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Dynamic Tyrosine Kinase-Regulated Signaling and Actin Polymerisation Mediate Aggregate Stability Under Shear

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Objective—Aggregate formation on collagen at arteriolar rates of shear is mediated by coordinated signaling between tyrosine kinase–linked and G protein–coupled receptors. We have investigated the role of these receptors and the actin cytoskeleton in maintaining aggregate stability under shear.

Methods and Results—Platelet aggregates are rapidly formed when blood is flowed over collagen at 1000 s^{-1} and remain stable over 20 minutes. A novel fibrin-independent mechanism of retraction against the direction of flow occurs at the aggregate front and recruits platelets into the main aggregate. Stable aggregates are not observed in the presence of cytochalasin D, which blocks de novo actin polymerization. When exposed to the Src family kinase inhibitor, PD0173952, preformed aggregates spread in the direction of flow and rounded platelets appear within the aggregate body and are lost in the direction of flow. A similar set of observations is observed in the presence of latrunculin A, which disrupts preexisting actin filaments, but not in the combined presence of inhibitors of ADP and thromboxane A_2 formation.

Conclusions—Maintenance of stable aggregates at high shear is a dynamic process mediated by Src kinases and actin polymerization. These signals maintain aggregates in a compact structure and prevent continuous streaming of platelets. (***Arterioscler Thromb Vasc Biol.* 2008;28:000–000**)

Key Words: platelet aggregation ■ collagen ■ cytoskeleton ■ Src kinase ■ shear

Collagen is a key component of the vascular basement membrane that surrounds blood vessels and the deeper vessel wall. On injury, the interaction of platelets with collagen plays a critical role in initiating thrombus formation and preventing excessive blood loss. Collagen is also a major component of atherosclerotic plaques and mediates the massive platelet activation that occurs on plaque rupture, leading to thrombotic disorders such as myocardial infarction and stroke.

The events underlying aggregate formation on collagen at arteriolar rates of shear in vitro have been studied extensively. Platelet tethering or capture is mediated by binding of collagen-adherent von Willebrand factor (vWF) to the glycoprotein (GP) Ib-IX-V complex. Subsequent activation via the collagen receptor GPVI converts integrins αIIbβ3 and α2β1 to high-affinity states that support binding to vWF and collagen, respectively, giving rise to stable adhesion.[1,2] Under these conditions, GPVI is critical for aggregate formation,[3,4] whereas the role of α2β1 is supportive because of redundancy with αIIbβ3.[5] The secondary mediators, ADP and thromboxane A_2, support aggregate growth through activation of integrin αIIbβ3 and subsequent crosslinking via vWF and fibrinogen. The aggregate is further stabilized by "late-stage signaling events" through a variety of surface proteins, including integrins, ephrins and Eph kinases, cell adhesion molecules (eg, ESAM and JAM-A), and semaphorin 4D.[6] The presence of anticoagulant prevents conversion of fibrinogen to fibrin and further stabilization through clot retraction.

The actin cytoskeleton has a critical role in supporting aggregate formation. We have reported that the Rho family small GTPase Rac1 is required for lamellipodia formation in platelets and that aggregates from Rac1-deficient mice embolize on a monolayer of collagen to leave a single layer of adherent platelets.[7] Further, inhibition of stress fiber formation using selective inhibitors or myosin IIa–deficient mice leads to reduced aggregate formation on collagen at arteriolar shear.[8,9] Rac and myosin IIa also contribute to thrombus stability in vivo, although this may additionally reflect their role in clot retraction.[8–10]

Collagen signaling is maintained over several hours in platelets and is required for sustained lamellipodia formation in vitro.[11] However, it is not known whether sustained signaling by collagen or continuous actin polymerization contributes to aggregate stability at arteriolar rates of flow. The present study has addressed these questions by monitoring newly formed aggregates on collagen exposed to arteriolar shear. The results demonstrate that continuous signaling by Src kinases and dynamic actin polymerization are required for aggregate stability on collagen. Furthermore, inspection of the events early on in aggregate formation reveals a novel

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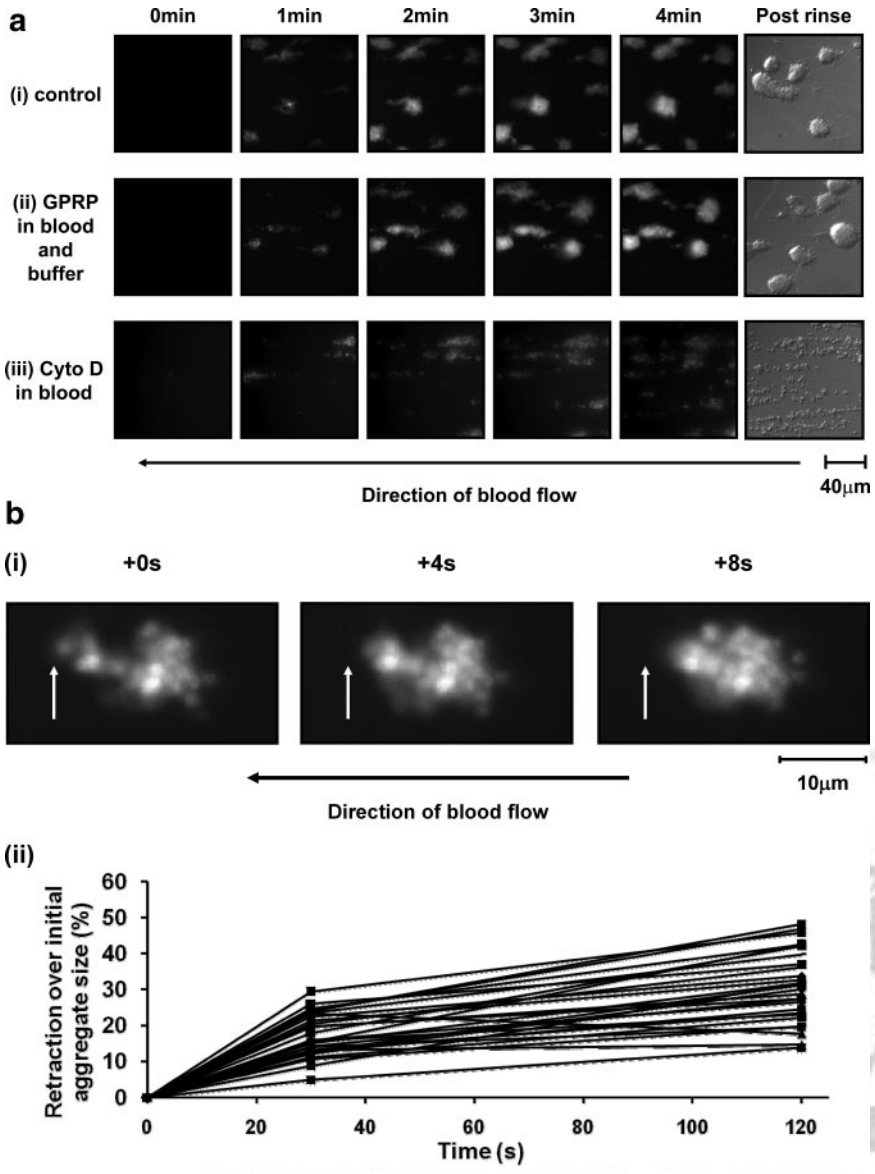

Figure 1. Identification of a fibrin-independent, actin polymerization-dependent process of aggregate retraction. a, Blood, preincubated with 2 μmol/L $DiOC_6$ and 0.1% DMSO (control), 0.1% DMSO and 10 mmol/L GPRP, 10 μmol/L cytochalasin D, 25 μmol/L PD0173952, or 10 μmol/L indomethacin and 2U/mL apyrase, for 10 minutes was flowed over collagen at 1000 s^{-1} for 4 minutes before rinsing with Tyrode for 5 minutes, except for studies using GPRP, which was also present throughout. Fluorescent images were captured at specified times (i), and DIC images recorded subsequent to rinsing with Tyrode (ii). b, i, Example recording of aggregate retraction, in the absence of inhibitors. White arrows represent a fixed point. Images are separated by 4-s intervals, beginning 15 s after onset of aggregation. ii, Distance moved by the outermost platelet after 15 seconds of aggregate formation, as a percentage of initial aggregate size. Mean results of aggregate retraction for all aggregates in field of view from 22 donors are shown.

actin-dependent mechanism of retraction that recruits platelets into the main aggregate.

Methods and Materials

Collection of blood, fluorescent labeling with $DiOC_6$, and measurement of aggregate formation on collagen at 1000 s^{-1} was as described.[7,12] Measurement of protein tyrosine phosphorylation in aggregates was achieved by SDS-PAGE[3] and Western blotting with the antiphosphotyrosine antibody, 4G10. Image analysis was performed using ImagePro Software[5] and confocal microscopy. Results are from a minimum of 3 experiments performed on different donors. For more extensive procedures, see the online supplement, available at http://atvb.ahajournals.org.

Results

Identification of a Fibrin-Independent Mechanism of Aggregate Retraction on Collagen at Arteriolar Shear

When P-PACK-anticoagulated human blood is flowed over collagen at 1000 s^{-1}, which is representative of an intermediate arteriolar rate of shear, platelets adhere to the fibers and form aggregates in a time-dependent manner. This process can be followed in real-time by fluorescently labeling the platelets as illustrated in Figure 1a (i). "Islands" of aggregated platelets can be detected within 60s of blood flow and increase steadily in size over time. These aggregates are stable when washed for several minutes with Tyrode's buffer (Figure 1a, ii).

Real-time monitoring of aggregate formation using fluorescently labeled platelets reveals retraction of platelets at the aggregate front into the main body of the aggregate, against the direction of blood flow (supplemental Movie I). Aggregate retraction was observed in all 22 donors used in this study (Figure 1b, i and ii). Moreover, all newly formed aggregates, independent of size and shape, undergo retraction against the direction of flow. Retraction is most marked within the first 30 seconds of recording but continues for several minutes (Figure 1b, ii). The degree of retraction over 2 minutes was calculated as 30.7±9.8% (SD; n=22) of the aggregate size, 15 seconds after the onset of formation (Figure 1b, ii). Aggregate retraction can also be seen using

DIC microscopy (supplemental Movie II), demonstrating that it is not a consequence of fluorescent labeling. The quality of the DIC recording is limited by the presence of erythocytes in the blood stream, and so fluorescent images were used for quantitative analyses.

The presence of the anticoagulant thrombin inhibitor P-PACK suggests that the process of retraction is unrelated to fibrin formation. In line with this, we have been unable to detect fibrin formation in aggregates using a specific antibody (not shown). Furthermore, aggregate formation and retraction was not significantly altered in the presence of an inhibitor of fibrin polymerization, GPRP (n=3), relative to controls (not shown). GPRP was used in these studies at a concentration that prevents thrombin-induced coagulation in platelet-rich plasma (not shown).

Treatment of blood with the actin polymerization inhibitor, cytochalasin D, inhibits aggregate retraction and prevents formation of stable aggregates (Figure 1a and supplemental Movie III). In the presence of cytochalasin D, platelets are able to adhere to the collagen fibers and generate small aggregates, but rapidly embolize in the direction of flow with no evidence of retraction (n=10). Thus, after several minutes of rinsing with buffer, subsequent to flowing over collagen, there is a high level of adhesion of single unspread platelets and small groups of no more than 2 to 3 platelets. In comparison, the Src kinase inhibitor, PD0173952, or a combination of indomethacin and apyrase, completely block aggregate formation as measured in real-time. This is illustrated by the DIC images taken at the end of the recording in Figure 1a (ii).

These results demonstrate that actin polymerization is essential for formation of stable aggregates at an arteriolar rate of shear and reveal a novel mechanism of aggregate retraction at the aggregate front that may contribute to aggregate stability.

Sustained Src Kinase Signaling Is Required for Maintenance of Aggregate Stability

We have previously shown that aggregate formation on collagen is dependent on Src kinases, because when whole blood is treated with a Src kinase inhibitor before flow over collagen, aggregate formation is abolished[5] (Figure 1a, ii). This observation, however, does not address whether continuous signaling through Src kinases is required to maintain stability of newly formed aggregates, which is of particular significance in light of the observation that signaling through the collagen receptor GPVI is sustained.[11] To address this, blood was flowed over collagen for 4 minutes before washing with Tyrode buffer for 5 minutes (to allow clear visualization of aggregates using DIC microscopy as well as analysis of protein phosphorylation), and then washing with Tyrode buffer containing either vehicle (0.1% DMSO) or the Src kinase inhibitor PD0173952 (25 μmol/L). To establish the contribution of Src family kinases to the maintenance of tyrosine phosphorylation, platelet aggregates were lysed after 20 minutes perfusion and Western blotted for phosphotyrosine using the mAb 4G10. The Src kinase inhibitor PD0173952 completely inhibited tyrosine phosphorylation, whereas marked tyrosine phosphorylation was detected in aggregates perfused with DMSO for 20 minutes (Figure 2). These results demonstrate dynamic signaling through Src kinases over the course of the perfusion.

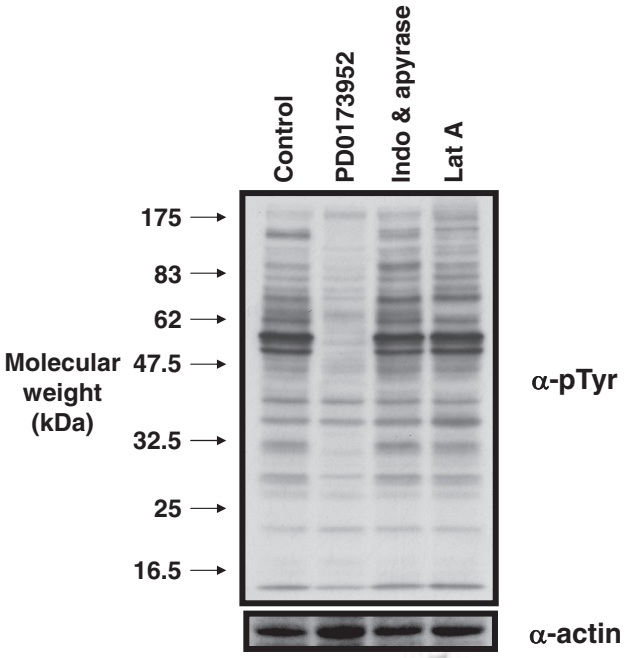

Figure 2. Phosphorylation of collagen-adherent platelets in aggregates is maintained via dynamic signaling by Src kinases. Blood was flowed over collagen at 1000 s^{-1} for 4 minutes, and aggregates were rinsed with Tyrode for 5 minutes. Aggregates were further rinsed with Tyrode containing 0.1% DMSO (control), PD0173952 (25 μmol/L), indomethacin (10 μmol/L) & apyrase (2U/mL), or latrunculin A (3 μmol/L) for 20 minutes. Lysates from capillaries were Western blotted for phosphotyrosine (upper) and reprobed for actin (lower).

Using the above protocol, we found that aggregates retain their compact, retracted structure throughout a 20-minute perfusion with DMSO (Figure 3a, i). Interestingly, however, careful inspection of supplemental Movie IV demonstrates movement of individual platelets around the front and side of the aggregates, which increases with time. Individual cells cannot be resolved in the main body of the aggregate, however, because of the compact structure. In contrast, washing in the presence of the Src kinase inhibitor PD0713952 leads to a marked increase in rounded platelets at both the front and sides of the aggregates, and also at later times, in the main body, such that the aggregates increase in size and can be seen to slowly spread in the direction of flow (Figure 3a, ii and supplemental Movie V). These morphological changes are clearly illustrated in the DIC image taken after the 20-minute washing period in comparison to that of the control (Figure 3b). The control image has a compact appearance with few identifiable single platelets, although those that can be seen have visible lamellipodia (Figure 3b, i). In contrast, the PD0173952-rinsed aggregates are spread-out and contain many single platelets, very few of which have visible lamellipodia (Figure 3b, ii). These results demonstrate that Src kinase activity is essential for maintenance of the compact aggregate structure and retention of lamellipodia under flow, suggesting a possible causal relationship. The same results

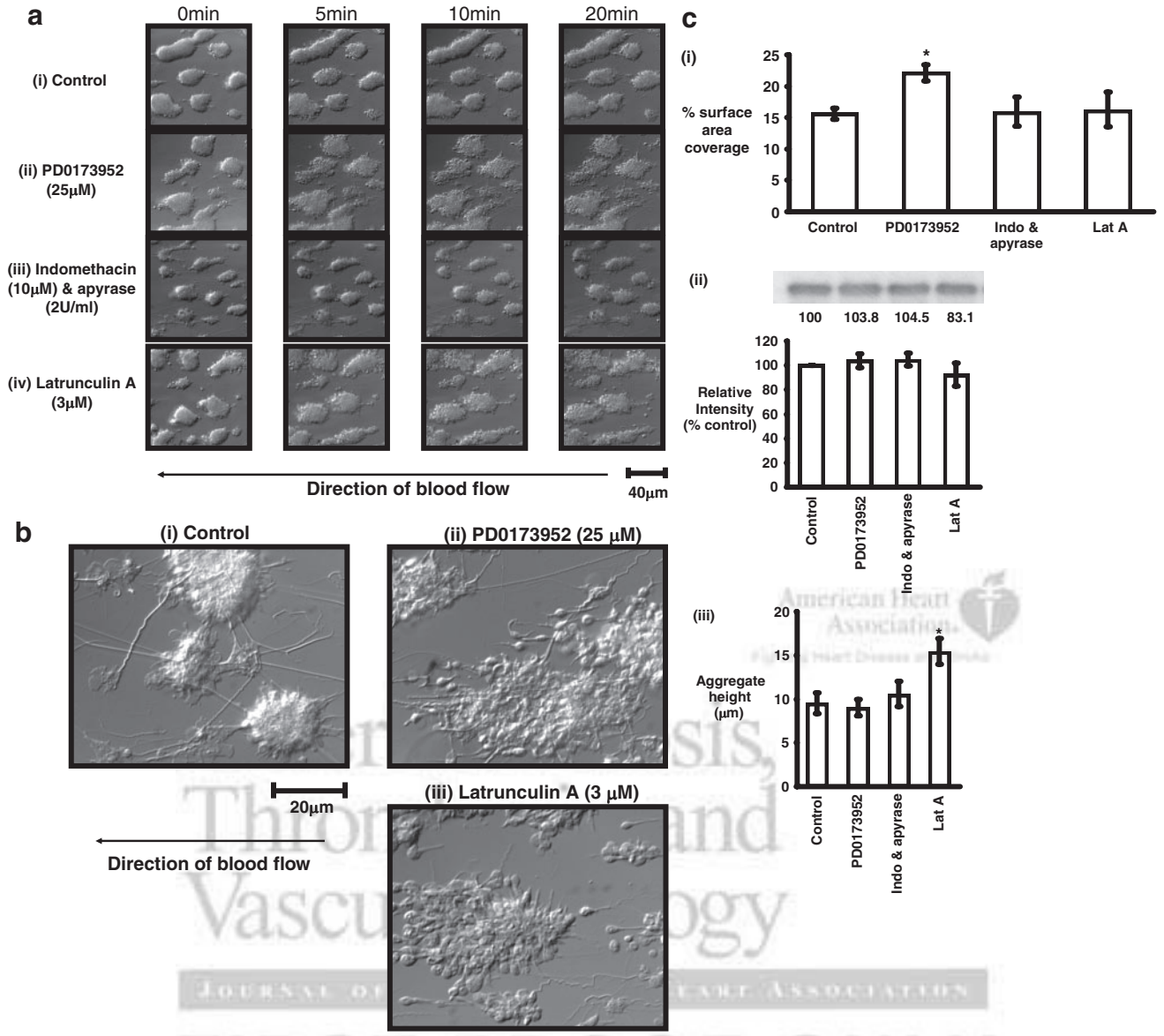

Figure 3. Inhibition of Src kinases or actin polymerization leads to instability in preformed aggregates on collagen. Blood was flowed over collagen at 1000 s^{-1} for 4 minutes, and aggregates were rinsed with Tyrode for 5 minutes and then for a further 20 minutes in the presence of inhibitors as described in Figure 2. a, DIC images (of a single field of view) were recorded during rinsing. b, DIC images of adherent platelets were recorded after 20 minutes of rinse (higher magnification).

were observed in the presence of the structurally distinct Src kinase inhibitor, PP1 (not shown).

The above changes were analyzed quantitatively at the end of the 20-minute perfusion by measurement of surface area, thrombus height, platelet number, and number of visibly detaching platelets. PD017395 induced a significant ($P<0.05$) increase in surface area of the aggregates of approximately 30% compared with the controls (supplemental Figure Ia), although surprisingly there was no significant difference in the amount of platelets as measured by Western blotting for the integrin subunit, αIIb, or change in height of the aggregates (supplemental Figure Ib and Ic). These results can be accounted for by the analysis of the DIC movies, which reveals streaming away of approximately 15 to 20 rounded platelets per field/focal plan of view during 10 minutes rinsing in the presence of the inhibitor, which is similar to the number lost in controls. This represents an exceedingly low proportion of the number of platelets that are present in the aggregates. The results therefore indicate that the increase in surface area represents a looser packing of platelets in the horizontal axis and thereby emphasize the role of Src kinase in maintaining the compact structure of the aggregates over time.

Sustained Secondary Mediator Signaling Is Not Required for Maintenance of Aggregate Stability

Several studies have demonstrated that the feedback agonists, ADP and TxA_2, play a critical role in the formation of platelet aggregates on collagen at arteriolar rates of shear. In confirmation of this, we have observed that the presence of apyrase and indomethacin in whole blood before flow over collagen leads to abolition of aggregate formation (Figure 1a, v). To

investigate whether ADP and TxA_2 are required for maintenance of the compact aggregate structure, we perfused newly formed aggregates on collagen with buffer containing apyrase and indomethacin for 20 minutes. The inclusion of the two inhibitors had no significant effect on the appearance of single platelets at the edge of the aggregates (Figure 3a and supplemental Movie VI) or on platelet surface area and platelet number (supplemental Figure Ia through Ic). Analysis of DIC movies reveals no difference in the number of detaching platelets compared with the control (15 to 20 platelets per field of view over a period of 10 minutes). Moreover, a similar set of results was obtained after perfusion with the $P2Y_{12}$ antagonist AR-C69931MX (10 μmol/L) for 20 minutes (not shown). Thus these results demonstrate that neither ADP nor TxA_2 is required for maintenance of aggregation on collagen under flow.

The Actin Cytoskeleton Contributes to Aggregate Stability

The contribution of the actin cytoskeleton to the stability of newly formed aggregates was investigated using the above protocol and the inhibitors cytochalasin D and latrunculin A. These 2 inhibitors differ in that the former prevents further growth of existing actin filaments but does not affect their breakdown, whereas the latter promotes a more rapid breakdown of filamentous actin through binding to monomeric actin.[13] The rapid streaming of single rounded platelets from the newly formed aggregates in the direction of flow can be seen throughout the 20 minutes wash in the presence of latrunculin A (supplemental Movie VII) whereas this is less apparent with cytochalasin D (not shown) and minimal in controls. Additionally, many of these platelets remain attached to the aggregates by long tethers before either release or, occasionally, their return to the main body of the aggregate as illustrated in supplemental Movie VII and in Figure 3b (iii). These tethers are similar in appearance to those that have been previously described for platelets flowing over vWF, which have also been shown to increase on inhibition of actin polymerization.[14] Analysis of DIC movies reveals approximately 4 times as many platelets visibly detaching from the rinsing aggregates over the control (60 to 80 per 10 minutes rinse with latrunculin A), although a significant number of these can be seen to readhere to aggregates downstream of their point of release. The relatively low number of released platelets and the fact that a proportion of these are caught by aggregates lower down in the direction of flow accounts for the lack of a significant change in the overall platelet mass as measured by Western blotting for integrin αIIb (supplemental Figure Ib). In line with this, there was no increase in surface area (supplemental Figure Ia) despite the change in shape. However, analysis of aggregate height revealed a significant increase in the presence of latrunculin A of approximately 50% of the control size (supplemental Figure Ic), representing aggregate destabilization and spreading out of platelets in the vertical plane ($P<0.05$). However, in contrast to the effects of PD0173952, latrunculin A did not inhibit tyrosine phosphorylation of platelet proteins, indicating that the collagen signaling pathway was unaffected by the loss of actin polymerization (Figure 2).

Discussion

The current study demonstrates that maintenance of stable compact platelet aggregates on collagen at arteriolar rates of flow is a highly dynamic process that is regulated by Src family kinases and the actin cytoskeleton, but not by the secondary mediators, ADP and thromboxanes. This reveals a fundamental difference between the mechanisms that mediate aggregate formation and those that maintain aggregate stability at arteriolar rates of flow. In addition, the present study reports the novel observation that platelets captured at the front of an aggregate are brought into the main body by a process that we have termed "aggregate retraction." This retraction is mediated by the actin cytoskeleton and is independent of fibrin formation. Continuous signaling through Src family kinases and dynamic actin polymerization are required to limit the loss of platelets from the front of the aggregate and maintain platelets in a fully spread morphology. These events may play a crucial role in vivo in limiting the loss of platelets from aggregates in the arteriolar system.

An inhibitor of Src kinases or an agent that promotes rapid disruption of the actin cytoskeleton promotes the appearance of single rounded platelets at the edge of the aggregate and causes spreading of the aggregate in the direction of flow or into the capillary lumen. This suggests loosening of the packing of the aggregate, presumably attributable to loss in strength of platelet-platelet interactions. This could be a result of reduced activation of integrin αIIbβ3 (or other receptors that mediate attachment of platelets to each other), or attributable to a change in platelet morphology forcing a greater distance between adjacent platelets in an aggregate. The dependence on Src family kinases indicates that these changes are mediated by loss of tyrosine kinase-dependent signals from the major GP receptors, most notably integrin αIIbβ3 and GPVI. The stabilizing effects of Src kinases could also involve $\alpha 2\beta 1$, which has previously been shown to be important in the early formation of stable aggregates on collagen under shear.[15] These findings are consistent with the recent observation that dynamic signaling through Src kinases is required for sustained lamellipodia formation on collagen under static conditions.[11] Interestingly, signaling by ADP and TxA_2 is dispensable for aggregate stability, most likely because of emptying of dense granules and reduced activation of phospholipase A_2, respectively.

We have previously reported that Rac1-deficient mouse platelets, which are unable to form lamellipodia, generate unstable aggregates that rapidly embolise at arteriolar rates of shear.[7] The current study demonstrates that blockade of actin polymerization (and the corresponding inability of platelets to spread) leads to a loss in aggregate retraction and subsequent aggregate instability, raising the possibility that lamellipodia formation plays a critical role in the retraction process. The observation that loss of aggregate stability in the presence of either Src kinase blockade or actin disruption is associated with retraction of lamellipodia and the appearance of single rounded platelets leads us to speculate that Src kinases

contribute to aggregate stability through the actin cytoskeleton, especially bearing in mind the wealth of evidence for direct regulation of actin polymerization by integrin αIIbβ3 and GPVI.[8,16,17]

Several group have reported stable thrombus formation at arteriolar rates of shear in vitro, as observed in the present study.[15,18,19] On the other hand, over the past few years, the use of various in vivo thrombosis models has revealed unstable aggregate formation in the absence of many platelet receptors or receptor mutants, including the α_{2A}-adrenergic receptor, SLAM receptors, CD40L, and the diYF mutant of the integrin β3 subunit.[20–23] In many cases, the molecular mechanism underlying the thrombus instability is unknown and could even be mediated through a nonplatelet mechanism. For example, in the case of the tetraspanin TSSC6 and Gas6 receptors, aggregate instability may be related to involvement of these proteins in fibrin clot retraction.[24,25] On the other hand, the discovery that absence of either coagulation factor XI or XII leads to increased embolization in vivo reinforces the importance of coagulation in thrombus stability.[26] A recent study by Goto et al demonstrated that inhibition of $P2Y_1$ or $P2Y_{12}$ receptors leads to breakup of the platelet aggregate.[27] The probable explanation for the difference with the findings of the present current study is that Goto et al administered the two P2 receptor antagonists much earlier after aggregate formation, when presumably dense granules are continuing to release ADP. Together these studies emphasize the importance of understanding the molecular mechanisms that underlie aggregate stability, the way in which these change, and their interaction with the coagulation system.

An important consideration based on the present study is whether inhibiting Src kinases or actin polymerization in vivo could facilitate the breakup of life-threatening thrombi in occluded arteries and arterioles, without generating emboli that themselves would cause major clinical problems. Certainly, the streaming away of individual rounded platelets is unlikely to have a major effect on blood vessel patency, but on the other hand, the rate of dissolution would appear to be too slow to have a significant effect on thrombus size over short periods of time, although it could be of potential benefit over longer periods. Promoting thrombus breakdown in this way could also be of benefit in cases of small thrombi that have formed on damaged vessels, including atherosclerotic plaques, and which in turn might lead to the generation of larger, life-threatening thrombi.

Note Added During Revision

During the course of revision of this study, Ono et al also described fibrin-independent contraction in platelets and further demonstrated that this also occurs in vivo.[28]

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Disclosures

None.

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