Platelet function under high-shear conditions from platelet concentrates

Petra Jilma-Stohlawetz, Michaela Horvath, Beate Eichelberger, Daniela Koren, Bernd Jilma, and Simon Panzer

BACKGROUND: Platelet (PLT) collection and storage affect the functional capacity of PLTs in PLT concentrates (PCs). Therefore, PLTs' functional quality should be studied before transfusion.

STUDY DESIGN AND METHODS: PCs (n = 15) were collected by a standard apheresis procedure (Trima, Gambro BCT) and were stored for 7 days. Samples were taken to assess PLT adhesion and aggregate formation by a cone and plate analyzer (Impact-R, DiaMed) on Days 1, 3, 5, and 7 after harvesting. This device allows testing PLT function under high-shear stress close to physiologic conditions. Concomitantly, P-selectin expression and the residual responsiveness to TRAP-6 were determined by flow cytometry.

RESULTS: PLT adhesion, as measured by surface coverage, decreased during the entire observation period; likewise, the size of aggregates was significantly lower on Days 5 and 7 compared to Day 1 ($p < 0.02$). P-selectin expression increased from Day 5 to Day 7 ($p < 0.04$), whereas TRAP-6–inducible expression remained stable until Day 5 of storage and decreased significantly on Day 7 ($p = 0.04$).

CONCLUSIONS: Our results show that high-shear–induced PLT adhesion and aggregation on the polystyrene surface deteriorate upon storage, suggesting decreased PLT function in vivo. Thus, the Impact-R may be a useful tool to assess the functional capacity of PLTs under various PLT harvesting and storage procedures.

Platelet (PLT) transfusions are used to prevent or treat bleeding episodes in patients with thrombocytopenia, for example, after high-dose chemotherapy. The process of PLT collection and storage may affect the functional capacity of PLTs in PLT concentrates (PCs). Therefore, a variety of in vitro methods have been extensively investigated for their validity and feasibility to test and guarantee the function of PLTs that were prepared for their transfusion.[1] Results from in vitro tests were correlated with the corrected count increment (CCI) of PLTs 1 or 24 hours after transfusion to estimate the validity of these tests clinically.[2-4] Although the CCI is most often used to estimate the success of PLT transfusions, it does not always correlate with PLT survival.[1] Very few in vitro tests performed on PLTs from PCs, however, correlate with PLT viability after their transfusion to healthy individuals,[4,5] and these tests have not been further validated after transfusion to thrombocytopenic patients. Further, the CCI does not evaluate PLTs' hemostatic activity. Thus, in vitro evaluation of PLT function in PCs is desirable before transfusion. According to the European guidelines, only the pH must be measured (6.4-7.4) and the swirling phenomenon must be demonstrated after 5 days of storage.[6]

ABBREVIATIONS: APC = allophycocyanin; AS = average size; GP = glycoprotein; PC(s) = platelet concentrate(s); SC = surface coverage.

From the Departments of Blood Group Serology and Transfusion Medicine and Clinical Pharmacology, Medical University of Vienna, Vienna, Austria.

Address reprint requests to: Petra Jilma-Stohlawetz, MD, Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria; e-mail: petra.jilma@meduniwien.ac.at.

PJS and MH have contributed equally to this study.

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PLTs adhere to damaged endothelium and aggregate in response to physiologic stimuli, such as adenosine diphosphate (ADP), thrombin, and collagen to form a "PLT plug." Key receptors and ligands, such as glycoprotein (GP)Ibα, von Willebrand factor, GPVI, and collagen, facilitate adhesion under high-shear conditions to the subendothelium. Thus, PLTs exert their function mainly under high-shear conditions. The currently used parameters, however, do not assess PLT function of PCs under high-shear conditions.

Recently, a cone and plate analyzer, the Impact-R (DiaMed, Cressier sur Morat, Switzerland), became commercially available for the estimation of PLT function.[7] This device allows evaluation of PLT function under close to physiologic conditions in a whole-blood assay. PLT adhesion and aggregation can be determined in anticoagulated blood under arterial flow conditions wherein a cone and plate viscometer induces laminar flow with a uniform shear stress over a plastic plate by the rotating cone. Results can then be evaluated with an image analyzer. We evaluated in a pilot study the feasibility to use this device for the estimation of the function of PLTs from PCs that were stored for 5 to 7 days.

MATERIALS AND METHODS

PCs were collected from 15 healthy male donors (median age, 43 years; range, 29-56 years) with the same automated collection system (Trima Accel collection system, Version 5.0, Gambro BCT, Lakewood, CO) following the manufacturer's directions. Donors provided informed consent for standard plateletpheresis donations. The median PLT yield was 4.6×10^{11} (range, 2.0×10^{11}-6.5×10^{11}). After the collection, the PLT additive solution SSP (Macopharma UK Ltd, Twickenham, UK; composition 69.3 mmol/L NaCl, 28.2 mmol/L NaH_2PO_4/Na_2HPO_4, 10.8 mmol/L trisodium citrate; 32.5 mmol/L sodium acetate, 5.0 mmol/L KCl, and 1.5 mmol/L $MgCl_2/MgSO_4$) was added to the PLTs (on Day 0) with a plasma:SSP ratio of 35:65. The PCs were stored at 20 to 24°C on a flatbed PC storage system (PC4200i, Helmer, Baxter Vertriebs GmbH, Vienna, Austria) for 5 to 7 days.

Sample preparation

Aliquots of 2 mL were drawn from each concentrate on the day after apheresis (Day 1), 2 days thereafter (Day 3), and an additional 2 days later (Day 5). Samples were also collected from eight concentrates on Day 7. A total of 150 to 250 µL of these aliquots was further diluted with 250 to 350 µL of PLT-poor plasma from the same donor and added to 600 µL of blood group O (null) leukoreduced red cells (RBCs) in SAGM to a final volume of 1 mL. A blood count was then determined with a hematology analyzer (Cell-Dyn 3500R, Abbott, Vienna, Austria). The hematocrit (Hct) level was a median of 35 percent (range, 33%-38%), and the PLT count was a median of 247×10^9 per L (range, 203×10^9-291×10^9/L). In separate experiments we aimed to exclude the influence of plasma storage and its dilution with SSP. Therefore, we compared the results from the Impact-R from six PCs' samples with or without depletion for the plasma:SSP mixture. PLTs were sedimented onto RBCs by centrifugation to deplete for plasma:SSP and resuspended in fresh-frozen plasma (FFP).

Impact-R

PLT-RBC preparations were used for the evaluation of PLT function under close to physiologic conditions in a whole-blood assay. High shear was applied with a cone and plate analyzer, the Impact-R (DiaMed), as described previously.[8] In brief, 130 µL of the sample was placed onto a polystyrene plate onto which a Teflon cone was perfectly fitted and shear was immediately applied (2050/sec) for 2 minutes. Plates were then washed with tap water and stained with May-Grünwald solution according to the manufacturer's manual. Samples were analyzed with an inverted light microscope connected to an image analyzing system (Galai, Migdal Haemek, Israel). PLT adhesion and aggregation were recorded by examination of the percentage of total area covered with PLTs, designed surface coverage (SC, %), and the average size (AS, μm^2) of surface-bound objects. Seven images were collected from each run. Medians of these were calculated by the analyzing system. All samples were tested by the same person (BE), to reduce the variation from one operator to the next. Thereby, an intraassay coefficient of variation (CV) of 10 percent (n = 15) and a day-to-day variation of 15 percent were seen for the SC (n = 10), and both CVs were 10 percent for the AS.

Flow cytometry

The mixed sample was used for all whole-blood flow cytometric evaluations, as described previously.[9] Anti-CD41a (clone HIP8), allophycocyanin (APC)-conjugated, was purchased from PharMingen (San Diego, CA), and anti-CD62P (anti-P-selectin, clone CLB-Thromb/6), phycoerythrin (PE)-conjugated, was purchased from Immunotech, Beckman Coulter (Fullerton, CA). The PLT agonist TRAP-6 (Bachem, Bubendorf, Switzerland) was used at a concentration of 6.66 µmol per L to induce P-selectin expression as a sensitive marker for PLT activation. Acquisition was performed on a flow cytometer (FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, CA). PLTs were gated in a side scatter versus fluorescence 4 (CD41a, APC). Ten-thousand events were acquired within this gate. The gated events were further analyzed in histograms for fluorescence 2 (PE) for the detection of P-selectin. Single-color experiments were

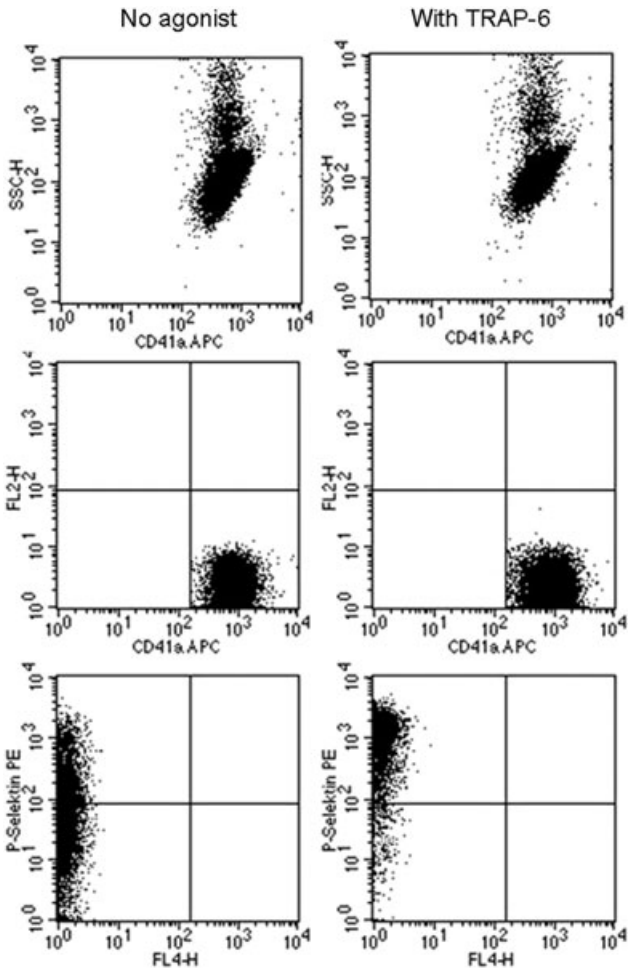

Fig. 1. A representative sample of a reconstituted PC before and after TRAP-6 activation. Fluorescence 4 and side scatter profile (top panel), single-color experiments with anti-CD41a (APC, middle panel), and anti-P-selectin (PE, lower panel). PLTs were gated based on their fluorescence 4 (CD41a-APC) versus side scatter profile. Activation did not result in the exclusion of any events. Next, quadrants were set based on the isotype-matched controls. There was no signal crossover in the nonactivated sample or after the addition of TRAP-6.

used to rule out that signal of one channel is not uncompensated bleedover from another channel (Fig. 1). Further, because PLTs may aggregate upon TRAP-6–induced activation, all events within FL4 (APC) were included for the analysis of P-selectin expression (Fig. 1). The geometric mean fluorescence intensity (MFI) of all gated events was used for statistical analyses. The interday CV was 7.8 percent (n = 10).

Statistical analysis

Data were analyzed by computer software (Statistica 6.1, StatSoft, Inc., Tulsa, OK). Data are presented as medians and interquartile ranges and were analyzed by nonparametric tests. After Friedmann analysis of variance, post hoc comparisons were performed by the Wilcoxon signed rank test. A p level of less than 0.05 was considered significant.

RESULTS

PCs were stored at room temperature under continuous constant agitation, and all samples were analyzed on Days 1, 3, and 5. Only those PCs with PLTs that still adhered with a SC of greater than 1.5 percent by the Impact-R on Day 5 were assayed again on Day 7 (n = 8). In preliminary experiments we have seen that PLTs from PCs without RBCs barely adhere to the polystyrene well (data not shown). We therefore added RBCs to obtain a close to physiologic Hct level. With these preparations 1 day after the apheresis procedure, adherence and the size of aggregates were within the normal range of whole blood from healthy controls (SC, median 8.4%, range 3.8%-19.3%; and AS median 37%, range 20%-124%), which was tested 1 to 2 hours after blood drawing. In preliminary experiments we substituted the plasma:SSP from the concentrate by centrifuging down the PLTs onto a RBC cushion and adding FFP. There was no difference in SC and AS compared to results without this depletion procedure ($p > 0.05$; data not shown). Therefore, all samples were prepared without depleting plasma:SSP. In all PCs, the pH was within the limits (6.8-7.4) recommended by the European Council of Europe.[6]

Adhesion (SC, Fig. 2A) decreased during the entire observation time; likewise, the size of aggregates (AS, Fig. 2B) was significantly lower on Days 5 and 7 compared to those of Day 1. Adhesion deteriorated already after 3 days significantly and dropped further toward Day 5. Only the PCs with the best SC on Day 5 were also investigated on Day 7. PLTs from these PCs maintained their adhesion capacity until Day 7. Aggregate formation was maintained until Day 3 and decreased significantly thereafter, particularly from Day 5 to Day 7. A representative example of P-selectin expression from Day 1 to Day 7 is shown in Fig. 3. Thereby, during the observation time the expression of P-selectin increased. To estimate the residual functional capacity to express P-selectin, PLTs were activated with suboptimal concentrations of TRAP-6. TRAP-6–induced expression did not change until Day 5, but declined on Day 7. These observations are confirmed by analyzing the data from all concentrates together: P-selectin expression increased from Day 5 through Day 7 (Fig. 4A), but a significant reduction of TRAP-6–inducible P-selectin was seen only on Day 7 (Fig. 4B).

DISCUSSION

PCs are obtained by apheresis, the PLT-rich plasma method, or the buffy-coat method.[10] Each of these has

advantages and disadvantages and may affect the hemostatic capacity of the PLTs in different ways.[11] Further, storage conditions and duration of storage also influence the success of PLT transfusions.[1,12] We used apheresis PCs, which were all obtained under the same standardized conditions with the same harvesting equipment for the evaluation of their PLT function. Thus, variables that may affect the results, like the type of the harvesting procedure, were all the same. Thereby we can demonstrate that the function of the PLTs in the PCs can be determined regarding their adhesion and aggregate formation under high-shear conditions. These parameters are further useful to monitor the function of the PLTs during storage.

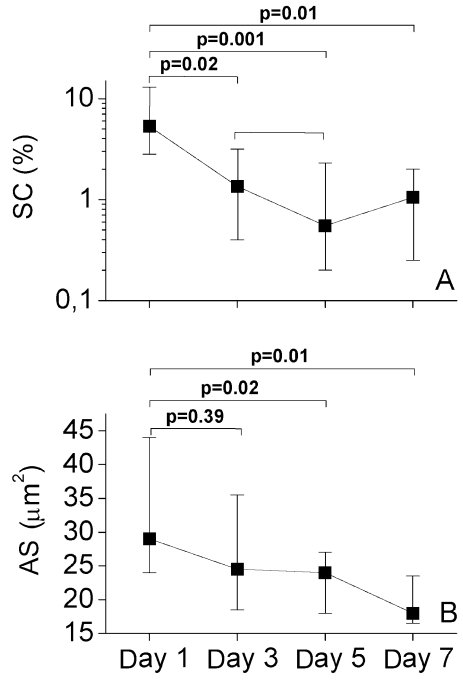

Fig. 2. Evaluation of the effects of storage on PLT function under high-shear conditions. High-shear–induced adherence (SC, A) and aggregate size (AS, B) were determined from PCs that were reconstituted with RBCs. Data were determined on the first day after harvesting (Day 1) and at intervals of 2 days thereafter. The median and upper and lower quartiles (bars) from 15 concentrates (Days 1-5), and from 8 concentrates (Day 7) are shown.

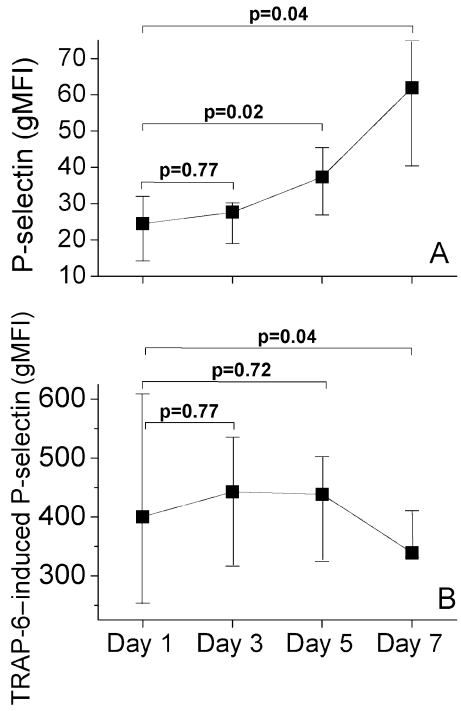

Fig. 4. P-selectin expression (A) and TRAP-6–inducible P-selectin (B) of PLTs in PCs. gMFI = geometric mean fluorescence intensity. Design as in Fig. 2.

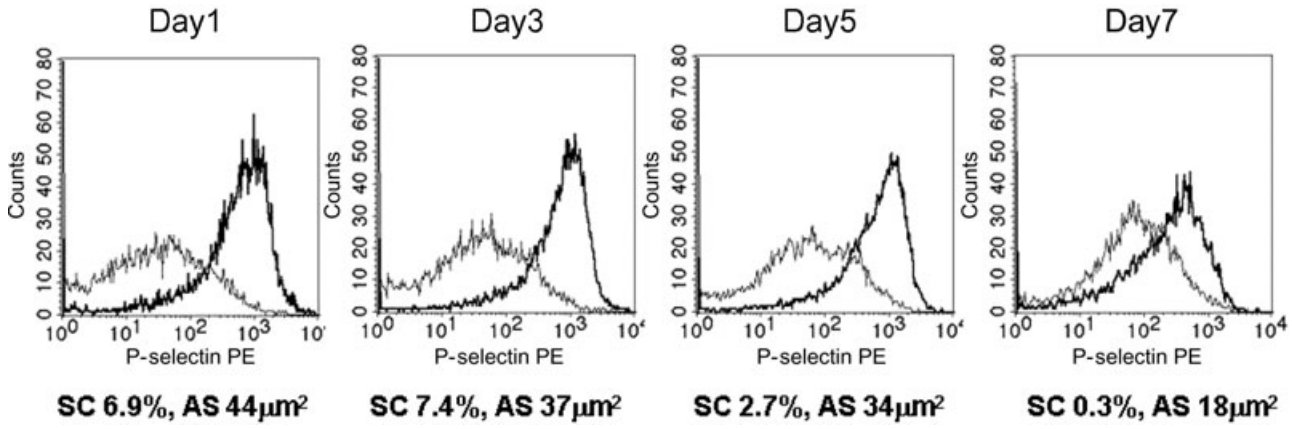

Fig. 3. PLT P-selectin expression from a concentrate stored for 7 days. The expression of P-selectin was determined before (gray line) and after the addition of TRAP-6 (black line) on Day 1, 3, 5, and 7. The corresponding data with the Impact-R (SC and AS) are also shown.

A prerequisite for the evaluation of PLT function by the Impact-R was to add back RBCs to the PLTs. It is known from previous experiments that under shear rates of 2600 per second both adhesion and thrombus formation increase continuously as the Hct level increases.[13,14] In line with these earlier findings, Peerschke and colleagues[15] have demonstrated that RBCs are required for the adhesion of PLTs to a polystyrene surface. In their experiments, increasing the Hct resulted in an increased deposition of PLTs. Most likely, RBCs support under arterial shear conditions the engagement of P2Y1 and P2Y12 ADP receptors and P2X1 ATP receptors, which play a role in stable PLT adhesion and thrombus formation.[15,16] Based on these experiments, we used Hct levels close to the normal range. Likewise, Borzini and associates[17] have reconstituted PLTs from PCs with RBCs to test these samples by the PLT function analyzer PFA-100. Similar to the Impact-R, PLTs were tested under high-shear laminar condition.[17] Some adaptations of the capillary and coating system may be required for its application. More sophisticated, the deposition of PC-derived PLTs to collagen has been tested in a capillary-flow chamber with reconstitution with RBCs to a Hct of 40 percent.[16]

On Day 1, adhesion and aggregate formation were close to those seen in healthy individuals that were tested on the day of blood collection. Thus, the manipulation to obtain the PC and its storage for 18 to 20 hours barely influenced its PLTs' functional properties. Both adhesion and aggregate formation, however, significantly decreased on Day 5. Indeed, only 8 of 15 concentrates had SC values of more than 1.5 percent, which was arbitrarily considered the cutoff for further evaluations.

We have concomitantly evaluated the expression of P-selectin, which has been used as a marker for storage lesion in many previous studies.[18-21] Thereby, we selected for APC-stained anti-CD41a to identify PLTs and PE for P-selectin. These fluorochromes do not bleed over from one channel to the other one (Fig. 1), thus allowing the exact determination of P-selectin. Further, by gating on the CD41a-positive events, PLTs were identifiable, even if aggregated upon TRAP-6 activation. Our data are in line with previous reports showing an increase of P-selectin expression during storage.[1,22] Whether or not this increase of P-selectin is detrimental to PLT function is disputed.[23,24] P-selectin is rapidly shed, but P-selectin–negative PLTs still circulate and function.[21,25] Further, the correlating P-selectin expression with the in vivo recovery has yielded inconsistent results.[23,26,27] It has been shown that the concentration of thrombin necessary to induce the expression of P-selectin increases during storage. This indicates that the content of the αgranules depletes during storage. Alternatively, the PAR-1 receptor may be shed or the signal transduction be inhibited.[28] To our knowledge, this is the first report evaluating the PCs' residual capacity to respond to TRAP-6. The use of suboptimal, rather than optimal, concentrations of this agonist allowed assessing the residual capacity to respond. As shown in Fig. 4B, TRAP-6–inducible expression of P-selectin dropped on Day 7, whereas no effect was discernible before. Thus, the in vitro agonist–induced expression of P-selectin may reflect only in part the PLTs' hemostatic capacity, because high shear is not applied. The data also suggest that storage induces continuous depletion of P-selectin from the αgranules until their eventual exhaustion. It has been shown in rabbits transfused with human PLTs that high P-selectin triggers particularly early fast PLT sequestration (within 1 hr), whereas GPIbα governs their later ongoing clearance (8-12 hr after transfusion).[29] Proteolytic cleavage of GPIbα from PLTs occurs already within 3 days of storage.[30] Because intact GPIbα is required for adhesion to the polystyrene surface, its cleavage explains a significantly reduced adherence on Day 3 compared to Day 1. Aggregate formation appears to be affected later (Day 7), possibly characterizing further or final storage lesion. Thus, the combined determination of P-selectin and shear-induced PLT function complement each other.

In summary, the results from this pilot study indicate that the function of PLTs from PCs can be estimated under close to physiologic conditions by the cone and plate analyzer. After submission of this article an article was published by Morrison and colleagues[31] showing essentially the same results with regard of feasibility to monitor PLT function from PCs with the Impact-R. Further studies are needed, however, to investigate whether this device is useful to evaluate PLT function under various PLT harvesting and storage procedures.

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