Thrombosis

Platelet Heterogeneity
Variation in Coagulation Complexes on Platelet Subpopulations

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Objective—Previous work has shown that platelets stimulated with the combination of thrombin and convulxin, a glycoprotein VI agonist, develop 2 populations with different levels of α-granule factor V bound to the platelet surface. To evaluate whether this phenomenon is restricted to α-granule components or is a feature that can be generalized to other coagulation factors, we studied the binding of factors V, VIII, IX, and X on the surface of platelets stimulated by convulxin and thrombin.

Methods and Results—The relative amount of factors V, VIII, IX, and X on the surface of platelets stimulated with thrombin or convulxin plus thrombin was measured using flow cytometry. Simultaneous measurements of factor Xa and thrombin generation were obtained and correlated with the binding of coagulation factors on the platelet surface. The binding of factors V, VIII, IX, and X all increased on a subpopulation of platelets when stimulated with the combined agonists. The development of this platelet subpopulation is dependent on convulxin concentration and correlates with increases in factor Xa and thrombin generation.

Conclusions—The development of increased coagulation factor binding to a subpopulation of platelets is not limited to α-granule components. Convulxin-induced increases in thrombin generation are regulated by the proportion of platelets with increased coagulation factor binding. (Arterioscler Thromb Vasc Biol. 2005;25:861-866.)

Key Words: thrombin generation ▪ platelets ▪ glycoprotein VI ▪ COAT ▪ platelet subpopulation

Platelets activated by strong agonists develop an outer surface, which, in the presence of calcium, supports the binding of coagulation factor complexes leading to thrombin generation. Two important physiological agonists of platelet activation are collagen and thrombin. Although collagen and thrombin can individually lead to maximal platelet aggregation, the combination is superior to either agent alone in stimulating platelet procoagulant activity. Platelets have multiple collagen receptors including integrin α2β1 and glycoprotein VI (GPVI). GPVI is thought to be the dominant receptor mediating platelet activation. Although the lack of GPVI leads only to a phenotype of minor bleeding in humans and mice, GPVI-deficient mice appear to be protected against thrombosis.

The combination of collagen and thrombin leads to increased exposure of phosphatidylserine (PS) on the platelet surface. However, despite the clear role of PS in platelet procoagulant activity, there is disagreement regarding the precise components on the platelet surface that are necessary for the assembly of the tenase (VIIa/Xa) and prothrombinase complexes (Va/Xa). Some investigators have postulated the presence of additional components important in coagulation factor binding such as specific binding sites for coagulation factors. Previous studies have shown that the combination of convulxin, a GPVI agonist, and thrombin leads to 2 platelet subpopulations with distinct levels of α-granule factor V (FV), and this may be related to serotonin and fibrinogen binding. Further evidence of discordance between platelet procoagulant activity and PS was demonstrated when the addition of FV equalized thrombin generation on collagen- and fibrinogen-adherent platelets despite persistent differences in PS. These observations suggest that features other than PS exposure may be important in coagulation factor binding and thrombin generation.

In this report, we investigated the effect of combined stimulation of the collagen GPVI receptor and thrombin receptors on the binding of FV, FVIII, FIX, and FX on the platelet surface. These data show that development of a subpopulation of platelets with high levels of coagulation factor binding is not limited to components released from α-granules. The number of platelets in this subpopulation correlates with increased factor Xa (FXa) and thrombin generation, suggesting that the number of platelets in the subpopulation with increased binding of coagulation factors regulates the extent of increased thrombin generation.

Materials and Methods

Proteins and Reagents
Tos-Gly-Pro-Arg-pNA (thrombin substrate), Pefachrome Xa (FXa substrate), recombinant hirudin, and convulxin were purchased from...
FXs and Thrombin Generation in a Cell-Based Model System

A cell-based model system simulating tissue factor (TF)-initiated coagulation was used as described previously. Components of the model system included adherent monocytes stimulated with lipopolysaccharide to express TF, gel-filtered unactivated platelets, and plasma levels of the following coagulation factors: 100 μg/mL prothrombin; 7 μg/mL FV; 0.5 μg/mL FVII; 1 U/mL FVIII; 4 μg/mL FX; 8 μg/mL FX; 5 μg/mL FXI; 0.1 μg/mL TF pathway inhibitor (TFPI); and 200 μg/mL AT. The zymogens were incubated with inhibitors (TFPI, AT, and C1 esterase inhibitor) overnight to minimize small amounts of contaminating proteases. Isolation of cellular components was performed in accordance with the local institutional review board guidelines. Monocytes were prepared from freshly drawn human blood from healthy donors as described previously. Platelets were isolated from healthy donors who had not received antiplatelet agents for 7 to 10 days before phlebotomy and prepared as described previously.

In the model system, coagulation was initiated by combining the TF-bearing monocytes, unactivated platelets (1 × 10^11/mL), and coagulation factors in the presence of CaCl_2 (3 mmol/L). In the model system, a small amount of thrombin is first generated on the TF-bearing monocyte. This small amount of thrombin primes the system by activating platelets and clotting factors. The activated platelet in conjunction with activated clotting factors can then generate a burst of thrombin. Thus, thrombin is present in all model system experiments. When convulxin is added, platelets are stimulated by the combination of thrombin and convulxin. To assay FXa and thrombin generation, samples were removed at timed intervals and diluted 1:10 in assay buffer to give final concentrations of 20 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, and 1 mmol/L Pefachrome Xa or thrombin substrate. In the assays using Pefachrome Xa, hirudin (100 mmol/L) was used in the assay buffer to inhibit thrombin cleavage of the FXa chromogenic substrate. The reaction was stopped by adding an equal volume of 50% (by volume) acetic acid after 9.5 minutes. Absorbance of 405 nm was measured using a SoftMax plate reader (Molecular Devices) and converted to a concentration of thrombin or FXa by comparison to a standard curve.

Measurement of FV, VIII, IX, and X Binding on the Platelet Surface in the Cell-Based Model System

Samples were removed at defined time points from microtiter wells and added to an equal volume of paraformaldehyde at a final concentration of 1% as described previously. Platelets were incubated with a primary antibody for 60 minutes at room temperature. With the exception of FVIII, polyclonal antibodies from different species were used to facilitate dual staining. Polyclonal antibodies were used at the following dilutions: anti-FV 1:500 to 2000; anti-FX 1:250; and anti-FIX 1:250. Monoclonal anti-FVIII antibody was used at a concentration of 1 μg/mL. Secondary antibodies were used at the follow dilutions: fluorescein-labeled goat anti-rabbit and donkey anti-sheep 1:30; PE-labeled goat anti-rabbit and donkey anti-sheep 1:40; and PE-labeled rabbit anti-mouse 1:100, as described previously. Secondary antibodies were titrated using platelets activated with convulxin plus thrombin to determine concentrations that minimized background staining. Each platelet sample was diluted in 10 volumes of HEPES/buffered saline and analyzed on a FACScan flow cytometer. A total of 5000 events per sample were analyzed. Platelets were identified by forward and side scatter. Platelet microparticles were not included in analysis. Sub-populations were identified by visual inspection of contour plots and determined to be present when there were 2 distinct contours. Gates were used to determine the percentage of platelets in each population were set at the time point at which 2 contours were most distinct. These gates were then used to evaluate all other time points. When 2 coagulation factors were stained on the same platelet, 1 coagulation factor was stained first with primary and secondary antibody, followed by the second coagulation factor primary and secondary antibody. Appropriate dual-color compensation was performed. In all experiments, control platelet samples were taken from platelet preparation in the presence of coagulation factors before initiation of coagulation in the model system and thus are unactivated platelet controls. In 2 separate experiments, additional controls using irrelevant or preimmune antibodies were also performed. The rabbit IgG was used to estimate nonspecific binding by the rabbit anti-FIX and anti-FX antibody. Similarly, the sheep IgG and mouse monoclonal IgG2a estimated nonspecific binding by the anti-FV and anti-FVIII antibodies, respectively. Staining with each control antibody was performed using unactivated and activated platelet samples from model system experiments at multiple time points.

Detection of PS on the Platelet Surface

Aliquots (10 μL) were obtained from microtiter wells during a model system assay. Background fluorescence was determined on unactivated platelet preparations. Fluorescein-labeled annexin V (5 μL) was incubated for 2 minutes with platelet samples. It was determined that this concentration of annexin V and this duration of incubation gave optimal staining. Samples were diluted with 500 μL of HEPES/buffered saline containing 2.5 mmol/L CaCl_2 and were analyzed immediately on a FACScan flow cytometer.

Statistical Analysis

Data are reported as mean (±SE).

Results

Effect of Convulxin on FXa and Thrombin Generation

We used an established cell-based model of FXa and thrombin generation to investigate the effect of GPVI stimulation on platelet procoagulant activity. The addition of a GPVI agonist, convulxin, in this system led to increased FXa and thrombin generation (Figure 1A and 1B). In 11 experiments with 8 different platelet donors, all donors showed an increase in peak FXa (mean 14-fold [± 2.3]) and thrombin generation (mean of 3.5-fold [± 0.3]) in the presence of convulxin (300 ng/mL; Figure 1C). The dose response to convulxin as measured by peak FXa and thrombin followed a single-site ligand model consistent with convulxin interacting with a single receptor. CRP (10 μg/mL), which interacts specifically with GPVI, led to increases in thrombin generation similar to those seen with convulxin (n = 2; data not shown), supporting the idea that convulxin is acting predominantly through the GPVI receptor in this system. The addition of convulxin did not affect monocyte TF-VIIa activation of FX (data not
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7) during 10), FVIII (n/H11005), FIX (n/4), and FX (n/H11005/n5).

To investigate the changes in platelet surface binding that may lead to increased generation of FXa and thrombin, we used flow cytometry to examine the surface appearance of FV.

Effect of GPVI Stimulation on Appearance of Coagulation Factors on Activated Platelet Surfaces

To determine the percentage of total platelets that were in each subpopulation, gates were set around each population on the contour plot when the 2 populations were most distinct. This gate was not changed during analysis of the remainder of the time points for that experiment. Subpopulations with higher fluorescence were readily seen for factors V, IX, and X (Figure 3). The development of FVIII subpopulations was more variable, but convulxin clearly increased the mean fluorescence of platelets in all experiments in which FVIII was measured (n=5). With staining of only FVIII, 2 distinct populations were seen in 2 experiments with 2 different platelet donors (Figure 3B). With the remaining 3 experiments, although the 2 histogram peaks were not as distinct, the histogram was wider and more diffuse in the presence of convulxin. The difficulty in detecting a distinct subpopulation with higher FVIII binding likely arises because of the low concentration of FVIII in solution relative to the other coagulation factors. CRP (10 μg/mL) also led to increased FV, FVIII, FIX, and FX on the platelet surface on a subpopulation of platelets similar to that seen with convulxin (n=2; data not shown).

Despite the marked difference in coagulation factor binding to the 2 populations; surface P-selectin at 15 minutes was similar in both platelet subpopulations (data not shown). This suggests that differences in α-granule degranulation do not account for differences in coagulation factor binding.

With maximal convulxin concentrations, ~50% of platelets demonstrated higher levels of surface FV, FIX, and FX (49±6.2%, 57±10%, and 52±6.5%). These factors showed a dose-dependent increase, with 20 ng/mL of convulxin giving ~30% platelets in the high fluorescence group and 5 ng/mL of convulxin giving ~7% of platelets in the high fluorescence.
burst of FXa and thrombin generation, and that the development of platelet subpopulation factor binding is dependent on the concentration of convulxin used, is correlated with peak FXa and thrombin generation (arbitrarily defined as the time at which thrombin levels reaches 10 nmol/L). For example, in the presence of 100 ng/mL of convulxin, the onset of the burst of thrombin generation occurred at 3.1 minutes (±0.3), which corresponded to 8%, 16%, and 4% of platelets in the subpopulation, with increased binding of FV, FIX, and FX, respectively. Additionally, at all convulxin concentrations, <10% of FXa had been generated by 3 minutes. Therefore, we can conclude that the number of platelets with increased coagulation factor binding is dependent on the concentration of convulxin used, is correlated with peak FXa and thrombin generation, and that the development of platelet subpopulations with increased coagulation factor binding precedes the burst of FXa and thrombin generation.

Colocalization of Coagulation Factors
Dual staining was performed to determine whether the same group of platelets had increased fluorescence for multiple coagulation factors. Results demonstrated that platelets with increased FV on their surface also have increased FVIII (n=5; Figure 5A, quadrant 2). Although subpopulations with increased FVIII binding were not detected by single staining on all donors, all donors demonstrated the pattern of colocalization with FV. Together, these findings are consistent with the presence of increased FVIII on subpopulations of platelets stimulated with the combination of convulxin plus thrombin. When platelets were stained for FV and FX (n=3) or FIX and X (n=3), coagulation factors colocalized to the same platelet population (n=3; Figure 5B and 5C, quadrant 2). Thus, the same population of platelets binds increased levels of FV, FVIII, FIX, and FX.

PS Exposure on Activated Platelets
To assess the effect of convulxin on PS exposure on the platelet surface in the model system, we assayed annexin V binding on samples obtained directly from model system experiments (Figure 6A and 6B). Although initially, increases in PS exposure and coagulation factor binding occur coincidentally, the dose response is different from that seen when peak thrombin generation is the end point (Figure 6C). At 9 minutes, in the presence of convulxin 5 ng/mL or in its absence, there is very little annexin V binding (0.5% and 3%, respectively). Comparatively, with 20 ng/mL or 100 ng/mL of convulxin, there is ≈53% and 51% of platelets binding annexin V, respectively. This dose response is different from that seen for thrombin generation, suggesting that the level of PS exposure is not the sole determinant of thrombin generation.

Discussion
In this report, we demonstrate that combined stimulation of the platelet collagen receptor (GPVI) and thrombin receptors leads to development of a subpopulation of platelets with increased binding of not only α-granule platelet FV, but also FVIII, FIX, and FX. As the percentage of platelets with higher coagulation factor binding increases, FXa and thrombin generation increases. Specifically, the appearance of a subpopulation of platelets with higher coagulation factor binding occurs just before the burst of FXa and thrombin. However, despite maximal convulxin concentrations, only half of the platelets are in the subpopulation with increased coagulation factor binding. These findings indicate that platelets are heterogeneous in their ability to respond to procoagulant stimuli. This work adds to that reported previously in which platelets stimulated with 2 agonists were found to bind low and high levels of platelet FV.

Other previous work examining coagulation factor binding on the platelet surface has focused predominantly on thrombin-stimulated platelets. However, 1 report compares FVIII binding on thrombin-stimulated platelets to collagen

![Figure 3](https://example.com/f3.png)

**Figure 3.** Factors V, VIII, IX, and X demonstrate a similar pattern of binding. In representative model system experiments with 100 ng/ml of convulxin, platelet samples were obtained and surface-bound FV (A), FVIII (B), FIX (C), and FX (D) were detected as described in Material and Methods. The solid arrow points to the center of the population with increased fluorescence. The dashed arrow points at the population with lower fluorescence. The inset is the corresponding histogram plot. Line histograms represent platelet controls (A, C, and D, Unstimulated platelet control; B, Stimulated platelet control using mouse monoclonal IgG2a primary antibody). FSC indicates forward scatter.

![Figure 4](https://example.com/f4.png)

**Figure 4.** Development of subpopulations is dose dependent. Each point represents the percentage of platelets (plts) in the subpopulation with high coagulation factor binding at a specific convulxin (Cvx) concentration and plotted as a function of time. n=5 (FV); n=3 (FIX); n=4 (FX). Graphed points represent the mean±SE.
plus thrombin-stimulated platelets and found no difference in binding. The difference compared with results presented here may be related to technique (ie, radioligand binding versus flow cytometry). Radioligand binding measures the platelet population as a whole, and thus, 2 populations will be averaged into 1 and subtle differences may be missed. Other studies using flow cytometry to examine coagulation factor binding have used only thrombin or individual protease activated receptor agonists to stimulate platelets, which resulted in only 1 level of coagulation factor binding. This is consistent with our study showing that 2 populations, 1 with low levels of coagulation factor binding and 1 with high levels of coagulation factor binding, do not occur when platelets are stimulated with thrombin alone. Further evidence that the combination of collagen plus thrombin produces a unique platelet signal was reported by Leo et al. They demonstrated that thrombin generation on platelets stimulated by either thrombin or collagen alone is independent of the adaptor protein SLP-76, whereas thrombin generation on platelets stimulated by thrombin plus collagen is SLP-76 dependent.

At maximal convulxin concentrations, the percentage of platelets that expose PS, as measured by annexin V binding, correlates with the percentage of platelets with increased coagulation factor binding. This is consistent with the known role PS has in platelet procoagulant activity. However, the possibility that other parameters, in addition to early PS exposure, are important is raised by the discordance in the percentage of platelets with increased fluorescence at submaximal convulxin concentrations (Figure 6C). Platelets stimulated with either 20 ng/mL or 100 ng/mL developed a similar percentage of PS-positive platelets; however, 20 ng/mL led to >25% less FXa and thrombin generation, suggesting that PS is not the only parameter that contributes to thrombin generation. Rather than correlating with PS, upregulation of thrombin generation correlated with the proportion of platelets recruited into the subpopulation with increased coagulation factor binding.

In our study, only a minority of platelets bound annexin V. This is consistent with other reported studies detecting annexin V binding by flow cytometry, whereas confocal microscopy of bound platelets typically yields higher levels of annexin V binding. It is unclear whether this difference is a result of studying platelets that are bound versus platelets in suspension or reflects a difference in the sensitivity of flow cytometry compared with confocal microscopy.

Despite the documented importance of PS, there is additional evidence suggesting that other membrane components play an important role in the ability of the platelet membrane to support coagulation. There are 3 lines of evidence. First, when tenase and prothrombinase activity were compared in 17 healthy individuals, there was no correlation between tenase activity, prothrombinase activity, and PS exposure. Second, there is substantial evidence to support the role for specific platelet membrane–binding proteins for activated coagulation factors. Third, purified phospholipids cannot completely substitute for platelets in clotting reactions.

In addition to PS, some have postulated that serotonin may contribute to coagulation factor binding. It was demonstrated previously that α-granule contents are conjugated to serotonin. Subsequently, it was postulated that fibrinogen and thrombospondin act as serotonin binding sites to anchor serotonin derivatized–platelet FV on the surface of platelets stimulated by thrombin plus convulxin. However, unstimulated platelets also contained a serotonin-conjugated macromolecular fraction that increased only 1.8-fold after platelet stimulation with convulxin plus thrombin. Furthermore, we demonstrated that increased binding on the surface of thrombin plus convulxin-stimulated platelets is not limited to α-granule contents but includes factors VIII, IX, and X. It is unknown whether factors VIII, IX, and X also become derivatized with serotonin.
In summary, these results show: (1) increased binding of coagulation factors on a subpopulation of convulxin plus thrombin stimulated platelets is not limited to α-granule components; (2) the proportion of platelets in the subpopulation with increased coagulation factor binding is well correlated with and potentially causative of upregulated FXa and thrombin generation; and (3) the level of PS exposure, as opposed to the percentage of platelets with increased coagulation factor binding, did not correlate with increases in FXa and thrombin generation on platelets stimulated with convulxin plus thrombin. These studies add to a growing body of literature that the combination of collagen and thrombin provide a unique platelet stimulus compared with collagen or thrombin alone.

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