Shear Induces a Unique Series of Morphological Changes in Translocating Platelets

Effects of Morphology on Translocation Dynamics

Mhairi J. Maxwell, Sacha M. Dopheide, Samantha J. Turner, Shaun P. Jackson

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

Original received October 26, 2005; final version accepted December 16, 2005.

From Australian Centre for Blood Diseases, Monash University, Prahran, Australia.

Correspondence to Shaun P. Jackson, Australian Centre for Blood Diseases, Monash University, 89 Commercial Rd, Prahran, Australia, 3181. E-mail shaun.jackson@med.monash.edu.au

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.

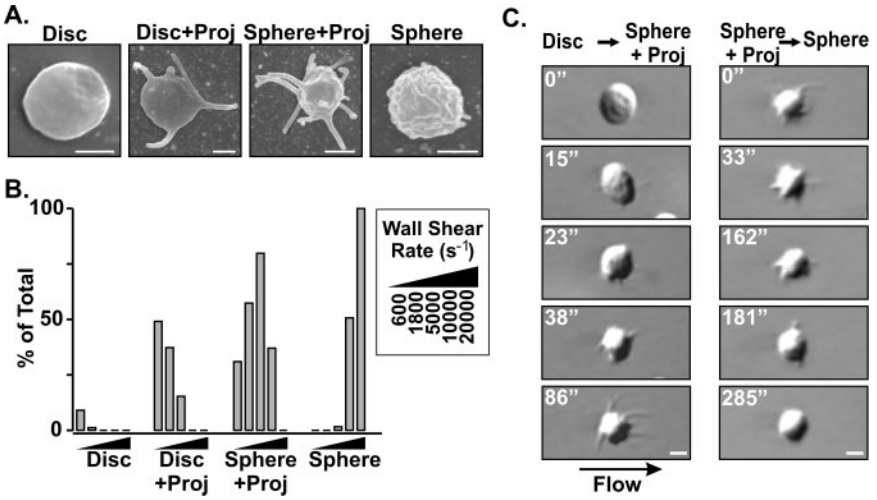

Figure 1. Shear-dependent morphological changes during platelet translocation on vWf. A, Scanning electron micrographs of platelet morphologies observed during surface translocation (scale bar=1 μm). B, The proportion of platelets adopting each morphology at each shear rate. C, DIC images showing a platelet 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-

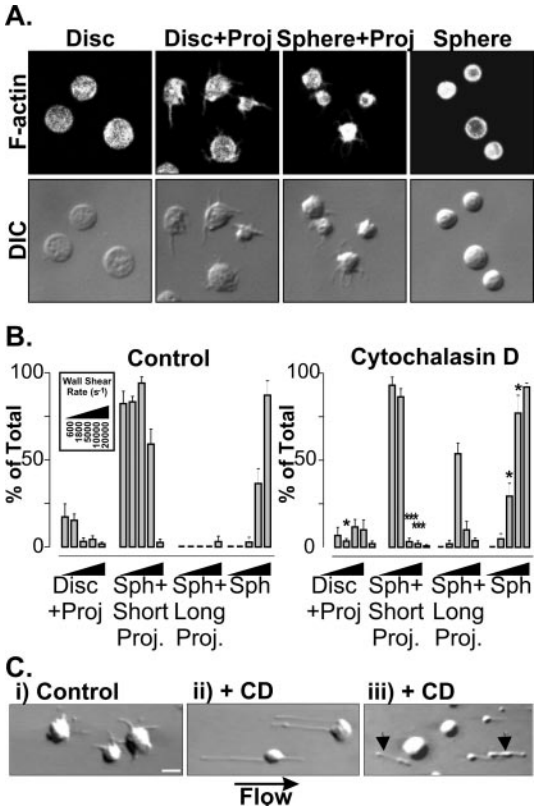

Figure 2. Role of actin polymerization in shear-dependent shape change. A, Filamentous actin stained throughout all platelet morphologies. B, Cytochalasin D inhibited filopodia, but not membrane tethers, which became abnormally elongated (sphere + long projections). C, DIC images of rolling platelets (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.

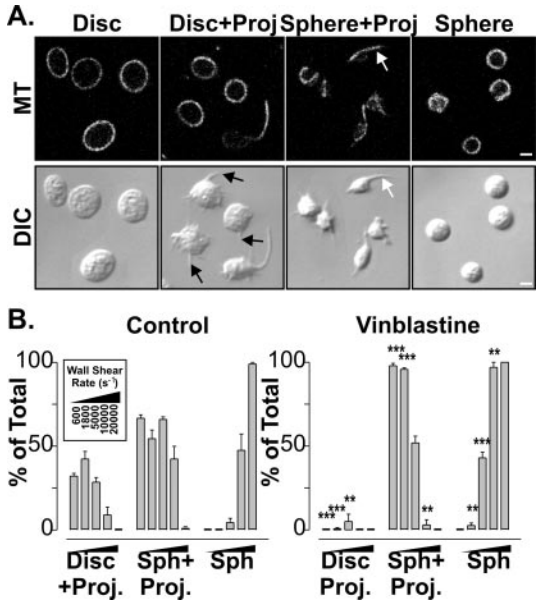

Figure 3. Role of microtubule remodeling in shear-dependent platelet shape change. A, Microtubule staining in platelets of different morphologies (scale bar=1 μm). B, Vinblastine-treated platelets tethered as irregular spheres and were still seen to extend membrane projections. Results show the mean±SEM 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-

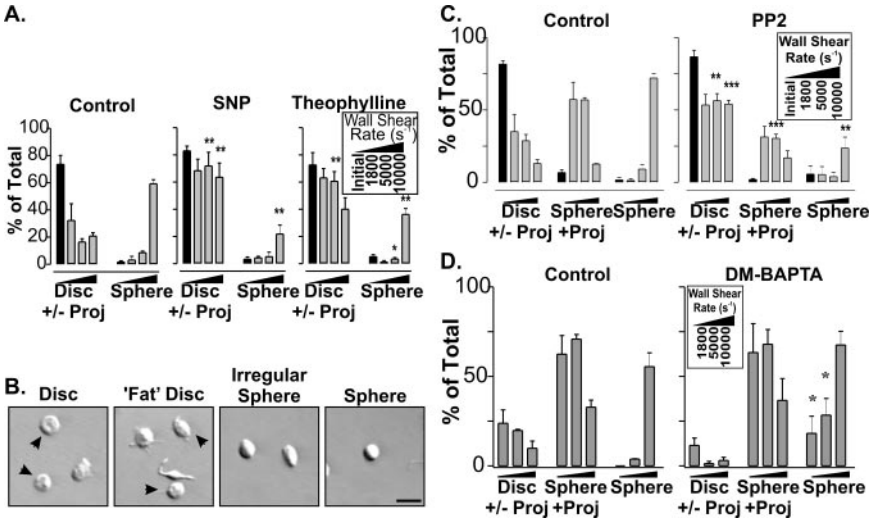

Figure 4. Role of platelet activation, Src kinases, and calcium flux in shear-dependent shape change. In all panels, results show the mean±SEM from 3 independent experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$). A and C, Sodium nitroprusside (SNP), theophylline, or PP2 promoted retention of discoid 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.

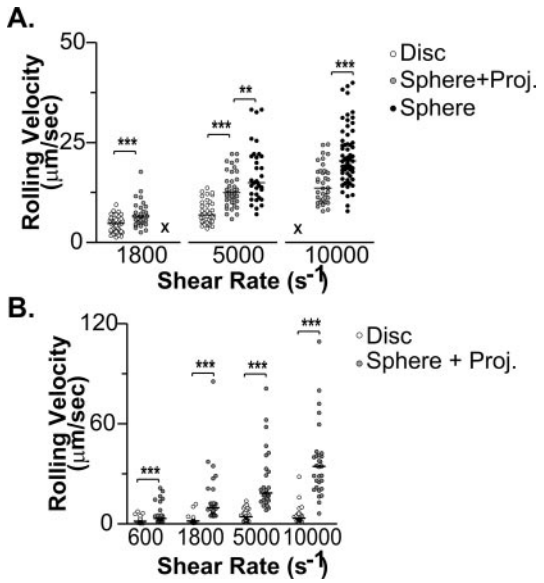

Figure 5. Effect of morphological change on adhesion to vWf. The rolling velocity of live (A) or fixed (B) platelets of discoid or spherical morphologies (n=35 to 40, bar represents median, **$P<0.01$, ***$P<0.001$). Please see http://atvb.ahajournals.org for detailed Figure legends.

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.

Acknowledgments

This work was supported by a grant from the National Health and Medical Research Council of Australia.

References

1. Cattaneo M. Inherited platelet-based bleeding disorders. *J Thromb Haemost*. 2003;1:1628–1636.
2. Andrews RK, Fox JE. Interaction of purified actin-binding protein with the platelet membrane glycoprotein Ib-IX complex. *J Biol Chem*. 1991; 266:7144–7147.
3. Fox JE. Identification of actin-binding protein as the protein linking the membrane skeleton to glycoproteins on platelet plasma membranes. *J Biol Chem*. 1985;260:11970–11977.
4. Okita JR, Pidard D, Newman PJ, Montgomery RR, Kunicki TJ. On the association of glycoprotein Ib and actin-binding protein in human platelets. *J Cell Biol*. 1985;100:317–321.
5. Kanaji T, Russell S, Ware J. Amelioration of the macrothrombocytopenia associated with the murine Bernard-Soulier syndrome. *Blood*. 2002;100: 2102–2107.
6. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol*. 1992;118:1421–1442.
7. Yuan Y, Kulkarni S, Ulsemer P, Cranmer SL, Yap CL, Nesbitt WS, Harper I, Mistry N, Dopheide SM, Hughan SC, Williamson D, de la Salle C, Salem HH, Lanza F, Jackson SP. The von Willebrand factor-glycoprotein Ib/V/IX interaction induces actin polymerization and cytoskeletal reorganization in rolling platelets and glycoprotein Ib/V/IX-transfected cells. *J Biol Chem*. 1999;274:36241–36251.
8. Kuwahara M, Sugimoto M, Tsuji S, Matsui H, Mizuno T, Miyata S, Yoshioka A. Platelet shape changes and adhesion under high shear flow. *Arterioscler Thromb Vasc Biol*. 2002;22:329–334.
9. Nichols TC, Bellinger DA, Reddick RL, Read MS, Koch GG, Brinkhous KM, Griggs TR Role of von Willebrand factor in arterial thrombosis. Studies in normal and von Willebrand disease pigs. *Circulation*. 1991; 83:IV56–IV64.
10. Dopheide SM, Maxwell MJ, Jackson SP. Shear-dependent tether formation during platelet translocation on von Willebrand factor. *Blood*. 2002;99:159–167.
11. Montgomery RR, Zimmerman TS. von Willebrand's disease antigen II. A new plasma and platelet antigen deficient in severe von Willebrand's disease. *J Clin Invest*. 1978;61:1498–1507.
12. Yap CL, Anderson KE, Hughan SC, Dopheide SM, Salem HH, Jackson SP. Essential role for phosphoinositide 3-kinase in shear-dependent signaling between platelet glycoprotein Ib/V/IX and integrin alpha(IIb)beta(3). *Blood*. 2002;99:151–158.
13. Yap CL, Hughan SC, Cranmer SL, Nesbitt WS, Rooney MM, Giuliano S, Kulkarni S, Dopheide SM, Yuan Y, Salem HH, Jackson SP. Synergistic adhesive interactions and signaling mechanisms operating between platelet glycoprotein Ib/IX and integrin alpha IIbbeta 3. Studies in human platelets ans transfected Chinese hamster ovary cells. *J Biol Chem*. 2000;275:41377–41388.
14. Schoenwaelder SM, Jackson SP, Yuan Y, Teasdale MS, Salem HH, Mitchell CA. Tyrosine kinases regulate the cytoskeletal attachment of integrin alpha IIb beta 3 (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin polymers. *J Biol Chem*. 1994;269:32479–32487.
15. Mody NA, Lomakin O, Doggett TA, Diacovo TG, King MR. Mechanics of transient platelet adhesion to von Willebrand factor under flow. *Biophys J*. 2005;88:1432–1443.
16. Wu Y, Asazuma N, Satoh K, Yatomi Y, Takafuta T, Berndt MC, Ozaki Y. Interaction between von Willebrand factor and glycoprotein Ib activates Src kinase in human platelets: role of phosphoinositide 3-kinase. *Blood*. 2003;101:3469–3476.
17. Mangin P, Yuan Y, Goncalves I, Eckly A, Freund M, Cazenave JP, Gachet C, Jackson SP, Lanza F. Signaling role for phospholipase C gamma 2 in platelet glycoprotein Ib alpha calcium flux and cytoskeletal reorganization. Involvement of a pathway distinct from FcR gamma chain and Fc gamma RIIA. *J Biol Chem*. 2003;278:32880–32891.
18. Glogauer M, Arora P, Chou D, Janmey PA, Downey GP, McCulloch CA. The role of actin-binding protein 280 in integrin-dependent mechanoprotection. *J Biol Chem*. 1998;273:1689–1698.
19. Stossel TP, Condeelis J, Cooley L, Hartwig JH, Noegel A, Schleicher M, Shapiro SS. Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol*. 2001;2:138–145.
20. Raucher D, Sheetz MP. Characteristics of a membrane reservoir buffering membrane tension. *Biophys J*. 1999;77:1992–2002.
21. Frojmovic MM, Milton JG. Human platelet size, shape, and related functions in health and disease. *Physiol Rev*. 1982;62:185–261.
22. Escolar G, Leistikow E, White JG. The fate of the open canalicular system in surface and suspension-activated platelets. *Blood*. 1989;74: 1983–1988.