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Segregation of Platelet Aggregatory and Procoagulant Microdomains in Thrombus Formation Regulation by Transient Integrin Activation

Imke C.A. Munnix, **Marijke J.E. Kuijpers**, **Jocelyn Auger**, **Christella M.L.G.D. Thomassen**, **Peter Panizzi**, **Marc A.M. van Zandvoort**, **Jan Rosing**, **Paul E. Bock**, **Steve P. Watson**, and **Johan W.M. Heemskerk**

From the Departments of Biochemistry and Biophysics (I.C.A.M., J.J.E.K., C.M.L.G.D.T., M.A.M.v.Z., J.R., P.E.B., J.W.M.H.), CARIM, Maastricht University, The Netherlands; the Centre for Cardiovascular Sciences (J.A., S.P.W.), University of Birmingham, United Kingdom; and the Department of Pathology (P.P., P.E.B.), Vanderbilt University School of Medicine, Nashville, Tenn.

Abstract

Objective—Platelets play a dual role in thrombosis by forming aggregates and stimulating coagulation. We investigated the commitment of platelets to these separate functions during collagen-induced thrombus formation in vitro and in vivo.

Methods and Results—High-resolution 2-photon fluorescence microscopy revealed that in thrombus formation under flow, fibrin(ogen)-binding platelets assembled into separate aggregates, whereas distinct patches of nonaggregated platelets exposed phosphatidylserine. The latter platelet population had inactivated αIibβ3 integrins and displayed increased binding of coagulation factors. Coated platelets, expressing serotonin binding sites, were not identified as a separate population. Thrombin generation and coagulation favored the transformation to phosphatidylserine-exposing platelets with inactivated integrins and reduced adhesion. Prolonged tyrosine phosphorylation in vitro resulted in secondary downregulation of active αIIbβ3.

Conclusions—These results lead to a new spatial model of thrombus formation, in which aggregated platelets ensure thrombus stability, whereas distinct patches of nonaggregated platelets effectuate procoagulant activity and generate thrombin and fibrin. Herein, the hemostatic activity of a developing thrombus is determined by the balance in formation of proaggregatory and procoagulant platelets. This balance is influenced by antiplatelet and anticoagulant medication.

Keywords

aggregation; coagulation; microdomains; platelets; integrin activation

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Activated platelets have a dual role in hemostasis and thrombosis. They form the building blocks of a thrombus and provide the membrane surface for coagulation factor activation, which results in thrombin and fibrin formation.[1] Once formed, thrombin greatly enhances platelet activation and aggregation. Given the strong interdependency of thrombin generation and platelet activation, it is intuitively assumed that those platelets that participate in aggregate formation are also involved in the coagulation process, but this has not been investigated.

Correspondence to Dr J.W.M. Heemskerk, Department of Biochemistry, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands. E-mail jwm.heemskerk@bioch.unimaas.nl.

Disclosures

None.

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The mechanism(s) by which platelets contribute to thrombin generation and coagulation have been investigated for decades. Kinetic evidence shows that collagen/thrombin-activated platelets expose phosphatidylserine (PS) at their outer surface and then bind Gla domain-containing coagulation factors, mediating factor Xa and thrombin generation.[2] However, even after activation with strong agonists, only a subpopulation of the platelets tends to expose PS.[3] Conversely, it has been argued that PS exposure alone is insufficient to explain the procoagulant contribution of platelets.[4,5] There is also evidence that subfractions of activated platelets have different roles in the coagulation process. Several reports indicate an imperfect relation of PS exposure and the binding of coagulation factors Va, VIIIa, IXa, and Xa to the platelet surface.[6–8] Another report characterizes a subfraction of activated platelets according to their so-called SCIP morphology (for *s*ustained *c*alcium-*i*nduced *p*latelet); these platelets are targets for transglutaminases and partly express PS.[9] Another subpopulation are the so-called *co*llagen-*a*nd *t*hrombin-activated (COAT or "coated") platelets, which express serotonin binding sites at their outer surface, and appear to function in the transglutaminase-mediated assembly of sheets of fibrinogen, von Willebrand factor (vWF), and factor Va.[10,11] However, it has been questioned whether transglutaminase activity is necessary for the coated platelet phenotype.[12] Thus, although there is substantial evidence that individual platelets can differently respond to agonists like collagen/thrombin in binding to coagulation factors and other plasma proteins, the mechanisms of origin and the functions of this heterogeneity is still unclear.

Recently, we and others have shown that platelets which adhere to collagen under high shear flow conditions are heterogeneous with respect to Ca^{2+} signaling.[9,13] We now extended this work and hypothesized that differences in intracellular signaling properties determine the "fate" of a platelet to become either procoagulant or proaggregatory. Here, we provide evidence from in vitro and in vivo models of thrombus formation, that platelets in a thrombus segregate into distinct microdomains or patches with aggregatory or procoagulant functions. A key discriminatory event in this segregation is activation and subsequent inactivation of the integrin αIIbβ3.

Materials and Methods

Origin of coagulation factors and collection of blood was as described before.[14,15] Thrombus formation under flow and in vivo was performed as described elsewhere.[16] Two-photon laser scanning microscopy (TPLSM) and confocal microscopy were used to record confocal images. For more extensive procedures, see the online supplement, available at http://atvb.ahajournals.org

Results

Microdomains of Aggregated and Procoagulant Platelets in Thrombi Formed Under Shear Stress

In the absence of coagulation, high-shear flow of human or murine blood over collagen results in GPVI-mediated platelet activation and gradual development of thrombi.[15,16] Using PPACK-anticoagulated blood, labeled with OG-fibrinogen (0.2 mg/mL) or OG-annexin A5 (0.5 μg/mL), kinetic measurements showed that the labeled fibrinogen slowly, but steadily incorporated into platelet aggregates that were formed on collagen (supplemental Figure I). In contrast, the OG-annexin A5 label, which probes platelets with exposed PS, started to accumulate after a delay of several minutes. When coagulation was triggered by coperfusion of citrate blood with tissue factor and $CaCl_2$, the formation of platelet aggregates was followed by that of fibrin fibers and larger clots, trapping erythrocytes. In this case, the OG-fibrinogen incorporated in the aggregates at a higher rate, which even more increased at the time point of

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clot formation (about 3 minutes). Typically, the labeling of OG-annexin A5 greatly further increased once the clots were formed.

Multicolor TPLSM with high optical resolution and high penetration power was used to better localize the bound OG-fibrin(ogen) and AF647-annexin A5 on human and murine thrombi. Comparison of bright-field contrast images and 2-color fluorescent images indicated that OG-fibrinogen was only present on aggregated platelets, whereas AF647-annexin A5 bound a distinct population of single nonaggregated platelets (Figure 1). Triggering of coagulation with tissue factor/$CaCl_2$ did not influence the labeling of OG-fibrin(ogen), which still bound to aggregated platelets. Again, AF647-annexin A5 labeled a different population of platelets around the clots. The same staining pattern was obtained, when 1 of the 2 labels was omitted (data not shown), excluding the possibility of mutual repulsion of the labels when binding to platelets. Time-dependent scans showed that the heterogeneity in annexin A5 labeling pattern was already present from the start phase of thrombus formation (supplemental Figure II).

"Coated" platelets are recognized by their property to bind multi-amine serotonin-like compounds such as BPA-sBSA, and may participate in coagulation.[10] To detect the formation of coated platelets under flow, thrombi were formed on collagen in the presence of labeled BPA-sBSA, whereas AF647-annexin A5 was present to detect PS-exposing platelets. Surprisingly, the BPA-sBSA labeling pattern differed markedly from that of annexin A5 in both human and mouse blood, with many of the fluorescent BPA-sBSA corresponding to platelets in aggregates (Figure 1, lower panels). Clot (thrombin) formation affected this labeling pattern slightly, in that some of the BPA-sBSA label coincided with annexin A5. This points to a clear difference between coated and PS-exposing platelets. Control experiments indicated that the BPA-sBSA labeling was abolished after heat treatment of the probe and, importantly, after preincubation of the blood with serotonin, demonstrating its specificity for serotonin binding sites (supplemental Figure IIIA through IIIC).

The biphasic increase in annexin A5 labeling under coagulant conditions suggested that platelets start to expose PS after interaction with collagen or with clot material (eg, fibrin). This was indeed visible in high magnification images (supplemental Figure IV). In the absence of clots, or before their formation, only isolated, collagen-bound platelets with a bleb-shaped appearance bound annexin A5. A few minutes after coagulation, strings or patches of annexin A5-positive, bleb-shaped platelets appeared around the clots. Interestingly, the majority of aggregated platelets did not form blebs nor stained positively for annexin A5. This suggested that PS exposure was accompanied by decreased adhesion and diminished platelet-platelet contact.

Heterogeneity of Integrin Activation and PS Exposure after Stimulation of Collagen and Thrombin Receptors

To search for the mechanism responsible for this heterogeneity of aggregated and PS-exposing platelets, dual labeling flow studies were performed with fluorescein isothiocyanate (FITC)-PAC1 mAb (detecting activated αIIbβ3) and AF647-annexin A5 (for PS exposure), again under coagulant conditions. TPLSM showed that platelets which were assembled into aggregates and clots expressed activated aIIb β3 (Figure 2A). These platelets were well separated from the annexin A5-binding platelets, which assembled around the clots and did not bind PAC1 mAb. Control experiments indicated that the annexin A5-binding platelets bound normally to an anti-CD61 mAb, directed against activation-independent epitopes of aIIbβ3, but not to a control IgG, thus indicating that αIIbβ3 integrins were still present on the population of PS-exposing platelets (Figure 2A). Heat-treated annexin A5 did not bind to the platelets (supplemental Figure III).

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To study the integrin inactivation in more detail, platelets in suspension were stimulated with the GPVI agonist, convulxin, alone or in combination with thrombin, and analyzed by 2-color flow cytometry. In the *FL1* channel, this resulted in a clear separation of 2 populations of FITC-PAC1 positive and negative platelets, which were detected as AF647-annexin A5 negative and positive platelets in the *FL4* channel, respectively (Figure 2B). Time-dependent measurements indicated that, shortly after stimulation with convulxin with/without thrombin, most of the platelets were able to bind FITC-PAC1 and were negative for AF647-annexin A5 binding. In the next 15 minutes, PAC1 binding downregulated in platelets that started to bind annexin A5 (Figure 2C).

Characterization of Microdomains of PS-Exposing Platelets Within Thrombi

It has been established that a PS-exposing membrane functions as assembly site for the prothrombinase complex to form thrombin.[17] To investigate prothrombinase complex formation under flow conditions, blood was labeled with each one of the factors of this complex, ie, AF488-factor Va, OG-factor Xa, or OG-prothrombin, always in the simultaneous presence of AF647-annexin A5. Without coagulation, only little green labeling of each kind incorporated at sites of aggregated platelets. However, in the presence of tissue factor and coagulation, the labeling with these factors increased markedly. After 4 minutes, the amount of incorporated AF488-factor Va fluorescence per pixel increased by 2.3 ± 0.84 fold ($n = 4$). High-magnification images showed that particularly patches of (bleb-formed) platelets were double-stained with annexin A5 and any of the coagulation factors (supplemental Figure V).

Further evidence that especially the PS-exposing platelets have a function in coagulation came from quantitative overlap analysis of the sets of 2-colored fluorescence images. The Pearson correlation coefficient (R_r) was calculated as a measure of the pattern overlap of complementary images of red annexin A5 and green probe. Strikingly, R_r was low (around 0.2) for green fibrinogen, PAC1, or BPA-sBSA, but markedly higher for green factor Va, factor Xa, prothrombin, or anti-CD62 (P-selectin) mAb (Figure 3). Similar results were obtained when the overlap coefficient (R) was calculated for the same image sets (not shown), in spite of the fact that this parameter is sensitive for color intensity variation. Similar analysis showed a high overlap of BPA-sBSA and anti-CD62 staining with a R_r of 0.39 ± 0.03, $n = 7$ (supplemental Figure V).

Two-color flow cytometric analysis was performed, using platelets stimulated with convulxin and thrombin, to confirm that this activation can result in two platelet populations with different in coagulation factor binding. After stimulation, the population of annexin A5-negative platelets only weakly bound fluorescent-labeled factor Va, factor Xa, and prothrombin, whereas the annexin A5-positive platelets displayed increased binding of these labeled factors by 2.7, 3.1, and 2.0 fold, respectively (supplemental Figure VI). Both populations of activated platelets were similarly high in CD62 (P-selectin) expression.

To further characterize the populations of AF647-annexin A5 positive and negative platelets, threshold levels of green fluorescence were set to estimate the increased activation-induced binding of labeled antibodies and coagulation factors (Figure 4). We found that hardly any of PS-exposing platelets bound PAC1, whereas only a minority bound BPA-sBSA (probing coated platelets). A substantial fraction of the PS-exposing platelets showed increased binding of factor Va, factor Xa, and anti-CD62 mAb. On the other hand, the PS-negative platelets preferentially bound PAC1, anti-CD62, and to a moderate extent PBA-sBSA, but they were low in factor Va and factor Xa binding. Taken together, these data strongly suggest that, on activation, platelets segregate into cells that bind fibrin(ogen), PAC1, and BPA-sBSA and, at the other hand, PS-exposing cells with increased binding of all components of the prothrombinase complex (factor Va, factor Xa, and prothrombin) at the expense of integrin activation.

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Importance of Tyrosine Phosphorylation State for Platelet Heterogeneity

The observation that in platelets stimulated by convulxin and thrombin, αIIbβ3 inactivation is linked to PS exposure may implicate that a common signaling factor is involved in either event. To investigate this further, platelets in suspension were pretreated with the αIIbβ3 blocker lotrafiban,[13] before stimulation with convulxin/thrombin. Lotrafiban treatment abolished PAC1 binding, whereas annexin A5 binding remained high (Figure 5), indicating that the integrin activation state does not regulate PS exposure. As collagen and thrombin receptors activate platelet tyrosine kinases, we investigated the effects of modulation of the tyrosine phosphorylation state. Pretreatment of platelets with the protein tyrosine phosphatase inhibitor, phenylarsine oxide (PAO), resulted in increased tyrosine phosphorylation (not shown), which was accompanied by severe reduction in PAC1 binding and unchanged PS exposure (Figure 5). By contrast, platelet preincubation with the general protein tyrosine kinase inhibitor genistein or the Src-kinase inhibitor PP2 left PAC1 binding virtually unchanged, whereas they reduced the number of PS-exposing cells. Yet, in all conditions, the majority of the platelets that bound PAC1 did not bind annexin A5, and vice versa. Together, this suggested that a prolonged high tyrosine phosphorylation state (eg, by Src kinase activity) reduced integrin activation, but stimulated PS exposure.

The importance of protein tyrosine phosphorylation was further examined in flow studies (no coagulation). Preincubation of blood with lotrafiban increased the number of PS-exposing platelets, but reduced the total number of adherent platelets, as aggregate formation was abolished (supplemental Figure VII). Gel electrophoresis of the platelet proteins, followed by immunoblotting with anti-phosphotyrosine 4G10 mAb, indicated that lotrafiban treatment specifically increased the tyrosine phosphorylation levels of proteins of about 7, 38, 72, and 105 kDa. Other evidence for an increased tyrosine phosphorylation in PS-exposing platelets was obtained by direct staining of collagen-bound platelets on coverslips with FITC-4G10 mAb. Comparison of brightfield and confocal fluorescent images indicated that, typically, the single (AF647-annexin A5 positive) platelets were more fluorescent than adjacent platelets in aggregates. Accordingly, high tyrosine phosphorylation levels accompany PS exposure, but not integrin activation.

Heterogeneity in Fibrin(ogen) Binding and PS Exposure of Thrombi Formed In Vivo

We questioned how the platelet heterogeneity as observed in thrombi formed in vitro relates to the in vivo situation. To investigate this, anesthetized mice were coinfused with fluorescently labeled OG-fibrinogen and AF568-annexin A5. Damage of the carotid arteries was then provoked by 2 different techniques. In one method, the artery was ligated, which results in mechanical exposure of subendothelial collagen.[18] Alternatively, $FeCl_3$ was locally applied, which causes free radical formation and also endothelial denudation.[16,19] After thrombi were formed in vivo for 10 minutes, injured and control (undamaged) vessels of the animals were scanned using the high penetration power of TPLSM. Fluorescence from both probes was detected only at sites of ligation or $FeCl_3$ application, and was essentially absent in control arteries. Optical cross-sections showed local spots of OG-fibrin(ogen) at the wall-lumen interface, which to a small extent overlapped with spots of AF568-annexin A5 (Figure 6A, first row). Three-dimensional reconstruction of the images at thrombotic sites showed distinct patches of fluorescent fibrin (ogen) and annexin A5, again only at the damaged vascular lumen (Figure 6B and supplemental Movie I). After scanning, the still intact arteries were poststained for vessel wall cells with the nuclear stain Syto-44, and then rescanned. Optical cross-sectioning demonstrated absence of Syto-44 fluorescence at the sites of ligation or $FeCl_3$ application, thus confirming the local damaging effect of the prothrombotic interventions (Figure 6A, second row). No fluorescence was detected after coinjection of heat-denatured fibrinogen and annexin A5 into the mice (supplemental Figure VIII). Furthermore, coinjection of autologous CFSE-labeled platelets and AF-568-annexin A5 showed that annexin A5 binding

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was mostly confined to (part of) the CFSE-labeled platelets in thrombi. Other mice were injected with rhodamin 6G to label leukocytes; however, fluorescent leukocytes were not detected at the thrombotic site (not shown). Together, these data show that fibrin(ogen)-rich and annexin A5-binding platelets appear at adjacent but distinct locations during in vivo thrombus formation.

Discussion

The present data indicate that during thrombus formation under flow different clusters of platelets contribute to aggregate formation and to coagulant activity. This points to a spatial separation of these 2 key functions of platelets. In thrombi that are formed on collagen under conditions of high shear-rate and coagulation, we can distinguish at least 2, and likely 3 forms of activated platelets: (1) platelets packed in fibrin-containing aggregates (clots) with activated αIIbβ3 integrin at their surface; (2) strings of nonaggregated platelets with inactivated αIIbβ3, exposing PS and binding components of the prothrombinase complex; and (3) platelets exposing serotonin binding sites, which overlap with the aggregated platelets.

The microdomains of aggregated platelets appear to be rich in fibrin(ogen) and were most readily distinguished by high binding of PAC1 mAb, which stains the activated conformation of αIIbβ3. Part of these platelets express CD62 at their surface, indicating they have undergone secretion, such in agreement with electron microscopic studies showing heterogeneous levels of platelet degranulation in aggregates.[20] Typically, these clustered platelets do not expose PS, as apparent from their inability to bind annexin A5. Both confocal microscopy and flow cytometry show that annexin A5 negative platelets are low in binding factors Va and Xa and prothrombin.

The second form consists of mostly single, bleb-shaped platelets, which bind annexin A5 and often appear as strings at the edge of a clot. Overlap analysis of 2-color fluorescence images points to a clear separation of these annexin A5-binding platelets from the fibrinogen/PAC1-binding platelets in aggregates or clots. Strikingly, the PS-exposing platelets fail to bind PAC1; their round morphology and lack of pseudopods is indeed compatible with a low integrin activation state and thus diminished adhesion. Likely, these platelets play an important role in thrombin generation, as they show increased binding of factors Va and Xa and prothrombin.

The formation of bleb-shaped PS-exposing platelets has already been reported for platelets interacting under shear with collagen via GPVI.[15] A novel finding is that this platelet type becomes much more frequent in the presence of thrombin generation and coagulation. Under coagulant conditions, it appears that strings of platelets at the surface of clots jointly transform into PS-exposing cells. The result is the development of thrombi of a patched structure with microdomains of platelet/fibrin aggregates, alternated with loose clusters of PS-exposing platelets. It is tempting to suggest that the PS-exposing platelets on collagen contribute to the initial phase of thrombin generation, whereas the massively appearing PS-exposing platelets around clots mediate the second, propagation phase of thrombin generation. A patched structure of fibrin(ogen)- and annexin A5-binding platelets is also observed by TPLSM imaging of the thrombi formed in murine arteries in vivo. This makes it likely that a similar structure of separate microdomains of aggregated/clotted and coagulation-active platelets is present in human thrombi formed in vivo as well.

Our data also distinguish a third group of platelets, though with less characteristic binding properties. We observe appreciable labeling of platelet aggregates and to a smaller extent of single PS-exposing platelets with the probe BPA-sBSA, which specifically detects serotonin binding sites. This labeling is prevented in the presence of serotonin, confirming that it identifies those platelets that fulfill the original description of coated (or COAT) platelets.

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These are proposed to be platelets that are covered with the serotonin-derivatized proteins, fibrin-(ogen), and thrombospondin in association with the granular proteins vWF, factor V, and fibronectin.[10] Pearson correlation coefficients of TPLSM images indicate a high degree of costaining of BPA-sBSA with CD62 ($R_r = 0.39$) and fibrinogen ($R_r = 0.54$), ie, higher than the costaining with annexin A5 ($R_r = 0.21$). This indicates that coated platelets express CD62 but not annexin A5. The observation that aggregated platelets are labeled with BPA-sBSA is not surprising, given the initial description of coated platelets as cells covered with fibrinogen and other plasma proteins.

We investigated the possibility that intracellular signaling pathways are responsible for platelet heterogeneity in thrombus formation. The current data indicate that protein tyrosine kinase activity favors PS exposure (likely via stimulation of Ca^{2+} mobilization via the GPVI/phospholipase C-γ2 pathway),[16] but inhibits persistent integrin αIIbβ3 activation. This was concluded from the relatively high tyrosine phosphorylation state of PS-exposing PAC1-negative platelets on collagen, and also from the reducing effect of tyrosine phosphatase inhibition on PAC1 binding with unchanged PS exposure. Further support comes from perfusion experiments in the presence of αIIbβ3 antagonist, which leads to an increase in tyrosine phosphorylation of several platelet proteins along with the number of PS-exposing platelets on collagen. Together, these data point to a new pathway of secondary inactivation of integrin αIIbβ3 via tyrosine kinase activity.

In summary, we conclude that in collagen- and thrombin-induced thrombus formation, differences in intracellular signaling state control the activation of αIIbβ3 integrin and accomplish a spatial separation of microdomains of aggregated and PS-exposing platelets. Typically, in PS-exposing platelets, downregulation of αIIbβ3 is accompanied by up-regulation of coagulation factor binding. This generates a balance, in which coagulant activity suppresses integrin activation, whereas conversely aggregate formation reduces the procoagulant activity of platelets. This balance is influenced by antiplatelet and anticoagulant medication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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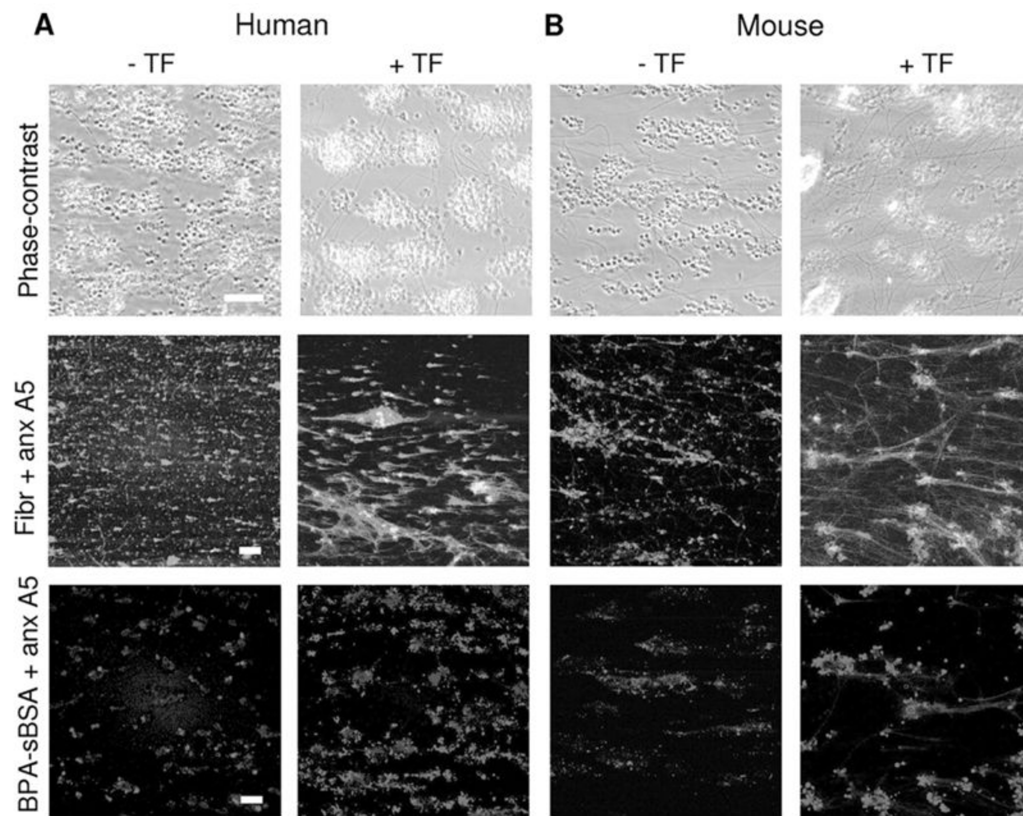

Figure 1.

Heterogeneity in human and murine thrombi formed on collagen. Flow experiments were performed with human (A) or murine (B) blood in the absence of tissue factor (−TF) using PPACK-anticoagulated blood (left columns); or with citrate blood that was perfused together with tissue factor (+TF, 2 pM, f.c.) and $CaCl_2$ (2 mmol/L free Ca^{2+}, f.c.) to allow coagulation (right columns). Standard perfusion time was 4 minutes at a shear rate of 1000 s^{-1}. Blood was preincubated with 0.2 mg/mL OG-fibrinogen. Alternatively, preincubation was with 50 μg/mL BPA-sBSA and postlabeling with 1 μg/mL AF532-labeled streptavidin. In both cases, AF647-annexin A5 was also present. Upper panels, Bright-field phase-contrast images after perfusion. Middle panels, TPLSM images of OG-fibrinogen (green) and AF647-annexin A5 (red) fluorescence (different fields of view). Lower panels, TPLSM images of BPA-sBSA (blue) and AF647-annexin A5 (red) staining. Images are representative of 4 to 8 experiments; bars indicate 20 μm.

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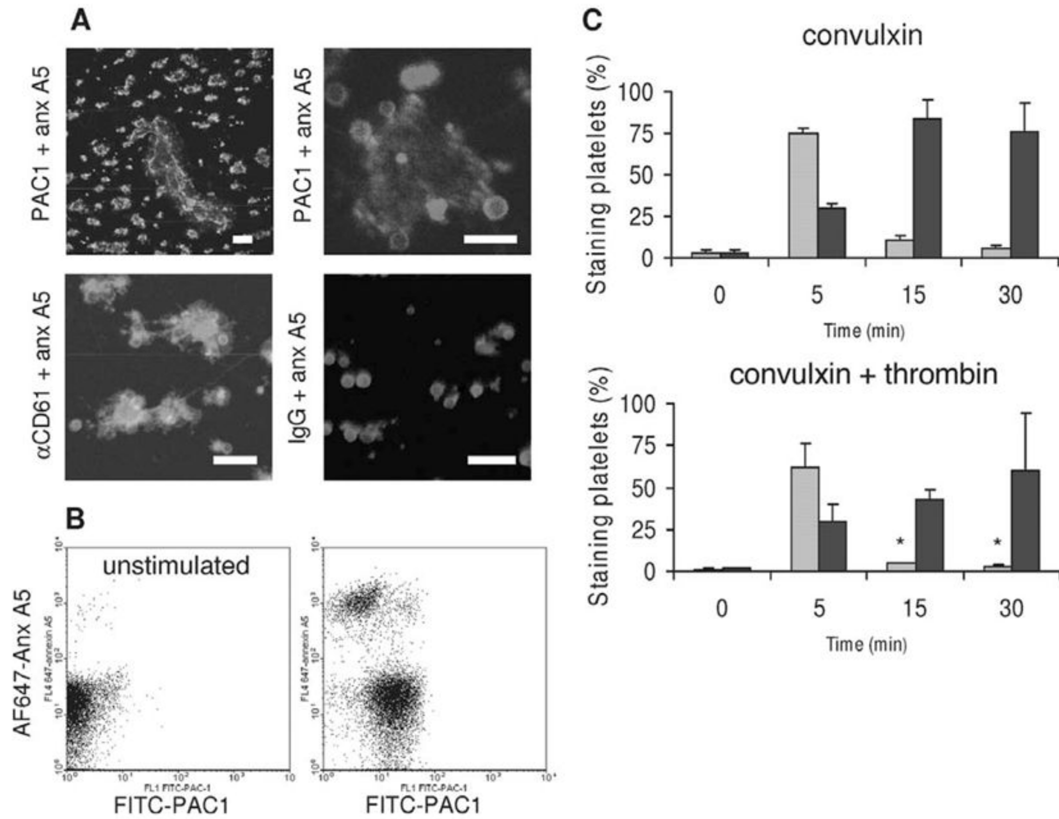

Figure 2.

Temporary integrin activation state in procoagulant platelets. A, Human thrombi were formed on collagen in the presence of tissue factor/$CaCl_2$ (see Figure 1). Blood was labeled with 0.5 μg/mL AF647-annexin A5 plus either 0.6 μg/mL FITC-PAC1, 1.25 μg/mL FITC-α-CD61, or 1.25 μg/mL FITC-IgG. Representative TPLSM images are shown of FITC (green) and AF647 (red) fluorescence at lower and higher magnifications. Optical zoom 1 to 4×; bars indicate 20 μm. B and C, Washed platelets were stimulated in the presence of 2 mmol/L $CaCl_2$ with 50 ng/mL convulxin alone or with 4 nmol/L thrombin, for 5 to 30 minutes without stirring (1×10^7 platelets/mL). B, Representative dot plots are given of *FL1* (FITC) vs *FL4* (AF647) after 5 minutes of stimulation with convulxin. C, Two-color flow cytometry after costaining at indicated times with FITC-PAC1 and AF647-annexin A5, showing transient appearance of PAC1 binding sites and persistent PS exposure. Data are percentages of platelets staining with FITC-PAC1 (gray bars) or OG-annexin A5 (black bars). Platelets were selected according to their forward/side scatter characteristics. Mean ± SD (n = 4), *$P < 0.05$ compared with t = 5 minutes.

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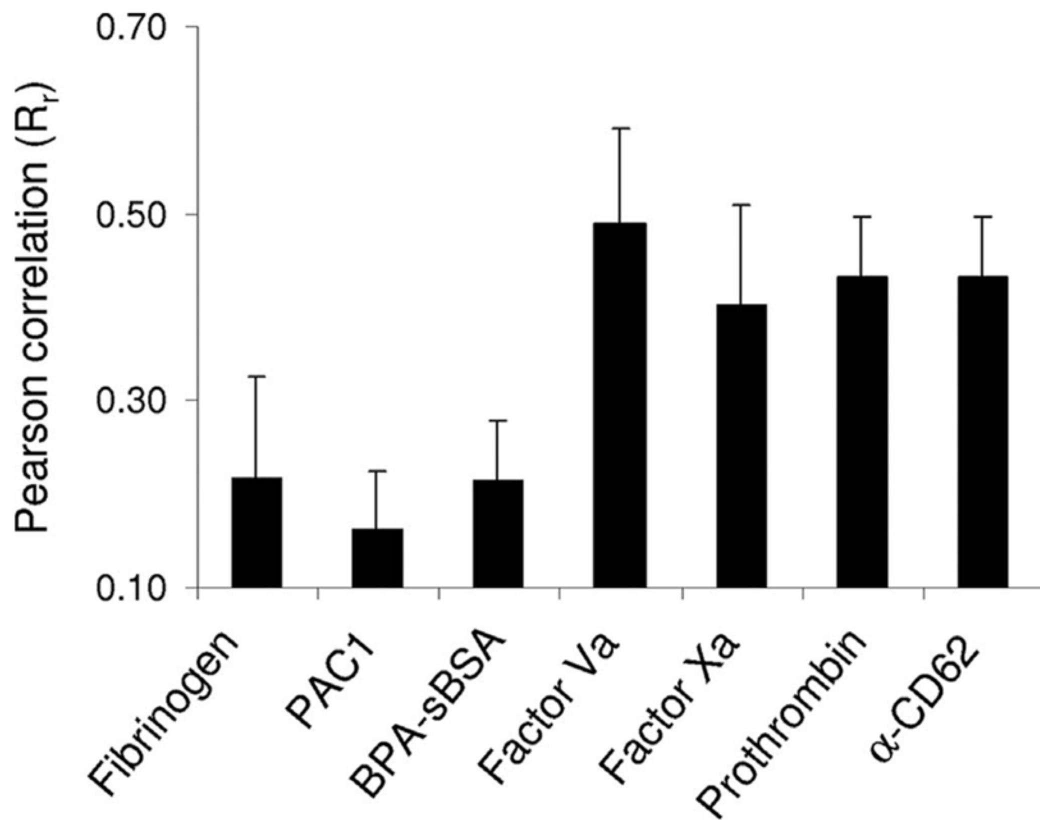

Figure 3.

Partial overlap of binding of coagulation factors and annexin A5 to platelets in thrombi. Human thrombi were generated on collagen under flow and coagulant conditions (see Figure 1) in the presence of AF647-annexin A5 (red) plus either 20 nmol/L AF488-factor Va, 200 nmol/L OG-prothrombin, 100 nmol/L OG-factor Xa, or 1.25 μg/mL FITC-α-CD62 mAb (green). Histograms give pattern overlap analysis of TPLSM images for annexin A5 (red) and indicated factor or antibody (green); shown is the Pearson correlation coefficient (R_r) of corresponding green and red bit maps (n = 4 to 7).

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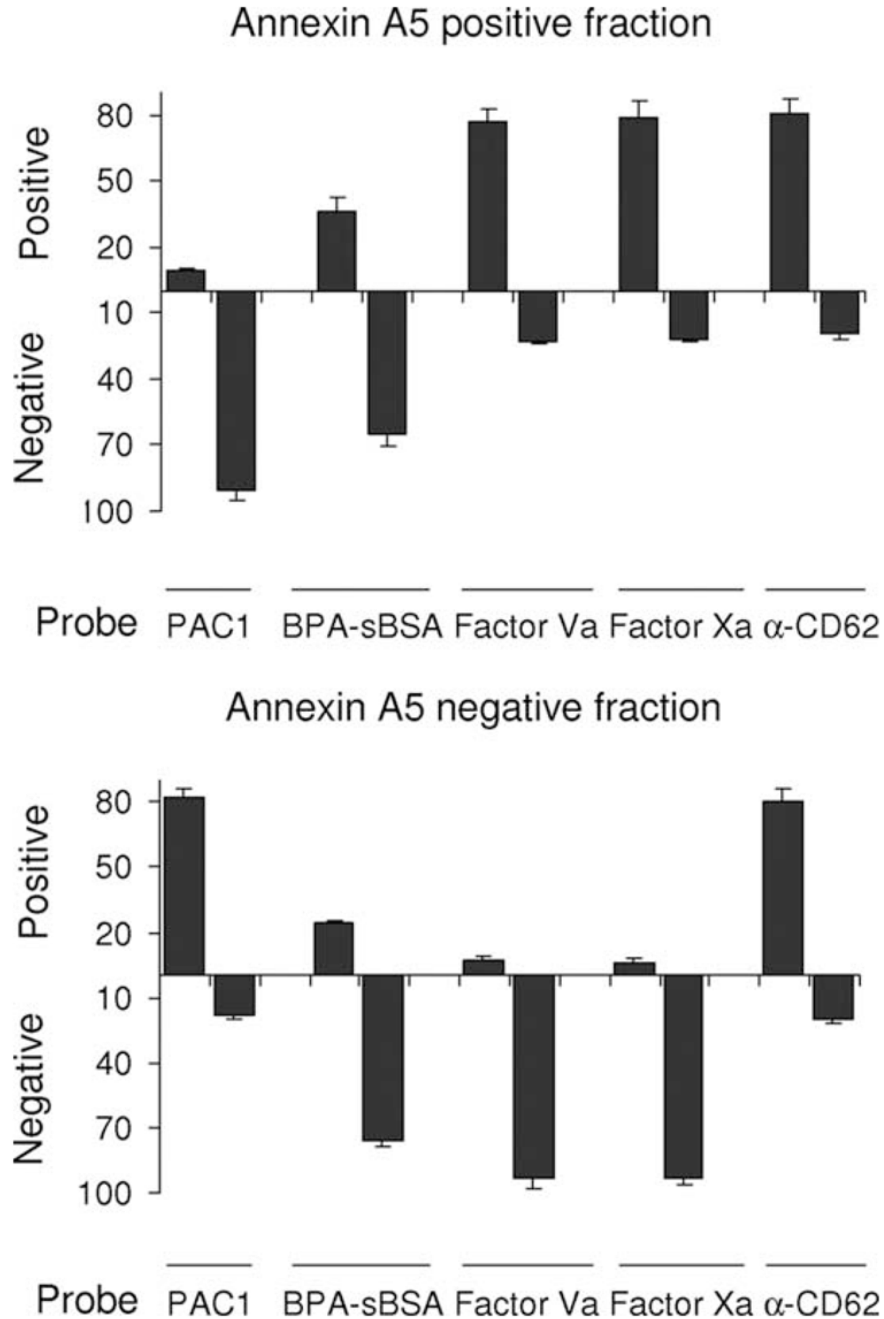

Figure 4.
Heterogeneity in binding properties of PS-exposing platelets. Platelets in suspension were either unstimulated or stimulated with 50 ng/mL convulxin, 4 nmol/L thrombin and 2 mmol/L $CaCl_2$. Two-color flow cytometry was performed in the presence of AF647-annexin A5 combined with either 20 nmol/L AF488-factor Va, 100 nmol/L OG-factor Xa, 200 nmol/L OG-prothrombin, or 1.25 μg/mL FITC- α-CD62 mAb. Histograms show binding properties of the annexin A5 positive and negative platelet fractions after 10 minutes of activation: percentages of cells staining positively (upside bars) or negatively (downside bars) with indicated green probe: FITC-PAC1, AF532-streptavidin BPA-sBSA, AF488-factor Va, OG-factor Xa, or FITC-α-CD62. Mean ± SD (n = 4).

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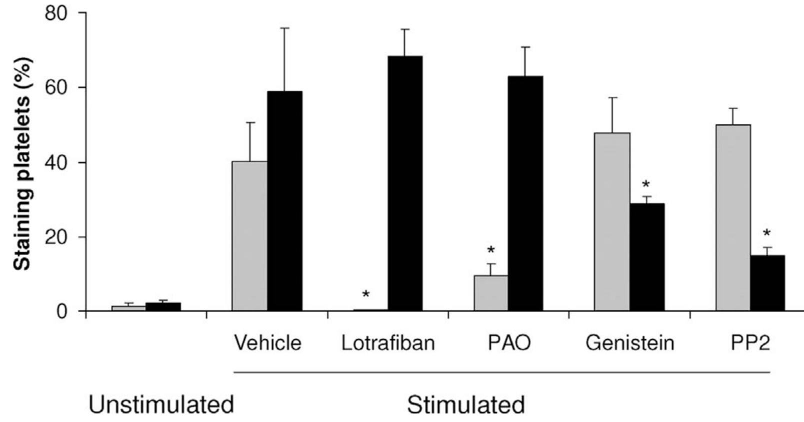

Figure 5.

Modulation of protein tyrosine phosphorylation alters integrin activation and PS exposure. Human washed platelets were stimulated with 50 ng/mL convulxin and 4 nmol/L thrombin in the presence of 2 mmol/L $CaCl_2$. The cells were preincubated for 10 minutes with vehicle, 200 μmol/L phenylarsine oxide (PAO), 100 μmol/L genistein, 20 μmol/L PP2, or 10 μmol/L lotrafiban. Colabeling was with a mixture of FITC-PAC1 and AF647-annexin A5. Data are expressed as percentages of platelets staining with FITC-PAC1 (gray bars) or OG-annexin A5 (black bars). Mean ± SD (n = 4), $^*P < 0.05$ compared with stimulation in the presence of vehicle.

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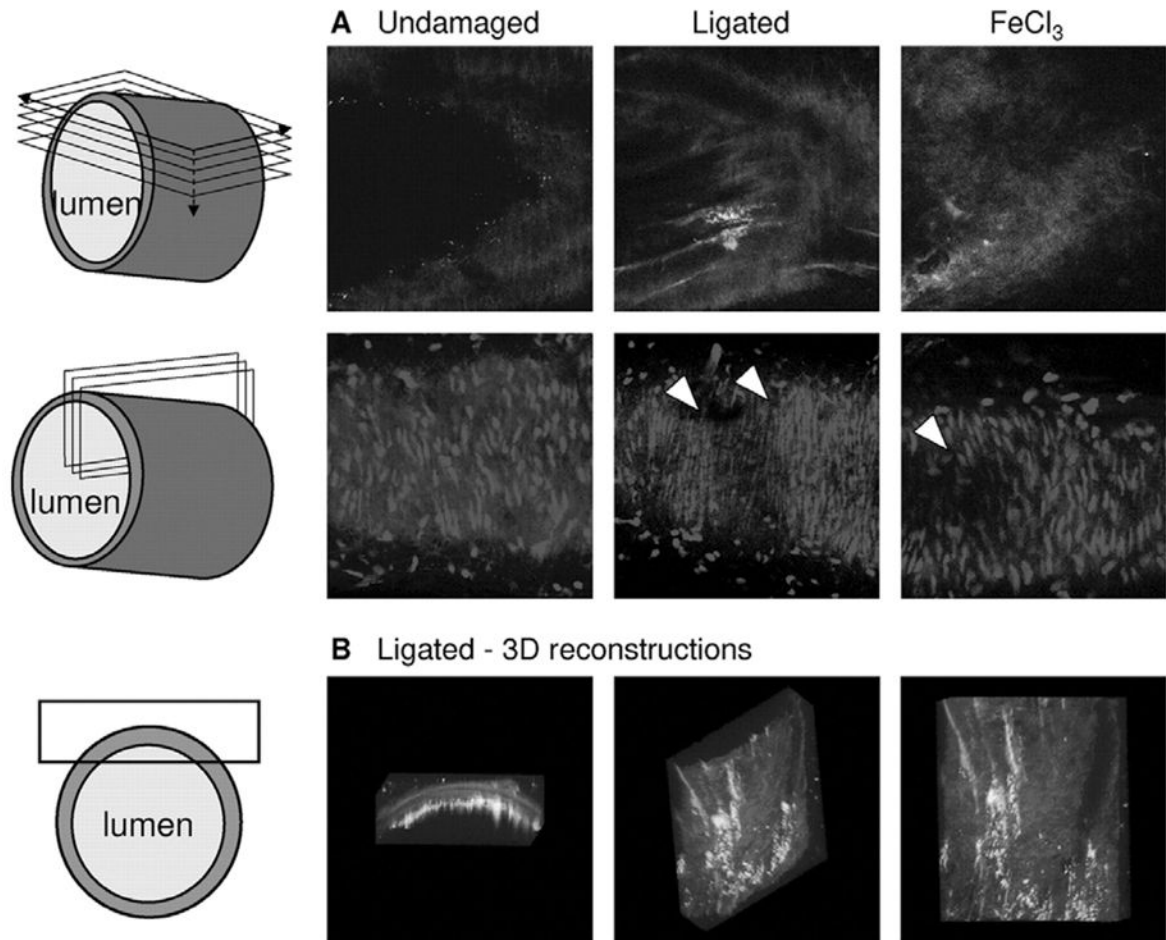

Figure 6.

Heterogeneity in arterial thrombi induced by carotid ligation or by $FeCl_3$ application. Mice were infused with OG-fibrinogen and AF568-annexin A5 (200 μg each). A, left panels, Undamaged control carotid artery. Middle panels, One carotid artery was damaged by tight ligation at the bifurcation for 5 minutes to induce vascular damage. Right panels, In other animals, 1 carotid artery was damaged by local application of saturated $FeCl_3$. Thrombus formation proceeded for 10 minutes, after which fluorescence at the luminal side of the artery was recorded by TPLSM. Upper row, Images of fibrin(ogen) (green) and annexin A5 (red) fluorescence at the damaged side of the vessel wall. Note distinct patches of green and red fluorescence; also note blue autofluorescence, indicating the demarcation of the vessel wall with the lumen. Second row, Images of Syto-44 fluorescence (blue) at cross section through the vessel wall. Arrow heads indicate absence of cell nuclei at side of damage. B, Three-dimensional reconstruction of fibrin(ogen) and annexin A5 fluorescence within the ligated artery: side view through vessel wall, turned view, and view from inside of vessel lumen. Data are representative of 3 to 4 vessels. Cartoons indicate way of optical sectioning (images 206 $\times$ 206 μm).