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Flow cytometric detection of endothelial microparticles (EMP): Effects of centrifugation and storage alter with the phenotype studied ☆

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ABSTRACT

Introduction: Endothelial microparticles (EMP) are released into the circulation in case of endothelial disturbance, and are therefore increasingly investigated as a biomarker reflecting disease activity. Numerous pre-analytic methods have been proposed for their flow cytometric enumeration, but standardization is still lacking. In this study we evaluated the influence of centrifugation and storage conditions on EMP quantification.

Materials and Methods: Platelet-poor plasma (PPP) from 10 healthy volunteers was prepared by centrifugation at 1 550 g for 20 minutes twice. A first aliquot of PPP was analyzed immediately, a second after storage at 4 °C for 7 hours. A third and fourth aliquot were snap-frozen and stored at -80 °C for 7 and 28 days. A final aliquot was further centrifuged at 10 000 g for 10 minutes and analyzed immediately. EMP were defined as CD31+CD42b-, CD62E+, CD144+ or CD144+CD105+ particles, smaller than 1.0 µm.

Results: High speed centrifugation led to a significant loss of CD31+CD42b- EMP ($p = 0.004$). A good correlation between PPP and high speed centrifuged PPP was only found for CD144+ EMP (Kendall tau $b = 0.611$, $p = 0.025$).

Storage at 4 °C did not affect EMP quantification. However, freezing at -80 °C increased CD31+CD42b- and CD62E+ EMP counts, and lowered CD144+ EMP ($p < 0.05$). Nevertheless, the agreement among the different storage conditions was relatively good (Kendall coefficient of concordance >0.487; $p < 0.05$).

Conclusion: The flow cytometric detection of EMP varies with the centrifugation protocol and the storage method used, and these changes also depend on the phenotype studied. The results of this study caution against comparing study results gathered with different EMP laboratory protocols.

Introduction

Endothelial microparticles (EMP) are small membrane vesicles, < 1.0 µm diameter, released from activated, damaged and apoptotic endothelial cells [1]. EMP can be characterized by the presence of endothelial-specific surface antigens. These membrane markers vary with the microparticle (MP) generating process: CD31+, CD105+ and Annexin V+ EMP are generated mainly during apoptosis; while CD62E, CD54 and CD106 expression are increased on EMP released upon activation [2,3]. To date, growing evidence indicates that EMP are more than simple markers of the endothelial status [1,4]. EMP likely have an important function in various physiological processes, including the modulation of inflammation, coagulation and vascular function [1,4]. As such, there is now growing interest in the detection of EMP as a novel biomarker of endothelial disturbance.

Circulating EMP are commonly measured in plasma samples by flow cytometry, due to its ability to perform multiparametric analysis of a high number of events in a very short time period [5,6]. A generally accepted uniform flow cytometry protocol to enumerate EMP is lacking. Moreover, reliable EMP enumeration poses a technical challenge, as their size is near the detection limit of currently available flow cytometers, resulting in different instrument settings [5,7]. In their search for the superior marker in a particular disease, laboratories have used diverse combinations of antibodies for the detection of EMP [5,6]. Finally, there is no inter-laboratory agreement on the pre-analytic preparation of plasma samples [5,6]. In fact the anticoagulant, needle bore size, centrifugation technique, storage time and temperature have been shown to affect the flow cytometric

Abbreviations: ACD, acid citrate dextrose; EMP, endothelial microparticles; FITC, fluorescein isothiocyanate; MP, microparticle; PBS, phosphate buffered saline; PE, phycoerythrin; PPP, platelet poor plasma.

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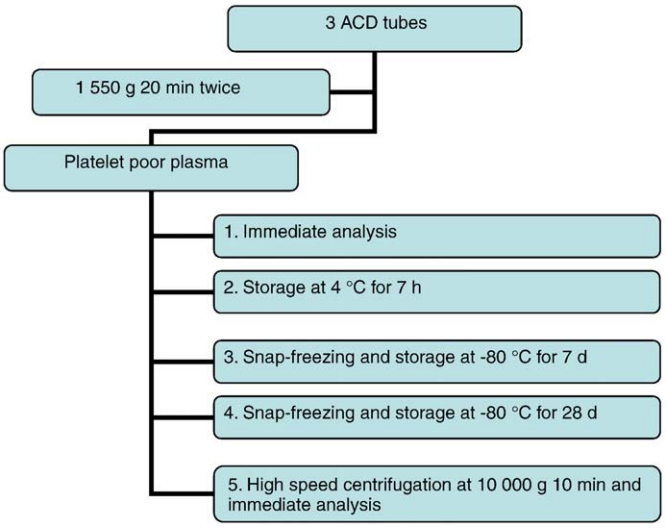

Fig. 1. Sample treatment. ACD = Acid citrate dextrose, d = days, h = hours, min = minutes.

recovery of MP in general, and platelet MP in particular, thereby further complicating standardization procedures [8–10].

As opposed to a rapidly growing number of papers describing EMP in different diseases, little attention has been given to the pre-analytic factors that may affect their flow cytometric detection. The objective of the present study was to examine the impact of centrifugation and storage conditions on EMP recovery from plasma samples in healthy individuals. Since the effect of these pre-analytic variables among different phenotypes of circulating EMP has never been investigated, we focused on four EMP subsets that are frequently applied in the current literature: CD31+CD42b-, a non-specific EMP marker; and CD62E+, CD144+ and CD144+CD105+ EMP, more specific EMP markers.

Materials and Methods

Study population

Ten healthy individuals (5 males, 5 females), with a median age of 42 (25th-75th percentile: 34-45) years, participated. None of them had significant cardiovascular risk factors, medical history, active disease or pharmaceutical treatment. Written informed consent was obtained and the study protocol was approved by the local research and ethics committee corresponding to the principles outlined in the Declaration of Helsinki.

Blood sampling

After an overnight fast, peripheral blood was collected in acid citrate dextrose (ACD) vacutainer tubes, using a 21-gauge needle (BD Diagnostics, Erembodegem Belgium). The first 3 ml of blood were discarded to avoid contamination with EMP due to vascular injury [11]. A tourniquet was left in place no longer than 1 minute, to avoid frank endothelial damage and activation [5].

Preparation and storage of plasma samples

Blood samples were processed within 30 minutes after collection, as outlined in Fig. 1. Two different centrifugation protocols were compared [5,12]. For the first protocol, samples were centrifuged on an Eppendorf (Hamburg, Germany) 5 810 centrifuge for 20 minutes at 1 550 g without acceleration or brake. Next, the upper part of the plasma was collected, without disturbing the white buffy coat layer on top of the cell compartment, and centrifuged for another 20 minutes at 1 550 g. Again the upper part of plasma was collected resulting in platelet poor plasma (PPP). For the second protocol the collected PPP was further centrifuged at high speed, using a Beckman Coulter Centrikon with a T 1 080 rotor type 65 (Fullerton, California, USA) at 10 000 g for 10 minutes. The supernatant was used for MP analysis and had a ±65% reduction in platelet count compared to PPP (data not shown).

PPP and high speed centrifuged PPP were stained for flow cytometric analysis within one hour. In addition, aliquots of 600 µl of PPP were stored at 4 °C for 7 hours or snap-frozen in liquid nitrogen and stored at -80 °C for 7 and 28 days before analysis. In analogy with Shah M. et al, samples were thawed on ice for 2 hours and gently vortexed for 20 seconds prior to labeling with antibodies [8].

Detection of endothelial microparticles using flow cytometry

Plasma samples were measured using a BD FACSCantoII flow cytometer and BD FACSDIVA software version 1.2.6 (BD Biosciences, Erembodegem Belgium). EMP were defined as CD31+CD42b-, CD62E+, CD144+ or CD144+CD105+ particles, smaller than 1.0 µm. (For an example of the flow cytometric dot plots see Fig. 2) A logarithmic scale was implemented for forward scatter signal, side scatter signal and each fluorescent channel; size calibration was done with 0.9 µm beads and forward scatter signal (BioCytex, Marseille, France). A volume of 50 µl plasma was incubated with the different fluorochrome-labeled antibodies for 20 minutes at 4 °C in the dark. Three different antibody combinations were tested: CD31-phycoerythrin (PE, clone WM59, 4 µl/test) with CD42b-fluorescein isothiocyanate (FITC, clone HIP1, 4 µl/test), CD62E-PE (clone 68-5H11, 4 µl/test) and CD144-PE (clone 55-7H1, 4 µl/test) with CD105-FITC (clone 166707, 4 µl/test). (see Table 1) All antibodies were obtained from BD Biosciences, with the exception of CD105-FITC that was purchased from R&D Systems (Abingdon, UK). Samples were diluted with 1 ml of 0.22 µm filtered phosphate buffered saline (PBS) before flow cytometric analysis. Fluorescence minus one (FMO) controls and non-stained samples were used to discriminate true events from noise, and to increase the specificity for MP detection. The contamination of CD31+CD42b- EMP with CD45+CD31+CD42b- leukocyte MP was tested using the pan-leukocyte marker CD45. Overall, the amount of CD45+CD31+CD42b- leukocyte MP was low (3.56% in PPP and 2.34% in high speed centrifuged PPP). These percentages were well within the ranges found by other groups [13,14].

Samples were acquired on a medium flow rate for 180 seconds. Due to the low amount of positive events, CD144 samples were analyzed for 600 seconds. The flow rate was quantified using Trucount Beads (BD Biosciences), run each time as separate samples in parallel. Using these beads we calculated that, on medium flow rate, a mean sample volume of 58.5 µl/min (coefficient of variation of 5.18%) was processed. EMP counts per µl plasma were determined using the following formula (example for CD31+CD42b- events):

$$\frac{\text{Number CD31} + \text{CD42} - \text{events}}{\text{Volume sample analyzed}} * \frac{\text{Total volume of the sample}}{\text{Amount of PPP}}$$

Where: the total volume of the sample equals 50 µl PPP stained with 4 µl CD42b-FITC and 4 µl of CD31-PE diluted with 1 000 µl PBS; the volume of the sample analyzed by the flow cytometer in 3 minutes equals 3* 58.5 ml; and the amount of PPP used for the analysis is 50 µl.

Statistical analysis

Statistical analysis was performed using R-statistics version 2.8.1. Since our data were not normally distributed, results were expressed as medians (25th-75th percentile), and non-parametric statistics with exact significance calculation were performed. For comparison of the two different centrifugation protocols, Wilcoxon signed rank to compare medians and Kendall's tau b to measure correlation were