Examination of Platelet Function in Whole Blood Under Dynamic Flow Conditions With the Cone and Plate(let) Analyzer

Effect of Erythrocytosis and Thrombocytosis

Ellinor I. Peerschke, PhD,1 Richard T. Silver, MD,2 Babette B. Weksler, MD,2 Wei Yin, PhD,1 Bernard Bernhardt, MD,3 and David Varon, MD4

Key Words: Platelets; Cone and plate(let) analysis; Platelet function; Polycythemia vera; Essential thrombocythemia

Abstract

We studied cone and plate(let) analysis (CPA) for evaluating global platelet function in whole blood under arterial flow conditions (~1,800 seconds⁻¹). CPA allows direct visualization and quantitation of platelet adhesion (surface coverage [SC]) and determination of average aggregate size (AS) following brief shearing of a small blood sample (3.2% sodium citrate) in plastic wells. By using blood from healthy volunteers manipulated to alter platelet or RBC counts and blood from patients with myeloproliferative disorders (MPDs), quantitative and qualitative changes in SC and AS were observed. Thrombocytosis resulted in increased SC, whereas erythrocytosis increased AS. The RBC volume (mean corpuscular volume) had no effect. It is interesting that differences in CPA AS were discerned among subgroups of patients with MPD undergoing different treatment regimens. These studies suggest that CPA platelet deposition patterns may provide novel insight into global platelet function during hemodynamic flow.

Platelet function testing is an important component of evaluating patients with bleeding and thrombotic disorders and monitoring antiplatelet therapy. However, traditional platelet aggregometry is time-consuming, requires a substantial blood volume, and examines platelet function in the absence of RBCs and WBCs and at low shear rates (<100 seconds⁻¹).1 In recent years, a number of ex vivo platelet function analyzers have been developed to examine platelet function in whole blood (eg, Ultegra, Accumetrics, San Diego, CA; Platelet Works, Helena Laboratories, Beaumont, TX) and under hemodynamic blood flow conditions (PFA-100, Dade Behring, Deerfield, IL).2

The cone and plate(let) analyzer (CPA) provides unique measures of platelet function in whole blood, under physiologic blood flow conditions, using a venous blood sample that can be analyzed within 4 hours of blood collection.3,4 An aliquot of anticoagulated whole blood is placed into a polystyrene well, and a defined shear rate is applied using a Teflon-coated cone. Under arterial shear conditions, only platelets adhere to the surface. Adherent platelets are visualized after removal of blood, washing, and staining with May-Grünwald solution. In addition to direct visualization of platelet deposition patterns, CPA provides 2 unique measures of platelet function, surface coverage (SC) and aggregate size (AS). The percentage of the well surface covered by stained platelets and the average AS of adherent platelets (µm²) are quantified by using an image analyzer. In normal blood, platelet deposition is a shear- and time-dependent process, reaching maximal levels within 2 minutes at shear rates typical of arterial blood flow.5

Platelet adhesion requires plasma von Willebrand factor and fibrinogen immobilization on the plastic surface, platelet
activation, and quantitatively and qualitatively normal levels of platelet membrane glycoprotein (Gp)IIb-IIIa and GpIb-IX complexes. CPA has been used to detect von Willebrand disease and Glanzmann thrombasthenia, to assess platelet function in people with diabetes and people with thrombocytopenia, and in newborns, for whom the small whole blood requirement is a significant advantage over traditional aggregation studies. It is interesting that CPA has been reported to be more effective than platelet aggregation in monitoring platelet inhibition by GpIIb-IIIa antagonists.

Similar to in vivo platelet function screening using the bleeding time or other ex vivo tests of platelet function in whole blood under high shear stress conditions, platelet deposition during CPA is affected by thrombocytopenia and anemia. The effects of erythrocytosis and thrombocytosis, however, are less well characterized.

Erythrocytosis and thrombocytosis have been associated with increased thrombus formation in in vitro and ex vivo systems under high blood flow conditions. Moreover, shear produced by arterial blood flow over stenotic vessels has an important role in platelet adhesion, activation, and accretion at sites of vascular injury.

The present study was designed to investigate global platelet function by CPA using blood from patients with polycythemia vera (PV) or essential thrombocythemia (ET). Distinct platelet deposition patterns were observed for patients with PV or ET. These observations provide the background for prospective clinical studies to assess the physiologic relevance of platelet deposition patterns generated by CPA.

Materials and Methods

Patients and Blood Sampling

This study was approved by the institutional review board at Weill Medical College of Cornell University, New York, NY. Procedures followed were in accord with the ethical standards set forth in the Helsinki Declaration of 1975. Venous blood samples were obtained from healthy adult volunteers or a population of adult patients being followed up for PV or ET. Healthy volunteers had not taken medication known to affect platelet function for at least 10 days before blood sampling. Patients with myeloproliferative disorders (MPDs) were undergoing treatment with various regimens, including anagrelide hydrochloride (Agrylin), imatinib mesylate (Gleevec), hydroxyurea (Hydrea), interferon alfa, and phlebotomy and/or aspirin. Values for hematocrit and platelet count are summarized in Table 1. Patients had no history of bleeding or thrombotic complications.

Blood was collected into 3.2% sodium citrate (blood/anticoagulant ratio, 9:1) using Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). None of the patients in this study had hematocrit levels that exceeded 50% (0.50), and, thus, no adjustment of anticoagulant volume was required to maintain the 9:1 blood/anticoagulant ratio. CBC analysis was performed on a variety of automated clinical laboratory cell counters, including Sysmex R3000, Sysmex America, Mundelein, IL; Bayer Advia 120, Bayer, Tarrytown, NY; and Roche Cobas Minos STX, Roche Diagnostics, Basel, Switzerland.

Processing and Reconstitution of Normal Blood Samples

To obtain normal blood samples with varying RBC and platelet counts, washed platelets, packed RBCs, and platelet-poor plasma (PPP) were prepared from the same person’s blood, as described previously. Whole blood was first centrifuged (160 g; 8 minutes), and the platelet-rich plasma (PRP) was removed. The remaining blood sample was further centrifuged (1,000 g; 20 minutes) to obtain PPP and packed RBCs. The packed RBC concentration was measured by microhematocrit determination. Washed platelet concentrates were prepared from PRP by centrifugation (1,000 g; 20 minutes) in the presence of EDTA (final concentration, 13 mmol/L). The resulting platelet pellet was resuspended in a small volume of 0.01 mol/L of Hepes-buffered modified

| Table 1 |

<table>
<thead>
<tr>
<th>Patient Group/Treatment</th>
<th>N</th>
<th>Platelet Count, × 10^3/µL (× 10^9/L)</th>
<th>Hematocrit, % (Proportion of 1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers</td>
<td>26</td>
<td>283 ± 46 (283 ± 46)</td>
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<td>MPD</td>
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<td>Anagrelide</td>
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<td>Phlebotomy and/or aspirin</td>
<td>20</td>
<td>530 ± 231 (530 ± 231)</td>
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</tbody>
</table>

MPD, myeloproliferative disorder.

* Data are given as mean ± SD.
Tyrode solution\textsuperscript{19} to achieve a 5- to 10-fold increase in platelet count relative to PRP. Finally, the desired volumes of packed RBCs, PPP, and washed platelets were combined to reconstitute normal blood samples for CPA. CBC counts were obtained on the reconstituted samples using Sysmex R3000 automated analyzers for confirmation of cell counts.

Cone and Plate(let) Analysis

A small volume (200 \( \mu \)L) of anticoagulated native or reconstituted blood was applied to a polystyrene plate (Nunc\textregistered Surface, Nalge Nunc International, distributed by VWR Scientific, West Chester, PA) and subjected to a defined shear rate of 1,800 s\(^{-1}\) for 2 minutes. After removal of blood, the wells were washed with tap water and their surfaces exposed to May-Grünwald stain, as previously described.\textsuperscript{14} Adherent platelets were visualized microscopically (\( \times 100 \)) and quantified using an image analysis program to calculate SC and AS.\textsuperscript{3,4} Each blood sample was sheared in duplicate wells. Platelet deposition in each well was analyzed in 4 quadrants. Final SC and AS results reflect the average of these 8 measurements. Numeric results for SC and AS in the present study are lower than values reported by others,\textsuperscript{4,7-9} and likely reflect differences in software applications.

Results

\section*{Figure 1} depicts the relationship between CPA SC and platelet count. Results from normal blood, manipulated to mimic thrombocytosis, were compared with results obtained with native blood from patients with MPD. As previously described,\textsuperscript{3,17} a positive correlation was observed between SC and platelet count for normal blood samples. A similar correlation was seen with blood from patients with MPDs and platelet counts up to approximately 600 \( \times 10^3/\mu \)L (\(-600 \times 10^9/L\)). At higher platelet counts, significant variation in SC was noted, and a subpopulation (circled) of patient samples demonstrating lower than expected SC could be identified.

\section*{Image 1} depicts representative adhesion patterns obtained following CPA of blood from a healthy volunteer, a patient with thrombocythemia, and a patient with erythrocytosis due to PV. Increased SC can be appreciated in the adhesion pattern of the sample shown in Image 1B (platelet count, 1,027 \( \times 10^9/\mu \)L \((1,027 \times 10^9/L)\)). In contrast, a different adhesion pattern was produced by the blood sample shown in Image 1C with an elevated hematocrit (46.7% \((0.47)\)); a distinct increase in AS was evident. In general, thrombocythemia (platelet count, 500-1,200 \( \times 10^9/\mu \)L \([500-1,200 \times 10^9/L]\)) in patients with MPDs with hematocrit values less than 45% \((0.45)\) increased SC but did not affect AS. In contrast, erythrocytosis was associated with an increase in CPA AS.

As summarized in \section*{Table 3}, statistically significant \((P < .001)\) increases in AS were observed with blood samples from patients with elevated hematocrit values of 41% to 50% \((0.41-0.50)\); AS, 14.9 \(\pm\) 2.1 \(\mu m^2\)) compared with normal hematocrit values of 30% to 40% \((0.30-0.40)\); AS, 12.7 \(\pm\) 1.4 \(\mu m^2\)). All platelet counts for patients in the 2 comparison groups were within normal limits \((150-450 \times 10^3/\mu \)L \([150-450 \times 10^9/L]\)). It is interesting that despite similar hematocrit values, patients treated with imatinib mesylate, hydroxyurea, or interferon alfa demonstrated AS levels that were lower than those of patients treated with anagrelide hydrochloride or phlebotomy and/or aspirin \section*{Table 3}. Aspirin therapy was previously reported to have no effect on unmodified CPA.\textsuperscript{20}

Regardless of treatment regimen, CPA results obtained with blood from patients with MPDs demonstrated slightly decreased SC compared with blood from healthy volunteers reconstituted to mimic similar platelet counts and hematocrit values. This was particularly evident when platelet counts exceeded 600 \( \times 10^3/\mu \)L \((600 \times 10^9/L)\). Rare cases were found with extremely high or low CPA SC \((P < .001)\). Platelet aggregation studies were not performed to further evaluate platelet function.

In addition, more marked increases in AS were noted for normal blood samples reconstituted to yield elevated hematocrit values \((Table 2)\) compared with samples from patients with MPD with erythrocytosis. It is unclear from these data whether these differences reflect intrinsic platelet function defects in
patients with MPD or ex vivo artifact arising from the manipulation of normal blood samples to increase RBC or platelet counts, although mock reconstitution of normal blood samples had no discernible effect on SC or AS (data not shown).

Because blood flow is affected not only by hematocrit but also by RBC size and shape, we further examined the effect of mean corpuscular volume (MCV) on platelet deposition patterns following CPA. In our MPD population, the MCV ranged from 60 to 110 µm³ (60-110 fL). However, no effect on SC or AS was demonstrable as a function of MCV.

Discussion

CPA is a simple, rapid platelet function screening tool that requires extremely low volumes of blood compared with traditional platelet aggregation studies. The ability to evaluate platelet function in whole blood under dynamic blood flow conditions provides a more physiologically relevant testing system. Moreover, the ability to visualize platelet deposition patterns and to quantitate platelet adhesion (SC) and AS may add additional information to platelet function testing.

We previously noted distinct platelet deposition patterns associated with thrombocytosis and erythrocytosis following CPA of reconstituted blood from healthy volunteers. Results from the present study extended these observations to native blood from patients with MPDs (PV and ET). Thrombocytosis appears to be associated with marked increases in SC, whereas erythrocytosis increases AS. Results from these studies suggest that CPA patterns may be predictive of enhanced platelet function. Indeed, recent clinical studies have demonstrated the ability of CPA to identify patients with antiphospholipid antibody syndrome who are at increased risk for thrombosis.

**Image 1** Representative images of platelet deposition patterns after cone and plate(let) analysis (2 minutes, 1,800 seconds – 1). Wells were stained with May-Grünwald stain and examined by light microscopy (×100 magnification). Depicted are typical results for blood from a healthy volunteer (A, platelet count, 191 x 10³/µL [191 x 10⁹/L]; hematocrit, 36.8% [0.37]; surface coverage [SC], 6.1%; aggregate size [AS], 12.4 µm²); a patient with essential thrombocythemia (B, platelet count, 1,027 x 10³/µL [1,027 x 10⁹/L]; hematocrit, 37.4% [0.37]; SC, 11.2%; AS, 10.9 µm²); and a patient with polycythemia vera (C, platelet count, 202 x 10³/µL [202 x 10⁹/L]; hematocrit, 46.7% [0.47]; SC, 8.2%; AS, 16.1 µm²).
The effect of RBCs on platelet deposition on vascular surfaces has been extensively studied (summarized by Hathcock). RBCs not only influence the laminar flow properties of blood, but also directly affect platelet function via molecular cross-talk. Because patients with PV may develop iron-deficient erythropoiesis and, hence, microcytic RBCs, whereas people treated with hydroxyurea develop macrocytic RBCs, this patient population was ideally suited to evaluate the effect of MCV on platelet function by CPA. No differences in SC or AS were discerned among samples with a normal hematocrit value (35%-45% [0.35-0.45]) and an MCV ranging from 60 to 100 µm³ (60-100 fL).

Despite decades of clinical and laboratory research, relatively little information exists concerning the identification of risk factors for thrombosis or bleeding in patients with MPDs. In PV, the thrombogenic effects of an elevated hematocrit value are well established. It is interesting that results from the present study demonstrate a direct positive correlation between hematocrit value and CPA AS. In contrast, thrombocytosis is more often associated with bleeding than thrombosis. In the present study, thrombocytosis was associated with an increase in CPA SC but not AS. In both conditions, advanced age and the presence of a prior thrombotic event.

**Table 2**

<table>
<thead>
<tr>
<th>Platelet count, 150-450 × 10⁹/µL (150-450 × 10⁹/L)</th>
<th>MPD†</th>
<th>Normal‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, &lt;40% (&lt;0.40)</td>
<td>12.9 ± 1.7 (n = 23)</td>
<td>12.7 ± 1.4 (n = 22)</td>
</tr>
<tr>
<td>Hematocrit, &gt;41% (&gt;0.41)</td>
<td>14.9 ± 2.1 (n = 19)†</td>
<td>16.8 ± 2.1 (n = 36)†</td>
</tr>
<tr>
<td>Platelet count, 500-1,200 × 10⁹/µL (500-1,200 × 10⁹/L)</td>
<td>13.2 ± 2.3 (n = 19)</td>
<td>12.4 ± 1.6</td>
</tr>
<tr>
<td>Hematocrit, &lt;40% (&lt;0.40)</td>
<td>13.8 ± 2.1 (n = 6)</td>
<td>Not done</td>
</tr>
</tbody>
</table>

AS, aggregate size; CPA, cone and plate(let) analysis; MPD, myeloproliferative disorder.

† Hematocrit in blood samples from patients with MPD ranged from 32% to 50% (0.32-0.50).
‡ Hematocrit in normal blood samples was manipulated to range from 33% to 67% (0.33-0.67).
§ Statistically significant difference between groups; Student t test.

**Table 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet Count, × 10⁹/µL (× 10⁹/L)</th>
<th>Hematocrit, % (Proportion of 1.0)</th>
<th>Surface Coverage (% of 1.0)</th>
<th>Average Size (µm²)</th>
</tr>
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<tr>
<td>Healthy control subjects (n = 26)</td>
<td>283 ± 46 (283 ± 46)</td>
<td>35 ± 5 (0.35 ± 0.05)</td>
<td>4.5 ± 1.5</td>
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<td>Phlebotomy or aspirin (n = 20)</td>
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<td>430 ± 262 (430 ± 262)</td>
<td>40 ± 4 (0.40 ± 0.04)</td>
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<td>Imatinib mesylate (n = 9)</td>
<td>543 ± 182 (543 ± 182)</td>
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</tr>
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* Data are given as mean ± SD. All blood samples were native samples.
† Indicates statistical significance relative to healthy control subjects based on the Student t test (P < .05).

**Figure 2**

Effect of MCV on platelet function by CPA. Each data point represents 1 patient.
identify thrombosis-prone patients. A minority of patients display decreased levels of high-molecular-weight von Willebrand factor multimers in their plasma during periods of thrombocytosis, and this may explain the bleeding diathesis. Although the majority of patients with ET have abnormalities of platelet function, thus far, no measure of platelet function has demonstrated predictive value, particularly for thrombosis. Recently, increases in megakaryocytic colony-stimulating factor overexpression, or the presence of the Jak2V61F mutation in patients with MPDs have been associated with increased thrombotic risk.

We hypothesize that changes in SC or AS, as determined by CPA, are associated with thrombotic or hemorrhagic risk. Thus, platelet adhesion patterns and quantitation of SC and AS may offer new insights into platelet function. In this regard, it is interesting to note that treatment with hydroxyurea was recently reported to be superior to anagrelide in clinical studies of patients with ET at high risk for vascular events. Although the results are preliminary, in our study, blood samples from patients undergoing treatment with hydroxyurea demonstrated statistically significant lower CPA AS than patients treated with anagrelide hydrochloride. CPA AS in patients treated with anagrelide hydrochloride or phlebotomy and/or aspirin was significantly increased compared with AS in healthy control subjects and patients undergoing other treatment regimens. No statistically significant differences in platelet count or hematocrit value were observed between treatment groups to account for the results. These observations require confirmation in larger prospective studies but are highly suggestive of the potential usefulness of CPA SC and AS in thrombohemorrhagic risk assessment in patients with MPDs. It will be particularly interesting to correlate CPA findings with PRV-1 or megakaryocytic colony-stimulating factor overexpression or Jak2 mutational status in these diseases.

CPA represents a rapid test of global platelet function that provides unique information derived from analysis of platelet adhesion patterns following exposure of whole blood to arterial flow conditions. Further studies are required to evaluate the clinical diagnostic usefulness of AS and SC for the prediction of thrombosis risk in patients with MPDs. It is of interest in this regard that CPA was shown to predict bleeding risk in thrombocytopenic patients and thrombotic risk in patients with antiphospholipid antibody syndrome.

References


