Platelet Activation Test in Unprocessed Blood (Pac-t-UB) to Monitor Platelet Concentrates and Whole Blood of Thrombocytopenic Patients

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Keywords

Flow cytometry · Platelet, Platelet activation · Platelet concentrates · Platelet function · Platelet storage · Platelet transfusion · Platelets · Thrombocytopenia

Summary

Background: Platelet concentrate transfusion is the standard treatment for hemato-oncology patients to compensate for thrombocytopenia. We have developed a novel platelet activation test in anticoagulated unprocessed blood (pac-t-UB) to determine platelet function in platelet concentrates and in blood of thrombocytopenic patients. **Methods:** We have measured platelet activity in a platelet concentrate and in anticoagulated unprocessed blood of a post-transfusion thrombocytopenic patient. **Results:** Our data show time-dependent platelet activation by GPVI agonist (collagen related peptide; CRP), PAR-1 agonist (SFLLRN), P2Y₁₂ agonist (ADP), and thromboxane receptor agonist (U46619) in a platelet concentrate. Furthermore, pac-t-UB showed time-dependent platelet activation in unprocessed blood of a post-transfusion patient with thrombocytopenia. Testing platelet function by different agonists in relation to storage show that 3-day-old platelet concentrates are still reactive to the studied agonists. This reactivity rapidly drops for each agonists during longer storage. **Discussion:** Pac-t-UB is a novel tool to estimate platelet function by different agonists in platelet concentrates and in unprocessed blood of thrombocytopenic patients. In the near future, we will validate whether pac-t-UB is an adequate test to monitor the quality of platelet concentrates and whether pac-t-UB predicts the bleeding risk of transfused thrombocytopenic patients.

Introduction

Platelet concentrates are transfused prophylactically to prevent bleeding in thrombocytopenic hemato-oncology patients. At present, thrombocytopenia is the most important indication for platelet transfusions. However, the ‘clinical efficacy’ of prophylactic transfusions is often only judged on the basis of corrected count increments (CCI). Neither count nor CCI are reliable indicators for prevention of bleeding [1]. Therefore, bleeding itself is still the most relevant endpoint for studies investigating platelet product quality or platelet transfusion triggers. Altogether, there is an urgent need for platelet function measurements to improve the indication for prophylactic treatment of thrombocytopenic hemato-oncology patients with platelet concentrates.

Several platelet function tests are currently on the market, which all serve specific applications. Platelet aggregation measurements, such as the classical light transmission aggregometry and the newer Multiplate[®] assay have proven to be reliable platforms for diagnosis of bleeding disorders [2]. Neither of them can be used to measure platelet function in platelet concentrates, nor handle the low platelet numbers of thrombocytopenic patients. Platelet function analyses are commonly used to pre-screen patients with a hemorrhagic diathesis, but are not reliable for the measurement of platelet function in thrombocytopenic patients or in platelet concentrates [2]. The VerifyNow[®] is an established platform to monitor aspirin and P2Y₁₂ inhibitor therapy, but it is not suitable for monitoring platelet function in platelet concentrates or thrombocytopenic patients [2]. Finally, thrombelastography (TEG) measurements after transfusion have been shown to correlate with storage time of platelet concentrates [2]. However, using TEG for direct measurements of platelet concentrates has been contra-indicated due to conflicting results of in vitro studies [2].

We have designed a novel platelet activation test in unprocessed blood (pac-t-UB). The test is based on platelet activation induced by addition of a specific agonist to whole blood. Activation state of platelets was measured on a flow cytometer by P-selectin expression on the outer membrane of platelets and fibrinogen binding to $\alpha_{IIb}\beta_3$, a membrane glycoprotein which is activated via platelet inside-out signaling [3, 4]. The read-outs of pac-t-UB give specific insight in the granule release capacity and in the aggregation potential of platelets. Platelets have several independent activation pathways. The most important pathways include: the thrombin activation pathway via PAR1, the collagen activation pathway via GPVI, the ADP activation pathway via P2Y₁₂, and the thromboxane activation pathway via the TP receptor. The main advantage of pac-t-UB is that all activation pathways can separately be investigated in a single experiment using only a minimal amount of unprocessed blood. The pac-t-UB has been validated by our laboratory in several studies on the relation between platelet function and (secondary) cardiovascular disease incidents as well as on the storage of untreated versus Mirasol-treated platelets in plasma [5]. Applications for monitoring P2Y₁₂ and aspirin treatment and diagnosis for (un) known bleeding disorders are ongoing.

The aim of the current study was to investigate if pac-t-UB can be used to measure platelet function in a platelet concentrate and whether it can be used to monitor platelet function in thrombocytopenic patients transfused with platelet concentrates.

Material and Methods

Patient

A patient receiving weekly platelet transfusions gave written informed consent to donate two tubes of citrated blood, one before and one after each transfusion. Blood was drawn from the patient's totally implantable venous access system in addition to the blood collected as part of the transfusion protocol and after flushing the intravenous line after transfusion. One discard tube was drawn after flushing and before blood collection. According to routine clinical practice, the empty platelet bag was disconnected from the intravenous line after which it was transferred immediately to the laboratory for processing. With minimal handling of the bag approximately 2 ml of platelet concentrate was collected. Cells were counted using a CELL-DYN Sapphire (Abott Diagnostics, Wiesbaden, Germany).

Material

A platelet concentrate (250 ml CPD anticoagulated platelet-rich plasma derived upon centrifugation, sedimentation, and filtration of 5 buffy coats from identical ABO/RhD donors) were ordered from Sanquin Blood Supply (Zwolle, the Netherlands) for clinical use, FITC-conjugated anti-fibrinogen was purchased from DAKO (F0111, Dako, Glostrup, Denmark), RPE-conjugated anti-P-selectin was purchased from BD-Biosciences (555524; BD Pharmingen™, Franklin Lakes, NJ, USA). Platelet agonist include the P2Y₁₂ agonist ADP (01897; Sigma-Aldrich, Zwijndrecht, the Netherlands), the PAR-1 agonist SFLLRN (H-2936; Bachem, Weil am Rhein, Germany), the GPVI agonist cross-linked collagen-related peptide (XL-CRP, a generous gift by Professor Farndale), and the thromboxane receptor agonist U46619 (Cayman Chemical, Ann Arbor, MI, USA).

Assay Optimization

The optimal concentration of agonists for the pac-t-UB was titrated from a platelet activation experiment in blood of 6 healthy volunteers using dilution series of CRP (2,500, 625, 156, 39, 10, 2, 0.6, 0.2 ng/ml) SFLLRN (625, 156, 39, 10, 2.4, 0.6, 0.2 μ mol/l) ADP (125, 31, 8, 2, 0.5, 0.1, 0.03, 0.01 μ mol/l) and U46619 (288, 72, 18, 4.5, 1.1, 0.3, 0.1 μ mol/l) to measure both P-selectin expression and $\alpha_{IIb}\beta_3$ activation. From the dose-effect curves, we selected 200 ng/ml CRP, 6 μ mol/l SFLLRN, 4.5 μ mol/l ADP, and 18 μ mol/l U46619 for time-dependent platelet experiments. To test the stability of the ready-to-use pac-t-UB reaction mix, we prepared a SFLLRN concentration range ready-to-use pac-t-UB reaction mix and stored it for 1 week in the -20 °C freezer. We compared the platelet response of this mix with an identical freshly prepared mix.

Pac-t-UB

A platelet concentrate or whole blood of a patient was added to a reaction mix consisting of agonist (200 ng/ml CRP, 6 μ mol/l SFLLRN, 4.5 μ mol/l ADP, or 18 μ mol/l U46619), 10 μ l/ml FITC-conjugated anti-fibrinogen, 40 μ l/ml RPE-conjugated anti-P-selectin in HEPES-buffered saline (HBS; 10 mmol/l HEPES, 150 mmol/l NaCl, 1 mmol/l MgSO₄, 5 mmol/l KCl, pH 7.4) in a 1:18 ratio (5 μ l concentrate/whole blood in 90 μ l assay). Reactions were stopped by adding 10 μ l of assay/concentrate or assay/whole blood-mix into 190 μ l 0.2% formaldehyde in 0.9% NaCl after 1, 2, 4, 8 and 16 min of incubation. Flow cytometry was used to distinguish between platelets and other cells on forward and sideward scatter pattern. Fluorescent intensity in the RPE gate was selected to determine P-selectin density, and fluorescent intensity in the FITC gate was used to determine fibrinogen binding, which indicates $\alpha_{IIb}\beta_3$ activation.

Platelet Concentrate Storage

Stability of platelets in platelet concentrates was investigated by storing a platelet concentrate according to the manufacturers' recommendations. Platelet numbers were counted and platelet reactivity to 200 ng/ml CRP, 6 μ mol/l SFLLRN, 4.5 μ mol/l ADP, and 18 μ mol/l U46619 was measured in a concentrate at day 1, 2, 3, 6, 7, and 10.

Patient Measurements

To study the efficiency of platelet concentrates, we collected blood from a thrombocytopenic patient before and after he was treated with platelet concentrate transfusions. Efficiency of platelet concentrate treatment was monitored by measuring platelet counts in whole blood and by platelet reactivity to 200 ng/ml CRP, 6 μ mol/l SFLLRN, 4.5 μ mol/l ADP, and 18 μ mol/l U46619 before and after he received platelet concentrates.

Results

In whole blood samples, platelets can be distinguished from red blood cells and white blood cells on the basis of forward and sideward scatter plots derived by flow cytometry (fig. 1a). Within the platelet gate, platelet activation can be quantified by P-selectin expression and by fibrinogen binding. Non-activated platelets do not express P-selectin on the surface and are therefore not recognized by RPE-conjugated anti-P-selectin antibodies (fig. 1b), while activated platelets express high numbers of P-selectin that bind to RPE-conjugated anti-P-selectin antibodies (fig. 1c). A similar pattern was observed for FITC-conjugated anti-fibrinogen antibodies (not shown). Platelets have several independent activation pathways. The most important pathways are: the ADP activation pathway via P2Y₁₂, the thrombin activation pathway via PAR1, the collagen activation pathway via GPVI, and the

Fig. 1. Flow cytometry technique to measure platelet activation in whole blood. **a** Platelets are separated from red blood cells and white blood cells by forward-side scatter characteristics. Platelet activation can be quantified by fluorescence intensity in the platelet gate of RPE-conjugated antibody binding to the platelet activation marker P-selectin or FITC-conjugated antibody against fibrinogen. **b** Resting platelets show no signal of platelet activation markers, while **c** activated platelets show high intensity of platelet activation markers. The concentration-dependent platelet reactivity experiments to different agonists (ADP (**d, e**), SFLLRN (**f, g**), XL-CRP (**h, i**) and U46619 (**j, k**)) show dose-dependent reaction curves both for P-selectin expression (**d, f, h, j**) and for fibrinogen binding (**e, g, i, k**). The experiments in figure 1 are done in six healthy donors.

Fig. 2. Determination of the concentration agonist (SFLLRN, ADP, XL-CRP, and U46619) to study time-dependent platelet activation of each agonist was done in 6 healthy donors. Serial dilutions of SFLLRN, CRP, ADP, and U46619 were used to measure platelet activation by P-selectin expression on the platelet surface. From the dose-response curves we titrated 4.5 $\mu\text{mol/l}$ ADP, 6 $\mu\text{mol/l}$ SFLLRN, 200 ng/ml CRP and 18 $\mu\text{mol/l}$ U46619 as optimal concentrations for further experiments (**a–d**). **e** shows the reactivity of platelets of three healthy donors to a serial dilution of SFLLRN using a freshly prepared reaction mix that was stored in complete reaction mix for one week, while **f** shows the identical experimental setting using reaction mix that was stored in complete reaction mix for 1 week.

thromboxane activation pathway via the TP receptor. Typical measurements of concentration-dependent platelet activation of normal individuals by different agonists (ADP, SFLLRN, XL-CRP, and U46619) are plotted in fig. 1d–k). Dose-dependent reaction curves are measured both for P-selectin expression (fig. 1d, f, h, j) and for fibrinogen binding (fig. 1e, g, i, k).

We titrated agonist concentrations of ADP to trigger the P2Y_{12} activation pathway (fig. 2c), SFLLRN to trigger the PAR1 activation pathway (fig. 2a), CRP to trigger the colla-

gen activation pathway (fig. 2b), and U46619 to trigger the thromboxane receptor pathway (fig. 2d). From the outcome of these experiments, we titrated 200 ng/ml CRP, 6 $\mu\text{mol/l}$ SFLLRN, 4.5 $\mu\text{mol/l}$ ADP, 18 $\mu\text{mol/l}$ U46619 as optimal concentrations for further experiments (fig. 2a–d). The reaction mix is stable for at least 1 week (fig. 2e, f).

A platelet concentrate was triggered by addition of 200 ng/ml CRP, 6 $\mu\text{mol/l}$ SFLLRN, 4.5 $\mu\text{mol/l}$ ADP, or 18 $\mu\text{mol/l}$ U46619, and time-dependent P-selectin expression and fibrino-

Fig. 3. Forward side-ward scatter plot (**a–c**) of flow cytometry measurements of platelet activation by SFLLRN (PAR1 agonist) in platelet concentrates. Time response curves of platelet activation by 4.5 μ mol/l ADP (**d, e**), 6 μ mol/l SFLLRN (**f, g**), 200 ng/ml CRP (**h, i**), and 18 μ mol/l U46619 (**j, k**) were used to measure platelet activation by RPE-conjugated anti-P-selectin antibody binding to the platelet surface (**d, f, h, j**), and FITC-conjugated anti-fibrinogen antibody binding to platelets (**e, g, i, k**).

gen binding was quantified by flow cytometry (fig. 3). As expected, the forward side-ward scatter plot of the flow cytometry analysis of the platelet concentrate shows a pure platelet population, without other cells or aggregates (fig. 3a). Furthermore, platelet activation in the platelet concentrate can be quantified

with both P-selectin expression and with fibrinogen binding to platelets (fig 3b, 3c). Both P-selectin expression and fibrinogen binding were low in the non-activated platelet concentrate (fig. 3b) while a strong increase of both activation markers was observed upon platelet activation by PAR-1 (fig. 3c). The time-

Fig. 4. Platelet activation of platelet concentrates by 4.5 $\mu\text{mol/l}$ ADP (**a, b**), 6 $\mu\text{mol/l}$ SFLLRN (**c, d**), 200 ng/ml XL-CRP (**e, f**), and 18 $\mu\text{mol/l}$ U46619 (**g, h**) after 20 min reaction time. Platelet function in platelet concentrates was measured at day 1, 2, 3, 6, 7 and 10.

Fig. 5. Forward side-ward scatter plots (a–c) of flow cytometry measurements of platelet activation by SFLLRN (PAR1 agonist) in blood of a platelet concentrate transplanted patient with cell count of $20 \times 10^9/l$. Time response curves of platelet activation by $4.5 \mu\text{mol/l}$ ADP (d, e), $6 \mu\text{mol/l}$ SFLLRN (f, g), 200 ng/ml CRP (h, i) and $18 \mu\text{mol/l}$ U46619 (j, k) were used to measure platelet activation by RPE-conjugated anti-P-selectin antibody binding to the platelet surface (d, f, h, j), and FITC-conjugated anti-fibrinogen antibody binding to platelets (e, g, i, k).

dependent increased expression of P-selectin and binding of fibrinogen upon stimulation with ADP, SFLLRN, XL-CRP, and U46619 indicates that flow cytometry can be used to measure platelet excitability in concentrates (fig. 3d–k).

To further study the excitability of stored platelets in platelet concentrates, we investigated the effects of age on platelet function. A platelet concentrate was stored at routine conditions and a sample was taken from the concentrate at day 1, 2, 3, 6, 7, and 10 to check platelet reactivity by 20 min of incubation with 6 $\mu\text{mol/l}$ SFLLRN (fig. 4a, b), 200 ng/ml CRP (fig. 4c, d), 4.5 $\mu\text{mol/l}$ ADP (fig. 4e, f), and 18 $\mu\text{mol/l}$ U46619 (fig. 4g, h). Our data show that a 3-day-old platelet concentrate is still reactive to the studied agonists. This reactivity rapidly drops for each agonist during longer storage. These findings indicate that pac-t-UB is an adequate test to measure the quality of platelet concentrates.

The acceptance of platelet concentrates by the thrombocytopenic patient was measured with platelet excitability in blood of a patient with thrombocytopenia before and after transplantation with a platelet concentrate. Before treatment, the platelet counts in blood of the patient with thrombocytopenia was too low to obtain enough platelets for a reliable quantification of P-selectin expression or fibrinogen binding. After transfusion the platelet counts increased to $20 \times 10^9/\text{l}$, which was sufficient for a reliable quantification of platelet excitability by different agonists on the flow cytometer (fig. 5). We followed one patient with thrombocytopenia who received three platelet concentrate transfusions. Platelet reactivity in the platelet concentrate and in the post-transfusion blood samples was plotted in the same graph (fig. 5). The data show that the platelet transfusion resulted in a successful recovery of excitable platelets by the thrombin activation pathway via PAR1, the collagen activation pathway via GPVI, the ADP activation pathway via P2Y₁₂, and the thromboxane activation pathway via the TP receptor (fig. 5a–k). Remarkably, the response of platelet concentrates to stimulation by ADP is mild, while the reactivity of platelets from unprocessed blood of the transplanted thrombocytopenia patient seems completely recovered. Furthermore, platelets from stored concentrates react strongly to stimulation by SFLLRN and CRP, while the reactivity of platelets to those agonists from unprocessed blood of the transplanted thrombocytopenia patient seems normal. Neither the platelet concentrate nor the unprocessed blood of the transplanted patient seemed to be sensitive to activation by U46619. Altogether, pac-t-UB is a new opportunity to monitor patients on the effectivity of treatment with platelet concentrates.

Discussion and Conclusion

We introduce a novel test to measure platelet activity in unprocessed blood (pac-t-UB). Pac-t-UB is a flow cytometry-based test that measures platelet activation by P-selectin expression on the outer membrane of platelets and fibrinogen binding to $\alpha_{\text{IIb}}\beta_3$, which is activated via inside-out signaling of

platelets. Our data show that platelet activation by GPVI agonist (CRP), PAR-1 agonist (SFLLRN), P2Y₁₂ agonist (ADP), and thromboxane receptor agonist (U46619) can be used to determine platelet function in platelet concentrates. We also tested platelet reactivity to agonists in whole blood of a thrombocytopenic patient post transfusion, showing that pac-t-UB is a promising tool for the monitoring the effect of transfusion of platelet concentrates to patients with thrombocytopenia.

Pac-t-UB blood has been validated in large population studies on platelet function and (secondary) cardiovascular disease outcomes [3]. The test provides quantitative insight for a broad range of platelet agonists with a broad concentration range. A major advantage of pac-t-UB is that it can be prepared as a ready-to-use mix and in large quantities and can be stored as stable test for several months at -20°C . This makes the test accessible for a broad range of applications, including clinical studies in fresh patient blood, but also for future diagnostics purposes. Pac-t-UB is an extremely simple to use test, which requires a minimal amount of equipment. Furthermore, only very basic laboratory handlings are required to do the test in a clinical setting. Freshly collected blood needs to be transferred to four different tubes of master mix solution, and after five fixed time points a fraction of the blood reaction mixture needs to be transferred to a stop solution. With regard to conventional platelet function tests, pac-t-UB is a cheap, sensitive, reproducible, and customer friendly technique to measure platelet function in whole blood.

Currently, prophylactic transfusions are given at fixed platelet count triggers and their ‘clinical efficacy’ is often only judged on the basis of CCI. Neither count nor CCI show sufficient correlation with prevention of bleeding. Therefore, bleeding itself is still the most relevant end point for studies investigating platelet product quality or platelet transfusion triggers. Our data indicate that pac-t-UB can be used to monitor platelet function in stored platelet concentrates, which is novel opportunity to prejudge the potential efficacy of the concentrates. Both platelet product-oriented studies and studies of transfusion triggers would benefit tremendously from a method that can predict clinical bleeding.

In summary, we have introduced a novel test to measure platelet function in platelet concentrates and in post-transfusion blood of thrombocytopenic patients that are treated with platelet concentrates. The combined measurement of CCI and pac-t-UB platelet function after transfusion may give an indication for the potency to predict bleeding. Our data show that the novel pac-t-UB estimates platelet function to different agonists in thrombocytopenic patients after transfusion. The next step will be a prospective evaluation about the predictive value of combined measurement in relation with clinical outcome, such as bleeding and transfusion dependency.

Disclosure Statement

The authors declared no conflict of interest.

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