Shear Induces a Unique Series of Morphological Changes in Translocating Platelets : Effects of Morphology on Translocation Dynamics

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Effects of Morphology on Translocation Dynamics

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Objective—The platelet glycoprotein (GP) Ib/V/IX complex plays an important role in regulating the morphology of resting platelets and can induce shape change during adhesion to immobilized von Willebrand factor (vWf). In this study we have examined the effects of fluid shear stress on GPIb-dependent changes in platelet morphology during translocation on vWf.

Methods and Results—We demonstrate that translocating platelets undergo a unique series of morphological changes in response to increasing fluid shear stress. Under moderately low shear conditions (600 s^{-1}), initial shape change involved extension of membrane tethers and/or filopodia from the platelet surface. With increasing shear rate, platelets adopted a spherical morphology with numerous surface projections (1800 to 5000 s^{-1}). At high wall shear rates (10000 to 20 000 s^{-1}), translocating platelets retracted filopodia, developing a smooth ball-like appearance. These changes in morphology were dependent on reorganization of the actin and microtubule components of the cytoskeleton and were regulated by intracellular signaling processes linked to Src kinases. Functionally, alterations in platelet shape had a major effect on translocation dynamics in that conversion from discs to spheres resulted in a 3- to 8-fold increase in rolling velocity.

Conclusions—These studies demonstrate that platelets undergo shear-specific morphological changes during surface translocation on vWf that may serve to regulate translocation dynamics under flow. (***Arterioscler Thromb Vasc Biol.* 2006;26:663-669.**)

Key Words: GPIb/V/IX ■ platelets ■ shape change ■ shear ■ vWf

Platelet adhesion and aggregation at sites of vascular injury is essential for hemostatic plug formation and vessel wall repair but can also contribute to pathological thrombosis, precipitating diseases such as acute myocardial infarction and ischemic stroke. Adhesion and aggregate formation is a complex process, regulated through the interplay of numerous adhesive receptors and ligands. The GPIb/V/IX receptor plays a key role in this process by recruiting platelets to the site of vascular injury through specific engagement of the A1 domain of immobilized von Willebrand factor (vWf). This adhesive interaction has intrinsically rapid binding kinetics that readily supports initial platelet tethering and translocation; however, it is insufficient to support firm adhesion in the absence of a second adhesive step, typically involving platelet integrins. The importance of the vWf–GPIb/V/IX interaction in platelet function has been well-established and is underscored by the severe bleeding disorder experienced by individuals with qualitative or quantitative abnormalities in either vWf or GPIb/V/IX.[1]

In addition to its adhesive function, the GPIb/V/IX complex also plays an important role in regulating the cytoskeletal architecture of resting platelets. The GPIb/V/IX complex is physically anchored to the membrane skeleton through a specific noncovalent interaction between the cytoplasmic tail of GPIbα and the actin binding protein, filamin A (ABP-280).[2–4] This is proposed to maintain the compact structure of the membrane skeleton, because perturbations to the GPIb-filamin-A interaction undermine the submembranous actin superstructure.[5,6] The GPIb/V/IX complex is a unique receptor in that it not only maintains the normal cytoskeletal organization of resting platelets but also can induce platelet cytoskeletal remodeling following engagement of vWf, resulting in the conversion of flat discoid platelets into spherical forms expressing multiple filopodia.[7,8] The potential importance of vWf in promoting cytoskeletal remodeling in vivo has been highlighted from studies on pigs with von Willebrand disease (vWD).[9] In these studies, platelet adhesion to injured coronary arteries of normal pigs was associated with platelet filopodial extension and cell spreading. In contrast, platelets from vWD pigs extended few filopodia and failed to spread after adhesion to the subendothelium, indicating that cytoskeletal reorganization of adherent platelets under these conditions in vivo is primarily a vWf-dependent process. Despite the potential importance of vWf-induced

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cytoskeletal reorganization, there is currently limited information on the mechanisms by which the vWf-GPIb/V/IX interactions regulates cytoskeletal reorganization and the effects of shear on this process.

A recently identified platelet morphological change that is partially responsible for regulating the stop–start phase of translocation involves the pulling of thin membrane tethers from the surface of discoid platelets.[10] Membrane tethers are elongated structures that extend from small, localized adhesion contacts under the influence of flow, and are distinct from filopodia in that they do not require active actin filament elongation.[10] Tether formation is shear-dependent and can develop from adhesion contacts mediated solely by vWf–GPIb/V/IX bonds. All aspects of tether dynamics, including the percentage of platelets forming membrane tethers, the rate of tether elongation, and life-time of tether bonds is influenced by the shear environment,[10] suggesting that these structures are intimately linked to shear-dependent platelet adhesive function. Despite the potential importance of membrane tethers and platelet shape change in regulating platelet adhesion dynamics under flow there is currently limited information on the functional relationship between these morphologically distinct platelet forms.

In the current study we have examined the effect of shear on platelet shape change during surface translocation on immobilized vWf. We demonstrate that increasing shear induces a unique series of morphological changes in platelets that involve reorganization of the actin and microtubule components of the cytoskeleton. We show that under moderately low shear conditions (600 s^{-1}) initial platelet shape change involved extension of membrane tethers and/or filopodia from the surface of platelets. With increasing shear rate, the majority of platelets eventually adopted a spherical morphology with numerous surface projections (1800 to 5000 s^{-1}). In response to high wall shear rates (10000 to 20 000 s^{-1}) platelets adopted a smooth spherical morphology due to retraction of filopodia into the cell body. Functionally, alterations in platelet shape had a major effect on platelet translocation dynamics in that conversion of platelets from disc to spheres significantly increased platelet rolling velocity. These studies suggest that shear-dependent platelet morphological changes may play a potentially important role in regulating platelet translocation dynamics under flow.

Methods

Materials

All reagents were from sources described previously.[7] Please see Figure I at http://atvb.ahajournals.org for additional details for all Methods.[11]

Blood Collection and Platelet Preparation

Blood was taken from healthy adults, and approval was gained from the Monash University Human Ethics Committee for all experiments performed. Whole blood was anticoagulated with hirudin (200 U/mL); washed platelets and red blood cells were prepared as described previously.[10,12] For some studies, platelets were fixed with 4% paraformaldehyde in a resting discoid form, or in a cold-activated shape changed form.

In Vitro Flow Studies

Flow studies were performed according to a modified method of Yap et al.[13] In brief, whole blood or washed platelets were treated with the anti-integrin $\alpha_{IIb}\beta_3$ antibody, c7E3 Fab (20 μg/mL), or the peptidomimetic Aggrastat (500 nM) and perfused through vWf-coated microcapillary tubes (100 μg/mL) at 600 s^{-1}. The wall shear rate was incrementally increased to 1800, 5000, 10 000, and 20 000 s^{-1} and translocating platelets were classified according to morphology. Where indicated in the text, platelets were also treated with the following inhibitors: vinblastine (10 μg/mL), cytochalasin D (5 μmol/L), theophylline (10 mmol/L), sodium nitroprusside (SNP) (10 μmol/L), PP2 (10 μmol/L), or DM-BAPTA (70 μmol/L).

Immunofluorescence and Scanning Electron Microscopy

Platelets were fixed and actin filaments and microtubules were fluorescently labeled. Platelets were processed for scanning electron microscopy (SEM) as described previously.[14]

Statistical Analysis

Significant differences were determined using an unpaired Student *t* test.

Results

Translocating Platelets Undergo a Distinct Series of Morphological Changes in Response to Increased Wall Shear Stress

To investigate the effects of shear on platelet morphology during surface translocation on vWf, anticoagulated whole blood was perfused through vWf-coated microcapillary tubes. All experiments were performed in the presence of a blocking anti-integrin $\alpha_{IIb}\beta_3$ antibody (c7E3 Fab) to prevent stable platelet adhesion. Platelets initially tethered to vWf as flat discs with no observable membrane protrusions (Figure 1A, Disc). However, during translocation, platelets underwent a distinct sequence of morphological transitions, many of which were influenced by the level of shear. At lower shear rates (600 s^{-1}), the majority of translocating platelets retained their discoid morphology with ≈30% of platelets converting to a spherical form with multiple filopodia (Figure 1A, 1B, 1C, Sphere + Proj). Membrane projections were readily observed on the surface of discoid platelets, and were caused by the formation of membrane tethers (Figure 1A, Disc + Proj), because these structures were not inhibited by pretreating platelets with cytochalasin D. At 600 s^{-1}, platelet sphering and filopodial extension occurred slowly, ranging from 30 seconds up to several minutes after initial adhesion. Increasing the wall shear rate (1800 to 5000 s^{-1}) converted the majority of discoid platelets into spiny spheres (Figure 1B, 1C). These studies confirmed that the vWf–GPIb/V/IX interaction is sufficient to induce morphological changes in translocating platelets and furthermore suggest that the rate of shape change is shear-dependent. It should be noted that a small proportion of platelets (<5%) exhibited a spindle-like morphology at shear rates between 600 and 5000 s^{-1}; however, none of these cells was observed to convert from an initial discoid morphology during translocation, but instead appeared to be present within the blood sample before perfusion. This morphology was not investigated further in this study.

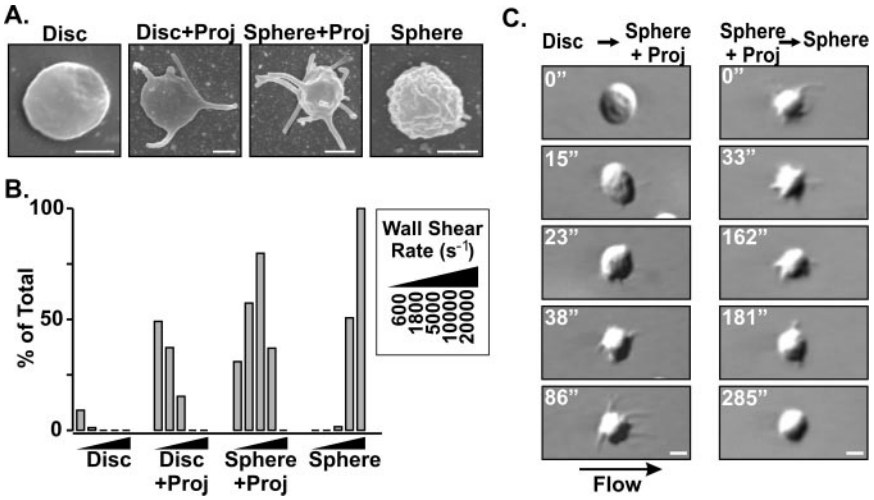

Figure 1. Shear-dependent morphological changes during platelet translocation on vWf. A, Scanning electron micrographs of platelet morphologies observed during surface translocation (scale bar=1 μm). B, The proportion of platelets adopting each morphology at each shear rate. C, DIC images showing a platelet converting from disc → sphere + projections (1800 s^{-1}), or sphere + projections → smooth sphere (10 000 s^{-1}) (scale bar=1 μm). Please see http://atvb.ahajournals.org for detailed Figure legends.

Analysis of translocating platelets exposed to high wall shear rates (10 000, 20 000 s^{-1}) revealed that a large proportion of platelets converted from spiny spheres to smooth spherical cells, totally lacking membrane projections (Figure 1A, 1B). This transformation primarily resulted from the retraction of filopodia and membrane tethers, rather than their detachment from the cell surface (Figure 1C), although in a small percentage of cases some membrane tethers were observed to detach. Complete retraction of these projections was a relatively slow process, requiring >60 seconds for full incorporation back into the cell body. Adoption of a smooth spherical morphology was dependent on reaching a threshold level of shear as subjecting platelets to lower shear rates (1800 s^{-1}) for up to 20 minutes did not result in filopodial retraction (unpublished data, 2004). Once fully spheroidal, platelets retained this morphology even after reducing flow to 5000 s^{-1} or 1800 s^{-1} (unpublished data, 2004), indicating that maintenance of high shear was not essential to sustain this morphology. In control studies, we confirmed that similar shear-dependent morphological changes occurred in Glanzmann's thrombasthenic platelets (congenitally lacking integrin $\alpha_{IIb}\beta_3$), excluding an important role for integrin $\alpha_{IIb}\beta_3$ in this process (Figure III, available online at http://atvb.ahajournals.org). Furthermore, a similar temporal sequence of morphological changes was observed (membrane tether formation from discoid platelets → conversion to spiny spheres → retraction of filopodia producing smooth spheroidal platelets) when anticoagulated whole blood was continuously perfused through vWf-coated microcapillary tubes (unpublished data, 2004). Notably, retraction of filopodia occurred at lower wall shear rates with whole blood (5000 s^{-1}) presumably because of the higher viscosity and shear forces induced by red blood cells. These control studies confirmed that shear-dependent morphological changes were not dependent on platelet isolation or inhibition by theophylline, nor were they dependent on the washout of nonadherent platelets and red cells. In further control studies, we confirmed that these morphological changes occurred in both untreated and 7E3-treated whole blood, confirming that these changes can occur even when integrin $\alpha_{IIb}\beta_3$ engagement of vWf is allowed to occur (unpublished data, 2004). Overall, these studies demonstrate that translocating platelets undergo a distinct series of morphological changes in response to shear.

Role of the Cytoskeleton in Mediating Platelet Morphological Change

To investigate the relationship between actin remodeling and shear-dependent platelet morphological change, platelets were fixed during translocation on vWf and filamentous actin was stained with fluorescein isothiocyanate (FITC)-conjugated phalloidin (see Methods). As shown in Figure 2A, filamentous actin was distributed evenly throughout all platelet morphological forms, and also within membrane projections (filopodia and membrane tethers). Inhibition of actin polymerization with cytochalasin D (CD) abolished filopodia extension; however, membrane tether formation and cell sphering was not affected (Figure 2B). Inhibiting actin polymerization severely impacted on the structural integrity of the plasma membrane, such that at shear rates in excess of 1800 s^{-1} membranes became increasingly unstable, resulting in the formation of greatly elongated bulbous membrane tethers (Figure 2C, ii). These tethers were commonly observed to detach from the platelet body, resulting in the premature formation of smooth spherical-shaped platelets at wall shear rates of 5000 s^{-1} (Figure 2B, 2C, iii). These findings demonstrate an important role for the actin cytoskeleton in preserving platelet shape and membrane integrity under conditions of high shear.

To examine the relationship between microtubule reorganization and shear-dependent platelet morphological changes, translocating platelets were fixed and microtubules stained with a FITC-labeled anti-β-tubulin antibody (see Methods). As demonstrated in Figure 3A, discoid platelets displayed the characteristic microtubule coil beneath their circumferential surface membrane. As platelets converted to spherical forms with filopodia, microtubule staining became progressively more diffuse throughout the cytoplasm. Tubulin staining was not observed in all membrane projections, with preferential staining in thick filopodia (Figure 3A). The smooth spherical platelets observed at high shear rates (10 000 to 20 000 s^{-1}) was associated with the reformation of the microtubule ring, the diameter of which (1.38±0.04 μm; n=16) was signifi-

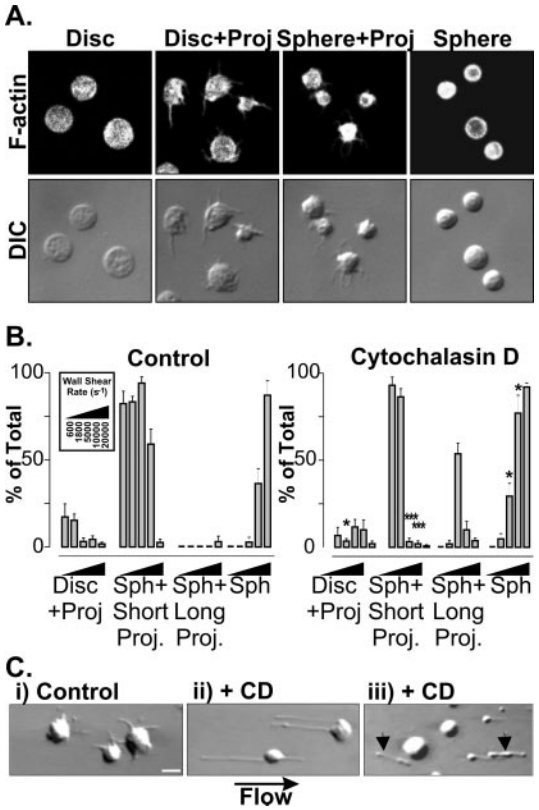

Figure 2. Role of actin polymerization in shear-dependent shape change. A, Filamentous actin stained throughout all platelet morphologies. B, Cytochalasin D inhibited filopodia, but not membrane tethers, which became abnormally elongated (sphere + long projections). C, DIC images of rolling platelets (5000 s^{-1}) showing typical spherical cells in control studies (i), in comparison to CD-treated platelets forming elongated membrane tethers (ii), which often detached (iii, see arrowheads) (scale bar=1 μm). Please see http://atvb.ahajournals.org for detailed Figure legends.

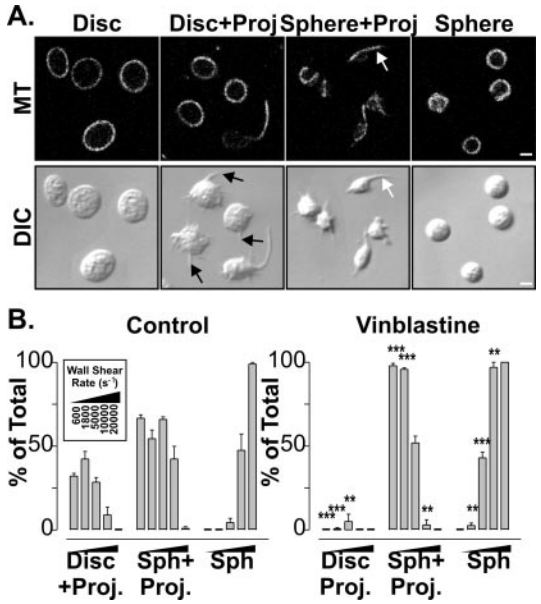

Figure 3. Role of microtubule remodeling in shear-dependent platelet shape change. A, Microtubule staining in platelets of different morphologies (scale bar=1 μm). B, Vinblastine-treated platelets tethered as irregular spheres and were still seen to extend membrane projections. Results show the mean±SEM from 5 independent experiments (**$P<0.01$, ***$P<0.001$). Please see http://atvb.ahajournals.org for detailed Figure legends.

cantly ($P<0.0001$) smaller than that observed in resting discoid platelets (2.05±0.09 μm; mean±SEM n=26). These findings suggest a potential role for shear in regulating microtubule assembly in platelets. After disruption of microtubule assembly with vinblastine, resting platelets adopted a spherical morphology; however, these platelets tethered normally to vWf and extended membrane projections in a similar manner to untreated platelets. The major effect of vinblastine was at high shear rates, which reduced the shear threshold required to induce filopodial retraction, with 50% of the population at 5000 s^{-1} converting to smooth spheres compared with 4% in controls (Figure 3B). In addition, the membrane of these platelets remained intact even up to shear rates of 10 000 s^{-1}. These studies suggest a potentially important role for microtubules in regulating platelet morphological changes under high shear conditions.

Signaling Processes Regulating Platelet Morphological Change

To investigate the signaling mechanisms regulating shear-dependent morphological changes in platelets, flow studies were performed with platelets treated with pharmacological regulators of cAMP (theophylline) or cGMP (sodium nitroprusside, SNP). Previous studies have demonstrated that vWf-induced cytoskeletal remodeling under low shear conditions is regulated by cAMP-dependent signaling processes.[7] As demonstrated in Figure 4A, theophylline or SNP markedly inhibited platelet shape change under flow with the majority of platelets maintaining their discoid morphology up to a shear rate of 5000 s^{-1}. However, at 10 000 s^{-1}, ≈25% of cells converted to a spherical form through a process involving gradual deformation from disc →"fat disc" → irregular sphere → smooth sphere (Figure 4B). Although theophylline and SNP-treated platelets did not extend filopodia, membrane tether formation still occurred resulting in the majority of platelets forming fine surface projections at high shear. Similar results were obtained with forskolin or PGE1 treatment (unpublished data, 2004), and combining these inhibitors with theophylline had no greater inhibitory effects then when used individually (unpublished data, 2004). These studies indicate that shear-dependent morphological change is influenced by signaling processes linked to the regulation of cAMP and cGMP.

Previous studies have defined an important role for Src kinases in transducing signals downstream of GPIb/V/IX. To investigate a potential role for Src kinases in shear-dependent platelet shape change, platelets were pretreated with the Src kinase inhibitor PP2. As demonstrated in Figure 4C, PP2 reduced the proportion of platelets converting from flat discs to spherical forms at all shear rates examined. However, up to 30% of platelets became spherical and extended membrane projections, primarily through the development of membrane tethers. Similar to theophylline and SNP-treated platelets ≈25% of platelets retracted surface projections and adopted a smooth spheroid morphology at high shear. These cytoskel-

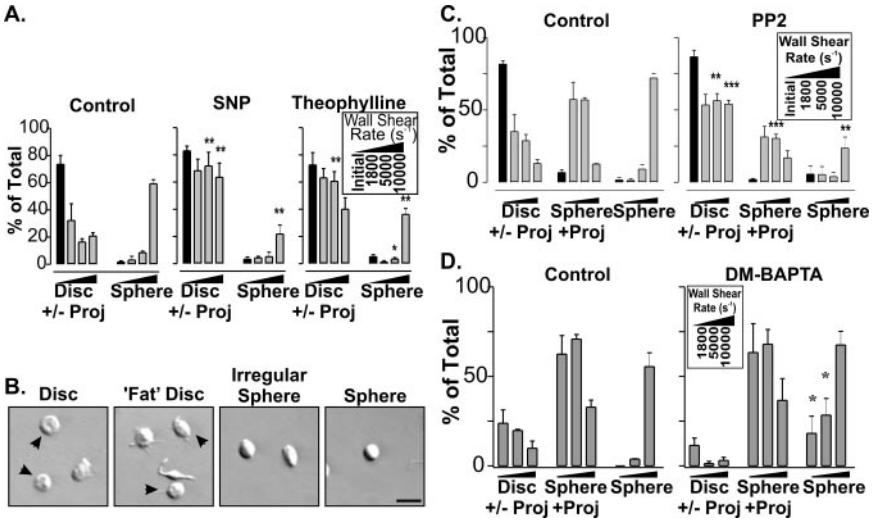

Figure 4. Role of platelet activation, Src kinases, and calcium flux in shear-dependent shape change. In all panels, results show the mean±SEM from 3 independent experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$). A and C, Sodium nitroprusside (SNP), theophylline, or PP2 promoted retention of discoid morphology at all shear rates. B, DIC images show the transition of a representative SNP-treated platelet from disc to "fat disc" (1800 s^{-1}), to irregular sphere (5000 s^{-1}), to sphere (10000 s^{-1}) (scale bar=5 μm). D, Inhibiting calcium flux did not prevent high-shear morphological changes. Please see http://atvb.ahajournals.org for detailed Figure legends.

etal changes were partly mediated through the mobilization of cytosolic calcium as chelating intracellular calcium reduced the number of filopodia formed per platelet (unpublished data, 2004). However, at elevated shear rates (1800 to 10 000 s^{-1}) platelet sphering and the extension of membrane projections was still apparent, indicating that cytosolic calcium flux was not essential for these processes (Figure 4D).

Shear-Dependent Morphological Change Regulates Platelet Translocation Dynamics

To investigate whether changes in morphology influences platelet adhesion dynamics under flow, we investigated the translocation velocity of discoid, spherical with filopodia, and smooth spherical platelets at wall shear rates of 1800, 5000, and 10 000 s^{-1} (Figure 5A). Direct comparison of discoid platelets with spiny spheres was only possible up to 5000 s^{-1} because discs were not prevalent above this shear rate. As demonstrated in Figure 5A, discoid platelets translocated significantly slower than spheres at 1800 and 5000 s^{-1} and typically exhibited a stop–start translocation behavior, with the duration of the stop phase inversely proportional to the shear rate.

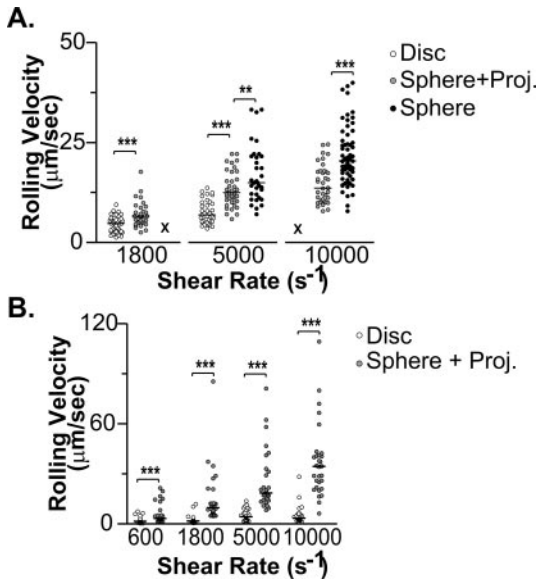

Figure 5. Effect of morphological change on adhesion to vWf. The rolling velocity of live (A) or fixed (B) platelets of discoid or spherical morphologies (n=35 to 40, bar represents median, **$P<0.01$, ***$P<0.001$). Please see http://atvb.ahajournals.org for detailed Figure legends.

To determine whether differences in shape per se was responsible for these altered translocation dynamics, flow studies were performed on paraformaldehyde-fixed platelets. A significant advantage of this approach is that individual platelets retain their morphology regardless of the shear conditions. For these studies, 2 distinct platelet populations were prepared: a resting platelet preparation (>95% discoid) and a second shaped changed population of platelets (see Methods for details). Cells from these 2 fixed platelet preparations were combined to yield a population consisting of 65% discoid platelets and 35% spherical platelets with filopodia. When perfused over immobilized vWf, an equal proportion of each morphological type adhered over a range of wall shear rates (600 to 10 000 s^{-1}) (Figure IV, available online at http://atvb.ahajournals.org), demonstrating that morphology does not affect the ability of platelets to initially tether to this matrix. Furthermore, when this mixed platelet population was perfused over vWf, the translocation velocity of discoid platelets was similar to that measured in the live platelet studies, suggesting that fixation does not overtly affect the ligand binding capacity of GPIb/V/IX (Figure 5B).

Examination of the effects of increasing shear on translocation velocity revealed a marked difference between discoid and shape-changed platelets. Whereas only a small increase in translocation velocity was apparent with discoid platelets from 600 to 10 000 s^{-1}, the velocity of spherical platelets increased 3- to 8-fold. Further evidence that platelet morphology influences translocation behavior was obtained from comparative analysis of spiny spherical platelets with smooth spherical platelets. As demonstrated in Figure 5A, the presence of filopodia slowed translocation velocity of platelets at both 5000 and 10 000 s^{-1}. This difference was likely caused by filopodial participating in the adhesion process directly, rather than differences in shape per se, as filopodia were

observed to form transient adhesion contacts with the matrix and retard the normal smooth rolling phenotype of spherical platelets. Overall, these studies define a potentially important role for platelet morphological change in regulating translocation dynamics under flow.

Discussion

The studies presented here demonstrate that platelets undergo a distinct series of morphological changes during translocation on vWf. These alterations in shape are highly dependent on the wall shear rate and are initiated by GPIb/V/IX-induced cytoskeletal rearrangements through signaling pathways involving Src kinases. Platelet shape changes in response to soluble agonist stimulation typically involve sphering of the cell body and extension of multiple filopodia. Whereas similar changes were also observed during translocation on vWf at low–moderate wall shear rates (600 to 1800 s^{-1}), exposing platelets to high shear stresses (5000 to 20 000 s^{-1}) induced retraction of filopodia, a novel functional change not previously identified in platelets. In addition, adoption of different morphological types were found to be associated with altered translocation properties. These studies suggest that platelet shape change may be a potentially important variable regulating platelet translocation under flow.

It has not previously been possible to examine in real-time the temporal sequence of events associated with shear-dependent platelet morphological change. Previous attempts with whole blood have involved labeling platelets with fluorescent dyes to enable real-time monitoring of membrane projections.[7,8] This method does not allow easy discrimination between membrane tethers and filopodia and cannot distinguish discoid from spherical platelets. By using high-magnification differential interference contrast microscopy we have been able to examine platelet morphological changes during initial adhesion from whole blood without the need for platelet isolation or surface dye labeling. Furthermore, through simple washout of nonadherent red blood cells and platelets we have been able to obtain high resolution images of translocating platelets, enabling accurate assessment of the dynamics of surface membrane projections. These studies have enabled detailed analysis of the effects of shear flow on platelet morphological change and have demonstrated for the first time a potentially important role for platelet shape in regulating translocation dynamics under flow.

The difference in translocation behavior of distinct platelet morphologies observed in this study is likely to reflect differences in cell shape, rather that altered inherent binding properties of GPIb, as we have observed no significant differences in the level of GPIb/V/IX expression on the surface of shape changed platelets nor any deleterious affect on their ability to bind vWf under static or flow conditions (unpublished data, 2004). Furthermore, the demonstration that fixation of platelets preserved the marked differences in translocation behavior of discoid and shape changed platelets strongly suggests that altered shape, rather than dynamic changes in GPIb/V/IX receptor function, is the dominant mechanism influencing translocation behavior. In addition, the demonstration that similar shear-dependent morphological changes occurred when integrin $\alpha_{IIb}\beta_3$ was allowed to engage the vWf matrix, and that shape change under these conditions also lead to a marked increase in translocation velocity (unpublished data, 2004), suggests that our findings are likely to have physiological relevance. Cell shape may influence translocation dynamics in several ways. For example, whereas flat oval disc is not ideal for a cell undergoing rotational motion (rolling), it is ideal for cell sliding, a translocation behavior that may have relevance to membrane-tethered discoid platelets. Rotating flat discs experience sudden changes in forces during side-to-side "flipping,"[15] increasing the likelihood of cell detachment from the adhesive surface. In contrast, a spherical form is ideally suited to "rolling," and platelets of this morphology had the most rapid translocation velocities. Interestingly, the presence of filopodia significantly reduced rolling velocity. This suggests that filopodia slow platelet translocation by providing additional adhesive bonds with the vWf substrate. Regardless of the precise mechanism(s) by which platelet shape change regulates translocation dynamics, our studies clearly establish that shear-dependent morphological change is a potentially important variable regulating platelet translocation under flow.

Our studies suggest that shear-dependent morphological change is dependent on intracellular signaling processes, potentially linked to GPIb/V/IX. However, even in the presence of potent platelet activation inhibitors some sphering could still occur at very high shear rates, suggesting that deformation from physical forces may also contribute to shape change. Experiments using the pharmacological inhibitor PP2 established that shear-dependent platelet morphological change was highly dependent on Src kinases, a finding consistent with previous studies demonstrating an important role for these enzymes in GPIb/V/IX signaling.[16] Src kinases promote activation of one or more PLCγ isoforms[17] and we have provided evidence that calcium flux is important for GPIb-dependent platelet shape change.[7] The demonstration of platelet sphering and formation of membrane projections in dimethyl-BAPTA–treated platelets under high shear indicates that cytoskeletal changes can occur independent of calcium flux. However, the interpretation of such experiments is complicated by the artifactual promotion of cytoskeletal deformation as result of lowering the basal cytosolic calcium levels. Similarly, it is well known that slow actin filament elongation can still occur in calcium-chelated platelets and as a result dimethyl-BAPTA treated platelets can form abnormal U-shaped membrane projections after agonist stimulation.[6] The challenge ahead will be to identify the precise intracellular events regulating cytoskeletal remodeling during surface translocation and to define the mechanisms by which platelets sense alterations in their shear environment to induce specific cytoskeletal responses.

The rate of change in morphology of translocating platelets is slow relative to agonist-stimulated platelets, presumably reflecting the weak platelet activating properties of GPIb/V/IX. Even in the absence of integrin $\alpha_{IIb}\beta_3$ receptor blockers, platelet shape change occurred slowly on vWf (unpublished observations), suggesting that co-stimulation by soluble agonists is probably necessary for rapid cytoskeletal remodeling in vivo. These observations, in combination with in vivo studies demonstrating reduced platelet shape change in pigs

with vWD pigs,[9] suggest that optimal cytoskeletal remodeling in vivo may require the interplay of various adhesive and agonist input signals. In addition, our studies suggest that shear per se is a potentially important variable regulating platelet cytoskeletal remodeling. The mechanism for this remains unknown, although it is tempting to speculate that the physical link between GPIbα and filamin A is important for shear-induced cytoskeletal changes in platelets. Filamin A has previously been demonstrated to mediate force-induced actin accumulation in nucleated cells, potentially as a result of its direct effects on actin polymerization, actin filament cross-linking.[18] Filamin A may also promote changes in the cytoskeleton through indirect means, as a result of coordinating the formation of cytoskeletal signaling complexes and through the regulation of stretch-activated ion channels that are themselves linked to the underlying cytoskeleton.[18,19]

An unexpected finding from this study was the observation that platelets retract filopodia at pathological wall shear rates (10 000 to 20 000 s^{-1}). Whereas the reason for this shear-specific difference in morphology remains unclear, one possibility is that it may be a direct result of the level of tension experienced by the membrane bilayer. For example, cells which undergo changes in membrane organization and/or geometry may use up internal membrane reserves to protect the cell from sudden changes in bilayer tension that might otherwise rupture the cell.[20] The projection of filopodia from the platelet surface may function in a similar manner as membrane reserves stored within the invaginations of the surface connecting canalicular system (SCCS) are used to fill these membrane projections.[21,22] Furthermore, as filopodia are also capable of forming adhesive interactions with the vWf surface, they may facilitate adhesion by allowing a greater number of GPIb/V/IX–vWf bonds to form. Although the exact reason why platelets retract filopodia remains unclear, it is possible that the platelet is attempting to increase its membrane stores as a means of reducing membrane stretching at high shear forces. This may promote a smooth rolling behavior at high shear rates.

In conclusion, these studies demonstrate that platelet cytoskeletal remodeling and morphological change represents a shear-sensitive platelet functional response. Given the critical role of shear in regulating platelet adhesion and thrombus growth, these findings may have potential pathophysiological significance. Our findings add further complexity to the understanding of shear effects on platelet adhesive function and suggest that platelets may be a useful model system to investigate mechanosensory signaling systems regulating cytoskeletal remodeling.

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SUPPLEMENTARY FIGURE I: DETAILED METHODS

Materials - Cytochalasin D, vinblastine, paclitaxel, theophylline, FITC-conjugated phalloidin, and anti-β-tubulin mAb were purchased from Sigma. The anti-integrin $\alpha_{IIb}\beta_3$ antibody, chimeric Fab fragment of mAb 7E3 (c7E3 Fab - Abciximab) was from Eli Lilly (Centocor). Aggrastat was from Merck. Hirudin was purchased from Novartis AG. von Willebrand factor was purified from plasma cryoprecipitate according to the method of Montgomery and Zimmerman.[11] PP2 was purchased from Calbiochem. All other reagents were from sources described previously.[7]

Blood collection and platelet preparation – Blood was taken from healthy adults, and approval was gained from the Monash University Human Ethics Committee for all experiments performed. Whole blood was collected directly into syringes to a final concentration of 200 U/ml hirudin. Washed platelets were prepared as described previously[10] and suspended in platelet washing buffer (PWB) (4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, pH 6.5, 113 mM NaCl, 5.5 mM glucose, 0.5% (w/v) bovine serum albumin and 10 mM theophylline) at 1×10^9/ml. Red blood cells were isolated and washed as described previously.[12]

Preparation of Fixed Platelets - Washed platelets in PWB were treated with EDTA (2 mM) then incubated at for 45 minutes at either 37ºC or 4ºC to obtain a resting and activated population, respectively. We found that these experimental conditions were optimal for inducing platelet shape change, without altering the normal adhesive function of the GPIb/V/IX complex, as assessed by receptor surface expression and vWf-induced platelet agglutination (unpublished data, 2003). Platelets were fixed with 4% paraformaldehyde, washed three times with Tris-saline (20 mM Tris, 150 mM NaCl, pH 7.2), then stored at 4ºC prior to use.

In Vitro Flow Studies - Flow studies were performed according to a modified method of Yap *et al.*[13] Rectangular glass microcapillary tubes (Vitro Dynamics Inc.) were coated with human vWf (100 μg/ml) overnight at 4°C. In studies using washed platelets, the tubes were also blocked with 5% heat-inactivated human serum containing 50 μg/ml phenylmethylsulfonyl fluoride at room temperature for 60 min. In some studies, hirudin-anticoagulated (200 U/ml) whole blood was pretreated with

either the anti-integrin $\alpha_{IIb}\beta_3$ antibody, c7E3 Fab (20 μg/ml) or the peptidomimetic Aggrastat (500 nM) for 10 minutes and then perfused through microcapillary tubes at 600 s^{-1} for 30 seconds. Non-adherent cells were removed by perfusing with modified Tyrode's buffer (10 mM HEPES, pH 7.4, 12 mM $NaHCO_3$, 137 mM NaCl, 2.7 M KCl, 5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) containing either c7E3 Fab (20 μg/ml) or Aggrastat (500 nM) for 5 min. The morphology of translocating platelets was visualized by differential interference contrast (DIC) microscopy on an inverted microscope (Leica DMIRB; 100× PL APO objective, NA 1.4 - 0.7) and 5 random fields were video-recorded for off-line analysis. The wall shear rate was incrementally increased to 1800, 5000, 10000 and 20000 s^{-1} and maintained at each shear rate for 4 minutes prior to video recording. DIC images shown in figures were digitized using Microcomputer Imaging Device (MCID) software. Adherent platelets were classified according to morphology (discoid, discoid with membrane projections, spindles, spherical with membrane projections, or smooth spheres), and data was expressed as a percentage of the total number adherent. There was a small degree of inter-donor variability, reflected in the slightly different platelet morphology distributions for control platelets in different figures. However, the overall process of shape change was identical in all donors, and a minimum of 3 individual donors was used to generate data for each experiment. In some studies, whole blood was pretreated with Aggrastat or c7E3 Fab in addition to vinblastine (10 μg/ml for 20 minutes) or with cytochalasin D (5 μM for 10 minutes) prior to perfusion. In other studies, experiments were performed by perfusing washed platelets in PWB (containing 10 mM theophylline and 20 μg/ml c7E3 Fab) through vWf-coated microcapillary tubes. Theophylline was removed by perfusing with modified Tyrode's buffer containing c7E3 Fab (20 μg/ml) for five minutes at 600 s^{-1} prior to exposing adherent platelets to incremental increases in shear rate. Where indicated, platelets in PWB were treated with c7E3 Fab (20 μg/ml) in addition to sodium nitroprusside (SNP, 10 μM) or the Src kinase inhibitor PP2 (10 μM) for 10 minutes prior to perfusion through vWf-coated microcapillary tubes. To examine the effect of theophylline on shear-dependent shape change, washed platelets were perfused into vWf-coated microcapillary tubes in the presence of PWB, and then perfused with modified Tyrode's buffer containing theophylline (10 mM). In studies examining the role of intracellular calcium in morphological change, platelets in PWB were treated with Aggrastat (500 nM) and DM-BAPTA (70 μM) for 30 min at 37°C prior to perfusion.

In studies examining the adhesion of fixed platelets under flow conditions, a mixture of resting and activated fixed platelets were treated with Aggrastat (500 nM) and perfused through vWf-coated microcapillary tubes at 600, 1800, 5000 or 10000 s^{-1}, at a concentration of 3×10^8/ml in the presence of 50% (v/v) washed erythrocytes to facilitate adhesion. Non-adherent cells were washed out as described for whole blood. Translocating platelets were visualized and video-recorded for off-line analysis. Translocation velocity was determined by measuring the distance traveled (displacement) per unit time recorded.

Immunofluorescence Studies – Platelets translocating on vWf were fixed by perfusing with 4% formaldehyde in modified Tyrode's buffer and permeabilised with 2% Triton X-100. Actin filaments were stained with FITC-conjugated phalloidin and tubulin was stained with a monoclonal anti-β-tubulin antibody, followed by a CY5-conjugated anti-mouse antibody. Platelets were imaged by confocal microscopy (100× lens, Leica).

Scanning Electron Microscopy (SEM) - Translocating platelets were fixed by perfusion of 2% glutaraldehyde in SEM buffer (100 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) through microcapillary tubes for 2 min. The upper surface of the rectangular glass microcapillary tube was removed by scoring the glass with a diamond pen and the lower surface retained for SEM processing as described previously.[14] Samples were imaged on a Hitachi S570 electron microscope at 15 kV accelerating voltage (working distance of 3 mm).

Statistical analysis - Significant differences were determined using an unpaired Student's t-test using Prism software (GraphPAD Software for Science, San Diego, CA). Results are presented as mean ± SEM.

SUPPLEMENTARY FIGURE II: DETAILED FIGURE LEGENDS

Figure 1. Shear-dependent morphological changes during platelet translocation on vWf

Anticoagulated whole blood pre-incubated with c7E3 Fab was perfused through vWf-coated microcapillary tubes and non-adherent cells were removed by perfusing with modified Tyrode's buffer. The wall shear rate was incrementally increased from 600 s^{-1} to 1800, 5000, 10000 and 20000 s^{-1}. (**A**) Scanning electron micrographs of platelet morphologies observed during surface translocation (***Disc***, Discoid; ***Disc + Proj***, Discoid + Projections; ***Sphere + Proj***, Sphere + Projections; ***Sphere***, Spherical) (Scale bar = 1 μm). (**B**) The proportion of platelets adopting each morphology at each shear rat expressed as a percentage of the total number adherent. Results show one experiment, representative of 10. (**C**) Individual platelets were followed during translocation for the indicated periods (seconds) to observe conversion from disc → sphere + projections (1800 s^{-1}), or sphere + projections → smooth sphere (10000 s^{-1}) (Scale bar = 1 μm).

Figure 2. Role of actin polymerization in shear-dependent shape change

Anticoagulated whole blood treated with either vehicle alone (**Control**) or cytochalasin D (**CD**) was perfused through vWf-coated microcapillary tubes and adherent platelets exposed to incremental increases in shear rate as described in Methods. (**A**) Adherent platelets from control studies were fixed during perfusion and stained with FITC-conjugated phalloidin for simultaneous fluorescence and DIC imaging. Filamentous actin stained throughout all morphologies. (**B**) The proportion of platelets adopting each morphology was determined at each shear rate observed. In the presence of CD, an additional classification was necessary (**sphere + long projections**). Results show mean + SEM from four independent experiments (*$p<0.05$, ***$p<0.001$). (**C**) DIC images of rolling platelets (5000 s^{-1}) showing typical spherical cells in Control studies (**i**), in comparison to CD-treated platelets with unstable membranes forming elongated membrane tethers (**ii**) which often detached leaving a smooth spherical cell body (**iii – see arrowheads**). (Scale bar = 1 μm)

Figure 3. Role of microtubule remodeling in shear-dependent platelet shape change

Anticoagulated whole blood treated with either vehicle alone (control) or the microtubule depolymerizing agent vinblastine was perfused through vWf-coated microcapillary tubes and adherent platelets exposed to incremental increases in shear rate as described in Methods. (**A**) Platelets from Control studies were fixed during perfusion and microtubules were labelled with an anti-tubulin antibody. Microtubules formed a ring structure in discoid cells which disintegrated in spiny spheres, and reformed in smooth spherical platelets at high shear rates. (Scale bar =1 μm) (**B**) Vinblastine-treated platelets tethered as irregular spheres, and were still seen to extend membrane

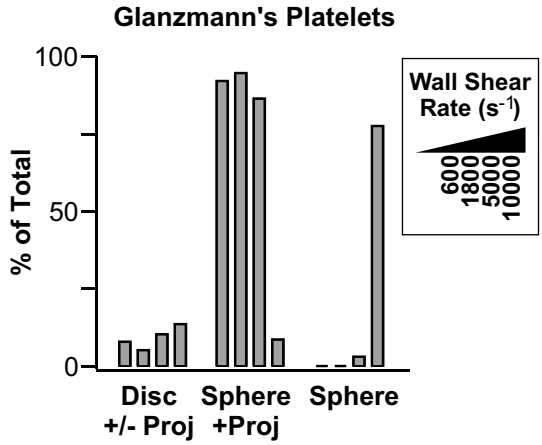
projections. Results show the mean + SEM from 5 independent experiments **p<0.01, ***p<0.001).

Figure 4. Role of platelet activation, Src kinases and calcium flux in shear-dependent shape change

Washed platelets in PWB were preincubated with c7E3 Fab in addition to sodium nitroprusside (SNP, 10 μM) (**A – SNP**), theophylline (10 mM) (**A – theophylline**), PP2 (10 μM) (**C – PP2**) or DM-BAPTA (70 μM) (**D – DM-BAPTA**) prior to perfusion through vWf-coated microcapillary tubes. PWB was removed by perfusing with modified Tyrode's buffer supplemented with each of the indicated inhibitors and adherent platelets exposed to incremental increases in shear rate. (**A**) Inhibiting platelet activation with SNP or theophylline resulted in the majority of platelets retaining their discoid morphology even at high shear rates. Results show mean + SEM from 3 independent experiments (*p<0.05, **p<0.01). (**B**) Approximately 25% of theophylline or SNP-treated platelets transitioned from discoid to sphere at the highest shear rate (10000 s^{-1}) without extending filopodia. DIC images show the transition from disc to 'fat disc' (1800 s^{-1}), to irregular sphere (5000 s^{-1}), to sphere (10000 s^{-1}). (Scale bar = 5 μm) (**C**) Inhibiting Src kinases with PP2 promoted retention of discoid morphology even at higher (5000 - 10000 s^{-1}) shear rates. Results show the mean + SEM from 3 independent experiments (**p<0.01, ***p<0.001). (**D**) Inhibiting calcium flux did not prevent high-shear morphological changes. Results show mean + SEM from 3 independent experiments (*p<0.05).

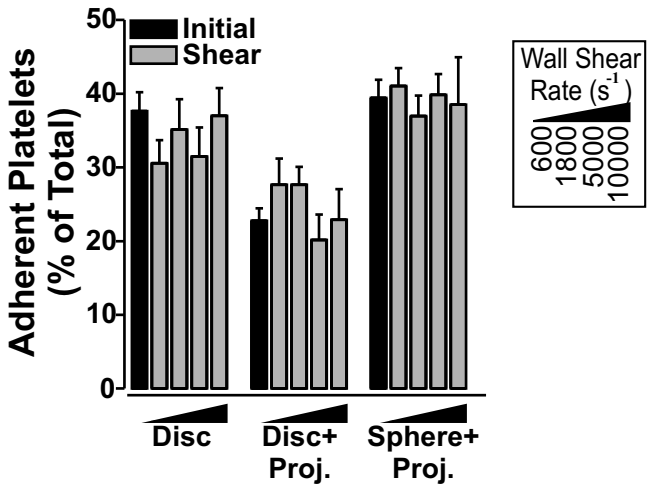
Figure 5. Effect of morphological change on adhesion to vWf

(**A**) c7E3 Fab-treated washed platelets in PWB (pH 6.5) were perfused through vWf-coated microcapillary tubes. PWB was removed by perfusing with modified Tyrode's buffer supplemented with c7E3 Fab and adherent platelets exposed to incremental increases in shear rate (see Methods). The rolling velocity was calculated in platelets of discoid, discoid + membrane projections and spherical morphology (n=35-40, bar represents median, **p<0.01, ***p<0.001). As discoid platelets were not prominent at 10000 s^{-1} and smooth spheres were not seen at 1800 s^{-1} translocation velocity could not be determined at these shear rates. (**B**) Washed platelets were fixed in a resting (discoid) or cold-activated (sphere + projections) morphology as described in 'Methods'. Platelets were treated with c7E3 Fab and then perfused through vWf-coated microcapillary tubes. Translocation velocity was calculated in discoid platelets or spheres with membrane projections at shear rates of 600, 1800, 5000 and 10000 s^{-1} (n = 30, bar represents median, ***p<0.001).



SUPPLEMENTARY FIGURE III. Shear-dependent shape change in Glanzmann's platelets

Whole blood from an individual with Glanzmann's thrombaesthenia was perfused through vWf-coated microcapillary tubes and adherent platelets were exposed to incremental increases in wall shear rate. The proportion of platelets adopting each morphology at each shear was expressed as a percentage of the total number adherent.



SUPPLEMENTARY FIGURE IV. Platelet morphology does not affect platelet adhesion to immobilised vWf

Washed platelets were fixed in a resting (discoid) or cold-activated (sphere + projections) morphology as described in 'Methods'. An equal number of cells from these two fixed platelet preparations were combined to yield a population consisting of 40% discoid, 25% discoid with membrane projections and 35% sphere with filopodia. Platelets were treated with c7E3 Fab and then perfused through vWf-coated microcapillary tubes at 600, 1800, 5000 or 10000 s^{-1}. DIC imaging revealed that an equal proportion of each morphological type adhered to the vWf matrix over a range of wall shear rates (600-10000 s^{-1}), indicating that morphology does not affect the ability of platelets to tether to immobilized vWf. Results show the mean + SEM, n=3.