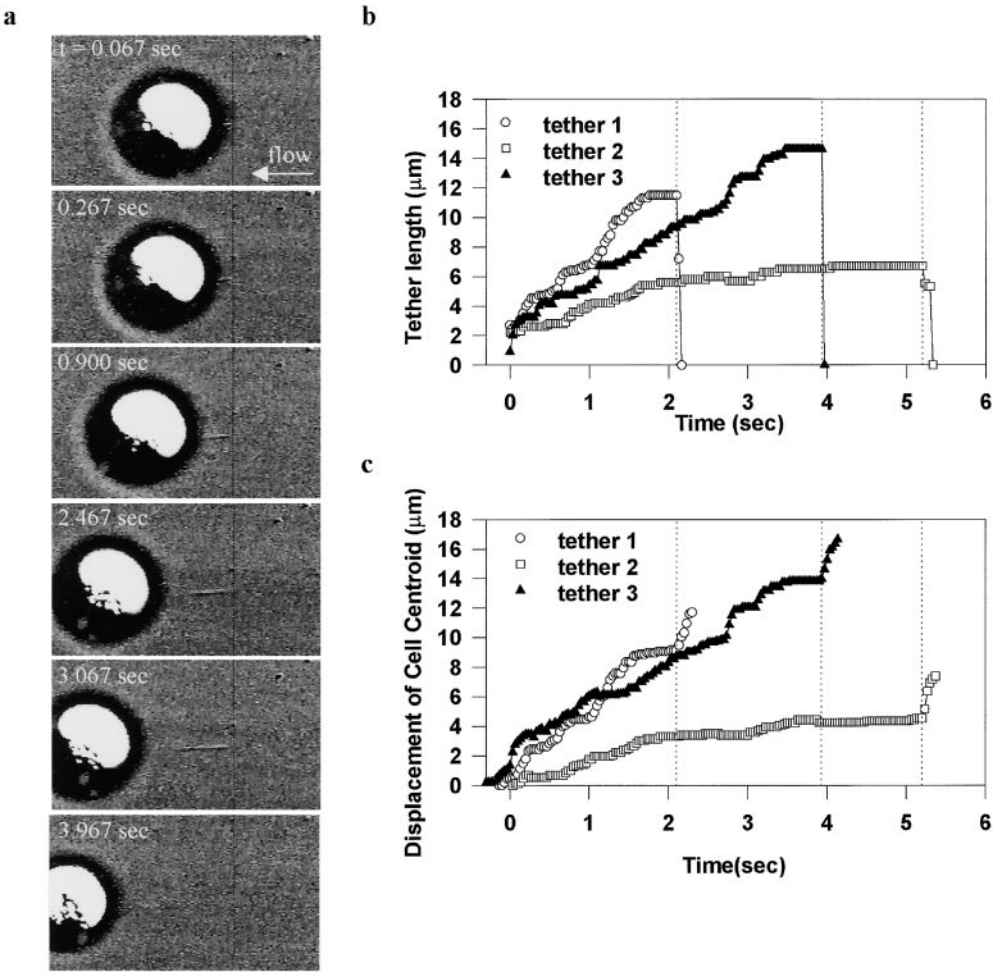


Figure 7. Tether formation during neutrophil rolling on P-selectin. (a) A single 14.6-μm tether was pulled over 4 s and recoiled fully back to the cell body ($\gamma_w = 150\ s^{-1}$, P-selectin site density of ~10 sites/μm^2). The instantaneous tether length (b) and cell centroid position (c) for three tethering events. The tethering event in a is shown as tether 3 in b and c. Distinct pausing in neutrophil rolling was observed in concert with the growth of membrane tethers, whereas the breaking of the tether (dashed line in b and c) caused a sudden movement forward.

on P-selectin–coated surfaces (data not shown). Clearly, P-selectin/PGSL-1 bonds were being formed and broken at the cell-surface contact area to cause pausing, as well as at the end of the tether to cause leaps forward when the tether was released. The force loading is difficult to model because of an uncertainty in contact area loading and bonding in the presence of a tether, which could also be multiply bonded to the surface (Fig. 8). The average tether length observed was 8.9 ± 8.8 μm (n = 31), average tether duration was 3.79 ± 3.32 s (n = 29), and the average tether growth rate was 1.7 ± 0.9 μm/s (n = 29). In contrast to tethering with platelets, where the neutrophil was translating ~0.3 μm above the surface, the neutrophil is attached to the surface by P-selectin bond(s) in the contact area as well as by bond(s) of the tether with the surface. This accounts for the long tether lifetime and slow tether growth rate observed with P-selectin–coated surfaces.

Multiple Point Attachments of Tethers on P-selectin–coated Surfaces

Frame-by-frame analysis revealed several unique features of the interaction between these membrane tethers and the P-selectin–coated surface never before directly visualized. Tethers were not always straight, but at times were kinked with sharp angles, which is indicative of multiple bonding events with the surface along the length of the tether. As the tether grew, these kinks were straightened as the linkages with the surface along the length of the tether were stressed and broken (Fig. 8 a). Also, tethers did not always recoil completely back to the neutrophil body when the attachment at the tip of the tether was broken. Fig. 8 b shows a tether growth sequence illustrating a tether with two kinks at points 2 and 3 (Fig. 8 b, t = 0 s) that loses its attachment at the original tethering site at point 1. Instead of the tether immediately snapping back its full-length to the cell body, it recoiled only to the position of nearest attachment at point 2 (t = 0.033 s). The crooked tether continued to grow as the neutrophil rolled for the next second (t = 0.033–1.033 s) at which time the bonding at point 3 was stressed and the attachment released at point 3 while remaining tethered at point 2 (t = 1.067 s). The angle of the tether from the surface to the neutrophil body can be appreciated in the last panel of Fig. 8 b at t = 1.067 s, where only the lower half of the tether is in the DIC focal plane. Adjustment of the focal plane allowed visualization of the upper part of the tether. The long duration of the neutrophil tethers on P-selectin–coated surfaces over many seconds is accounted for by the

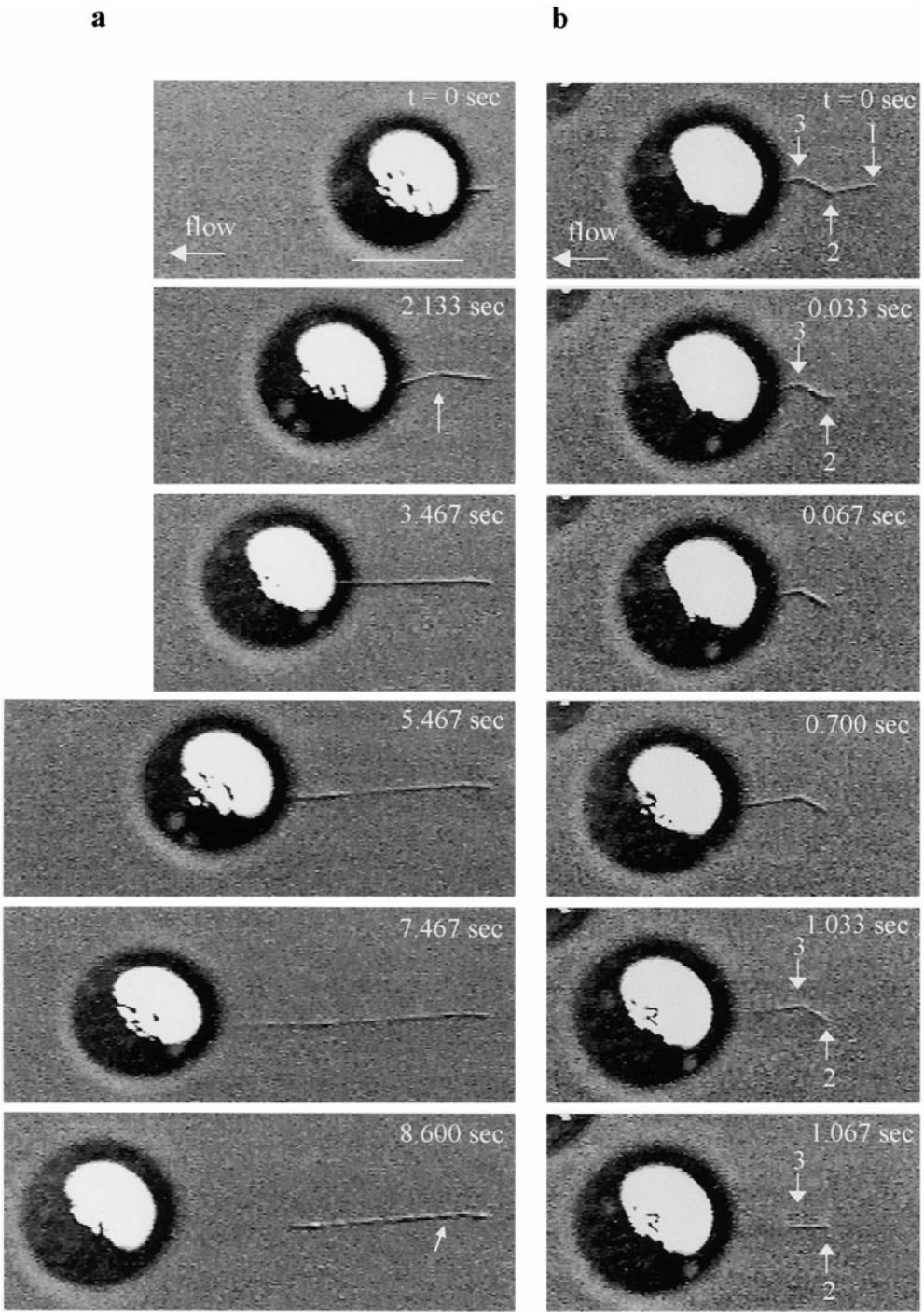


Figure 8. Multiple bonding of neutrophil tethers to a P-selectin–coated surface. (a) A single tether was pulled and displayed a kink because of a local attachment to the surface (arrow at t = 2.133 s). This attachment was subsequently broken (t = 3.467 s) while the tether continued to grow for over 8 s. The arrow at t = 8.600 s points to the formation of a beaded structure as the tether becomes elongated. (b) Multiple points of neutrophil tether attachment to the P-selectin–coated surface are formed and broken at a wall shear rate of 150 s^{-1}. The kinked tether at t = 0 s detaches at point 1 and, rather than fully snapping back, remains attached at points 2 and 3 (t = 0.033 s). The kink in the tether at point 3 remains as the tether grows until t = 1.033 s, at which point the tether detaches from point 3, yet remains bonded at point 2.

multiple bonding, the poor loading of the most distal bonds, and the opportunity to form new bonds in the tether contact area.

Tether Dragging, Multiple Tethering, Tether Beading, and Neutrophil String Formation on P-selectin

One unique feature of these tethers was, at times when the tether detached from the surface, it did not always snap back fully to the neutrophil body, but would drag along the surface and become reattached similar to the function of a grapple hook to bring the cell to arrest (data not shown). In addition, two or three elongated tethers that were pulled from a single neutrophil were occasionally observed (data not shown). Although most tethers snapped back, inspection of the P-selectin–coated surface after neutrophils rolled on the surface demonstrated the existence of detached tethers that often had a beaded appearance (Fig. 9 a). This is a flow-dependent mechanism of neutrophil microparticle formation and an example of cellular detachment by the failure of the membrane in which receptors are embedded. Fig. 9 b, which shows a single

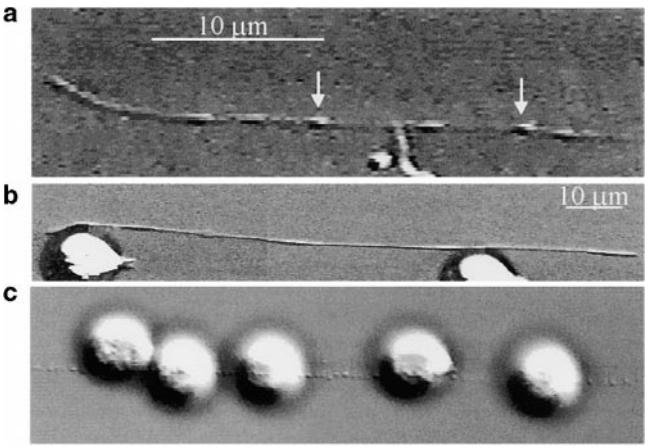


Figure 9. Deposition of membrane tethers on P-selectin–coated surfaces. Tethers could be deposited on the surface (a) and display a beadlike appearance (b). Neutrophil string formation on an ultralong-deposited and beaded neutrophil tether (c).

tether that was greater than four fields of view (>100 μm), illustrates that these tethers could grow to be extremely long, and demonstrated the tremendous excess of neutrophil membrane that can supply tether growth. Finally, it was observed that free-flowing neutrophils interacted and rolled on these deposited and beaded tethers, possibly through L-selectin/PSGL-1 interactions (Fig. 9 c).

Discussion

The recruitment of neutrophils to sites of inflamed endothelium or to platelets localized on the vessel wall during thrombosis is viewed as a multistep adhesion process that involves initial cell contact, rolling, firm adhesion, and transmigration. The initial contact made by the neutrophil is most likely to occur at the tip of the microvilli that protrude from its surface. Previous studies have shown that, unlike most other cell-surface molecules that are uniformly distributed, the molecules responsible for mediating initial neutrophil adhesion with other cells (i.e., L-selectin and PSGL-1) are localized at the tips of the neutrophil microvilli. It appears that localization of L-selectin and PSGL-1 at the microvilli tips, facilitates neutrophil tethering interactions by increasing their concentration and making them more accessible to their counter ligands. Therefore, a key question regarding the initial adhesion of neutrophils via these bonds is what happens when a force is imposed on them. Does the receptor–ligand bond break? Is either the ligand or receptor extracted from the cell membrane? Or does the cell membrane deform or break as the bond is loaded to reduce the force on the bond?

In this study, we investigated the initial interactions between neutrophils and platelets and report the first direct observation of the formation of elongated membrane tethers by neutrophils under physiological flow conditions. Elongated tethers were pulled from the neutrophil surface when free-flowing neutrophils were captured by spread platelets and when neutrophils rolled on P-selectin–coated surfaces. Tethering was completely blocked with mAbs against P-selectin and PSGL-1, whereas the mAb against CD18 had no effect. These observations extend the following recent measurements of: tether growth between neutrophils and beads coated with antibody using micropipette aspiration (Shao et al., 1998); moment arms of attached neutrophils in reversing flow (Alon et al., 1997); and neutrophil recoil in rapidly decelerated flow (Chen and Springer, 1999). Direct observation of the formation of these tethers under physiological flow was made achievable by using high speed, high resolution microscopy, a technique that makes it possible to directly observe cellular events with characteristic times as short as ~2 ms and length scales of ~100 nm.

In agreement with previous studies, the molecular bond responsible for the initial attachment and tethering of flowing neutrophils to platelets was the P-selectin/PSGL-1 interaction. This fact was demonstrated by the tethering being completely abolished after preincubation of platelets with anti–P-selectin or neutrophils with anti–PSGL-1, whereas incubation of neutrophils with anti-CD18 had no effect (Fig. 5). Similarly, preincubation of neutrophils with the metabolic inhibitor sodium azide, to prevent neutrophil activation during neutrophil–platelet interactions, also did not prevent tether formation, demonstrating that cell activation was not required. These observations, in conjunction with the tethers displaying lifetimes and strengths that are the signature of the P-selectin/PSGL-1 bond (Figs. 3 b and 4 d) and the reproduction of tethering in a purified system with P-selectin–coated surfaces, provide confirmation of the molecular mechanism of tether attachment.

Since cell–cell attachment depends on the balance between the dispersive hydrodynamic forces and the adhesive forces generated by the interactions between membrane bound receptors and their ligands, the effect of shear on tether formation was investigated. Both the tether growth rate and the lifetime of the tether were functions of the wall shear rate, while the average tether length of ~6 μm appeared as a plateau between 100 $s^{-1} < \gamma_w <$ 250 s^{-1}. Transient membrane tethers were not observed at shear rates ≤50 or ≥300 s^{-1}. During neutrophil tethering to adherent platelets, shear forces generated at a shear rate of 50 s^{-1} appeared insufficient to pull membrane tethers fast enough or long enough to be observed in our system, which could only resolve membrane tethers that were pulled beyond the cell's diameter (as opposed to microvilli extension). At a shear rate of 300 s^{-1}, the frequency of neutrophil capture was extremely low, and the force loading of the tether likely caused bond breakage before tethers could grow to an observable length beyond the cell's diameter.

The lack of observable membrane tethering below 50 s^{-1} or above 300 s^{-1} in our system is consistent with observations of Shao et al. (1998), Alon et al. (1997), and Chen and Springer (1999). Shao et al. (1998) estimated microvilli lengths between 0.35 and 1.0 μm at a shear rate of 22 s^{-1} (0.22 dyne/cm^2) since, at forces <45 pN, microvilli become extended but do not form tethers. Alon et al. (1997) detected lever arms between 2 and 4 μm corresponding to a tether length of 1.0 ± 0.32 μm at reversing flows of 0.3–0.8 dyne/cm^2 ($\gamma_w \sim$ 30–80 s^{-1}) during neutrophil rolling on P-selectin. For neutrophil rolling on E-selectin at a high wall shear stress of 16 dyne/cm^2

($\gamma_w \sim 1{,}600\ s^{-1}$), Chen and Springer observed neutrophil pull back of 1.4 μm upon instantaneous reduction of flow to 1 dyne/cm^2. Given these limits at low and high flow, the observed average tether length of ~6 μm in our study (Fig. 4 c) for shear rates between 100 and 250 s^{-1} represented a plateau to suggest that membrane tethering is most operative in this narrow range of shear rates.

After neutrophil tether breakage, the flowing neutrophil traveled in a plane closer to the wall. The height above the wall and the shear force on the cell, F_s were estimated at each wall shear stress using the results of Goldman et al. (1967). By assuming a single bond at the end of the fully grown tether and using the experimentally determined tether lengths (ℓ), we estimated the force on the bond to be $F_b = 86$ pN at $\gamma_w = 100\ s^{-1}$ (average bond life of 630 ± 380 ms, $n = 9$) and $F_b = 172$ pN at $\gamma_w = 250\ s^{-1}$ (average bond life of 133 ± 51 ms, $n = 10$). These bond strengths and lifetimes represent the first such estimates for the platelet P-selectin and neutrophil PSGL-1 bond pair, under conditions where the receptors reside in their respective cellular membranes with a definitive single point attachment between the two cells, and a direct measurement of the length of the lever arm for the force analysis.

Our observation that ~32% of neutrophils interacting with platelets formed tethers agrees with Shao's suggestion (Shao et al., 1998) that there may be heterogeneity in the association of the PSGL-1 ligand with the cytoskeleton. Shao et al. (1998) observed two different kinds of behavior, microvillus extension or tether formation, when a given force was applied to the microvilli of the neutrophil. The fact that the same force can result in two types of behavior suggests that they depend on their membrane–cytoskeleton association strength. Microvilli with a strong association between membrane and cytoskeleton can only be stretched a short distance (~1–2 μm), whereas microvilli with a weak association can be pulled out to form tethers. The average force required to extract an integral glycoprotein from the lipid bilayer of a cell has been estimated to be 100 pN (Bell, 1978). However, Evans et al. (1991) measured a force of 10–20 pN for glycophorin A extraction from a red cell membrane, whereas Shao and Hochmuth (1999) measured the forces of 25–45 pN for L-selectin extraction, 60–130 pN for β_2 integrin extraction, and 35–85 pN for CD45 extraction from neutrophils. However, this was a slow process requiring 1–2 s for L-selectin, 1–4 s for β_2 integrin, and 1–11 s for CD45 extraction. For tethering events that lasted under 1 s, we measured bond forces of >60 pN. This appears to agree with Shao (Shao et al., 1998; Shao and Hochmuth, 1999) and Alon et al. (1995) who have hypothesized that the cytoplasmic domains of selectins and their counterreceptors, in this case PSGL-1, help anchor these molecules to the cytoskeleton so that they can resist extraction.

When neutrophils interacted with surfaces coated with P-selectin under laminar flow conditions, they rolled with a characteristic jerky motion while forming observable membrane tethers to the surface. Thus, neutrophil PSGL-1 bonding with P-selectin can facilitate the formation of membrane tethers under venous flow conditions. In contrast to the tethers formed by flowing neutrophils with single platelets, membrane tethers formed by neutrophils rolling on P-selectin were of longer length and longer duration (Figs. 7 and 8). These longer lengths and durations were not due to β_2-integrin bonding since treatment of neutrophils with anti-CD18 did not inhibit tether formation. More likely they appear to be due to multiple binding interactions in the cell-surface contact area that would reduce the stress on the tether as well as the close proximity of the tether to the glass, which allowed multiple bonding of the tether to the absorbed P-selectin (as seen in Figs. 7 and 8).

During neutrophil tethering to P-selectin–coated surfaces, it appeared that detached membrane tethers were occasionally deposited on the surface. These deposited tethers may contain PSGL-1 and L-selectin to facilitate neutrophil (or platelet) interactions. Similarly, membrane tethers, if sufficiently long, may serve as a template for the formation of linear strings of neutrophils, a multicellular structure previously described by others (Alon et al. 1996; Walcheck et al., 1996; Lim et al., 1998). This tether-dependent mechanism of string formation would be distinct from neutrophil collision–assisted deposition via L-selectin, which would be expected to produce branched structures on the surface, not linear structures.

Adhesion of free-flowing neutrophils to activated endothelium or platelets is a key step in inflammation and thrombotic processes. The dynamics of selectin bonding determines whether a flowing neutrophil will adhere or detach. We present data that over 32% of neutrophil–platelet interactions under flow result in the formation of elongated membrane tethers. Similarly, we also demonstrated that neutrophils form elongated tethers when rolling on P-selectin–coated surfaces or platelet monolayers. The formation of these tethers is physiologically significant in that they shield the bond from full loading by changing the length and angle of the lever arm. Tether formation reduces the force on the bond by a factor of ~2 (Shao et al., 1998) with an expected reduction of k_{off} by a factor of ~2 for $F_b < 150$ pN (Smith et al., 1999). Given the reduction of stress on the bond due to the tethers and the consequent enhancement of the cellular interaction time, these tethered neutrophils are the ones with the greatest likelihood of transitioning to firm arrest in vivo. In addition, these tethers also may provide a mechanism by which neutrophils can translate between gaps between platelets and remain on or near the surface in close proximity to localized activating agents. By directly visualizing the formation of membrane tethers by neutrophils as they interact with platelets and P-selectin–coated surfaces, we have confirmed a widely postulated structure in cell–cell adhesion. The formation of these structures influences the dynamics of cell rolling as well as its biomechanical analysis and may have important implications for the rolling and adhesion of neutrophils in vivo.

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