Activation-induced changes in platelet surface receptor expression and the contribution of the large-platelet subpopulation to activation

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Abstract

Objective: Platelet surface receptors are also present subcellularly in organelle membranes and can be expressed on the surface upon platelet activation. However, some receptors were reported to be decreased after activation. We analyzed the mechanism of activation-dependent expression for different receptors.

Methods: Flow cytometry using platelet-rich plasma or washed platelets was used to analyze receptor-expression changes after platelet activation by glycoprotein (GP) VI-specific agonists, crosslinked collagen-related peptide (CRP-XL) and convulxin (Cvx), and thrombin. Platelets prelabeled with fluorescent antibody specific for a receptor were allowed to adhere on immobilized collagen or fibrinogen and post-stained with antibody against the same receptor labeled with another fluorophore, allowing us to differentiate preexisting receptors from newly expressed receptors.

Results: Surface expression of \( \alpha_{IIb}\beta_{3} \) increased in CRP-XL-, Cvx-, or thrombin-stimulated platelets, but GPIb decreased due to shedding and internalization. Both total and dimeric GPVI increased in thrombin-induced platelets, but decreased in platelets stimulated by Cvx, as a result of internalization. The larger platelets showed a greater increase in surface receptor (\( \alpha_{2}\beta_{1}, \alpha_{IIb}\beta_{3}, \) GPVI, GPIb) expression upon activation compared to the smaller ones. Pre- and postlabeling with antibody specific for the same receptor, but conjugated with different fluorophores, allowed us to differentiate the receptors expressed on the surface of resting platelets from receptors newly exposed to the surface upon platelet activation.

Conclusions: Increased receptor expressions after activation are mainly manifested in the larger platelets. On platelets adhered on fibrinogen, the newly expressed receptors, especially GPVI, are localized in the lamellipodia of the spread platelets.

Keywords:

blood platelets, glycoprotein Ib, platelet activation, platelet glycoprotein GPIb-IIIa complex, platelet membrane glycoprotein VI, platelet membrane glycoproteins, receptors
1 | INTRODUCTION

Platelets are anucleate small blood cells, but they have several intracellular organelles and membrane systems whose localization and morphology are changed upon platelet activation by various stimulants. Activation transforms the smooth disc-like shape of resting platelets to a disturbed spherical shape with numerous filopodial extrusions and lamellipodia, accompanied by marked changes in subcellular organelle localization. Secretory dense granules and α-granules extrude their contents to the extracellular medium or through the inside space of the open canalicular system (OCS), and granule membranes fuse with the plasma or OCS membrane.1-4 Major receptor proteins contained in the OCS and α-granule membranes, including glycoprotein (GP) Ib and αIIbβ3,1,2 become exposed to the surface when their membranes fuse with the platelet plasma membrane. This may explain increased surface αIIbβ3 expression in activated platelets,1,2 but a decrease in surface GPIbα upon activation suggests that other mechanisms may be involved.

Platelets are essential for primary hemostasis since they adhere to subendothelial collagen exposed by vessel injury, become activated, aggregate, and form a thrombus to arrest bleeding. Hyperactive platelets, however, lead to formation of unwanted thrombi, which can detach and travel to distal areas, causing ischemic stroke or cardiovascular disease (CVD). Larger platelet size, measured as increased mean platelet volume (MPV), is a risk factor for cardiovascular disease CVD.7,8 MPV increases with age in mice, which might explain the increasing CVD risk in the elderly.9 Circumstantial evidence suggests that large platelets are more active, but there is yet no direct evidence for this and why this may be so.

The aim of the present study is to compare larger platelets with the whole platelet population in terms of their surface expressions of receptors involved in thrombus formation in response to platelet activation using a clinically available method, flow cytometry. In resting platelets, surface expressions of GPIb, αIIbβ3, α2p1, and GPVI were higher in the larger platelets, commensurate with their larger surface area. Expressions of αIIbβ3 and α2p1 were increased in activated platelets, but GPIb and GPVI decreased due to shedding, internalization, or both. Increased exposure of intracellular receptors upon activation was most prominent in the larger platelets. These results suggest that platelets are a heterogeneous population, not only with respect to size but importantly with respect to activity and that the large platelets are the main determinants of platelet activation and function.

2 | MATERIALS AND METHODS

2.1 | Materials

GPVI dimer-specific, noninhibitory 204-11 Fab10 was previously reported. Other mouse monoclonal antibodies: 1G511 (anti-pan GPVI; Biocytex, Marseille, France), anti-GPIb antibodies AK2 (Novus Biologicals, Littleton, CO, USA) and clone 486805 (R&D Systems, Minneapolis, MN, USA); anti-αIIbβ3 (M148; Abcam, Cambridge, UK); anti-α2 (Gi9; Abcam), anti-CD62P (AK6; Abcam); fluorescein isothiocyanate–conjugated antiactivated integrin αIIbβ3 procaspase-activating compound-1 (PAC-1; BD Biosciences, San Jose, CA, USA). For some experiments, antibodies were labeled with Alexa Fluor-488 or -647 by an Invitrogen labeling kit (Thermo Fisher Scientific, Waltham, MA, USA). Convulxin (Cvx)12 and crosslinked collagen-related peptide (CRP-XL)13 were previously reported.

2.2 | Flow cytometry to measure surface receptor expression

Platelet-rich plasma (PRP), prepared from acid-citrate-dextrose-anticoagulated blood from healthy volunteers,10 was diluted 4-fold with modified HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)–Tyrode’s buffer (HT: 136 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH2PO4, 5.5 mmol/L glucose, 5 mmol/L HEPES, pH 7.4) or washed platelets (5 × 10^7 cells/mL, HT) were prepared as before.10 Platelets were activated by CRP-XL (5 μg/mL), Cvx (0.5 μg/mL), or thrombin (0.2 U/mL) for 4 minutes at 37°C. Samples were prepared for flow cytometry (Accuri C6, BD Biosciences, San Jose, CA, USA),10 with receptor-specific primary antibody and Alexa Fluor-488–conjugated anti-mouse Fab (50 μg/mL; Jackson Immuno-Research Laboratories, West Grove, PA, USA) as secondary antibody. The negative control was isotype control IgG or control mouse Fab. Platelet receptor expression was expressed as median fluorescence intensity (MFI) obtained for binding of receptor-specific antibody.

In each experiment, the whole platelet population (P1) of a donor’s platelets was divided into 2 subpopulations according to size by gating in the forward scatter/side scatter (FSC/SSC) plot: The larger half was defined as P2 (larger platelets) and the smaller half was defined as P3 (smaller platelets; Figure 1A shows an example). Numbers of platelets in each subpopulation are calculated as percentage of total events.
Washed platelets (200 μL, 5 x 10^8 cells/mL) were activated with CRP-XL (5 μg/mL), Cvx (0.5 μg/mL), or thrombin (0.5 U/mL) for 5 minutes at 37°C followed by 30 minutes at room temperature. Suspensions were centrifuged to separate supernatants and platelet pellets, which were dissolved with 200 μL 6 mol/L urea/1% SDS. Non-reducing Formaldehyde/glutaraldehyde solution was used, as it has less effect on cell membrane phospholipid distribution and better maintained platelet antibody binding.

2.4 Immunoblotting

Washed platelets (200 μL, 10^9 cells/mL) were activated with CRP-XL (5 μg/mL), Cvx (0.5 μg/mL), or thrombin (0.5 U/mL) for 5 minutes at 37°C followed by 30 minutes at room temperature. Suspensions were centrifuged to separate supernatants and platelet pellets, which were dissolved with 200 μL 6 mol/L urea/1% SDS. Non-reducing
Laemmlı’s SDS sample buffer was added to each sample and heated at 100°C for 1 minute. Each sample (5 μL for the pellet, 15 μL for the supernatant) was electrophoresed on a 3% to 12% acrylamide SDS gel, immunoblotted with 1G5 or clone 486805 and analyzed by the Odyssey CLx (LI-COR Biosciences, Lincoln, NE, USA).

2.6 | Statistical analysis

Paired Student’s t-test (Prism v8.9, GraphPad Software, La Jolla, CA, USA) was applied to compare differences between activated platelets and their corresponding resting platelets.

3 | RESULTS

3.1 | Large platelets have higher levels of surface receptor expression

From the SSC/FSC scatter plot, resting platelets of each donor were gated into the larger platelets (P2) of the total population (P1; Figure 1A, resting platelets). Because each donor’s platelets showed a different size distribution, gating was tailored to each donor’s FSC/SSC plot. P2 of resting platelets showed 1.5-fold higher binding of anti-GPVI dimer (204-11 Fab) than P1. CRP-XL activation slightly changed the scatter plot (Figure 1A, activated) and P2, defined by the same gating as used for resting platelets, exhibited 1.9-fold higher 204-11 binding than P1.

Thus, we determined if the P2 population also showed higher numbers of other platelet surface receptors, using specific primary antibodies: total GPVI (1G5), GPIb (AK2), αIIbβ3 (M148), and α2β1 (G9), using isotype IgG (control) to determine background fluorescence (black bar [control] in Figure 1B) under each condition (resting, CRP, Cvx, thrombin).

P2/P1 of control resting platelets was about 130%, showing that P2 bound more control IgG than P1, indicating that P2 platelets have a larger cell surface area. For all receptors, except for total GPVI, P2/P1 ratios were similar to that of the control. The higher P2/P1 for total GPVI, P2/P1 ratios were similar to that of the control. For CRP: dGPVI (P < .0001, n = 7), washed platelets, P < .0004, n = 5) is due to the ability of 1G5 to activate platelets.11

For CRP added with agonist, only some of the P2/P1 data were significantly higher than the respective resting control: for CRP: dGPVI (P < .0001, n = 5); for Cvx: dGPVI (P < .007, n = 5), α2β1 (P < .028, n = 4), GPIb (P < .020, n = 4), αIIbβ3 (P < .019, n = 5); for thrombin: dGPVI (P < .025, n = 4). Although the P2/P1 for each receptor was higher than the ratio in the resting platelets for each donor, there was a high variability among values for different donors. The high fibrinogen level in PRP may have had some effect on antibody binding in the activated platelets, so we measured P2/P1 in washed platelets for most of our other experiments.

In contrast to PRP, activated washed platelets show higher P2/P1 for the examined receptors, except for dGPVI and GPIb in Cvx- and CRP-induced platelets, respectively. Platelets are a heterogeneous population with respect to surface receptor expression in both the resting state and the activated state. The washed-platelet results suggest that among the activated platelets, the larger platelet population showed the highest increase in surface receptor expression.
3.2 | Receptor expression after platelet activation

Surface receptor expressions in platelets activated by CRP-XL, Cvx, and thrombin were determined and compared to expression of each receptor in resting platelets (Figure 2). For the total platelet population (P1) of PRP (Figure 2A), both total GPVI and GPVI dimer very markedly decreased after stimulation by Cvx (GPVI dimer: P = .03, n = 5; total GPVI: P = .000, n = 4), but no significant changes in expression of either in response to CRP-XL or thrombin.

\( \alpha_{2}\beta_{1} \) and \( \alpha_{IIb}\beta_{3} \) expressions tended to increase after activation but

**FIGURE 2** Changes of receptor expression after platelet activation. Receptor expressions are measured by flow cytometry using different agonists and receptor-specific antibodies. Receptor expressions in platelets activated by CRP, convulxin (Cvx), or thrombin (Thr) are compared to the respective value in resting platelets (rest). A, P1 (total platelet population) of platelet-rich plasma (PRP); B, P2 subpopulation of PRP (A); C, P1 of washed platelets; D, P2 subpopulation of washed platelets (C). Each bar in the graphs and their associated error bar show the mean ± SD of the indicated number of determinations. *P ≤ .05, **P ≤ .005, ***P ≤ .0005, ****P < .0001. Detailed descriptions of the statistical analyses are given in the Results section.
none of the agonists changed dimer, but not total GPVI, is slightly increased by thrombin ($P \leq .0001$, $n = 7$; total GPVI: $P \leq .0001$, $n = 6$). GPVI dimer, but not total GPVI, is increased by thrombin ($P = .0158$, $n = 13$). None of the agonists changed $\alpha 2\beta 1$ expression. $\alpha llb\beta 3$ expression tended to increase after activation (CRP-XL: $P = .03$, $n = 7$; Cvx: $P = .08$, $n = 6$; thrombin: $P = .003$, $n = 11$). Surface GPIb expression, however, strongly decreased in response to each agonist: CRP-XL ($P = .02$, $n = 7$), Cvx ($P < .0001$, $n = 7$), and thrombin ($P < .0001$, $n = 7$).

If we compare the differences in receptor expression in the P2 population of PRP and washed platelets (Figure 2B,D), which has higher expression of each receptor than the total population (P1), the changes in surface receptor expression upon activation are even more dramatic.

To confirm these results, we determined receptor expression in stimulated washed platelets that were subsequently fixed with formaldehyde/glutaraldehyde; 204-11 Fab was not used in these experiments, as it does not react with fixed platelets. These activated and fixed platelets showed similar receptor changes; Cvx, especially, strongly reduced GPVI expression (Figure 3A).

### 3.3 Time course and agonist-concentration dependency of receptor expression in activated platelets

Flow cytometry of activated platelets (Figure 3B) showed that total GPVI expression is decreased by Cvx but increased by thrombin in a similar time scale: changes were evident by 0.5 minutes in some experiments, well detectable by 1 minute, and maximal before 5 minutes. Thus, the receptor-expression changes are rapid, on the time scale of platelet aggregation.

Thrombin-induced changes in expressions of GPVI dimer, P-selectin, and activated $\alpha llb\beta 3$ in P1-P3 were measured using 204-11 Fab, anti-CD62P, and PAC-1 antibodies, respectively. All 3 receptors increased dose dependently with thrombin, but increases in P2 were much greater than those in P1 and P3 (Figure 4A). Increase of GPVI dimer expression in P3 platelets was much less, and expressions of CD62P and active $\alpha llb\beta 3$ in P2 were more than 2-fold those in P3. Since anti-CD62P and PAC-1 bind only to activated platelets, these results indicate that the
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**Figure 4** Concentration-dependent receptor expression of activation-dependent antigens P-selectin and activated αIIbβ3. All data shown in this figure are representative of 3 experiments, giving similar results, using platelets from different donors. A, Washed platelets were activated by increasing thrombin concentrations and expressions of GPVI dimer, P-selectin, and activated αIIbβ3 were measured using antibodies 204-11 Fab, AK6, and PAC-1, respectively. Expressions of receptors in whole platelets (P1), larger platelets (P2), and smaller platelets (P3) were measured. There are preferential and higher expressions of P-selectin and activated αIIbβ3 (PAC-1 binding) in P2 platelets compared to those in P1 and P3. B, Distribution of platelets to the large and small platelet populations. In the same experiments as panel A, the number of platelets in P1-P3 are calculated as a percentage of events from the flow cytometry data. All the data were determined twice by flow cytometer, and their mean values were plotted with standard deviation.

major contributor to the measured increase in activation marker expression in the total population of activated platelets is the P2 population. Increases of receptor expression observed at 0.005-0.01 units/mL thrombin were consistent with the thrombin sensitivity of a typical platelet aggregation assay.

Figure 4B shows the number of platelets distributed to P2 and P3 in terms of percentage of total events. If smaller platelets become larger upon activation (eg, platelet dimerization), the number in P2 should increase, while those in P3 should decrease. However, the percentage of total events increased slightly in P3 and decreased slightly in P2, suggesting that the platelets originally distributed in P2 would have increased their surface receptor expression. The slight decrease in the percentage of total events in P2 would be due to formation of aggregates.

### 3.4 Analysis of GPIb and GPVI shedding

Platelet activation by CRP-XL, Cvx, or thrombin decreased surface GPIb expression, and activation by Cvx strongly decreased surface GPVI (Figures 2A-D and 3A). Two mechanisms may cause these decreases—shedding or internalization. Shedding was assessed by activating washed platelets with CRP-XL, Cvx, or thrombin under the same conditions as those used for flow cytometry; platelet pellet and supernatant were
isolated and analyzed by western blotting. Western blotting with anti-GPIb (Figure 5A-C) detected low-molecular-weight GPIb (glycocalcin) in the platelet supernatant; its amount increased after platelet activation by any agonist. The pellet-fraction lane contained one-third the amount of loaded protein compared to the supernatant lane, so most of the GPIb is still in the platelets. However, the supernatant of resting or activated platelets contained similar amounts of faint bands of GPVI, with the same mobility as native GPVI and no degraded (shed) GPVI.16

3.5 | Internalization of GPIb and GPVI in activated platelets

Platelets, prelabeled with Alexa Fluor-488-conjugated antibodies against GPIb or GPVI, were activated by Cvx or thrombin, fixed, and immobilized on poly-L-Lys-coated dishes. The immobilized platelets were stained with Alexa Fluor-647-conjugated anti-αIIbβ3 antibody to clearly visualize the cell membrane. Cvx-activated platelets

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**FIGURE 5** Immunoblotting analyses to determine if glycoprotein (GP) Ib and GPVI shedding occurs. Washed platelets were activated under the same conditions as used for the receptor expression analysis by flow cytometry and separated into the supernatant and pellet (platelets) fractions by centrifugation. Pellets are dissolved by SDS/urea so that they were the same volume as the supernatant samples and analyzed by immunoblotting. A, The immunoblotting of platelet pellets and (B) shows the results from the supernatants. Lane 1 (resting platelets), Lane 2 (crosslinked collagen-related peptide [CRP-XL]-activated platelets), Lane 3 (convulxin [Cvx]-activated platelets), Lane 4 (thrombin-activated platelets) P (control platelets). C, The results from quantitation of these bands. Two SDS/immunoblotting analyses were done for the experiment and the graph indicates the means of band densities with standard deviation. *P ≤ .05, **P ≤ .005. These data indicate the shedding of GPIb from the activated platelets, but GPVI is not shed.
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stained for GPVI show strong granule-like fluorescence inside the platelets, while thrombin-activated or resting platelets show only diffuse staining over the whole cell (Figure 6A). Cvx- and thrombin-activated platelets stained for GPIb show similar strong staining inside the cells, and resting platelets show diffuse staining over the whole cell (Figure 6B). These results show that GPVI is internalized in Cvx-activated platelets and GPIb is internalized in Cvx- and thrombin-activated platelets are internalized. The images are typical of the results from four experiments; in each experiment, 5-8 fields of the sample were imaged for each condition.

**3.6 Exposure of intracellular receptors on platelet spreading**

Our flow cytometry data suggest that additional receptors are newly exposed on the platelet surface after platelet activation. Preexisting surface receptors were differentiated from newly exposed ones by allowing platelets labeled with Alexa Fluor-488–conjugated receptor-specific antibody to adhere and spread on immobilized collagen or fibrinogen and then post-staining with the same antibody conjugated with Alexa Fluor-647. For GPVI dimers, platelets were prestained with Alexa Fluor-488 204-11 Fab and post-stained with AlexaFluor-647-1G5, since 204-11 does not react with fixed platelets. Anti–GPVI dimer prelabeled platelets spread well on immobilized fibrinogen; prelabeled GPVI is localized on the cell body, but post-stained GPVI appears in the membrane, at the edges of the lamellipodia, of spread cells (Figure 8A). The GPIb-prestained cells are less spread but show differential staining similar to GPVI.

Platelets adhered to collagen (Figure 8B) have GPVI clusters along the collagen fibers, as previously reported, with prestained...
and post-stained GPVI colocalized. This suggests that both preexisting and newly exposed GPVI moved to collagen’s platelet-binding sites. Prestained GPIb is concentrated on the cell body, whereas post-stained GPIb is diffuse and mostly localized to the lamellipodia of the spread platelet; this is different from the platelets adhered to fibrinogen, as collagen strongly activated the platelets. However, prestained and post-stained αIIbβ3 are mainly colocalized, but strong prestaining is observed in the cell body. These results show that when platelets spread and a receptor does not interact with substrate, preexisting surface receptors reside at the cell body, while newly exposed receptors move to the edges of the lamellipodia in the spread platelet. If receptors react with substrate, preexisting and newly exposed receptors move to the binding site. Such conditions are typically present for GPVI on platelets adhering on fibrinogen and collagen.

**FIGURE 7** Analysis of glycoprotein (GP) VI internalization using TCEP (tris[2-carboxyethyl]phosphine). Platelets were prelabeled with 1G5-SS-biotin and Alexa Fluor-647–streptavidin and activated with convulxin (Cvx; 1 µg/mL) or thrombin (0.2 unit/mL). The platelets were reacted with 28 mmol/L TCEP in the presence of 5 mmol/L ethylenediaminetetraacetic acid for 10 min following with washing with citrate-buffered saline. The platelets were resuspended in modified HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)-Tyrode’s buffer, fixed with 1% paraformaldehyde, washed, plated on poly-Lys coated MatTek dishes, and analyzed with confocal microscopy. TCEP removes all the fluorescence in the resting or thrombin-treated platelets, indicating no internalization (ie, fluorescence confined to the platelet surface). However, the fluorescence of the Cvx-treated platelets remains intact, confirming internalization. The right-hand panel shows a schematic representation of how this experiment. This experiment was performed twice, with each giving similar results; in each experiment, 5-8 fields of the sample were imaged for each condition.

**DISCUSSION**

This study demonstrates that platelet activation changes the levels of major glycoprotein receptors, either decreasing or increasing their surface expressions depending on both the receptor and the agonist. PRP, washed platelets, and fixed platelets (Figures 2 and 3A) all showed increased expression of surface αIbβ2 upon activation by CRP-XL, Cvx, or thrombin, but surface GPIb is decreased. Total and dimeric GPVI expressions markedly decreased after Cvx-induced activation, but the other agonists induced similar or increased expression. 1G5 can activate platelets, so total GPVI expression in CRP-XL- or thrombin-induced determined with this antibody did not differ from the resting level. These results suggest that platelet activation increases surface expression of some receptors, like the integrins, but decreases expressions of GPIb and GPVI through other mechanisms.

GPIb and GPVI are susceptible to cleavage by metalloproteinases ADAM17 and ADAM10, respectively, after platelet activation, so their soluble forms, glycocalicin and 55-KD GPVI, respectively, may be released into the medium. Under the conditions of our flow cytometry assays, GPIb, as glycocalicin, was detectable in the supernatant, although most of it remained on the platelets (Figure 5), but almost no soluble GPVI was in the supernatant. No exogenous Ca2+ was added to our flow cytometry samples, so shedding of either glycoprotein should be minimized in our assay condition. Cvx-activated platelets have strong patch-like staining inside the cell, consistent with internalization of prestained GPIV (Figure 6). This was confirmed by our results showing that only the fluorescence of Cvx-stimulated 1G5-SS-biotin-streptavidin prelabeled platelets was resistant to cleavage by TCEP, whereas that of thrombin or resting platelets was not (Figure 7). Cvx- or thrombin-activated GPIb-prestained platelets showed similar internal fluorescent aggregates. Such GPIb internalization was previously observed, but this is the first report of Cvx-specific GPIV internalization. Platelets produce microparticles containing plasma membrane after activation, which could reduce the receptor level in the remaining plasma membrane, but expressions of other receptors increased under the
same conditions, so this would contribute little to decreases in GPIb and GPVI.

Increased receptor expression after activation could be explained by the merging of OCS and granule membranes with the surface membrane, which translocate intracellular receptors to the platelet surface. Thus, we can reasonably hypothesize that GPIb and GPVI expressions would first increase upon platelet activation and then decrease due to internalization, shedding, or both. Thrombin-induced increase in surface GPVI and its Cvx-induced decrease occur on similar time scales (Figure 3B), so GPVI internalization by Cvx must be rapid if intracellular GPVI is first cell-surface–expressed and then internalized. The observed differential expressions of surface GPVI induced by thrombin and Cvx raises several possibilities about the internalization process: GPVI expression may be increased by thrombin activation through a mechanism similar to that for the increase in αIIbβ3, while direct binding of Cvx to GPVI may induce GPVI internalization. Our results suggest that large-cluster formation may be required to induce strong internalization since CRP-XL produces little internalization. Cvx is a tetramer of heterodimers, and while CRP-XL is a crosslinked molecule, it is composed of single triple-helical peptides, which do not bind to GPVI. Thus, only Cvx would form dense GPVI clusters large enough to induce internalization.

In contrast to GPVI, many agonists have been reported to decrease GPIb expression, suggesting platelet activation in general would cause GPIb internalization. Electron micrographs suggested cytoskeletal involvement in GPIb internalization. However, αIIbβ3 binds to the cytoskeleton as well and is clustered after activation but does not internalize, so the precise mechanism of internalization remains inconclusive.

Using prestaining and post-staining with receptor-specific antibodies labeled with fluorophores that emit fluorescence of different wavelengths, we differentiated preexisting surface receptors in resting platelets from intracellular receptors newly exposed upon platelet activation. Prestained GPVI on fibrinogen-adhered platelets localizes to the cell body in the center of the spread cell, while prestained receptors localized to the cell body. On the other hand, both pre- and post-stained GPVI is essentially all confined to the clusters of GPVI adhered to the collagen fibers. This figure gives representative results from 1 of 3 experiments, all giving similar results; in each experiment, 5-8 fields of the sample were imaged for each condition.
post-stained GPVI (red) would move to the region of the membrane in contact with collagen fibers and become colocalized as membrane clusters on the fibers (yellow; Figure 8B). Distribution of prestained and post-stained GP Ib on platelets adhered to immobilized fibrinogen and collagen are essentially like that of GPVI on platelets adhered on fibrinogen, but since these platelets are not fully spread like the GPVI-stained platelets, many of the spread cells show only a small separation of post- and prestaining. These results suggest that intracellular receptors become exposed in the spread membrane (lamellipodia) of adhered platelets and localize separately from the originally expressed surface receptors when they do not interact with substrate.

The P2/P1 ratio is increased upon activation, consistent with the larger platelets expressing more receptors on their surface compared to the smaller platelets. These results suggest that larger platelets are more activated than smaller platelets, as reported before.25–28 Upon thrombin activation, P-selectin and PAC-1 expressions in the larger platelets are increased much more than in the total and smaller platelets (Figure 4A). At higher thrombin concentrations, P-selectin and PAC-1 expressions in P2 became about 2-fold and 3-fold higher than that of P1 and P3, respectively. Since gating by P-selectin and PAC-1 expressions in P2 became about 2-fold and 3-fold higher than that of P1 and P3, respectively. Since gating by P-selectin and PAC-1 expressions in P2 became about 2-fold and 3-fold higher than that of P1 and P3, respectively. Since gating by P-selectin and PAC-1 expressions in P2, these results suggest that larger platelets expressing more receptors on their surface compared to the smaller platelets, these results suggest that activation involving integrin activation and secretion would mainly occur in the larger platelet population.

The large-platelet fraction isolated by differential centrifugation or flow cytometry was reported to be more active,25–27 and a relationship between activity and platelet size was reported.28 Although young platelets were thought to be large and more active, platelet size and age were indicated to independently affect platelet function.29 30 Heterogeneity of platelet size was suggested to come from the heterogeneity of megakaryocytes31 and activation of the mechanistic target of rapamycin complex1 in megakaryocytes may contribute to elevated platelet volume,3 suggesting that megakaryocytes would produce different sizes of platelets and that the larger platelet subpopulation would have higher activities. Our data indicate that the larger subpopulation of activated platelets showed a higher level of agonist-induced receptor expression compared to the total platelet population, supporting the previous reports describing that larger platelets have higher activities. In this context, it is very interesting that a larger MPV has been indicated to be a risk factor for cardiovascular disease in both men and women7,8,32 or just in men.33 Our results importantly suggest that the large-platelet subpopulation would be the main contributor to higher platelet activity and our method, analyzing the receptor expression of resting and activated platelets by flow cytometry, would be a good method to analyze the risk factor of having larger platelets.

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RELATIONSHIP DISCLOSURE

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AUTHOR CONTRIBUTIONS

MM designed and performed experiments, analyzed data, made figures, and wrote the manuscript. RWF critically read the manuscript and discussed the results with the other co-authors. SMJ performed the confocal imaging, designed experiments, analyzed data, made figures, and wrote the manuscript.

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