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Differential Sensitivity of Various Markers of Platelet Activation with Adenosine Diphosphate

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Abstract
A number of techniques have been available to assess platelet activation, but their relative sensitivity is unknown and their usage is variable and not based on any rational criteria. Here, we compared the ability of several techniques based on morphological and biochemical markers to detect the first signs of ADP-induced platelet activation. Scanning electron microscopy of platelets was performed in parallel with flow cytometry to quantify the surface expression of P-selectin (marked by labeled anti-CD62P antibodies), active αIIbβ3-intergrin (assessed by the binding of labeled fibrinogen), and phosphatidylserine (assessed by the binding of labeled Annexin V). When expressed as a fraction of activated platelets, shape changes were the most sensitive to a low ADP concentration compared to the biochemical markers in the following order of sensitivity: morphological changes>fibrinogen binding capacity>P-selectin expression> phosphatidylserine exposure. These results suggest the greater sensitivity of platelet microscopy and the importance of its combination with flow cytometry used to detect surface expression of the molecular markers of platelet activation.

Keywords Platelet activation · Scanning electron microscopy · Flow cytometry

1 Introduction

Thrombotic disorders, such as myocardial infarction, ischemic stroke, and venous thromboembolism, are among the leading causes of death worldwide [1]. Platelet activation is one of the main mechanisms in the pathogenesis of thrombotic disorders [2–4]. Platelets can be activated by (patho)physiological agonists, such as collagen, adenosine diphosphate (ADP), thromboxane A_2, or thrombin [5]. ADP is an important platelet stimulant that works through purine receptors and induces major reactions associated with platelet activation that include the loss of platelet discoid shape and formation of pseudopodia or filopodia [5, 6], release and surface expression of pro-coagulant and adhesive substances, such as phopsphatidylserine, P-selectin and soluble CD-40L, activated platelet integrins [7], and generation of contractile forces that cause shrinkage of blood clots and thrombi [8].

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Platelet activation can be detected and measured by various methods. Soluble products specifically produced by stimulated platelets like PF4 or βTG [9] or soluble P-selectin [10] can be determined in a radioimmunoassay and enzyme-linked immunosorbent assays to characterize platelet activation. Alternatively, platelet activation can be assessed by flow cytometry based on platelet-specific membrane-associated markers. The most common surface markers used in flow cytometric analysis of stimulated platelets are the activated integrin αIIbβ3 capable of binding fibrinogen [11], procoagulant phosphatidylserine determined by the ability to bind annexin V [12], and the membrane-bound P-selectin (CD62p) expressed on the plasma membrane from the secretory α-granules [13]. However, the surface expression of P-selectin by activated platelets may be reduced [14, 15] because the membrane P-selectin is cleaved into soluble P-selectin [16]. The expression of phosphatidylserine or active αIIbβ3 has also been reported to occur only in a subset of activated platelets and with a differential response to various agonists [9]. Therefore, using a combination of various platelet activation markers is suggested to detect platelet activation.

Light transmission aggregometry remains the reference method for measurement of platelet function since it was first described by Born [17], but it is performed after robust platelet activation with a chemical stimulant and

does not allow for assessment of the background or initial threshold platelet activation in the absence or at very low doses of an activator. A direct approach to study platelet activation is using various types of light and electron microcopy, because platelet activation is followed by characteristic shape changes [6]. In our recent study, continuous platelet activation in the blood of patients with a prothrombotic status was revealed by scanning electron microscopy at the same time that flow cytometry assays did not show any signs of activation [2], suggesting that this discrepancy may reflect a differential sensitivity of morphological versus biochemical methods. Therefore, with a variety of techniques used to assess the extent of platelet activation, it remains unclear which are more sensitive and what is the threshold that determines the initial platelet activation.

In this study, we compared the sensitivity of four markers of platelet activation, one based on morphological and three on biochemical changes, by tracking them in parallel to detect human platelets activated by increasing concentrations of ADP. The results show that scanning electron microscopy reflects platelets activation at the lowest ADP concentrations, at which the flow cytometry assays remain unresponsive within the same time frame. Among the molecular markers of platelet activation studied with flow cytometry, the fibrinogen-binding capacity of the integrin αIIbβ3 is more sensitive than surface expression of P-selectin, while exposure of phosphatidylserine is the least informative.

2 Materials and Methods

2.1 Blood Collection and Processing

Blood was drawn by venipuncture from 4 healthy volunteers, not taking any medications known to affect platelet function for at least 14 days. Informed consent was obtained in accordance with a protocol approved by the Ethical Committee of Kazan Federal University. All procedures were carried out in accordance with the approved guidelines. To obtain platelet-rich plasma, the whole blood stabilized with 3.8% trisodium citrate (9:1 *v*/*v*) was centrifuged at 200*g* at 25 °C for 10 min within 30 min after blood withdrawal.

2.2 Platelet Activation

Platelet-rich plasma was diluted with Tyrode's buffer (4 mM HEPES, 135 mM NaCl, 2.7 mM KCl, 2.4 mM $MgCl_2$, 5.6 mM D-glucose, 3.3 mM NaH_2PO_4, 0.35 mg/ml bovine serum albumin, pH 7.4) to a final platelet concentration of 8×10^6/ml for flow cytometry or 20×10^6/ml for electron microscopy. Platelet count was performed in a hemocytometer with a 400× magnification. Platelets were activated by adenosine-diphosphate (ADP) for 5 min at room temperature at the following final concentrations: 0.05 μM, 0.1 μM, 0.25 μM, 0.5 μM, 1 μM, and 2 μM.

2.3 Monitoring Platelet Activation Using Flow Cytometry

Platelets from 4 healthy donors were stimulated by ADP and analyzed using flow cytometry. The extent of platelet activation was evaluated using flow cytometry by quantifying the expression of P-selectin, the binding capability of fibrinogen (activation of the integrin αIIbβ3) and the exposure of phosphatidylserine. Platelets (400,000 in 50 μl), untreated or treated with ADP, were incubated with one of the following fluorescent markers: (i) 1.5 μl anti-human CD62P R-phycoerythrin-labeled murine antibodies (BD Biosciences, USA) to assess expression of P-selectin; (ii) Alexa fluor 488-labeled human fibrinogen (5 μg/ml final concentration) (ThermoFisher Scientific, USA) to determine the fibrinogen-binding capacity; (iii) 1.5 μl FITC-labeled Annexin V (BioLegend, USA), diluted in 200 μl of calcium containing Hepes buffer (10 mM Hepes, 0.14 M NaCl, 2.5 mM $CaCl_2$), to measure the exposure of phosphatidylserine. Flow cytometry was carried out using the flow cytometer FacsCalibur connected to a computer supported with BD CellQuest™ software (BD Biosciences, USA). Platelets were gated based on the size and granularity using light forward scatter (LFS) and side scatter (LSS) channels. A total of 5000 cell signals were collected in each sample. A channel with the green filter was used to detect emission from Alexa fluor 488-labeled fibrinogen and FITC-labeled annexin-V, while a channel with the yellow filter was used for anti-CD62P PE-labeled antibodies. The data were analyzed using a FlowJo X program.

2.4 Monitoring Platelet Shape Changes Using Scanning Electron Microscopy

There were 1,000,000 platelets in 50 μl of diluted platelet-rich plasma (untreated or ADP-treated) fixed in glutaraldehyde at a final concentration of 2% for 90 min at room temperature. Fixed platelets were transferred on a carbon filter with a 0.4-μm pore size, then settled by centrifugation at 150 g for 7 min. Centrifuged samples were washed three times for 5 min by phosphate-buffered saline, then dehydrated by ascending concentrations of ethanol up to 100 vol%. Dehydrated samples were dried overnight with hexamethyldisilazane, and then coated by a thin layer of gold-palladium using a sputter coater (Quorum Q, 150 T ES, Quorum Technologies, UK). Micrographs of platelets were taken using a

scanning electron microscope Quanta FEI 250 (FEI, USA). Ten images containing 1355 unstimulated platelets and 72 images containing 6290 ADP-stimulated platelets from 2 independent donors were obtained and analyzed in parallel.

2.5 Statistical Analyses

Statistical analysis was carried out using GraphPad Prism 7.0. Significance of the different extent of platelets activation was analyzed using a chi-square test with $\alpha = 0.05$ for categorical data or with a paired t tests for numerical data (95% significance level, $p < 0.05$). Results are presented as mean ± SEM unless otherwise indicated.

3 Results

3.1 Morphological Changes in Platelets as a Function of ADP Concentration

Scanning electron micrographs of platelets showed that resting platelets had a discoid shape and smooth surface, while activated platelets demonstrated characteristic morphological changes with a loss of the discoid shape and formation of protrusions of the plasma membrane named pseudopods or filopodia (Fig. 1). To quantify the incidence of the observed morphological changes, scanning electron micrographs were analyzed to calculate percentage of platelets with altered morphology (activated platelets). Unstimulated platelet samples (total number of platelets analyzed $n = 1355$) showed 18% of individual platelets with more or less pronounced signs of activation. After stimulation with 0.05 μM ADP, the portion of activated platelets was significantly higher compared to the unstimulated cells (36%, $n = 988$, $p < 0.001$). As the concentration of ADP increased, the fraction of morphologically activated platelets also increased in a dose-dependent manner, reaching a plateau at 1 μM ADP (Table 1, Fig. 2).

3.2 Expression of P-Selectin, Fibrinogen-Binding Capacity, and Phosphatidylserine Exposure as a Function of ADP Concentration

In parallel with morphological changes, the extent of platelet activation was characterized biochemically based on the expression of P-selectin, fibrinogen binding capability, and exposure of phosphatidylserine. Unlike the shape changes, no initial activation of unstimulated platelets was detected by flow cytometry (Table 1), expression of the biochemical markers appeared and increased only in the presence of ADP in a dose-dependent manner. The fibrinogen-binding capacity of platelets increased significantly over the baseline at 0.05 μM ADP and ascended continuously with ADP concentrations up to 2 μM ADP. In contrast to the fibrinogen-binding capability, the expression of P-selectin over baseline started at the ADP concentration 0.05 μM, grew slowly until 0.5 μM ADP, but increased dramatically 15-fold after 0.5 μM ADP (Table 1, Fig. 2). Unlike the fibrinogen-binding activity and P-selectin expression, the effect of ADP on phosphatidylserine exposure detected using annexin V was not significant. At 2 μM of ADP, the percent of activated platelets detected by phosphatidylserine exposure was only 1.7 ± 0.1%, while it was 50 ± 2% by the fibrinogen-binding assay and 29 ± 6% by the P-selectin expression (Table 1, Fig. 2).

3.3 Comparison of the Biochemical and Morphological Markers of Platelet Activation

To compare directly the sensitivity of various markers of platelet activation, a fraction of activated platelets from total analyzed platelets detected by different methods was normalized by the initial activation without ADP using the following formula: $X_{normalized} = ((X\text{-}X_{min})/(X_{max}\text{-}X_{min})) \times X_{max}$ and plotted together as a function of ADP concentration (Fig. 2). This normalization was necessary to correct for the morphologically revealed background activation of platelets in the absence of exogenous ADP. At all ADP concentrations used, the fractions of activated cells detected by morphological changes were significantly higher compared to the results of

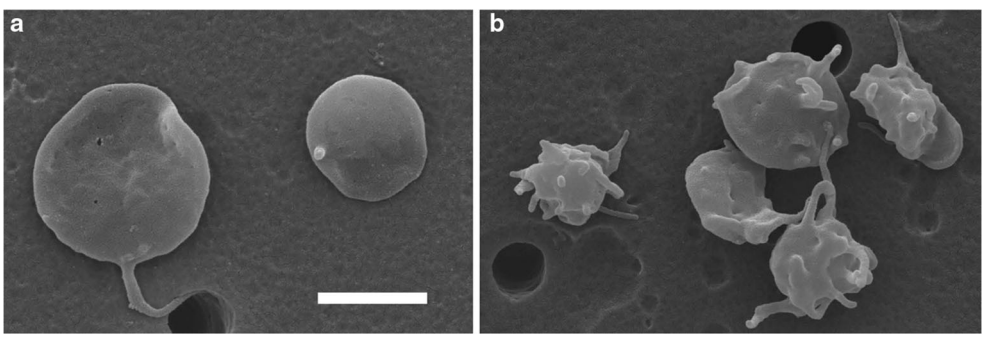

Fig. 1 Representative scanning electron micrographs of quiescent (**a**) and activated platelets (**b**), showing the loss of discoid shape and formation of pseudopods/filopodia in activated platelets. Magnification bar = 2 μm

Table 1 Fraction of activated platelets (%) detected by scanning electron microscopy and flow cytometry at various concentrations of ADP

Markers of platelet activation	ADP concentration, μM						
	0	0.05	0.1	0.25	0.5	1	2
Morphological changes	18* ($n = 1355$)	36* ($n = 988$)	46* ($n = 796$)	65* ($n = 1049$)	75* ($n = 2531$)	84* ($n = 311$)	85 ($n = 615$)
Fibrinogen-binding capacity	$0.7 \pm 0.1^{\S}$	$1.8 \pm 0.3^{\S}$	$3.9 \pm 0.4^{\S}$	$8.1 \pm 1.7^{\S}$	$18.2 \pm 2.2^{\S}$	$32.0 \pm 1.3^{\S}$	$49.7 \pm 2.1^{\S}$
P-selectin expression	0	$0.1 \pm 0.0^{\S}$	$0.7 \pm 0.1^{\S}$	0.9 ± 0.1	$3.3 \pm 0.5^{\S}$	$31.9 \pm 5.8^{\S}$	$43.2 \pm 8.1^{\S}$
Phosphatidylserine exposure	$0^{\S}$	$0.7 \pm 0.0^{\S}$	1.0 ± 0.2	0.9 ± 0.2	0.8 ± 0.2	$0.9 \pm 0.2^{\S}$	$1.7 \pm 0.1^{\S}$

*Statistical significance ($p < 0.05$, chi-squared test) of the differences in percentages of activated platelets between two successive ADP concentrations determined with scanning electron microscopy. In parenthesis, the number of individual platelets analyzed is shown (n) from at least 10 randomly selected micrographs of unstimulated platelets and 10 randomly selected micrographs of ADP-stimulated platelets obtained for each ADP concentration from each sample of 2 independent donors

§ Statistical significance ($p < 0.05$, paired t test) of the differences in percentages of activated platelets between two successive ADP concentrations determined with flow cytometry in experiments with platelets obtained from 4 independent donors

flow cytometry (Fig. 2). More importantly, at the lower concentrations of ADP (0.05, 0.1 and 0.25 μM), a substantial portion of platelets (36%, 46%, and 65%, respectively) was observed to be morphologically changed, although only a very small share of activated platelets was detected by flow cytometry (Table 1, Fig. 2). This finding suggests a sequential ADP concentration-dependent and/or time-dependent order of platelet activation reactions, in which morphological changes appear before the membrane-associated biochemical markers of activation.

4 Discussion

The main finding of this study is that there is a significantly larger fraction of activated platelets detected by shape change compared to biochemical markers of platelet activation at the lowest 0.05 μM concentration of ADP used as a platelet stimulant (Table 1, Fig. 2). The morphological changes in activated platelets are driven by outside-in signaling followed by rearrangements of the platelet cytoskeleton components, such as disassembly of microtubule rings and actin polymerization [18, 19]. These cytoskeleton dynamics have been reported to be essential for intracellular translocation of secretory granules and transportation of their components, including P-selectin and integrins, to the platelet membrane [20]. That is why, following binding of ADP with purine receptors and switching on intracellular signaling pathways, the morphological changes precede secretion and have been detected before the biochemical markers of platelet activation are expressed on the plasma membrane. The earliest response of shape changes to the ADP-induced platelet activation is also corroborated by an immediate short peak in optical density of a platelet suspension, which occurs right after adding a platelet activator in the light transmission aggregometry test in a typical aggregogram [21].

The reverse side of the high sensitivity to platelet activation is that the result of scanning electron microscopy can be affected by various pre-analytical factors, such as blood

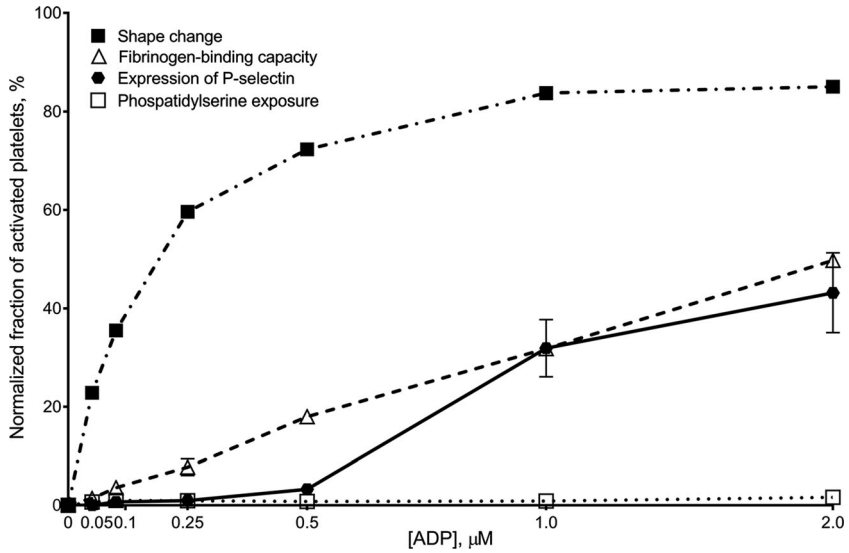

Fig. 2 Relative sensitivity of four methods in detecting platelet activation induced by increasing concentrations of ADP normalized by the initial background activation without ADP

collection and processing, platelet isolation, or platelet preparation for microscopy [7], which can lead to non-physiological platelet shape change and can explain why about one fifth of untreated isolated platelets display morphological signs of activation (Table 1). Another disadvantage of scanning electron microscopy is that it is labor intensive and time consuming, which complicates and may even preclude the use of electron microscopy as a conventional method to detect platelet activation in clinical laboratories. It is possible to watch the shape of live platelets using some types of light microscopy (e.g., phase contrast and differential interference contrast modes), but the resolution and sensitivity of these techniques are much worse than those of electron microscopy [22] and are not enough to detect the commencement of platelet activation.

When platelet activation is characterized by flow cytometry using membrane-associated markers (P-selectin and activated αIIbβ3), the fraction of activated platelets detected by fibrinogen binding capability grow faster as the ADP concentrations increase, while the percentage of activated platelets detected by the P-selectin expression grow more slowly until 0.5 μM ADP and then increase dramatically (Table 1, Fig. 2). Unlike the integrin αIIbβ3, which is mostly pre-located on the platelet membrane, although in an inactive form [23, 24], P-selectin is initially present only within α-granules [25] and has to be translocated to the plasma membrane. It is possible that the difference in the primary intracellular location of P-selectin and αIIbβ3 could explain the differential effects of ADP concentrations on the fibrinogen binding capacity (or αIIbβ3 activation) and the P-selectin expression, namely a higher sensitivity of the αIIbβ3 activation to lower ADP concentrations. In contrast to the P-selectin expression and fibrinogen binding, a very small extent of phosphatidylserine exposure in ADP-stimulated platelet was observed (Table 1, Fig. 2), which is consistent with the literature [26, 27]. This may be due to the strong dependence of the phosphatidylserine exposure on the nature of a platelet-activating stimulus, such that the impairment of membrane asymmetry followed by phosphatidylserine exposure is promoted by a sufficient increase in platelet [Ca^{2+}], which is triggered by other stimulants, such as collagen, but not by ADP [12, 28, 29].

5 Conclusions

Our results reveal that in response to platelet activation induced by increasing concentrations of ADP, the four techniques analyzed were found to be in the following order of sensitivity: morphological changes>fibrinogen binding capacity>P-selectin expression> phosphatidylserine exposure. Our result suggests using a combination of morphological and biochemical methods to assess platelet activation depending on their availability and conditions of platelet stimulation.

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