Monitoring of Platelet Activation in Platelet Concentrates Using Transmission Electron Microscopy

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Keywords
Quality control of platelet concentrates · Platelet activation · Ultrastructure · Transmission electron microscopy

Summary
Objective: The quality of platelet concentrates (PC) is important for the in vivo recovery of thrombostasis in patients suffering from bleeding disorders and in tumor patients after chemotherapy. In this respect, activated platelets (PLT) cannot display their full functionality in the recipient and even can cause adverse effects. Therefore, we developed a transmission electron microscopy (TEM) method for quality assessment of PC. Methods: Score values taken from panorama TEM images describe the progress of PLT activation. To exemplify this method, i) 19 apheresis PC isolated with the Baxter Amicus system (BA) were compared with 14 PC obtained from pooled buffy coats (BC). ii) The score values of 33 PC derived from BA as well from BC were compared with flow-cytometric CD62P determinations by cross correlation. iii) Changes in the score value profiles during storage of a single pathogen-reduced BA PC were monitored over a period of 7 days. Results: The TEM evaluation described allows for demonstrating particular PLT activation stages. i) Significant differences between the percentages of the score values 0, 1 and 2 could be demonstrated in both processing groups. No significant differences were found comparing these two groups. ii) A weak correlation could be shown when comparing the percentages of score values 2 plus 3 with the percentage of CD62P-positive PLT. iii) The pathogen reduction affected slightly the score profiles during storage due to an increase of dead PLT.

Introduction
Platelet concentrates (PC) are indispensable biopharmaceuticals for treatment of platelet-associated bleeding disorders. At room temperature and under gentle agitation, PC exhibit only short shelf life (usually 5 days) during which platelets (PLT) can be used for transfusion, reflecting the high sensitivity of these cell fragments. This sensitivity is related to the high reactivity of PLT in respect to their activation potential but also to their fragility.

PC can be produced by a variety of manufacturing processes which affect more or less the viability and reactivity of PLT. Several publications reported on this subject [1–13]. The reactivity of PLT in the recipient of the PC can only be estimated by the recovery of the hemostatic balance. To date, no function test is adequate to reliably predict PLT behavior in vivo following transfusion [14], and those which are available provide insufficient or conflicting results [15]. Therefore, it is obligatory in blood banking to determine only the quality of the PC before transfusion. Nevertheless, it is obvious that PLT activation during processing would impair the PLT functionality in vivo. It has been shown that activated PLT stored for 3 days can provoke activation of T cells, B cells, and monocytes of the recipient [16]. Furthermore, the formation

Conclusion: Our investigations demonstrate the unique detailed quality information of PC obtained by the TEM method. This method can be performed in every routine electron microscopy laboratory.
of platelet membrane microparticles (PMPs) may also be responsible for inducing adverse transfusion reactions by facilitating cell-to-cell interactions with cells of the recipient including signal transduction and even receptor transfer [17].

PMPs are thought to play a role in anaphylactic transfusion reactions [18] and in the development of transfusion-related lung injury (TRALI) [19].

The small size of PLT (diameter: about 2–3 µm, thickness: about 0.5 µm) prompted scientists to introduce transmission electron microscopy (TEM) to investigate them. Fundamental research about the complex ultrastructure of PLT under resting and activated conditions has been done in the three last decades of the 20th century [20–24]. Only few publications can be found after 2000 [25–29] also using advanced methods such as special morphometric techniques [30] as well as high-pressure cryofixation, and electron tomography [31].

The aim of this study is to underline the impact of TEM in the field of PLT research and to present a method for investigating the quality of PLT in PC that can be carried out in every electron microscopy laboratory equipped with a CCD camera, connected to a TEM and appropriate software able to align and stitch digital images. The usefulness of this method is exemplary demonstrated by comparing apheresis PC with PC obtained from pooled buffy coats (BC).

Material and Methods

Collection of Apheresis Platelets and Demonstration of Morphological Changes by TEM

In order to analyze the structural integrity of PLT in the course of manipulation during apheresis, we compared data from our quality assessment projects in cooperation with the Austrian Red Cross Blood Donation Center for Vienna, Lower Austria and Burgenland as well as the Department for Blood Group Serology and Transfusion Medicine at the Medical University of Graz. The respective samples were obtained using a Fenwal Baxter Amicus cell separator (Baxter Healthcare Corporation, Fenwal Division, Round Lake, il; USA) (19 PC) and from pooled BC (14 PC), both collected from healthy donors in 100% plasma containing acid citrate/dextrose (CPD) buffer as previously described [32].

Preparation of Blood Cells from Whole Blood Donations

BC-derived PC were prepared from 450 ml whole blood ± 10% which was obtained from healthy volunteer blood donors according to the Austrian regulations for blood donation and after informed consent. Whole blood was collected into triple bags containing 63 ml CPD buffer in the primary bag (Maco Pharma, AB,Pharmaceutiques, Tourcoing, France) and centrifuged at 4,000 × g for 10 min at 22 °C. After discarding the supernatant, the pellet was fixed with 10 ml of 2.5% glutaraldehyde (Fluka, Vienna, Austria) in cacodylate buffer, pH 7.2, for 90 min at 4 °C. After fixation, PLT were washed twice by centrifugation at 800 × g for 10 min at 22 °C. After discarding the supernatant, the pellet was fixed with 10 ml of 2.5% glutaraldehyde (Fluka, Vienna, Austria) in cacodylate buffer, pH 7.2, for 90 min at 4 °C. After fixation, PLT were washed twice by centrifugation at 800 × g for 10 min at 4 °C and transferred to 2 ml Eppendorf vials (Eppendorf AG, Hamburg, Germany), pelleted again and fixed with 1% OsO4 (Fluka) for 90 min. The pellets were dehydrated in a graded series of ethanol (50, 70, 90, 96 and 100%) and embedded in Epon (Serva, Heidelberg, Germany). Sections (70–80 nm) of Epon-embedded PLT were cut with an UltraCut-UCT ultramicrotome (Leica Inc., Vienna, Austria), transferred to copper grids, and routinely stained with uranyl acetate and lead citrate. The sections were viewed under an EM 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany), equipped with a 1k wide-angle slow-scan CCD camera, allowing distortion-free images for photomontages (Tröndle, Munich, Germany) at 80 kV. Panorama views consisting of 8 single digital images at a magnification of 3,000× providing a resolution of about 3,700 × 1,800 pixels. The software allows an exact alignment and stitching of the single images.

Flow Cytometry

Flow cytometry was performed using a FACSCanto flow cytometer (Becton Dickinson) with standard equipment. 10 µl PC was fixed with 90 µl buffered formaldehyde (BD CellFix™) for 10 min, incubated with monoclonal antibodies against CD41-FITC, CD62-PE and CD61-PerCP for 15 min, pelleted at 2,000 × g for 10 min at 22 °C on a flat-bed agitator under constant agitation at 60 rpm. In terms of time between collection and generation of the final pool and the centrifugation speed, both preparations were produced similarly.

The obtained PC were stored in additive solution for 1 day and prepared for TEM as well as for flow-cytometric (FC) investigations.

Pathogen Inactivation

An apheresis PC, isolated using the Baxter-Amicus centrifuge, was pathogen-inactivated using the Intercept™ blood system (Cerus Europe BV, Amersfoort, The Netherlands), stored in additive solution for a period of 7 days, and prepared for TEM on the days 0, 1, 5 and 7.

Transmission Electron Microscopy

Pre-fixation of PC was carried out by mixing 2 ml PC with 8 ml of a 10% buffered formaldehyde solution (BD CellFix™, Becton Dickinson, Vienna, Austria) in order to avoid shape changes of PLT by the subsequent preparation steps. Immediately after pre-fixation, the samples were centrifuged at 800 × g for 10 min at 22 °C. After discarding the supernatant, the pellet was fixed with 10 ml of 2.5% glutaraldehyde (Fluka, Vienna, Austria) in cacodylate buffer, pH 7.2, for 90 min at 4 °C. After fixation, PLT were washed twice by centrifugation at 800 × g for 10 min at 4 °C and transferred to 2 ml Eppendorf vials (Eppendorf AG, Hamburg, Germany), pelleted again and fixed with 1% OsO4 (Fluka) for 90 min. The pellets were dehydrated in a graded series of ethanol (50, 70, 90, 96 and 100%) and embedded in Epon (Serva, Heidelberg, Germany). Sections (70–80 nm) of Epon-embedded PLT were cut with an UltraCut-UCT ultramicrotome (Leica Inc., Vienna, Austria), transferred to copper grids, and routinely stained with uranyl acetate and lead citrate. The sections were viewed under an EM 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany), equipped with a 1k wide-angle slow-scan CCD camera, allowing distortion-free images for photomontages (Tröndle, Munich, Germany) at 80 kV. Panorama views consisting of 8 single digital images at a magnification of 3,000× providing a resolution of about 3,700 × 1,800 pixels. The software allows an exact alignment and stitching of the single images.

Analysis of the Panorama Images Obtained from PC

The morphological evaluation of all PLTs in the panorama view is based on a score classification of the individual PLT according to the following criteria:

- Score 0: unchanged discoid form (fig. 1a) showing the peripheral microtubular coil (MTC) in the equatorial plane (fig.1b) or in the cross section (fig. 1c)
- Score 1: formation of filopodia, and dilatation of the open canalicular system (OCS) (fig. 1d)

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Platelet Activation Visualized by Transmission Electron Microscopy

Fig. 1. This plate shows the particular stages of PLT activation used for quality evaluation of a PC: a Score 0: discoid shape; the PLT shows the microtubular coil (MTC), a prominent glycogen store (GL) and a normally developed open canalicular system (OCS); size bar 0.5 μm. b The MTC is visible in the equatorial plane; size bar 200 nm. c The MTC is visible in a cross section at high magnification; size bar 100 nm. d Score 1: Slight activation showing a dilated OCS and the formation of filopodia; size bar 1 μm. e Score 2: Strong activation with degranulation. The granules (G) deliver their content into the OCS; size bar 0.2 μm. f Score 3: Necrotic PLT showing remnants of granules and mitochondria as well as many vacuoles; size bar 0.5 μm. g Formation of PMP: one PMP is in a stage of budding (B) from a filopodium; size bar 1 μm. h Formation of a PLT aggregate. The PLT show many cell processes which are interlocked in an interdigitating manner; size bar 2 μm.

- Score 2: pronounced shape alterations, centralization of the MTC and processing degranulation (fig. 1e)
- Score 3: Degeneration and necrosis (fig. 1f).

In addition, also the budding and delivery of PMPs (fig. 1g) and the formation of aggregates can be visualized (fig. 1h).

PLT that are sectioned only marginally are neglected since in these cases no appropriate evaluation is possible. PLT representing a particular score value were counted using the analySIS® morphometry software (Soft Imaging System, Münster, Germany).

Statistical Analysis

The number of PLT representing a particular score value was calculated as percentage of the entire number of evaluated PLT. The H-test of Kruskal-Wallis was performed in order to see whether there are statistically significant differences between the PLT in the 3 score values in the two processing groups. Differences between the apheresis group and the pooled BC group of PC are shown in a Box and Whisker plot and statistically investigated using the parameter-free U-test of Mann-Whitney.

A comparison between the percentages of the percentages of TEM score-values 2+3 and the percentages of CD62-positive PLT in the FC analysis was done using a linear regression analysis.

All statistical calculations were performed with the help of the WinSTAT for Excel software R. Fitch (2CheckOut.com Inc, Columbus, OH, USA).

Results

The TEM evaluation of a PC preparation from a single donor represents a snapshot of the state of PLT prefixed with formaldehyde after processing (starting from blood donation via manipulations to the collection into a bag where the PC is stored).

As shown in the Box and Whisker plot of figure 2, a moderate PLT activation (score 1) is predominant and exceeds the percentage of unaffected PLT (score 0) as well as that of the strongly activated PLT (scores 2 and 3). Using the H-test of Kruskal-Wallis, highly significant differences between the percentages of the particular score values (p < 0.01) could be demonstrated. The U-test (Mann-Whitney) provided also highly significant differences (p < 0.01) comparing the different score values, both in the apheresis and the BC group, with exception of the comparison of the score values 2 and 3 in the apheresis group which did not show any significant difference.

The Box and Whisker plot indicates the medians, percentiles as well as minima and maxima. Means and standard deviations of percentages of score values related to the Baxter Amicus apheresis (BA) (n = 19) and the BC group (n = 14) were calculated as follows: score 0: 16.76 ± 12.87% for BA, 26.45±13.54% for BC; score 1: 67.66 ± 20.19% for BA, 67.12 ± 13.62% for BC; score 2: 5.72 ± 8.18% for BA, 2.17 ± 2.29% for BC; score 3: 6.60 ± 7.77% for BA, 3.89 ± 1.85% for BC.

Although there is a trend for a better preservation of PLT obtained from pooled BC compared with those from AB, no statistically significant difference could be demonstrated between the two processing groups by using the U test from Mann-Whitney. Nevertheless, variations in the moderate and pronounced activation are more pronounced in the AB group as demonstrated by higher standard errors.
As a further example, changes of the TEM score profiles during storage after pathogen reduction are shown in one preparation series of a single apheresis PC (fig. 4). Despite some fluctuations, the percentage of unaffected and activated PLT remains relatively constant over a period of 5 days while the percentage of degenerative and necrotic PLT (score 3) increased constantly with prolonged storage time. Most notably on day 7, an increased number of necrotic cells occurs where some of them form aggregates of 2–10 PLT. Representative images of PLT on day 0, 1, 5 and 7 are shown in figure 5.

The correlation between FC data of CD62P-positive PLT and TEM score values is not an easy task. Pre-experiments showed that the slight activation of PLT, reflected by a score value of 1, did not correlate with a CD62P expression on the surface of PLT. Taking together the data of both processing groups, we could only demonstrate a weak correlation comparing the percentage of score values 2 + 3 with the percentage of CD62P-positive PLT (fig. 3).

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Discussion

On the basis of a considerable amount of knowledge about the ultrastructural morphology of PLT, we developed a simple method to monitor PLT alterations due to preparation of PC. The pre-fixation step with formaldehyde prevents morphological changes during subsequent centrifugation steps. Glutaraldehyde cannot be used as pre-fixation reagent, since it would cause the formation of a plasma gel.

The evaluation of the state of PLT acquired from ultrathin sections can be performed using morphometry [30, 33] but implicates always interactive steps such as the choice of the PLT which can be assessed and of others that have to be neglected since they show predominantly too small fragments. The advantage of our score classification consists of the registration of alterations and different activation stages of PLT caused by the manipulations during production of PC. It can distinguish between slight and advanced PLT activation. The most striking activation event is the transition from the discoid into the amoeboid shape. Early changes are always demonstrated by a strong dilatation of the OCS and by the formation of surface projections while strongly activated PLT show distinct signs of degranulation and usually a budding of PMP or ectosomes. In addition, the presence of PLT aggregates is included in the evaluation. The assembled images of the photomontages allow further zooming and the analysis of subcellular features of single PLT in detail.

Nevertheless, there are also limitations of the TEM method. In contrast to flow cytometry, only a limited number of PLT can be investigated but with a high reliability since it is performed at a single cell level displaying many morphological details. The TEM investigation allows only a snapshot of the situation present in a PC. Instead of other methods such as laser confocal imaging or also flow cytometry, the TEM technique requires high vacuum and therefore fixed and dehydrated (or vitrified in a cryostage) samples. Metabolic changes such as calcium mobilization and signaling cannot be demonstrated using TEM. Nevertheless, the signaling events are followed by morphological changes which can be visualized by TEM. Another essential point concerns the fact that the MTC and its centralization during PLT activation are only visible in equatorial or in some cross-sections but cannot be demonstrated in every section of a PLT. In addition, we like to point out that the disadvantage of subjectivity of the score classification is significantly reduced by the enumeration of PLT representing a particular stage by the help of appropriate morphometry software.

The ultrastructural evaluation carried out in our laboratory is based on the classification of Canizares et al. [34] but has been further elaborated and refined. In this publication, the old concepts describing a loss of the MTC stabilizing the discoid shape of an unaltered PLT must be revised. It can be demonstrated that the MTC remains intact after exposure of PLT to agonists. In the course of activation, the MTC becomes constricted into a tight ring around centrally compressed granules. During the process of irreversible aggregation and clot retraction, the MTC becomes fractured, and groups of individual polymers appear in pseudopods or are oriented in the long axis of the PLT [35]. It has been shown that the MTC consists of a single polymer that is wound in 8–12 coils in the periphery of the cytoplasm [36]. However, 3D cryoelectron tomographic reconstructions of individually traced microtubules showed that some circumferential microtubules end at OCS invaginations, are sometimes incomplete and occasionally reveal physical interconnections [31].

The manufacturing process of PC and the storage conditions influence the viability of PLT and lead to the occurrence of dead PLT caused by necrosis but also by apoptosis [37]. In late stages of apoptosis and necrosis, the PLT fine structure is characterized by a loss of integrity of membranes and by extraction of the cytoplasm that contains remnants of granules and cytoskeleton. Prolonged storage of PLT can lead to the so-called storage lesion which is characterized by a diminished adhesion to adhesive surfaces caused by a variety of factors such as the alteration of the vWF receptor and the inhibition of the Ca²⁺ signaling pathway which can cause the clearance of affected PLT in the recipient [38]. The effect of storage has been investigated in several publications [30, 39, 40].

No significant differences could be demonstrated between the results of the two investigated processing groups (BA vs. BC) underlying that both groups are equivalent in respect to their quality. The percentage of the score values shows a similar profile. It can be demonstrated that the highest amount is represented by score 1 which is usually higher than the percentage of unaltered PLT (score 0). The percentage of the score 2 and 3 is usually lower than the score values 0 and 1. In our opinion, the score profiles are more sensitive than the FC determination of CD62P. Therefore, we could only find a weak correlation between the FC measurement and the sums of the percentages of score 2 and 3 representing advanced activation and degeneration of PLT.

Further investigations have to find out the morphological correlate to the CD62P expression at the surface of PLT during activation. In addition it is unclear thus far up to which state of PLT activation the CD62P expression and the formation of small aggregates are still reversible. Insofar, the impact of the FC assays remains a matter of discussion since they reflect different events in the course of PLT activation. While earlier studies suggested a negative correlation between CD62P expression and PLT survival and recovery, these data could not be confirmed using results from human as well as from animal experiments [15]. Therefore, in a future project we intend to perform TEM score profiles from CD62P-positive and CD62P-negative PLT as well as from PLT expressing other activation markers after FC sorting.

The second example where we used the TEM score profiles was the monitoring of PLT activation during storage after pathogen reduction using the Intercept system. Interest-
ingly the score values 1 and 2 remained relatively constant during a storage time of 7 days. This is not the case in PC which are not subjected to pathogen inactivation and in which PLT activation takes place with increasing storage time depending on the suspension medium (plasma or additive solution), leukodepletion, and the quality of storage containers [41, 42]. In contrast to cell activation, the number of dead PLT increased with storage time, a result which agrees with recently published papers demonstrating the induction of apoptotic signals [43] and cytokine accumulation [44]. Several alterations of PLT function have been described in pathogen-reduced BC-derived PC, e.g., an enhanced glucose consumption and lactate production as well as an early increase of activation markers [45].

In conclusion, we would like to stress that TEM investigations of PC are able to significantly supplement the routinely used quality assessment methods after different isolation procedures, above all if a new apheresis system is introduced in a blood bank. In addition, morphological changes during storage or due to antimicrobial treatment such as pathogen reduction or inactivation can be investigated. In this respect, the question whether or not TEM should be applied for quality control of PC must be answered in favor of the method. Of course, a blood bank is not equipped with an electron microscopy laboratory, but it could take efforts to cooperate with an institute or an appropriate core facility which is familiar with the respective methods.

Acknowledgement
The authors gratefully acknowledge the excellent technical assistance of Mag. Beatrice Mallinger and Mrs. Renate Wegscheider and like to cordially thank Mr. Thomas Nardelli and Mr. Ulrich Raindl for the photo-technical work.

Disclosure Statement
The authors did not provide a conflict of interest statement.

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