Platelet Shape Changes and Adhesion Under High Shear Flow

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Abstract—Recent studies have revealed that the platelet adhesive process under flow is tightly regulated by multiple ligand-receptor interactions. However, platelet morphological changes during this process, particularly its physiological relevance, remain unknown under blood flow conditions. Using epifluorescence and scanning electron microscopy, we evaluated the real-time changes in platelet morphology during a platelet adhesive process on a von Willebrand factor–coated surface under physiological high shear flow in a perfusion chamber. Here, we show that dynamic platelet shape changes occurring during distinct phases of the adhesive process are precisely regulated by “inside-out” and “outside-in” integrin signals and are also a key regulatory element in successful platelet thrombogenesis opposing rapid blood flow in vivo. (Arterioscler Thromb Vasc Biol. 2002;22:329-334.)

Key Words: platelet adhesion ■ von Willebrand factor ■ glycoprotein Ib ■ shape changes ■ high shear rate

Platelets flowing in the bloodstream respond to vessel damage by adhering to exposed subendothelial matrices of the vessel walls at sites of injury.1 This physiological defense mechanism in hemostasis can also trigger intravascular thrombosis, leading to myocardial infarction or stroke.2 The platelet adhesive process under physiological blood flow has been recently revealed to be tightly regulated by multiple ligand-receptor interactions. This process represents a 2-step event that involves initial “platelet rolling” followed by “firm platelet adhesion” on a thrombogenic surface.3,4 Platelet rolling is mediated by transient interaction of the platelet membrane glycoprotein (GP) Ib-IX complex with surface-immobilized von Willebrand factor (vWF),3,4 which is an essential constituent for in vivo platelet adhesion under high shear rates.5–7 During rolling, platelets become activated and begin firm adhesion to the surface by tight binding of integrins αIIbβ3 or αIbβ1 to vWF or other adhesive matrices such as collagen.3,4,8

Under experimental conditions designed to induce different activation stages, inherently disk-shaped platelets undergo morphological changes, becoming more spherical or swollen when they are stimulated by exogenous agonists such as ADP or collagen in the soluble phase, extruding filopods, and spreading when they adhere to a surface in a static adhesion assay.9–11 However, the physiological relevance, if any, of platelet shape changes has remained unclear because the essential role of blood flow in platelet plug formation in vivo was not considered in those experiments.

To address this question, we observed the real-time changes in platelet morphology on a thrombogenic surface during the platelet adhesive process in blood perfusion by using a parallel-plate flow chamber, epifluorescence, and scanning electron microscopy (SEM). We show that platelets dynamically change their shape at distinct phases of the adhesive process to complete successful thrombogenesis opposing physiological rapid blood flow.

Methods

Materials

The cytoplasmic marker calcein acetoxymethyl (AM), the visible-light–excitable cytoplasmic calcium indicator fura red AM, calcium green 1-AM, and intracellular calcium chelator BAPTA AM were products of Molecular Probes, Inc. The Fab fragment of human/mouse chimeric anti-integrin antibody c7E3, which totally inhibits the ligand-binding functions of αIIbβ3, monoclonal antibody c7E3, which totally inhibits the ligand-binding functions of αIIbβ3, and an anti-integrin β3 monoclonal antibody c7E3, which totally inhibits the ligand-binding functions of αIIbβ3 at concentrations up to 0.3 μmol/L, was purchased from Eli Lilly and Co.12,13 The anti-thrombin agent argatroban was supplied by Bristol-Myers Squibb, Inc. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K) and apyrase (grade VIII) was purchased from Calbiochem. Human native vWF containing the highest molecular weight multimers, as judged by SDS–1.5% agarose gel electrophoresis,14 was purified from cryoprecipitates, as described.15–17

Fluorescence Labeling of Platelets

Blood collection and preparation of platelet-rich plasma and washed platelet suspensions were performed as described.13,18,19 The washed platelet fraction was resuspended in HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl2, 3 mmol/L NaH2PO4, 5.5 mmol/L glucose, 0.35% albumin, and 3.5 mmol/L HEPES, pH 7.2) containing 5 μmol/L calcein AM and 3 U/mL apyrase and incubated at 37°C for 30 minutes. Labeled platelets were centrifuged again and resuspended in HEPES buffer. Erythrocytes were washed...
Flow Chamber and Epifluorescence Microscopy

The flow chamber used was a rectangular type (flow path was 1.9 mm wide, 31 mm in length, and 0.1 mm in height). Glass coverslips (24 mm x 50 mm, Matsunami Glass) were coated with 200 μL purified vWF (100 μg/mL) as described. The chamber was assembled and mounted on an epifluorescence microscope (BX60, BX-FLA, Olympus). The combination of optical lenses was as follows: ×100 objective lens (Up plan Apo 100× Oil Iris, Olympus), ×2 intermediate lenses (U-CA, Olympus), and ×10 contact lens connected to a silicon-intensified target camera (C2400-08, Hamamatsu). Reconstituted blood was aspirated through the chamber by a syringe pump at a constant flow rate of 0.285 mL/min, producing a wall shear rate of 1500 s⁻¹ at 37°C in a thermostatic air bath (model UI-50, Iuchi Inc.). The wall shear rate of 1500 s⁻¹ is considered to be a physiologically relevant rapid blood flow, and all experiments in the present study were performed at this shear rate. The platelet adhesive process was recorded with a Hi-8 videocassette recorder (VL-HL1, Sharp Inc), with a time resolution of 0.033 seconds.

Evaluation of Platelet Morphology, Movement, and Cytoplasmic Calcium Change

Videotape images were digitized by a frame grabber (DIG 98, Detect Corp) and DIPP-Motion software (Detect Corp). The horizontal view size of individual platelets interacting with the surface was evaluated as a measured area from the orthographic projection of each platelet, and the platelet height was expressed as a pixel value, a fluorescence signal intensity that is basically assumed to correspond to the object thickness, with the use of WinROOF software (Mitani Corp). Platelet morphology in epifluorescence microscopic images was evaluated as the ratio of the minor diameter to the major diameter of individual platelet cytoplast in digitized images. The rolling speed of platelets interacting with the surface was defined by digitized image frames that were superimposed (total 4.5 seconds) with the use of DIPP-Motion software. The [Ca²⁺]i change for platelets interacting with the surface was evaluated as described previously.

Scanning Electron Microscopy

Platelets interacting with the vWF surface were fixed by gradual exchange of reconstituted blood with fixation buffer (0.1 mol/L phosphate buffer containing 1% glutaraldehyde, pH 7.4) at 37°C under flow. The fixation process was started at 3 minutes after the initial platelet-surface interaction and continued for 30 minutes. The entire fixation process was performed under real-time observation with epifluorescence microscopy, in which each adhesive stage (ie, rolling, firm adhesion, and extensive spreading) was clearly seen during fixation. The preliminary experiments confirmed that glutaraldehyde can rapidly fix platelets, even when they are rolling, without affecting their morphology, whereas fixation by formaldehyde was rather slow, resulting in the failure of platelet fixation on the surface under flow. After the fixation, the perfusion chamber was disassembled, and platelets fixed onto the glass surface were dehydrated in an alcohol series and critical point–dried in CO₂. specimen were sputter-coated with platinum and viewed by SEM at a tilt angle of 45° (JSM 6301F, JEOL).

Results

Time-Course Changes of Platelet Shape During Platelet Adhesive Process

Because each phase of the platelet adhesive process (ie, rolling and firm adhesion) is more clearly observed on immobilized vWF than on immobilized collagens, we used a vWF-coated coverslip as a thrombogenic surface in the present study. Time-course images of the adhesive process of individual platelets in epifluorescence microscopy (Figure 1C and 1D) revealed star-shaped platelets rolling on the vWF surface at the earliest stage of platelet-surface interaction. In a few seconds of rolling, the platelets showed widened cytoplasts and flattening when they stopped on the surface. Some firmly adhering platelets became much flatter, as judged by the size and height after several minutes.

Figure 1. Time-course shape changes in an individual platelet during a real-time adhesive process under flow conditions with a shear rate of 1500 s⁻¹. A, SEM of a resting disk-shaped platelet (original magnification ×4000), B, SEM of 3 distinct shapes of platelets interacting with the vWF surface. Three distinct platelet shapes (left, ball-shaped with filopods; middle, hemisphere-shaped with filopods; and right, extensively spread) were observed by SEM analysis of vWF-coated coverslips fixed after 3-minute blood perfusion (see Methods). C, Videotape images by epifluorescence microscopy taken at the time points indicated by the arrows in panel D (original magnification ×1000). D, Time-course changes in rolling distance, size, and height of an individual platelet interacting with the surface, analyzed in videotape images by epifluorescence microscopy. E, Time-course changes in size and height of 11 platelets observed before and after the firm adhesion. Area size and height of each platelet at 10 seconds before, 10 seconds after, and 3 minutes after firm adhesion are shown. These real-time analyses of an individual platelet by epifluorescence microscopy revealed that star-shaped platelets roll on the vWF surface after a few seconds of rolling, the platelets showed widened cytoplasts and flattening when they stopped on the surface. Some of the stopping platelets became much flatter, as judged by the size and height after several minutes.
and 1B). These data, together with the real-time observations by epifluorescence microscopy, illustrate the typical scenario of morphological changes during the adhesive process of an individual platelet: ball-shaped platelets with filopods roll, gradually flatten to a hemispherical shape, and finally adhere to the surface. Then, some platelets that have firmly adhered to the surface are spread extensively over the surface under flow conditions. In the above scenario, it should be noted that the behaviors, including morphological changes of individual platelets observed in the field, were not always analogous. However, with regard to the ball-shaped–to–hemispherical shape changes, the statistical analysis of 54 platelets confirmed the 39±21% increase in the individual platelet area and the 27±13% decrease in the platelet height at the time point 10 seconds after the firm adhesion, partly supporting the above concept. In addition, we observed that 10% to 20% of firmly adhering platelets were extensively spread (diameter >3 µm) during 7-minute observation periods.

During the above adhesive process, the platelet [Ca^{2+}] was found to be drastically elevated just before the extensive spreading (Figure 2). In this regard, we have previously reported that the [Ca^{2+}] elevation occurs after firm platelet adhesion and before platelet cohesion (platelet–platelet interaction), raising a possibility that the [Ca^{2+}] elevation may be a crucial prerequisite for platelet cohesion. Although the [Ca^{2+}] elevation is certainly an upstream event of platelet cohesion, the present morphological study more precisely defined the event sequence during the adhesive process in which the [Ca^{2+}] elevation, which is not required for firm platelet adhesion, is crucial for the extensive spreading of a single platelet that could become a base of subsequent platelet cohesion. Recently, using similar experimental flow systems, Yuan et al observed the significant [Ca^{2+}] elevation during rolling and before firm adhesion, concluding that at least the immobilization of intrinsic calcium ions, not the calcium influx from the extracellular source, occurs as a function of the vWF–GP Ib interaction during rolling. The basis for these discrepant findings remains uncertain but may rest in the sensitivity difference between these 2 experimental systems for the detection of [Ca^{2+}] changes, as Yuan et al discussed in their report. However, it is important to reconsider that platelets are very photosensitive and can be easily activated under experimental conditions using fluorescence microscopy. Thus, the modest [Ca^{2+}] elevation during rolling observed in the study of Yuan et al must be carefully interpreted. Indeed, a transient and mild calcium elevation of some platelets can be seen during rolling even in our experimental system, although this observation could not be considered significant by our statistical analyses. Thus, we assume that the drastic [Ca^{2+}] elevation, which occurs after firm adhesion and could reflect the influx from the extracellular source, is relevant for the extensive spreading in the platelet adhesive process under high shear flow.

**Effects of Blockers for Platelet Functions on Platelet Shape During Adhesive Process**

To investigate the molecular mechanisms underlying the platelet shape changes in an adhesive process under flow, we performed inhibition studies using several blockers of intracellular signal transduction. Wortmannin significantly reduced filopodia extrusion and completely blocked the ability of platelets to maintain their ball shape against flow stress; ie, platelets were elongated in the direction of blood flow (Figures 3 and 4). Although the inside-out signals from GP Ib to integrin αIIbβ3 are still undefined, the 14-3-3 protein and the actin-binding protein are known to bind to the cytoplasmic tail of the GP Ib α-chain and are suggested to be involved in the signals from GP Ib,25,26. Because wortmannin is thought to affect either the actin reorganization or the PI3K signaling pathway (where the 14-3-3 protein plays a pivotal role), the observed effect of wortmannin is likely to reflect the blockage of signals from GP Ib–vWF interaction, suggesting that initial platelet shape changes (disk-shaped→ball-shaped with filopodia extrusion→hemisphere-shaped) are mediated by
signals from GP Ib during platelet rolling. Indeed, a recent study by Yuan et al.22 supports the above interpretation.

Although filopods appeared to be already extruded when the platelets started rolling in the control experiment, perhaps because of the limitation of time resolution in our system, the wortmannin inhibition studies revealed much slower and limited filopodia extrusion compared with the control studies (Figures 3 and 4). The observation that filopods of platelets pretreated with wortmannin were extruded in a direction of flow represents, at least in part, a unique physical mechanism to oppose rapid blood flow, a rheological force that peels off platelets from the surface.

To address the functional relevance of extensive spreading under flow, we examined the adhesive strength of platelets stopping on the surface. For this purpose, we used the "flow-stop" experiment, because a strained pressure to the surface by whole blood flow containing erythrocytes is considered to, at least in part, contribute to the platelet adhesiveness. The flow-stop experiment clearly demonstrated that platelets with a diameter >2.5 \( \mu m \) (total 100 platelets within a defined visual field) are detaching from the surface as a function of time, whereas those with a diameter <2.5 \( \mu m \) (total 100 platelets) remain adherent even at 8 minutes after the flow stop.

Mechanisms and Functional Relevance of Platelet Shape Changes During Adhesive Process

Figure 6 summarizes the event sequence, activation mechanisms, and functional relevance of platelet shape changes in the adhesive process on the basis of our observations. Thus, flowing disk-shaped platelets extrude filopods in a very short time when they interact with the vWF surface through the membrane receptor GP Ib. The platelets then become ball-shaped (with filopods) and begin to roll on the surface via the transient interaction of GP Ib with the vWF surface. Signals from GP Ib during rolling might be important for filopodia extrusion and the maintenance of ball-shaped cytoplasm as well as for the activation of integrin \( \alpha_{IIb}\beta_3 \). Rolling platelets are further activated by signals from GP Ib, thereby gradually assuming a hemispherical shape. Such hemispherical platelets...
then stop on and adhere firmly to the surface as a function of activated $\alpha_{\text{IIb}}\beta_3$ binding to the vWF surface. At present, the precise mechanism of the following events (the $[\text{Ca}^{2+}]$, elevation and extensive spreading) remains to be clarified, but it is most likely that outside-in signals from integrin $\alpha_{\text{IIb}}\beta_3$ occupied by vWF are transmitted to elevate $[\text{Ca}^{2+}]$. The platelet adhesive process under flow culminates in the extensive spreading over the surface in a calcium-dependent manner (Figure 6).

**Discussion**

Our results provide some insight into the platelet shape-function relationships in thrombogenesis under physiological blood flow that have never been sufficiently demonstrable in classic platelet functional studies. Indeed, the series of platelet shape changes demonstrated in the present study is precisely regulated by inside-out and outside-in integrin signals for platelet adhesive functions opposing rapid blood flow, a force that could dislodge platelets from the surface. The ball shape favors platelet rolling on a thrombogenic surface, and filopods may support this rolling, which is important for the activation of integrin $\alpha_{\text{IIb}}\beta_3$ and firm adhesion. The hemispherical shape changing in an increase of the area adjacent to the surface seems favorable for firm adhesion. It should be noted that at this time point, platelet adhesion is still reversible. Last, the final extensive spreading ensures firm adhesion to an irreversible mode and is essential in generating a greater thrombogenic base that favors the capture of adhesive proteins and platelets in a flow, leading to platelet aggregate accumulation on the surface.

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**References**


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