Identification of a Two-Stage Platelet Aggregation Process Mediating Shear-Dependent Thrombus Formation

Running Title: Shear-dependent Platelet Aggregation

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Authorship Statement

M.J. Maxwell designed and performed the majority of the research and experiments, and co-wrote the paper with S.P. Jackson. E. Westein collected and analysed data and assisted in manuscript preparation. W.S. Nesbitt collected data for figures 1 and 7. S. Giuliano collected data for figures 1 and 7. S.M. Dopheide collected data for figure 1. S.P. Jackson designed research, provided overall direction and co-wrote the paper with M.J. Maxwell.

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ABSTRACT

Disturbances of blood flow at sites of atherosclerotic plaque rupture is one of the key pathogenic events promoting platelet activation and arterial thrombus formation. Shear effects of platelets have been extensively investigated in vitro however the mechanisms by which shear promotes platelet aggregation in vivo remain incompletely understood. By employing high resolution imaging techniques to in vitro and in vivo thrombosis models we demonstrate a unique mechanism initiating shear-dependent platelet aggregation, involving aggregate formation between discoid platelets. These discoid platelet aggregates are initially unstable and result from the development of membrane tethers between co-adhering platelets. Tether formation involves the adhesive function of GPIb/V/IX and integrin $\alpha_{\text{Iib}}\beta_3$ and conversion of discoid platelet aggregates into stable aggregates requires released ADP. The efficiency of this process is regulated by three independent variables, including the reactivity of the adhesive substrate, the level of shear flow and the platelet density at the adhesive surface. These studies identify a new mechanism initiating platelet aggregation that is critically influenced by shear, physical proximity between translocating platelets and membrane tether formation. Moreover, they provide a model to explain how the discoid morphology of platelets facilitates the maintenance of adhesive interactions with thrombogenic surfaces under high shear stress conditions.
INTRODUCTION

Excessive accumulation of platelets at sites of atherosclerotic plaque rupture is one of the key pathogenic events precipitating arterial thrombus formation, leading to acute myocardial infarction, sudden death and ischemic stroke. This pathological process is responsible for more morbidity and mortality than any other disease process and as a consequence the platelet represents a major target for therapeutic intervention. Several factors contribute to the potent platelet activating properties of ruptured plaques including the high content of fibrillar collagens in the lesion, the presence of tissue factor, as well as the direct platelet activating effects of high shear stress caused by arterial narrowing. Rheological disturbances at sites of arterial stenosis are dynamic and complex and are highly influenced by the level of narrowing and altered geometry of the vascular lumen. Nonetheless, high shear is an inevitable consequence of progressive vascular occlusion, establishing a potentially hazardous cycle of further platelet activation and thrombus growth.

The mechanisms underlying platelet aggregation and thrombus formation have been extensively investigated and are highly influenced by the prevailing blood flow conditions. Under conditions of relatively low shear (0-1000 s^-1) platelet aggregation is primarily mediated by soluble fibrinogen, which physically cross-links platelets through engagement of integrin $\alpha_{\text{IIb}}\beta_3$. At progressively higher wall shear rates (1,000-10,000 s^-1) aggregation becomes more von Willebrand factor (vWf)-dependent, with fibrinogen playing a supportive role in stabilizing formed aggregates. Immobilized vWf on the surface of platelets is indispensable for the initiation of shear-dependent platelet aggregation, through its ability to rapidly engage glycoprotein Ib (GPIb). However, this adhesive interaction is rapidly reversible leading to platelet translocation in the direction of flow. Based on current models, platelets become activated during this process, stimulating integrin $\alpha_{\text{IIb}}\beta_3$ engagement of vWf and/or fibrinogen, leading to stable platelet aggregation. Recent studies have defined a unique mechanism initiating platelet aggregation that primarily operates at pathological levels of shear (>10,000 s^-1). This aggregation mechanism is exclusively mediated by vWf engagement of GPIb and does not require platelet activation or the ligand binding function of integrin $\alpha_{\text{IIb}}\beta_3$. While the pathophysiological significance of this new aggregation mechanism remains to be established, it none-the-less suggests that under certain experimental conditions, the vWf-GPIb interaction can potentially sustain platelet interactions with thrombogenic surfaces independent of other adhesive interactions.

Central to current models of platelet adhesion under flow is the concept that platelets become activated during interaction with thrombogenic surfaces, stimulating integrin activation and the formation of additional adhesion bonds that serve to reduce cell translocation velocity and promote cell arrest. Such a model has been well established for leukocyte adhesion under flow.
However, in the context of platelets, this model has potentially important implications for the mechanisms regulating surface translocation and aggregate formation. For example, in contrast to leukocytes which have a spherical morphology well suited to rotational motion (rolling), platelets have a flat discoid morphology that does not support smooth rolling interactions. One possibility is that signals generated during surface translocation induce morphological change sufficient to convert discoid platelets into spiny spheres, thereby facilitating a rolling phenotype. Such morphological changes have previously been described during platelet adhesion to vWF under flow. An alternative possibility is that platelets principally translocate as flat discs, through a rotational side-to-side flipping mechanism, or through a sliding mechanism that maximises surface contact (bond formation) with the adhesive surface. There is experimental evidence supporting the former mechanism, however the relationship between platelet morphological change and stable adhesion has not been defined. The potential mechanisms regulating platelet adhesion under flow are further complicated by the finding that platelets form thin membrane tethers during surface translocation on vWF. Membrane tethers are pulled from the surface of discoid platelets under the influence of hemodynamic drag forces. As such, they are preferentially formed under high shear conditions where they provide mechanical advantage to vWF-GPIb adhesive bonds. However, the physiological significance of membrane tethers has yet to be defined and it is currently unclear whether these structures primarily participate in platelet-vessel interactions or are also relevant to platelet aggregation.

In the present study we have employed high resolution imaging techniques to in vitro and in vivo thrombosis models to investigate the mechanisms regulating platelet aggregation under conditions of high shear stress. These studies demonstrate that platelets principally translocate on the surface of thrombi in vivo as flat, sliding discs and participate in the platelet aggregation process by forming transient adhesive contacts with other discoid platelets. In vitro perfusion studies suggest that the formation of discoid platelet aggregates involves the generation of membrane tethers between co-adhering platelets, via adhesive interactions mediated by GPIb and integrin αIIBβ3. We have identified three independent variables influencing the efficiency of conversion of reversible discoid platelet aggregates to stable aggregation, namely the reactivity of the thrombogenic surface, the level of shear stress and the density of platelets at the adhesive surface. In combination, these factors have a major impact on the rate of platelet aggregation. Overall, our studies define a new mechanism of shear-dependent platelet aggregation that is relevant to thrombus development in vivo. Furthermore, our studies provide a model to explain how the discoid morphology of platelets facilitates the maintenance of adhesive interactions with thrombogenic surfaces under conditions of high shear stress.
MATERIALS AND METHODS

Materials - Human vWF was purified from plasma according to the method of Montgomery and Zimmerman. Human fibrinogen was purified from plasma according to the method of Jakobsen and Kierulf. Heparin (enoxaparin sodium) was from Aventis Pharma (Australia). Hirudin (lepirudin) was from Pharmion (Australia). c7E3 Fab (Abciximab) was from Eli Lilly (The Netherlands). Apyrase, A3P5P, and MRS2179 were from Sigma (USA). ARC69931MX was from AstraZeneca (Australia). Oregon green 488 BAPTA-1, AM, Fura red, AM, DiOC6 and NP-EGTA were from Molecular Probes Inc. All other reagents were from sources previously described.

Intravital studies – Approval was gained from the Monash University Animal Ethics Committee for all experiments involving animals. Intravital studies were performed according to a modified method of Denis et al. and Kulkarni et al. Mesenteric arterioles (30-40 µm) of male Wistar rats were injured via photoactivation (550nm, 10-30 sec) of systemically administered Rose-Bengal (5 mg/kg). The dynamics of platelet interactions with the damaged vessel wall and with growing platelet thrombi were viewed by differential interference contrast (DIC) microscopy using a Leica DMIIRB microscope (×100 PL APO objective, NA 1.40-0.7) and recorded on video for off-line analysis.

Preparation of washed platelets and red blood cells – Blood was collected from healthy volunteers who had given informed consent, with the approval of the Monash University Human Ethics Committee. Washed platelets were prepared as described previously. Platelets were suspended at a concentration of 300×10⁶/ml in modified Tyrode’s buffer [12 mM NaHCO₃, 10 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 5.5 mM D-Glucose, 1 mM CaCl₂, 0.5 mg/ml BSA] containing 0.02 U/ml apyrase. Red blood cells were prepared as described previously. To inhibit residual thrombin and secreted ADP, the packed RBC preparation was supplemented with 200 U/ml hirudin and 0.02 U/ml apyrase.

In vitro perfusion studies - Perfusion assays were performed according to a modified method of Cooke et al. Micro capillary tubes (Vitrocom, USA) were coated with either purified vWF (50 µg/ml) or a mixture of vWF and fibrinogen (10 µg/ml vWF + 5, 10, 20 or 50 µg/ml fibrinogen), then blocked with 5% human serum (supplemented with 50 µg/ml phenylmethylsulfonylfluoride) prior to experimentation. Either whole blood (anticoagulated with 400 U/ml hirudin) or washed platelets (150×10⁶/ml) reconstituted with packed red blood cells (45% hematocrit) were perfused through micro capillary tubes at a shear rate of 600, 1800 or 5000s⁻¹. Where indicated, platelets were pre-
treated with c7E3 Fab (20 µg/ml), ARC69931MX (1 µM), A3P5P (1 mM), MRS2179 (100 µM) or PGE1 (0.5 µg/ml) prior to perfusion. Adherent platelets were visualised by DIC microscopy (×100 PL APO objective, NA 1.40-0.7) on a Leica DMIRB microscope. Adhesion was monitored over a 250 second period and video-recorded for off-line analysis. At 15, 30, 45, 60, 90 and 120 second time points, the number of adherent platelets within an optical field (dimensions 70 µm x 90 µm) was counted. An aggregate was defined as a cluster of at least 5 platelets maintaining physical contact for at least 3 seconds. Aggregate formation was quantitated by counting the number of aggregates present within a field of view, and recording the number of platelets within individual aggregates. An aggregate was classified as ‘unstable’ if it was composed of reversibly-adherent platelets. A ‘stable’ aggregate was classified as one composed of irreversibly adherent platelets.

In some experiments, washed platelets reconstituted with RBCs were perfused over a mixed vWf/fibrinogen matrix for 15 seconds to allow a small number of platelets to adhere (approximately 6-7 platelets per 70 µm x 90 µm field). Modified Tyrode’s buffer was perfused for a further 2 minutes to allow these platelets to adhere irreversibly and begin to spread. Washed platelets pre-treated with either c7E3 Fab (20 µg/ml) or cytochalasin D (5 µM) were reconstituted with RBCs as described above and then perfused over the adherent platelets. DIC microscopy was used to visualize subsequent interactions with the pre-adhered platelets.

**Platelet adhesion to monolayers** - Blood was collected from healthy donors or individuals with Glanzmann’s thrombasthenia (1% αIIbβ3). Confluent platelet monolayers (> 90% surface coverage) were obtained by allowing washed platelets (200×10^6/ml) to spread (30 minutes, 37°C) on glass micro capillary tubes. Anticoagulated whole blood (15 mM trisodium citrate, pH 7.4) was incubated with the fluorescent dye DiOC6 (1 µM, 10 minutes), then perfused over pre-formed platelet monolayers at 1800 s⁻¹ for 1 minute. The interaction of flowing platelets with pre-formed platelet monolayers was viewed in real time using fluorescence microscopy and recorded on video for off-line analysis. The number of platelets tethering to the surface of monolayers was analysed frame by frame (25 frames per second) over the first 5–10 seconds of flow. In all studies, any cell forming an adhesion contact for more than 40 milliseconds was scored as an adherent platelet.

**Analysis of cytosolic calcium flux under flow conditions** - Changes in intracellular calcium levels were monitored according to previously published methods 27,28. Briefly, platelets in PWB (1×10⁹/ml) were loaded with Oregon Green 488 BAPTA-AM (1 µM, emission wavelength 500-570 nm) and Fura Red-AM (1.25 µM, emission wavelength 600-710 nm) for 30 minutes at 37°C. Calcium dye-loaded platelets were subsequently incubated in PWB with 10 µM NP-EGTA for 30 min at 37°C. NP-EGTA-treated platelets were washed once with PWB and resuspended in modified...
Tyrode's buffer prior to experimentation. Platelets were combined with red blood cells (to a final concentration of 150×10⁶/ml or 20×10⁶/ml) for perfusion studies as described above. To examine changes in calcium flux, sequential images of adherent platelets were captured at a scan rate of 0.586 frames/sec for 200 frames. Real time calcium flux was calculated based on a ratio of signal intensity between the two dye channels, and converted to intracellular calcium concentrations as described previously ²⁷. NP-EGTA uncaging was carried out following 18 sec of reconstituted blood flow via exposure of platelets to a near UV (300–400-nm) light source generated by a 100-watt mercury lamp directed through the optical path of a Leica DMIRBE confocal microscope, for an interval of 0.6 sec. Control studies were carried out with unloaded control platelets and demonstrated that the brief UV exposure did not lead to photodynamic damage or activation of the platelets under flow.

**Scanning Electron Microscopy (SEM)** - Translocating platelets were fixed and prepared for SEM imaging as described previously ²⁹. Platelets were imaged on a Hitachi S570 scanning electron microscope (Tokyo, Japan) at 20 kV accelerating voltage, 3mm working distance.

**Statistical Analysis and Computer Programs Used** - Results are presented as mean ± standard error (SEM). Statistical significance was assessed by Student’s t-test. Schematic diagrams in Figures 2 and 4 were drawn using CorelDraw Graphics Suite (Corel Corporation). Video was digitized using Pinnacle Systems DV500 PLUS (Pinnacle Systems, Germany), and edited using Adobe Premiere Pro v1.5 (Adobe Systems Inc).
RESULTS

Analysis of platelet morphology during surface translocation in vivo

Platelets have been demonstrated to translocate on a vWf matrix in vitro as flat discs or as spiny-spheres \(^{14-16}\). To examine which of these morphologies is most relevant to thrombus growth in vivo we established a high magnification DIC imaging technique that has sufficient optical resolution to identify the morphology of individual platelets in the rat microcirculation (refer to supplementary online videos examining platelet thrombus formation in vivo for full appreciation of the dynamics of platelet aggregation). Vascular injury of mesenteric arterioles was induced by photoactivation of systemically-administered Rose-Bengal, a technique that induces vascular injury leading to the development of small non-occlusive thrombi \(^22\). Thrombi typically formed over a 1-5 minute period and a characteristic feature of thrombus growth in this model is the high proportion of translocating platelets on the thrombus surface \(^22\). As demonstrated in figure 1A, the majority of platelets (>95%) translocating on either the injured vessel wall or the surface of forming thrombi retained their discoid morphology (Supplementary Video I). While a small subset of platelets translocated in a flip-flop side-to-side motion, the majority typically translocated in a sliding motion, maintaining maximal surface contact with the thrombus surface (figure 1B). Only a small percentage of translocating platelets (<1%) exhibited a spherical morphology. Furthermore, the platelets residing in the superficial layers of the thrombi retained their discoid shape, and appeared to be loosely adherent. Sliding discoid platelets was a cardinal feature of thrombus development irrespective of the extent of vascular injury. While the optical resolution of the system was insufficient to enable clear visualisation of individual membrane tethers, their presence was implied by the finding that many discoid platelets displayed free movement or even rotation around a single tethered point (Supplementary Video III). Furthermore, individual platelets were observed to extend and recoil from a single contact point (figure 1C), a characteristic feature of membrane-tethered platelets (17) (Supplementary Video III). The distance that the discoid body of an adherent platelet extended from the single attachment point progressively increased, reaching up to 3 µm (mean 1.15 µm, range 0.5-2.8 µm). As demonstrated in figure 1C, despite no visible physical contact between their outer circumferential membranes, these platelets were clearly attached, as drag forces on downstream platelets were always transmitted to tethered upstream platelets (figure 1C, 0.02s), leading to coordinated propulsive and retractive platelet movement on the thrombus surface. As such, platelets in the superficial layers of forming thrombi were initially loosely tethered to each other such that under the influence of hemodynamic drag forces these cells readily detached from the thrombus surface, either as individual cells or small clusters of loose platelet aggregates (figure 1D, Supplementary Video II). These findings indicate that the majority of platelets retain their discoid
morphology during initial aggregate formation in vivo. Furthermore, they suggest a potentially important role for membrane tethers in regulating the initial reversible interaction between platelets.

Membrane Tethers Regulate Reversible Aggregation of Discoid Platelets

To investigate the potential relationship between membrane tethers and reversible aggregation of discoid platelets, in vitro perfusion studies were performed on a vWF matrix. By performing perfusion studies using anticoagulated whole blood and imaging adherent platelets with high resolution DIC microscopy, we were able to monitor in real-time the morphology of platelets throughout the entire adhesion/aggregation process without the need for platelet isolation or fluorescent membrane labelling. As shown in figure 2A and 2B, adhesion contacts between discoid platelets and the vWF matrix were initially mediated via thin membrane tethers and similar to our in vivo findings, we observed that the majority of initial platelet-platelet interactions occurred between discoid platelets and did not require platelet sphering during the translocation process. These initial platelet-platelet interactions were always reversible and as a consequence were associated with the development of unstable platelet aggregates (Supplementary Video IV). High magnification scanning electron microscopy imaging confirmed that the adhesion contacts between aggregating platelets were mediated through the development of thin membrane tethers (figure 2C). Similar findings were observed with either whole blood or washed platelets and under all conditions examined, reversible aggregate formation between discoid platelets was dependent on the development of membrane tethers. These studies demonstrate that under physiologically-relevant flow conditions, platelet aggregate formation on a vWF matrix is associated with the development of thin membrane tethers from the surface of discoid platelets.

Effect of Matrix Reactivity on the Reversible Aggregation of Discoid Platelets

vWF is a relatively weak thrombogenic surface supporting limited platelet aggregation and thrombus growth. In vivo, platelets are typically exposed to a range of adhesive proteins at sites of vascular injury with vWF and fibrinogen playing the major role in supporting thrombus growth. Previous studies have demonstrated that a combined vWF/fibrinogen matrix is considerably more reactive than matrices prepared with either protein alone. To examine the effect of increasing surface reactivity on the dynamics of platelet aggregation, perfusion studies were performed on a mixed matrix composed of a fixed concentration of vWF and increasing concentrations of surface-adsorbed fibrinogen. As demonstrated in figure 3A and 3B, perfusing washed platelets in the presence of red blood cells over a purified vWF matrix resulted in the development of reversible discoid platelet aggregates with limited conversion to stable aggregates. In contrast, increasing the surface concentration of fibrinogen with vWF increased the reactivity of the matrix, leading to
increased stable aggregate formation (figure 3A and B and supplementary videos V and VI). As demonstrated in figure 3Bi, the total number of unstable discoid aggregates was not dramatically altered by the presence of fibrinogen, however above 10µg/ml FGN there was a sharp increase in the rate of conversion of unstable aggregates into stable aggregates. Increasing the reactivity of the matrix had two major effects on the aggregation process, it significantly shortened the time to formation of both reversible and irreversible aggregates and markedly increased the efficiency of platelet recruitment into stable aggregates, leading to a much greater number of platelets per aggregate at all time points examined (figure 3Bii). Note, at the highest concentrations of fibrinogen (50 µg/ml) used, the rapid growth of stable aggregates meant that the number of platelets within individual aggregates could not be accurately determined (figure 3Bi). In addition, small aggregates often merged together, leading to a corresponding reduction in the total number of aggregates, but an increase in aggregate size (figure 3Bii). Scanning electron microscopy revealed that most platelets initially clustered around a central activated platelet through the formation of thin elongated membrane projections (see figures 3C and 2C). We confirmed that these projections were tethers, rather than filopodia, as they were not prevented by pretreating platelets with the inhibitor of actin polymerisation, cytochalasin D (data not shown). Furthermore, SEM imaging confirmed that conversion of reversible aggregates into stable aggregates was associated with platelet sphering and the extension of multiple filopodial projections (figure 3D). These studies define a major role for matrix reactivity in regulating the initial formation of discoid platelet aggregates and in promoting their conversion to stable aggregates.

*Effect of Shear on Reversible Platelet Aggregation*

Membrane tether formation in primary adherent platelets is regulated by shear \(^{17}\), raising the possibility that reversible aggregate formation between discoid platelets may represent a shear-dependent phenomena. As demonstrated in figure 4A, on a mixed vWF/FGN substrate at a wall shear rate of 600s\(^{-1}\), reversible aggregation between discoid platelets was negligible, with all stable aggregates developing from the recruitment of individual platelets to forming thrombi. Increasing the shear rates to 1,800 and 5,000s\(^{-1}\) resulted in a progressive increase in the number of discoid platelet aggregates with conversion of these reversible aggregates to stable aggregates occurring within 50-70 seconds of initial adhesion (figure 4B). These studies demonstrate that the formation of reversible aggregates between discoid platelets is a shear-dependent process, presumably mediated by the extension of membrane tethers.

*Role of Integrin αIIbβ3 in Regulating Membrane Tether Formation and Reversible Aggregation of Discoid Platelets*
Membrane tether formation can occur on an immobilized vWf matrix in the presence of integrin \( \alpha_{\text{IIb}} \beta_3 \) antagonists or on isolated vWf A1 domains \(^{12,17}\), demonstrating that the vWf-GPIb interaction alone is sufficient to promote this process. To investigate whether the vWf-GPIb interaction was sufficient to support membrane tether anchorage and reversible aggregation of discoid platelets, platelet perfusion studies were performed in the presence of the integrin \( \alpha_{\text{IIb}} \beta_3 \) antagonist, c7E3 Fab. Blocking ligand binding to integrin \( \alpha_{\text{IIb}} \beta_3 \) completely eliminated membrane tether formation and the development of reversible discoid platelet aggregates. However, this treatment also eliminated stationary adhesion, and in SEM studies (see figure 3C) it was noted that discoid aggregates were often clustered around a central stationary activated platelet. The potential for c7E3 Fab-treated platelets to aggregate around an activated platelet was examined in a two-stage perfusion assay in which an initial population of untreated washed platelets was allowed to firmly adhere to a vWf/fibrinogen substrate, followed by perfusion of a second population of c7E3 Fab-treated platelets. Under these conditions, translocating discoid platelets interacted only briefly (<3 seconds) with the immobilised platelets, preventing membrane tether formation and the development of reversible platelet aggregates (figure 4C). Similarly, perfusion of c7E3-treated platelets over preformed spread platelet monolayers abolished membrane tether formation and aggregate formation (data not shown). These studies define a major role for integrin \( \alpha_{\text{IIb}} \beta_3 \) in supporting the reversible phase of platelet-platelet interactions necessary for membrane tether formation.

Previous studies have suggested a potentially important role for membrane tethers in regulating the transient ‘stop-phase’ of platelet translocation on a vWf matrix \(^{17}\). To investigate the impact of inhibiting ligand binding of integrin \( \alpha_{\text{IIb}} \beta_3 \) on the duration of platelet-platelet adhesion contacts under flow, c7E3-treated platelets were perfused over preformed spread platelet monolayers. Inhibiting ligand binding to integrin \( \alpha_{\text{IIb}} \beta_3 \) and the subsequent development of membrane tethers, eliminating the stop-phase of surface translocation and dramatically reduced the duration of platelet-platelet adhesion contacts (data not shown). Similar findings were apparent using platelets congenitally-deficient in integrin \( \alpha_{\text{IIb}} \beta_3 \) (Glanzmann thrombasthenic (GT) platelets) (data not shown), and in reciprocal flow experiments, in which normal and GT platelets were perfused over normal and GT monolayers (respectively and vice versa), integrin \( \alpha_{\text{IIb}} \beta_3 \) on the surface of both immobilised and flowing platelets contributed to the duration of platelet-platelet adhesion contacts under flow (figure 4C).

It is known that integrin \( \alpha_{\text{IIb}} \beta_3 \) can engage fibrinogen independent of activation \(^{32,13}\). Therefore to determine whether reversible aggregate formation between discoid platelets was activation-dependent, platelet perfusion studies were performed on a mixed vWf/fibrinogen matrix in the presence of the potent platelet activation inhibitor, PGE\(_1\). As shown in figure 4D, platelet
interaction with the matrix was unaffected by PGE₁, however the reversible phase of platelet aggregate formation was completely inhibited. Collectively, these studies define a key role for platelet activation and integrin α₁bβ₃ bond formation in supporting the reversible phase of platelet-platelet interactions necessary for membrane tether formation.

**Role of ADP in Regulating the Reversible Aggregation of Discoid Platelets Under High Shear Flow**

The activation status of integrin α₁bβ₃ during platelet adhesion to immobilized fibrinogen and vWf is partially regulated by ADP. To investigate the requirement for ADP secretion in regulating membrane tether formation and reversible aggregation perfusion studies were performed in the presence of the ADP receptor antagonists A3P5P (P2Y1 antagonist) and ARC69931MX (P2Y12 antagonist). In contrast to c7E3 Fab, preventing ADP activation of platelets did not inhibit membrane tether formation or the initial reversible aggregation of discoid platelets (figure 4D). Rather, inhibition of the purinergic receptors led to a progressive increase in the number of unstable aggregates with most aggregates composed of a small number of loosely tethered platelets (typically <10 platelets/aggregate). The major effect of blocking the platelet activating effects of ADP was to prevent the transition from reversible to stable adhesion, leading to unstable platelet aggregation. These studies indicate that ADP is not essential for membrane tether formation and reversible aggregation, but is critical for the transition to stable aggregation.

**Importance of Platelet Density in Regulating the Transition from Reversible to Stable Aggregates**

Our studies have indicated that surface reactivity and shear flow are two important variables regulating the formation and transition of membrane-tethered platelets into stable aggregates. To investigate whether physical proximity between translocating platelets also plays an important role in this process, perfusion studies were performed at various platelet concentrations. As demonstrated in figure 5A, at very low platelet counts (5×10⁶/ml) there was low surface density of translocating platelets with minimal platelet-platelet interactions and no aggregation. With progressive increases in platelet count there was a concentration-dependent increase in the number of reversible platelet aggregates that peaked at 50×10⁶/ml and remained constant up to 800×10⁶/ml (supplementary video VI). The major effect of increased platelet count was to reduce the time required for formation of membrane-tethered reversible aggregates from 60-90 seconds at concentrations of 20-50×10⁶/ml to 15-30 seconds at 800×10⁶/ml. In addition, the lag time for stable aggregate formation was also considerably shortened from 120 seconds at 50×10⁶/ml down to 45 seconds at 800×10⁶/ml. Analysis of the total number of firmly adherent platelets in stable aggregates as a function of time revealed a steady concentration-dependent increase in firmly
adherent platelets up to 150×10⁶/ml, which increased markedly at 400 and 800×10⁶/ml (figure 5A). Significantly, a relatively modest increase in the density of platelets at the adhesive surface resulted in a disproportionate rise in the number of platelets incorporated into stable aggregates. For example, at the 60 second time point, the total number of platelets adherent at perfusion densities of 400 and 800×10⁶/ml were ~250 and ~325 platelets per field of view respectively (data not shown), while the number of platelets within stable aggregates were ~100 and ~300 platelets per field of view respectively (figure 5B). Thus, a 1.3-fold increase in the platelet surface density resulted in a 3-fold increase in those forming stable adhesive contacts. These studies demonstrate that platelet density has a major influence on aggregate formation, with higher platelet density correlating with not only an acceleration of the onset of unstable aggregate formation, but also the transition to firmly adherent stable aggregates.

**Increased Platelet Density Helps Sustain Platelet Nucleating Activity Necessary for Reversible Aggregate Formation**

One of the most striking effects of increased platelet surface density was the enhancement of primary platelet adhesion on the mixed vWf/FGN matrix, leading to the rapid formation of nucleation sites for subsequent platelet aggregation (see supplementary video VI). As demonstrated in figure 5C, the time required for the formation of stable primary platelet adhesion correlated inversely with the platelet surface density, such that in the absence of platelet-platelet interaction, primary adhesion was delayed 4-6 fold. This effect of platelet surface density was also apparent on a purified vWf matrix, with no firm adhesion apparent in the absence of platelet-platelet interactions (data not shown). To gain insight into the biochemical mechanisms by which platelet-platelet interactions facilitate primary platelet adhesion, cytosolic calcium flux was examined, as there is a close correlation between cytosolic calcium levels and firm platelet adhesion. In general, platelet calcium responses can be broadly classified into low, intermediate and high range calcium responses, with formation of sustained adhesion contacts associated with sustained high range calcium flux. As demonstrated in figure 6A, at low platelet surface densities (platelet count of 5×10⁶/ml), high range calcium responses were typically transient in nature with the duration of the calcium response correlating with the time of stationary adhesion. In the absence of platelet-platelet interactions, no sustained calcium flux or stable adhesion was observed (figure 6A). In contrast, at high platelet surface densities (platelet count of 150×10⁶/ml), approximately 60% of high calcium responders exhibited a sustained calcium flux (figure 6A), leading to the development of stable adhesion contacts (adhesion >30 seconds). These findings suggest that platelet-platelet interactions help promote sustained cytosolic calcium flux.
To investigate this phenomenon further, we performed perfusion studies on platelets loaded with the caged calcium chelator NP-EGTA. NP-EGTA is a caged calcium chelator that displays a marked increase in its $K_d$ for Ca$^{2+}$ upon photolysis with near UV light (300-400nm). This reagent can effectively be used to transiently release a relatively large concentration of free Ca$^{2+}$ in the cytosol within msec of UV activation. As demonstrated in figure 6B, at low platelet densities (platelet count of 5×10⁶/ml) the induction of a transient calcium response in translocating platelets resulted in immediate platelet arrest on vWF, however in all platelets the calcium signal was not sustained leading to resumption of translocation in 100% of platelets. In contrast, at higher platelet densities (platelet count of 150×10⁶/ml), induction of a transient calcium spike in a population of membrane-tethered reversible platelet aggregates triggered the onset of a sustained elevation in calcium levels that was sufficient to support prolonged adhesion of platelets and stable aggregation (figure 6C). It should be noted that in the absence of uncaging, platelet-platelet interactions on the vWF matrix were not capable of inducing sustained calcium flux in the forming aggregates and did not lead to stable aggregate formation (see figure 3). Stable aggregate formation following uncaging was ADP dependent as it was completely inhibited by apyrase (data not shown), suggesting that paracrine stimulation of platelets during initial aggregate formation is critical for sustaining cytosolic calcium responses necessary for stable primary platelet adhesion.
DISCUSSION

It has long been assumed that thrombus development in vivo involves the sequential accrual of individual platelets onto the surface of thrombi, leading to platelet shape change, integrin $\alpha_{\text{IIb}}\beta_3$ activation and firm adhesion $^{10,11,35-37}$. However, this concept has recently been challenged by the demonstration that under conditions of very high shear ($>10,000 \text{ s}^{-1}$), thrombus development can involve the initial formation and recruitment of reversible platelet aggregates onto the surface of forming thrombi, through a process mediated solely through vWf-GPIb bonds independent of platelet activation $^{12}$. Whether a similar mechanism operates under more physiologically-relevant shear conditions (1,000-10,000 $\text{ s}^{-1}$) has not been defined. By employing high resolution imaging techniques to in vivo and in vitro thrombosis models we have been able to identify the temporal sequence of events underlying platelet aggregation at physiological shear rates. We have demonstrated that the first phase of platelet aggregation involves shear-induced formation of membrane tethers between co-adhering platelets. This initial phase of aggregation principally involves discoid platelets, but is distinct from the mechanism described by Ruggeri and colleagues $^{12}$, in that it requires platelet activation and the adhesive function of both GPIb and integrin $\alpha_{\text{IIb}}\beta_3$.

The second phase of aggregation is associated with platelet shape change, irreversible platelet adhesion and is critically dependent on the release of ADP. We have demonstrated that the dynamics of this two-stage aggregation process are critically influenced by three variables, including high shear stress, platelet surface density and matrix reactivity, with the two former variables regulating reversible aggregate formation, and the latter regulating the transition to stable aggregation. Overall, these studies define the mechanisms regulating platelet aggregation under physiologically-relevant high shear flow and provide a mechanistic explanation as to how changes in matrix reactivity, in combination with high shear and platelet surface density, synergistically enhance platelet thrombus growth.

Rudolph Virchow described more than 150 years ago a triad of factors that contribute to pathological thrombosis, including changes (i) in the vessel wall; (ii) in blood flow and (iii) in the constituents of blood $^{38}$. In the context of arterial thrombosis, it is well defined that high shear, in combination with enhanced subendothelial matrix reactivity and heightened platelet responsiveness, are major contributors to this process $^{39-41}$. While the latter two elements have a clearly defined role in thrombogenesis, the mechanisms by which high shear forces promote platelet activation and aggregation remains less clearly defined. It is well established that shear enhances the adhesive and signalling function of GPIb $^{27,42}$, and that shear-induced binding of vWf to GPIb is essential for occlusive thrombus formation, however GPIb-derived signals are weak and only induce limited platelet activation in the absence of other co-stimuli $^{27,42,24}$. Consistent with this, exposing platelets to high shear (up to 10,000 $\text{ s}^{-1}$) during surface translocation on vWf does not promote rapid platelet
activation or efficient thrombus growth \(^{24}\). Similarly, exposing platelets in suspension to high shear (up to 10,000 s\(^{-1}\)) in the presence of soluble vWF leads to relatively slow development of macroscopic platelet aggregates \(^{43}\), suggesting that shear \textit{per se} is a relatively weak thrombogenic stimulus. We have demonstrated here that one of the major effects of shear is to initiate the formation of reversible platelet aggregates by inducing the development of membrane tethers between co-adhering platelets. On a weak thrombogenic surface such as vWF, this physical co-localization of platelets is inefficient at inducing platelet activation or stable aggregation independent of exogenous stimuli. As will be discussed below, one of the major effects of shear may be to enhance the physical interaction between platelets thereby increasing the probability of sustained platelet activation by soluble agonists.

Our studies demonstrate a central role for matrix reactivity in regulating the efficiency of conversion of membrane-tethered reversible aggregates into stable aggregates. It has been known for many years that highly thrombogenic surfaces, such as type I or III fibrillar collagens, are efficient at inducing primary platelet adhesion and subsequent aggregation \(^{3,44}\). Notably, only the primary adherent layer of platelets is in direct physical contact with the collagen fibres with all subsequent layers of platelets exposed to adhesive proteins expressed on the surface of aggregating platelets. The studies reported here suggest that the surface densities of vWF and fibrinogen are important in influencing the efficiency of stable platelet aggregation. These findings may have clinical relevance as the concentrations of fibrinogen and vWF used in these experiments are well within pathophysiological levels, and furthermore, elevated plasma levels of either fibrinogen or vWF increase the risk of arterial thrombosis \(^{45-49}\). Thus, it is tempting to speculate that increased surface adsorption of fibrinogen and vWF on the surface of developing thrombi, particularly under conditions of high shear stress, is one of the key mechanisms enhancing the conversion of discoid platelet aggregates into stable aggregates.

Our studies have defined an important role for platelet surface density in regulating the initial formation of reversible platelet aggregates, a not unexpected finding given that one of the important variables regulating the efficiency of the hemostatic process is the density of platelets at the adhesive surface \(^{50}\). Notably, increased physical interaction between platelets enhanced platelet aggregate formation by sustaining cytosolic calcium levels in adherent platelets. This is ADP dependent and is likely to represent the phenomenon of inter-cellular calcium communication, a previously described mechanism propagating activating signals throughout a developing thrombus \(^{20}\). Thus, shear-dependent tether formation and co-clustering of platelets is likely to potentiate platelet aggregate formation by at least two mechanisms; by increasing the efficiency of activation between co-adhering platelets; and by sustaining activation in the primary nucleating platelet, thereby providing a feedback amplification mechanism to propagate aggregate development.
One of the most striking features of platelet thrombus development \textit{in vivo} is the high proportion of reversible discoid platelet aggregates on the surface of thrombi. The shear-dependent clustering of discoid platelets may serve to facilitate paracrine platelet stimulation, by limiting the ‘wash-out’ effect of blood flow on generated soluble agonists. Such a mechanism may explain why most of the major soluble agonists regulating platelet activation, such as ADP, TXA2 and thrombin, are either released from the platelet themselves or generated in close proximity to the platelet surface. This is in marked contrast to leukocytes, in which many of the soluble chemokines and cytokines regulating leukocyte activation are generated from the vessel wall. Thus, through the formation of reversible platelet aggregates, platelets may have evolved a unique mechanism of facilitating localized and sustained platelet activation by soluble agonists in a high shear environment.

Our \textit{in vivo} studies shed new light on the mechanisms regulating platelet translocation at sites of vascular injury. In contrast to leukocytes, which have a spherical morphology well suited to a rolling interaction with the vessel wall, the discoid morphology of non-stimulated platelets is less well adapted to rotational movement. The only data on platelet morphology during surface translocation has been derived from \textit{in vitro} perfusion studies on a purified vWF matrix in which platelets undergo rotational movement as either flat discs (flipping platelets), following conversion to spiny spheres or after adopting a smooth spherical morphology at very high shear rates (10,000 s\(^{-1}\))\(^{15}\). Rotational translocation of flat discs is relatively inefficient, as this morphology experiences marked fluctuations in tensile force during rotational movement leading to cell detachment from the adhesive surfaces\(^{51}\), whereas adhesive bonds on spherical platelets experience more uniform force during surface translocation, producing a smoother, more sustained rolling interaction\(^{15}\). Relative to rotating discs or rolling spheres, sliding flat discs experience minimal drag force and have much lower tensile stresses on adhesive bonds. A flat disc also enables maximal surface contact area with the adhesive surface, increasing the potential for multivalent adhesive interactions. Additionally, membrane tethers increase the moment arm of adhesive bonds, thereby providing mechanical advantage to adhesion contacts and reducing platelet detachment from the thrombus surface\(^{17}\). Thus, the development of membrane tethers from the surface of flat, discoid platelets represents a unique mechanism of minimizing drag forces on adherent platelets, thereby enabling sustained platelet interaction with thrombogenic surfaces independent of substantial platelet activation.

The studies presented here, in combination with previous reports\(^{10,11,52}\), suggest that at least three distinct mechanisms can regulate the initiation of platelet aggregation, with the relative contribution of each mechanism dependent on the prevailing shear conditions. As demonstrated in figure 7, the traditional model of platelet aggregation that operates under relatively low shear conditions (<1,000
s⁻¹) involves platelet stimulation by one or more soluble agonists that induces platelet shape change and the upregulation in the affinity status of integrin αIIbβ3. Under relatively low flow conditions, activated integrin αIIbβ3 can engage fluid-phase fibrinogen, a dimeric molecule which physically bridges adjacent activated platelets. As demonstrated in detail in this report, under higher shear conditions (1,000-10,000 s⁻¹), the processes initiating aggregation are quite distinct, involving additional receptors, ligands and membrane tethers. This mechanism is unique in that it involves integrin αIIbβ3 adhesive interactions between discoid platelets, through an activation process independent of ADP. It is tempting to speculate that GPIb and integrin αIIbβ3-derived signals help facilitate localized integrin αIIbβ3 activation, necessary for sustained adhesion and membrane tether formation. Consistent with such a possibility, we have demonstrated a progressive low level increase in cytosolic calcium flux during platelet translocation and reversible aggregate formation. This calcium signal is ADP-independent and occurs prior to the onset of shape change and the sustained oscillatory calcium flux that is necessary for irreversible aggregation (unpublished observations I Goncalves and SP Jackson). Recently, a third mechanism initiating platelet aggregation has been described. This mechanism operates at extremely high shear conditions (>10,000 s⁻¹) such as those operating in severely stenosed arteries and requires both immobilized and soluble forms of vWF. In contrast to aggregation at physiological shear rates, aggregation does not require integrin αIIbβ3 or platelet activation and is exclusively mediated by vWF-GPIb adhesive bonds ¹². These aggregates are most likely initiated through the development of membrane tethers, however in contrast to our findings at physiological shear rates, these tethered aggregates develop between smooth spherical platelets, a unique platelet morphological form that develops under pathological shear conditions ¹²,¹⁵. Taken together, these studies highlight the unique relationship operating between platelet adhesive ligands, receptors and morphological forms relevant to shear-dependent platelet aggregation. Furthermore, they raise the possibility that targeting processes selective to platelet aggregation at different shear rates may lead to the development of novel shear-specific inhibitors of platelet aggregation.
REFERENCES


FIGURE LEGENDS

Figure 1. Dynamics of platelet thrombus formation in vivo.
Mesenteric arterioles of rats were injured via photoactivation of systemically administered Rose-Bengal, and platelet interactions with growing thrombi were visualized by DIC microscopy. (A) This single image (representative of 12) highlights the ability of this imaging technique to resolve single platelets during thrombus formation in vivo (see Supplementary Video II for analysis of thrombus development in real-time) (B) Representative example of a newly recruited platelet translocating over the thrombus surface with a sliding motion, maintaining maximal surface area contact (see supplementary video II). (C) Demonstrates a single adherent platelet (dotted outline) extending and recoiling from a fixed point of contact over the surface of another stationary adherent platelet. Such behaviour was often induced by transient contacts made with other translocating platelets (0.02s, dotted outline) (Supplementary Video III). (D) Platelets within the superficial layer of thrombi were loosely packed, such that platelets often detached from the thrombus surface in clusters. (Supplementary Video II). White scale bar = 5 µM, Black scale bar = 10 µM.

Figure 2. Formation of membrane tethers in vitro.
Washed platelets (150x10^6/ml) reconstituted with RBCs were perfused through microcapillary tubes coated with vWf (10 µg/ml) at 1,800s⁻¹. (A) DIC image and (B) Scanning electron microscopy (SEM) image of a single platelet forming membrane tethers with immobilized vWf. (C) SEM image of a forming discoid platelet aggregate. This image demonstrates that the adhesion contacts between aggregating platelets are mediated by membrane tethers. Platelets were fixed at 120 seconds and processed for SEM imaging as described in ‘Methods’. (Scale bar = 2 µm).

Figure 3. Reversible aggregation of discoid platelets in vitro is promoted by increasing matrix reactivity.
Washed platelets (150x10^6/ml) reconstituted with RBCs were perfused through microcapillary tubes coated with vWf (10 µg/ml) and varying concentrations of fibrinogen (5, 10, 20 or 50 µg/ml) at 1,800s⁻¹. (A) DIC images were taken at the indicated time points on a vWf (10 µg/ml) or vWf/fibrinogen matrix (10/50 µg/ml). Schematic diagrams highlight non-aggregated platelets (white), reversible aggregates (grey) and stable aggregates (black) (scale bar = 10 µm). On vWf, aggregates remained reversible, while on vWf/fibrinogen reversible aggregates (60s), eventually became stable (90-240 sec). (B) Aggregate formation was quantified by determining the number (i) and size (ii) of aggregates within a visual field. (i) Graph showing the number of unstable (open circles, broken line) and stable (solid circles, solid line) aggregates. Each point represents the mean
± SEM from at least 4 independent experiments. (ii) Each dot represents the number of platelets within an aggregate, showing data combined from 4 independent experiments (bar represents the median). *ND, quantitation was not performed because multiple small aggregates merged together into larger single aggregates (scale bar = 10 µm). (C) SEM image demonstrating a typical reversible discoid platelet aggregate forming on a mixed vWF/fibrinogen matrix. Note, all platelets cluster around a central activated platelet through the development of thin membrane tethers. Platelets were fixed at 60 seconds. Scale bar = 2 µm. (D) SEM images demonstrating the sequential platelet morphological changes associated with shear-dependent platelet aggregation. Platelets were perfused through vWF/fibrinogen coated microcapillary tubes (10/50 µg/ml) at 1,800 s⁻¹. These representative images demonstrate discoid platelets forming membrane tethers during surface translocation on the mixed matrix (30 sec), a typical unstable aggregate composed of several discoid platelets clustered around a central partially-spread platelet (60 sec) and platelets that have undergone classical shape change (sphering and extension of multiple filopodia extensions) during stable aggregate formation (90 sec). Scale bar = 2 µm.

**Figure 4. Formation of discoid platelet aggregates is shear-dependent and requires platelet and integrin αIIbβ3 activation.**

Washed platelets (150×10⁶/ml) reconstituted with RBCs were perfused through microcapillary tubes coated with vWF (10 µg/ml) and fibrinogen (50 µg/ml) at wall shear rates of 600, 1,800 or 5,000 s⁻¹. Aggregate formation was quantified by determining the number (A) and size (B) of aggregates within a 70 × 90 µm visual field at the indicated time points over a 240 second period. (A) An aggregate composed primarily of reversible-adherent platelets was classified as ‘unstable’ (open circles, broken line), while a ‘stable’ aggregate was classified as one composed of irreversibly adherent platelets (solid circles, solid line). Each point represents the mean ± SEM from at least 5 independent experiments. (B) The number of platelets within individual aggregates was counted at the indicated time points, and results show data combined from 5 independent experiments. The bar represents the median. *ND, quantitation was not performed because multiple small aggregates merge together into larger single aggregates. (C)(i) Washed platelets reconstituted with RBCs were perfused over a mixed vWF/fibrinogen matrix for 15 seconds to allow a small number of platelets to adhere. Washed platelets, untreated (control) or pre-treated with c7E3 Fab (7E3) were reconstituted with RBCs and then perfused over the adherent platelets. The time that translocating platelets interacted with the pre-adhered stationary platelets was analysed (control n=30, 7E3 n=16). (ii) Blood was collected from healthy donors (NBL) or individuals with Glanzmann’s thrombasthenia (GBL) (<1% αIIbβ3 by immunoblot) and perfused over confluent platelet monolayers made from...
normal platelets (NML) or Glanzmann’s platelets (GML). The interaction of flowing platelets (pre-labelled with the fluorescent dye DiOC6) with platelet monolayers was viewed in real time using fluorescence microscopy. The duration of adhesion contacts made by platelets tethering to the surface of monolayers was analysed as described in ‘Methods’. Results show data combined from 3 independent experiments, n = 60, bar represents the mean (***p<0.005). (D) Washed platelets (150×10⁶/ml) reconstituted with RBCs were perfused through microcapillary tubes coated with vWF (10 µg/ml) and fibrinogen (50 µg/ml) at 1,800s⁻¹ in the presence of the integrin αIIbβ₃ antagonist c7E3 Fab (7E3), the activation inhibitor PGE₁ (PGE₁), or a combination of the P2Y₁ and P2Y₁₂ receptor antagonists, A3P5PS and ARC69931MX (A3/ARC). The DIC images shown were taken at 120 seconds of perfusion (scale bar = 10 µm), highlighting the complete inhibition of aggregate formation in the presence of PGE₁ and the integrin αIIbβ₃ antagonist. In contrast, when ADP receptors were inhibited, reversible aggregates formed, but remained unstable (schematic diagrams indicate: white, non-aggregated platelets; grey, unstable aggregates; black, stable aggregates, scale bar = 10 µm). Each point represents the mean ± SEM from at least 4 independent experiments.

Figure 5. Aggregate formation is promoted by increasing platelet density.
Washed platelets (150×10⁶/ml) reconstituted with RBCs were perfused through microcapillary tubes coated with vWF (10 µg/ml) and fibrinogen (50 µg/ml) at 1,800s⁻¹. The platelet count was adjusted to 5, 20, 50, 150, 400 or 800 × 10⁶/ml. (A) Unstable and stable aggregate formation was quantified by determining the number of aggregates within a 70 × 90 µm visual field over a 240 second period. An aggregate composed primarily of reversible-adherent platelets was classified as ‘unstable’, while a ‘stable’ aggregate was classified as one composed of irreversibly adherent platelets. (B) Total amount of platelets recruited into stable aggregates. Each point represents the mean ± SEM from 3 independent experiments. *ND, quantitation was not performed because multiple small aggregates merge together into larger single aggregates. (C) Data from figure 5A was used to determine the time for the initial nucleating platelet to adhere to the vWF/Fibrinogen matrix. Data shown is representative of 5 independent experiments.

Figure 6. Increased platelet density helps sustain calcium flux in aggregating platelets.
Washed platelets loaded with the caged calcium chelator NP-EGTA and calcium indicator dyes (see Methods) were reconstituted with RBCs (to a final concentration of 150×10⁶/ml or 5×10⁶/ml), and perfused through micro capillary tubes coated with vWF (50 µg/ml) at 1,800s⁻¹. A transient elevation of cytosolic calcium was induced in translocating platelets by uncaging with brief exposure to near-UV light. (A) Proportion of high Δ[Ca²⁺]c platelets [displaying Transient or Sustained calcium oscillations at a platelet density of 5×10⁶/ml or 150×10⁶/ml (n=3)]. Note that all
of the cells were observed to undergo transient stationary adhesion events at a platelet density of 5x10^6/ml. (B) Single-channel Oregon green fluorescence images showing translocating platelets prior to uncaging (0 s), and the elevation in intracellular calcium levels following UV exposure (1.8 s). The left panels show a representative platelet uncaged at low density, where the platelet remains stationary for a period, then continues translocation when calcium levels decrease. The middle panels show a representative platelet at high density, where calcium levels remain elevated after uncaging, and the platelet remains stationary and becomes a focal point for recruitment of other platelets into an unstable aggregate (highlighted in the zoomed panels on the right hand side). (C) After uncaging, platelets remained stationary while calcium levels remained elevated. The duration of the stationary period was less in platelets at low density, compared to those uncaged at high density. (n=50, bar represents the median, cut-off time was 48 sec).

Figure 7. Model of platelet aggregation at various shear rates

A) Platelet aggregation at shear rates below approximately 1000 s^-1 is predominantly dependent on the activation of integrin \(\alpha_{IIb}\beta_3\) receptors and the presence of its ligand fibrinogen. (B) At shear rates up to \(\sim 10,000\) s^-1 platelet aggregation is a two stage process. Platelets are initially captured to a growing aggregate via the GPIb/V/IX and integrin \(\alpha_{IIb}\beta_3\) receptors binding to vWf and fibrinogen present at the aggregate surface. The formation of membrane tethers gives a mechanical advantage to adhesive bonds by reducing the level of force exerted on them\(^{17,53}\). (C) At extreme shear rates well above 10,000 s^-1, the aggregation of platelets is no longer dependent on the function of integrin \(\alpha_{IIb}\beta_3\) receptors and tether formation. Instead, immobilized vWF combined with soluble multimeric vWF is capable of initiating large unstable aggregates of non-activated platelets\(^{12}\).
Figure 2

A
Flow →

B
Flow →

C
Flow →
Figure 3

A

B(i)

B(ii)

C

D

For personal use only.
Figure 4

A

No. of Aggregates

Time (sec)

600 s⁻¹
1800 s⁻¹
5000 s⁻¹

B

No. of Platelets per Fibrin

Time (sec)

600 s⁻¹
1800 s⁻¹
5000 s⁻¹

C(i)

Platelet Aggregates

Time (sec)

CTE3
Control

C(ii)

Platelet Aggregates

Time (sec)

D

vWF/FGN + PGE₁ + 7E3 + A3I/ARC

No. of Aggregates

Time (sec)

Translocating platelets
Unstable aggregates
Stable aggregates
Figure 6

A

% High-Range [Ca^2+]

Platelet Count (x10^9/ml)

B

Low Density

High Density

Flow

C

Time stationary (sec)

Low Density

High Density
Figure 7

Characteristic features

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<th>Adhesion</th>
<th>&lt;i&gt;αIIb/3&lt;/i&gt;</th>
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Identification of a Two-Stage Platelet Aggregation Process Mediating Shear-Dependent Thrombus Formation

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