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Forces Required to Initiate Membrane Tether Extrusion from Cell Surface Depend on Cell Type But Not on the Surface Molecule

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Abstract: When a cell adhered to another cell or substratum via surface proteins is forced to detach, lipid membrane tethers are often extruded from the cell surface before the protein bond dissociates. For example, during the inflammatory reaction leukocytes roll on the surface of activated endothelial cells. The rolling adhesion is mediated by interactions of selectins with their ligands, e.g., P-selectin glycoprotein ligand (PSGL)-1, which extrudes membrane tethers from the surfaces of both leukocytes and endothelial cells. Membrane tether extrusion has been suggested to regulate leukocyte rolling. Here we examine several factors that may affect forces required to initiate membrane tethers, or initial tether force. It was found that initial tether forces were similar regardless of the presence or absence of the cytoplasmic tail of P-selectin and regardless of whether the tethers were extruded via binding to PSGL-1 or Fcγ receptors. Initial tether forces were found to depend on the cell types tested and were greatly reduced by treatment of latrunculin A, which inhibits actin polymerization. These data provide additional insights to the control of membrane tether extrusion, which should be taken into account when cellular functions such as rolling where tether extrusion plays a regulatory role are compared using different cell types expressing the same molecule.

keyword: Lipid membrane, cytoskeleton, membrane tethers, cell rolling, leukocytes.

1 Introduction

Trafficking of lymphocytes into secondary lymphoid tissues and recruitment of leukocytes to sites of infection or injury is a multistep process that includes initial attachment, rolling, firm adhesion, and transendothelial emigration [von Andrian, Chambers, McEvoy, Bargatze, Arfors, Butcher (1991)]. Initial attachment and rolling are primarily mediated by rapid and transient interactions between selectins and their ligands, e.g., P-selectin glycoprotein ligand 1, or PSGL-1 [McDonough, McIntosh, Spanos, Neelamegham, Goldsmith, Simon (2004)]. Firm adhesion and emigration are mediated by slower and stronger interactions of integrins with their ligands [Neelamegham, Taylor, Burns, Smith, Simon (1998)]. These are highly regulated processes since the accumulation of too many cells to one area can lead to tissue damage during an inflammatory response [Berton, Yan, Fumagalli, Lowell (1996)]. Both selectin/ligand and integrin/ligand interactions occur in the presence of hydrodynamic forces that have to be balanced by adhesive forces applied through the cell surface molecules, which often extrude lipid membrane tethers from the surfaces of both leukocytes and endothelial cells before the receptor/ligand bonds dissociate [Schmidtke and Diamond (2000); Girdhar and Shao (2004)]. Several findings indirectly suggest that membrane tether extrusion may regulate cell rolling as the relative ease or difficulty in forming these tethers affects the rolling velocity [Schmidtke and Diamond (2000); Girdhar and Shao (2004); Ramachandran, Williams, Yago, Schmidtke, McEver (2004)]. It is therefore relevant to identify and elucidate factors that impact the force required to extrude membrane tethers from cells. In the present study, we show that several cultured cells (HL-60, CHO, and K562) have different membrane tether behavior from that of neutrophils. These differences may impose additional parameters that have to be taken into account when determining the kinetics/forces of interactions between receptors/ligands involved in cell rolling.

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2 Materials and Methods

2.1 Cell, antibodies, and bead preparation

Human promyelocytic leukemia cell line HL-60 (from Ameican Type Culture Collection, Rockville, MD) was cultured in sterile cell culture flasks in RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin, and 1% L-glutamine. Human erythroleukemia cell line K562 transfected with human PSGL-1 [Yago, Leppanen, Qiu, Marcus, Nollert, Zhu, Cummings, McEver, (2002)] was grown in medium containing 0.2 mg/ml Hygromycin B. Chinese hamster overy (CHO) cells transfected to express a wild-type human P-selectin protein on the surface or a truncated P-selectin containing only the first seven residues of the 35-residue cytoplasmic domain [Setiadi, Disdier, Green, Canfield, McEver (1995)] were cultured in DMEM (Mediatech, Herndon, VA) , 10% FBS, using 400 μg/ml of the selection agent G418. Neutrophils were collected from healthy donors using venipuncture or finger prick as previously described [Shao and Hochmuth (1996)].

Microspheres coated with goat anti-mouse IgG Fc monoclonal antibody (mAb) were purchased from Sigma (St. Louis, MO). Two drops of uniformly mixed beads (approximately 100 μl) were placed in an Eppendorf tube, washed with 1 ml Dulbecco's phosphate buffered saline (PBS) (Life Technologies, Gaithersburg, MD), and resuspended in 200 μl of PBS. Next, 2 μl anti-PSGL-1 (PL1), anti-P-selectin (S12) mAb [Moore, Patel, Bruehl, Fugang, Johnson, Lichenstein, Cummings, Bainton, McEver (1995)], or irrelevant mAb (HPC4) [Stearns, Kurosawa, Sims, Esmon, and Esmon (1988)] were added to the bead solution. The antibody-bead mixture was incubated at 37°C for one hour on a rotating shaker. Finally, the beads were washed to remove excess antibodies and resuspended in PBS.

2.2 Micropipette manipulation apparatus

The apparatus used for membrane tether extrusion experiments is similar to the one used by Marcus and Hochmuth (2002). For experiments performed on HL-60, CHO, or K562 cells, the chamber contained the same culture media, but without selection agents. For experiments performed on neutrophils, the chamber contained Hanks Balanced Salt Solution (HBSS) with 1% human plasma. The micropipettes were pulled from glass tubes (0.75mm $\times$ 0.4mm $\times$ 75.2mm; A-M systems, Inc., Everett, WA) using a vertical pipette puller (Model 720; David Kopf Instruments, Tujunga, CA). A microforge was used to cut each microneedle at a desired position to obtain a diameter of $\sim$5 μm for the pipette that held the cultured cells/neutrophils and $\sim$9 μm for the pipette that held the bead.

2.3 Determination of Initial Tether Forces

Membrane tethers were extruded from cells using the protocol as previously described by Marcus and Hochmuth (2002). Briefly, one pipette holds the antibody-coated bead while the other pipette holds a cell in a stationary position. A precise suction pressure was created in the bead pipette by lowering the water-filled reservoir and measuring the difference in height from the equilibrium position at which the bead is stationary. A small positive pressure is applied to drive the bead to touch the cell, which allows infrequent and therefore most likely single bond formation between the cell and bead through specific receptor/ligand or antibody/antigen interaction. Then the pressure is suddenly dropped to a predetermined negative level, driving the bead away from the cell and pulling a membrane tether from the cell surface (Fig. 1). The presence of a (usually invisible) membrane tether is detected by the slower velocity of the bead moving inside the pipette than that predicted from the fluid mechanics theory. The force that extrudes the membrane tether from the cell surface is calculated from this velocity differential [Shao and Hochmuth (1996)]:

$$F = \Delta P \bullet A[1 - (V_t/V_f)], \tag{1}$$

where ΔP is the suction pressure in pN/μm^2, A is the cross-sectional area of the pipette in μm^2, V_t is the velocity of the tether in μm/s and V_f is the free velocity of the unconstrained bead in μm/s.

The experimental process, together with a simultaneous time display, was recorded on videotape at a speed of 30 frames per second. Distance was measured with a video caliper (Model 305, Vista Electronics, La Mesa, CA.). For each condition, several tethers were extruded over a range of suction pressures and the bead velocity (distance/time) was plotted against the tether force, which appeared linear (see example in Fig. 2) as observed previously [Marcus and Hochmuth (2002)]. The y-intercepts of the linear fits are referred to as initial tether forces as they represent the forces required to initiate tether extrusion. Initial tether forces were analyzed

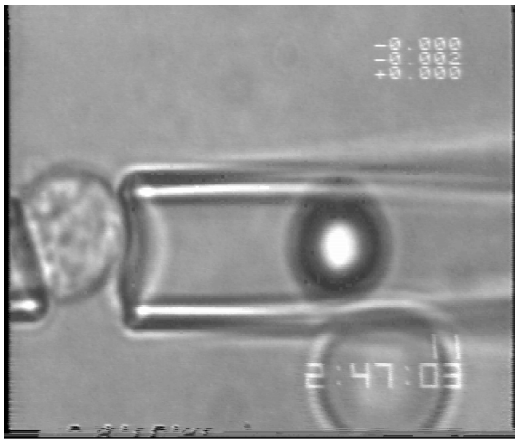

Figure 1 : Video micrograph of tether extrusion experiment.

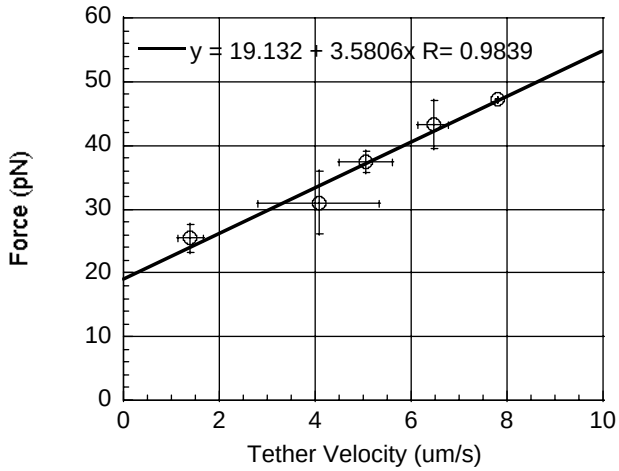

Figure 2 : Sample plot of tether extrusion experiment. The equation on the plot represents a linear fit (line) to the data (points). The error bars represent s.e.m.

with the least squared error method using KaleidaGraph 3.08 software (Synergy Software, Reading, PA). The statistical significance between the data was analyzed using a two-tailed Student *t*-test.

3 Results

3.1 PSGL-1-mediated initial tether force for neutrophils is greater than those for HL-60 and K562 cells

Using a latex bead coated with anti-PSGL-1 mAb (PL1) as the force transducer, membrane tethers were extruded from neutrophils, HL-60 cells and K562 cells with the micropipette technique described in the Methods. Tether forces were determined from Eq. 1 and plotted against tether velocity (of Fig.2). A linear function is fit to the data to extrapolate the force at zero velocity. This "zero velocity" force is interpreted as the minimum force required for initiating the formation of membrane tethers and is referred to as the initial tether force [Hochmuth and Marcus (2002)]. Initial tether forces were determined for neutrophils, HL-60 cells and K562 cells (Fig. 3). Neutrophils and HL-60 cells constitutively express PSGL-1 on their surfaces. K562 cells were transfected with a wild-type PSGL-1. The initial tether force for neutrophils is 47 $\pm$ 5 pN. This is more than twice the force required for tether formation from HL-60 cells (23 $\pm$ 3 pN) and K562 cells (19 $\pm$ 2 pN).

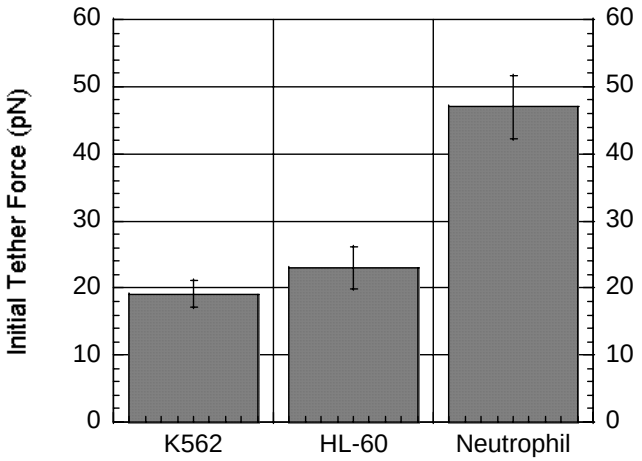

Figure 3 : Initial tether forces for the various cells. The error bars are standard errors and 5-7 cells per type were used.

3.2 Initial tether forces do not depend on cytoplasmic domain of targeted surface protein

To examine the contribution from the cytoplasmic connection of a cell surface molecule to the initial tether force mediated by that molecule, we compared P-selectin-mediated initial tether force for CHO cells transfected with the wild-type P-selectin and a mutant P-selectin with its cytoplasmic domain deleted (tailless P-selectin). Latex beads coated with S12 (anti-P-selectin) were used as force transducers. Surprisingly, the val-

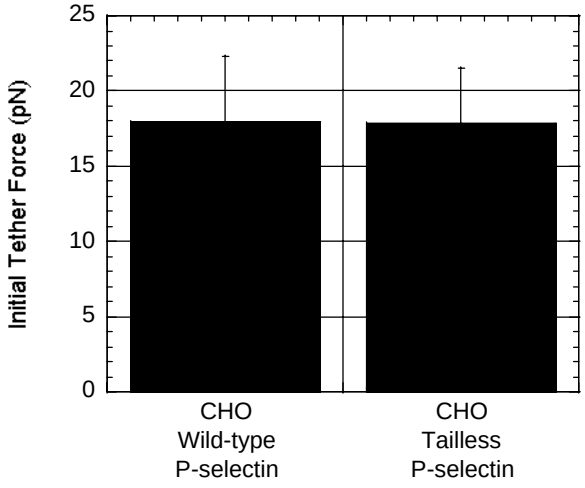

Figure 4 : Comparison of initial tether forces for CHO cells with wild-type and cytoplasmic tail deletions of P-selectin.

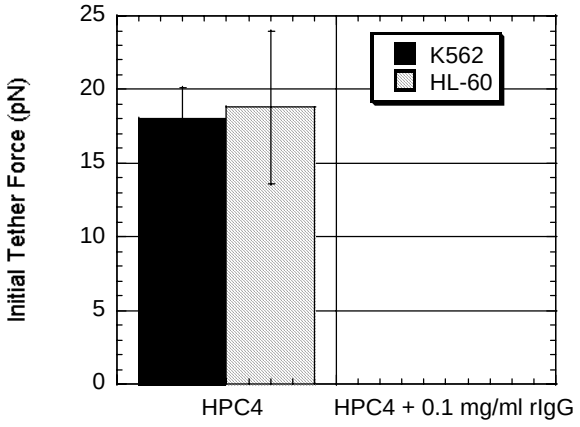

Figure 5 : Initial tether forces of K562 and HL-60 cells mediated by FcγR-HPC4 Fc interaction. No tethers were formed when using HPC4 after cells are incubated with 0.1 mg/ml of rabbit IgG to block the FcγRs (second column).

ues for the initial tether forces of wild-type and tailless P-selectin CHO cells (Fig. 4) were quite similar (18 $\pm$ 4.3 pN and 17.8 $\pm$ 3.7 pN, respectively), suggesting that the strength of cytoskeletal linkage of the cell surface molecule where the pulling force is applied had no effect on the initial tether force.

3.3 FcγRs Mediate similar initial tether forces as PSGL-1

The data in the preceding section suggest that the initial tether forces are insensitive to the cell surface molecules to which the pulling forces are applied. To further test this contention, we measured initial tether forces mediated by Fcγ receptors (FcγRs) - proteins that recognize the Fc segment of IgG's. Latex beads coated with a mAb, HPC4, whose antigen is not expressed on HL-60 or K562 cells were used to measure initial tether forces for these cells, which express FcγRs that bind to the Fc portion of HPC4. Figure 5 shows that membrane tethers can be extruded by the interaction of FcγRs on HL-60 and K562 cells with HPC4 on the beads. Membrane tether extrusion was completely abolished when 0.1 mg/ml rabbit IgG, which, like mouse IgG, cross-reacts with human FcγRs, were used to block FcγRs on HL-60 and K562 cells. By comparison, the presence of rabbit IgG in the media had no effects on either the frequency of tether formation or the initial tether forces when PL1-coated beads were used to extrude tethers from neutrophils (data not shown), indicating that these tethers were extruded via specific binding of PL1 to PSGL-1. It was found that the FcγR-mediated initial tether forces were also similar to the PSGL-1-mediated initial tether forces (Figs. 3 and 5). Tether extrusion experiments for K562 and HL-60 cells were repeated using a PL1-coated bead and using 0.1 mg/ml rIgG to block the FcγRs. However, no significant differences in initial tether forces were seen (data not shown). These data further indicate the lack of dependence of initial tether force on the cell surface molecule to which the pulling force is applied to extrude the membrane tether.

3.4 The actin microfilaments play a major role in the initial tether force

The cytoskeleton's contribution to the initial tether force was investigated by using latrunculin A, a known actin filament distruptor [Coue, Brenner, Spector, Korn (1987)]. In these experiments, the target cell was placed in a chamber containing the appropriate media, described in the methods, but now also containing 0.3 μM latrunculin A for at least 20 minutes before initiating membrane tether extrusion. Initial tether forces for untreated and treated cells are compared in Fig. 6. The neutrophil's initial tether force went from 47 $\pm$ 5 pN to 13 $\pm$ 4 pN, a significant reduction. K562 cells had a reduction in initial tether force from 19 $\pm$ 2 pN to 8 $\pm$ 3pN, and HL-

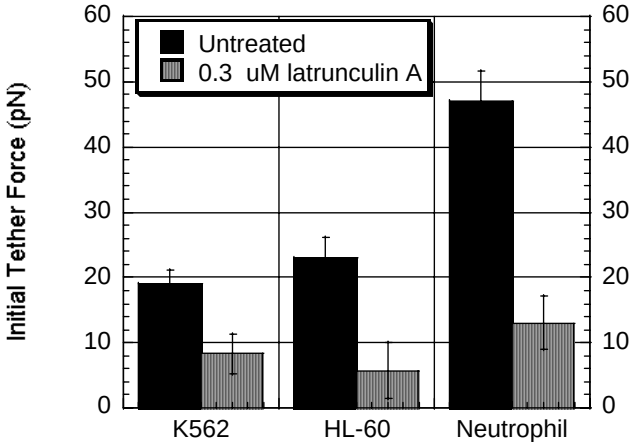

Figure 6 : Comparison of initial tether forces for untreated cells and cells treated with 0.3μM latrunculin A.

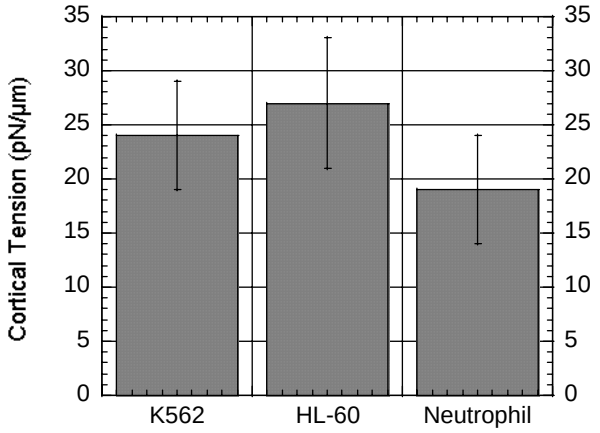

Figure 7 : Cortical tensions of the listed cell types. The error bars are standard deviations.

60 cells had a reduction from 23 $\pm$ 3 pN to 6 $\pm$ 4 pN. All of the reductions in initial forces from untreated to treated cells are statistically significant. However, no statistically significant differences were seen among the initial tether forces for the different treated cells.

3.5 Lack of correlation between initial tether force and cortical tension

The cortical tension [Ting-Beall, Lee, Hochmuth (1995)] of the different cell types was determined to see if this tension causes the differences in initial tether forces. Cortical tensions are determined by noting the suction pressure required to form a hemisphere in a pipette for a particular cell type. The following equation is then used to determine the cortical tension:

$$T = \Delta P \bullet 2(R_p^{-1} - R_o^{-1}), \tag{2}$$

where ΔP is the suction pressure in pN/μm^2, R_p is the radius of the pipette in μm, and R_o is the radius of the portion of the cell that is outside of the pipette in μm. The data collected for these experiments are shown in Fig. 7. The value of the cortical tension in neutrophils (n=9) is 19 $\pm$ 5 pN/μm. The value for HL-60 cells (n=8) is 27 $\pm$ 6 pN/μm; and the value for K562 cells (n=5) is 24 $\pm$ 5 pN/μm. There are no statistically significant differences among these values.

4 Discussion

Several studies have suggested that membrane tether extrusion occurs *in vivo* during leukocyte rolling, which may regulate rolling velocity [Schmidtke and Diamond (2000); Girdhar and Shao (2004); Ramachandran, Williams, Yago, Schmidtke, McEver (2004)]. Motivated by findings of these studies, we set out to identify factors that impact the initial tether force. We found that forces required for initiating membrane tethers, a measure for the degree of ease or difficulty of tether extrusion, depended on the cell type. Since cell lines of various types are commonly used to study adhesion receptors transfected to these different cells, different initial tether forces are expected for cells transfected to express the same molecule, which may affect the observed rolling behavior.

Our experiments indicate that more than twice as much force is required to initiate membrane tether formation from neutrophils as it takes for CHO, K562 and HL-60 cells (Fig. 3 and 4). To explore the mechanism controlling the initial tether force, we tested the effects of the actin disruptor latrunculin A and found that latrunculin A greatly reduced the initial tether forces for all the cells tested to approximately the same levels (Fig. 6). This suggests that actin microfilaments are required for maintaining the level and the differences in initial tether forces among the various cells tested.

From a mechanical standpoint, there are at least two ways that the actin microfilaments can impart greater difficulty

for membrane tether extrusion. One way is by imparting more tension to the cell's lipid membrane and making it more difficult to "pinch" off the membrane required for tether formation. Mechanical analysis has predicted a quantitative relationship between the initial tether force, an assumed energy per unit surface area of adhesion between the membrane and the underlying cytoskeleton structure, and a far field membrane tension [Hochmuth; Shao, Dai, Sheetz (1996); Hochmuth and Marcus (2002)]. Neutrophils have been modeled mechanically as a bag of highly viscous viscoelastic fluids enclosed by a pre-stressed cortical tension [Dong, Skalak, Sung, Schmid-Schonbein, Chien (1988); Evans and Yeung (1989)]. We therefore measured the cortical tension of neutrophils, HL-60 cells, and K562 cells (Fig. 7). However, the lack of correlation between the cortical tension and the initial tether force suggests that the initial tether force is not governed exclusively by the cortical tension.

Another way for the actin microfilaments to impart greater initial tether force is for there to be either a stronger cytoskeletal linkage of the molecule to which the point force is directly applied to extrude the membrane tether or a stronger connection between other cell surface proteins in the neighborhood where the membrane ether is extruded, which provides greater resistance to the flow of lipid bilayer into the tether to allow it to grow. It is also possible that a combination of these factors impacts the initial tether force. To test the role of the cytoskeletal linkage of the protein that directly exerts force to extrude membrane tethers, we used beads coated with anti-Pelectin-1 mAb (S12) to extrude tethers from CHO cells transfected to express either the wild-type P-selectin or a truncated P-selectin containing only the first seven residues of the 35-residue cytoplasmic domain. Our data show that the initial tether forces are independent of the cytoplasmic tail (Fig. 4). We note that the initial tether force is the extrapolation to zero-velocity of the tether force vs. tether velocity plot measured at nonzero velocities (Fig. 2). Therefore, tether forces at lower tether velocities than those measured could still be different for cells expressing wild-type and tailless surface proteins. Regardless, our results suggest that the resistance to tether growth arises from a stronger connection between cell surface proteins other than the one to which pulling force is directly applied. This hypothesis is further supported by the finding that initiation of membrane tether extrusion through FcγRs on HL-60/K562 cells requires similar forces as through PSGL-1 (Figs. 3 and 5). The lack of dependence of initial tether force on the neutrophil protein that mediates the tether extrusion has also been seen in a previous study, which show similar initial tether forces for tethers extruded through β_2 integrins, L-selectin, and CD45 [Shao and Hochmuth (1996)], which are similar to the initial tether forces from neutrophil PSGL-1 (this study). The same conclusion has been obtained by a recent study pulling membrane tethers from endothelial cells, the initial tether force of which is also independent of the protein used to impose the point forces [Girdhar and Shao (2004)]. In conclusion, initial tether force is cell-specific but not molecule-specific.

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