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Full Length Article

Correlative analysis of nanoparticle tracking, flow cytometric and functional measurements for circulating microvesicles in normal subjects☆

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ABSTRACT

Introduction: Circulating microvesicles (MV) can be analysed using a number of different techniques. The aim of this study was to evaluate the correlation between functional procoagulant based assays including thrombin generation, factor Xa activation test (XaCT), and phosphatidylserine factor Xa-activity by ELISA with optical MV enumeration by flow cytometry and nanoparticle tracking analysis.
Methods: Citrated blood samples were collected from 60 healthy volunteer blood donors after informed consent. Platelet free plasma was prepared using a standardized published protocol. MV subsets were enumerated by flow cytometry (BDFACS Canto) after staining with specific antibodies for platelets (CD41), endothelial cells (CD105), red cells (CD235) monocytes (CD14), tissue factor (CD142) and for phosphatidylserine expression by binding to annexin V. A standardized protocol using counting beads was employed. Nanotracking analysis was performed on both scatter and fluorescent settings after MV staining with quantum dot stain, Qdot 655. Procoagulant function was assessed by the XaCT assay on an automated coagulation analyser and by thrombin generation assay measuring endogenous thrombin potential (ETP), lagtime, peak (PEAK) and time to peak (ttPEAK) using a Calibrated Automated Thrombogram (CAT). The statistical analysis was carried out with Statistica 12 software using non-parametric tests (Spearman rank order correlations, with significance set at $p < 0.05$).
Results: In normal healthy subjects, thrombin generation parameters correlated with levels of MV measured by flow cytometry. ETP, lagtime, ttPEAK and PEAK correlated with MV expressing phosphatidylserine (r_s, Spearman rank order correlation was 0.29, 0.40, 0.31 and 0.34 respectively, $p < 0.05$), and MV expressing tissue factor (r_s was 0.29, 0.40, 0.31 and 0.34 respectively, $p < 0.05$), whilst red cell derived MV correlated with lagtime, ttPEAK and PEAK (r_s was 0.35,0.30 and 0.3, respectively, $p < 0.05$). Lagtime and ttPEAK negatively correlated with the clot based XaCT test (r_s was −0.34 and −0.30 respectively, $p < 0.05$) and positively correlated with the ELISA MP-activity assay ($r_s = 0.42$ for both, $p < 0.05$). In addition, endothelial MV levels weakly correlated with white cell counts ($r_s = 0.27$, $p < 0.05$).
Conclusions: Thrombin generation and flow cytometry for phosphatidylserine or tissue factor expressing MV correlate well as markers for procoagulant activity. A combination of optical or non-optical enumeration as well as functional methods may be required for a complete profiling of circulating MV.

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1. Introduction

Circulating microvesicles (MV) are currently a focus of intense research, having been implicated in both physiological and pathophysiological processes [1]. With specific reference to coagulation, they are now deemed to play a role in both normal and abnormal haemostasis [2]. However, the accurate identification, characterisation and enumeration of such small particles remain a challenge. Although a number of

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studies have evaluated the pre-analytical variables that can affect MV measurements, very few have addressed the correlation between functional assays and those that focus on enumeration and/or identifying the cell of origin.

The terminology for small circulating, cell derived particles, in the micron or submicron range, namely 'microparticles' (designated as MP) or 'microvesicles' has been used interchangeably in the literature. In contrast, the term 'exosomes' is often reserved for vesicles that are actively secreted by cells and are <50 nm in size [3–5]. For the purpose of this study, we have used the term 'microvesicle' to designate any cell derived anucleate particle in circulation that is 1000 nm or less in size.

Microvesicles can be measured and analysed in a number of different ways. Flow cytometry is a popular and widely used tool due to the ease of the technique and advantage of being able to identify expression of particular antigens that may reveal the cell of origin [6,7]. The deficiencies of flow cytometry include pre-analytical, as well as gating variables, in addition to its limitations in detecting MV < 200 nm in size. Nanotracking analysis (NTA), another optical approach, is capable of accurately measuring these small MV – challenges with this technique include reduced sensitivity in measuring larger particles in a polydisperse sample [8,9].

A variety of functional tests are utilized to evaluate MV, ranging from annexin V capture based ELISA to clot based assays and thrombin generation. Whilst the ELISA measures the factor Xa activity of phosphatidylserine (PS) expressing MV, the clot based and thrombin generation assays measure the contribution of MV to global haemostasis. The functional assays are capable of investigating the ability of the MV to contribute to the process of coagulation, in contrast to optical enumeration assays such as flow cytometry or NTA.

This study was undertaken to evaluate the correlation between functional procoagulant activity of MV with optical measurements using flow cytometry and nanotracking analysis. To date, no articles have been published looking at the correlation between nanotracking measurements and flow cytometry in conjunction with functional assays, in normal healthy subjects.

2. Methods

2.1. Subject characteristics

Blood samples were collected from 60 healthy volunteer blood donors, after receiving informed consent. The average age was 38 ± 16.3 years. The average BMI was 26.1 ± 3.5 kg/m^2. There were 30 males and 30 females in the cohort.

2.2. Sample preparation

Peripheral blood was collected in 0.109 M tri-sodium citrate. Platelet-free plasma was obtained by double centrifugation of whole blood for 15 min at 2500 ×*g*. All samples were processed within 2 h of collection, and aliquots were stored at −80 °C until analysis. Samples were then thawed at 37 °C within 15 min prior to testing. All samples were treated in a similar fashion and thawing was undertaken once, just before testing. None of the samples were refrozen, as different aliquots from the same subject were used where necessary.

2.3. Flow cytomtery

A 10 μl aliquot of platelet-free plasma was incubated (in the dark) at room temperature for 15 min with combinations of CD41a-PE (Clone HIP8, BD Biosciences, CA, USA), CD235a-APC (Clone GA-R2, BD Biosciences, CA, USA), CD105-PE (Clone IG2, Beckman Coulter, Marseille Cedex, France), and CD14-PE (Clone TUK 4, Miltenyi Biotec, CA, USA) and TF-FITC (CD142, Clone VD8, American diagnostics Inc., CT, USA) or appropriate isotype controls in a final volume of 100 μl of PBS. For experiments with annexin V-APC (eBioscience, CA, USA) the incubation was done in a total of 50 μl of binding buffer. The sample was then diluted to 400 μl with filtered PBS or 450 μl of calcium rich buffer for annexin V binding experiments. A known number of 10 μm enumeration beads (CountBright beads, Molecular Probes, Life Technologies, Oregon, USA) were added prior to analysis. The flow cytometer used in these experiments was standardized during the 'ISTH workshop for standardization of flow cytometry for Microparticles' as a participating site [10]. The gating was set as per recommended strategies using Megamix beads (Biocytex, Marseille, France) on a BDFACS Canto instrument (BD, New Jersey, USA). The data was collected and analysed using FACSDiva software.

2.4. Functional coagulation based studies

The factor Xa activation test (XaCT) is a clot based assay which detects procoagulant activity of microparticles/microvesicles, based on the ability of vesicles to generate Xa, using the commercially available XaCT test kit (Haematex, Australia) [11]. It was performed in duplicate according to the manufacturer's instructions on an automated BCS coagulation analyser (Dade Behring, Marburg, Germany). A dilution of plasma calibrator (provided by the manufacturer) generated a standard curve from which the individual sample results were read by the instrument's software expressed in ng/ml.

2.5. Functional ELISA based analysis (Zymuphen phosphatidylserine Xa-activity)

The functional assay for the measurement of MV procoagulant activity in plasma was performed using the Zymuphen PS Xa-Activity ELISA kit (Hyphen Biomed, Neuville-sur-Oise, France) according to the manufacturer's instructions. Essentially, MV in the sample were allowed to bind to the annexin V coated on the surface of the microplate wells. Washing process removed unbound particles and prothrombin added along with factor Xa- factor Va in the presence of calcium.

The production of thrombin was measured via cleavage of a chromogenic thrombin substrate producing absorbance at 405 nm and results derived from a standard curve of known MV concentration expressed in nM phosphatidylserine (PS) equivalent. Thrombin generation is directly related to the phospholipid concentration in the plasma. All samples were analysed in duplicate. Only replicates that were at least 85% concordant were used in the final analysis.

2.6. Calibrated Automated Thrombogram measurements

Thrombin generation experiments were carried out as per the manufacturer's instructions on the Calibrated Automated Thrombogram (CAT) and data analysed on the Thrombinoscope software version 3.0029 (Thrombinoscope, Stago Group, Maastricht, The Netherlands). In summary, 80 μl of sample was incubated with 20 μl of calibrator or 20 μl of specific MP-reagent (Thrombinoscope, Maastricht, The Netherlands). The MP-reagent is reportedly sensitive to tissue factor bearing microparticles/microvesicles [12]. The CAT automatically dispensed the prepared fluorescent substrate and buffer. The parameters evaluated for measuring thrombin generation included endogenous thrombin potential (ETP), lagtime, the peak (PEAK) and time to peak (ttPEAK).

2.7. Nanoparticle tracking analysis (NTA)

The enumeration of MV by nanotracking was undertaken on a Nanosight NS500 instrument (Malvern instruments, Malvern, United Kingdom). The scatter was undertaken on plasma samples and the fluorescence recorded after incubation with a quantum dot, Qdot 655 stain (Qtracker® 655 cell labelling kit, Life Technologies/ThermoFischer Scientific, MA, USA) in a dilution of 1:100 with PBS as per the manufacturer's instructions. The scatter settings and fluorescent capture settings, including camera, focus and gain, were optimised so that

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