Immunology Letters 194 (2018) 79–84

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Standardization of a fast and effective method for the generation and detection of platelet-derived microparticles by a flow cytometer

Daniela Recabarren-Leiva[a], Marcelo Alarcón[b,c,1,*]

[a] Parral, Maule, 3630000, Chile
[b] Department of Clinical Biochemistry and Immunohematology, Faculty of Health Sciences, Chile
[c] Interdisciplinary Excellence Research Program on Healthy Aging (PIEI-ES), Universidad de Talca, Talca 3460000, Chile

ABSTRACT

The Flow Cytometry is the principal method used to measure platelet-derived microparticles (PDMPs), by fluorescent properties analysis. PDMPs (0.1–1.0 μm) are abundant in circulation, accounting for approximately 90% of the microparticles and are associated with Cardiovascular Disease, the leading cause of death in the world.

We set the PDMPs gate limits using size-calibrated fluorescent beads (0.5, 1.0 and 3.0 μm). The protocol was performed on volunteer samples with Thrombin 0.5 IU/mL (platelet agonist) and the detection were done using anti-GP IIIa antibody (CD61) on BD Accuri™ C6 Flow Cytometer.

It was possible to standardize the method of generation and detection of PDMPs through the flow cytometer (BD Accuri™ C6). By stimulating for 60 min with the platelet agonist thrombin 0.5 IU/mL, the generation of PDMPs was obtained over 80% ($p < 0.0001$). Finally, we evaluated this protocol for detecting the inhibition of PDMPs generation, prior to the addition of thrombin were incubated washed platelets with acetylsalicylic acid (10 μM), we found an inhibition of 6.3-fold in PDMPs generation.

In conclusions, the present strategy provides a new and easy protocol for PDMPs generation and analysis by Flow Cytometry. For future could be used for evaluating possible treatments in diseases like atherothrombosis.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the world and therefore one of the most important public health priorities. High blood pressure, hyperlipidemia and platelet hyperactivity, among other, are recognized risk factors for CVD [1–3].

Atherothrombosis is a CVD, where inflammatory immune responses and platelets activations participate in the early development of atheroprogression and increasing CVD [4]. In this context, researchers have been shown the presence of microvesicles or microparticles released by platelet as a trigger for this pathology.

Microparticles (MP) are small vesicles derived from membranes or generated during the activation of platelets *in vitro* by platelet agonist as thrombin and collagen of a size ranging from 0.1 to 1.0 μm in diameter [5]. The microparticles can be originating from endothelial cells, platelets, leukocytes and erythrocytes [6,7], but the platelet-derived microparticles (PDMPs) are the most abundant, accounting for approximately 70–90% [8–11]. In addition, PDMPs are surrounded by a phospholipid bilayer consisting mainly of phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine [12], in contrast to cells, MPs can expose negatively charged phospholipids to their surface [9]. In Addition, elevated levels of PDMPs are frequently associated with CVD [13–15]. In this context, Suades et al. [7] indicate that a greater number of MPs in human blood and, more specifically, PDMPs improve platelet adhesion and thrombus formation in the vessel wall, with vascular injury. Small-sized PDMPs generated collectively by platelets and monocytes, modulate thrombogenesis via P-selectin [16].

Flow Cytometry is the most commonly used method for measure MPs in biological samples [17]. It is an optical method for the quantitative analysis of cells by physical and fluorescent properties analysis [18]. In brief, measures absorption and light scattering properties of the cell or subcellular particle, and fluorescence emitted by fluorochromes bound to cellular components of interest, induced by appropriate illumination [19]. However, analysis the PDMPs is difficult by to generation protocol, the PDMPs size definition and flow cytometer resolution detection. In this context, Accuri™ C6 is a flow cytometer that uses a peristaltic pump can measure absolute cell counts with a low-pressure pumping system that drives the fluidics that allows and high-resolution

* Corresponding author at: Platelet Research Laboratory, Faculty of Health Sciences, Universidad de Talca, Casilla, 747, Talca, Chile.
E-mail address: malarcon@utalca.cl (M. Alarcón).
[1] http://www.thrombosisresearchcenter.utalca.cl.

https://doi.org/10.1016/j.imlet.2018.01.003
Received 2 January 2018; Accepted 8 January 2018
Available online 09 January 2018

of the samples [20]. This cytometer has been validated to measure platelet and microparticles in many types of research [21,22], but the different protocol was a very laborious process and with a high price.

The main objective of the present study was to standardize the detection of the PDMPs counts by a Flow Cytometry (BD Accuri™ C6), using size-calibrated fluorescent beads. In the present research, we describe a rapid and low cost-effective protocol of PDMPs generation and detection by Flow Cytometry.

2. Materials and methods

2.1. Reagents

It was used as an agonist Thrombin 0.5 UI/mL (Pacific Hemostasis), for platelet stimulation in vitro. Calibration of PDMPs detection was performed with calibrated commercial beads of 0.5 µm (Latex beads Carboxylate-modified polystyrene, fluorescent red, Sigma-Aldrich); 1.0 µm (Latex beads Amine-modified polystyrene, fluorescent red, Sigma-Aldrich) and 3.0 µm (Spherotech 6-Peak Validation beads FL4, polystyrene beads, BD Bioscience) and acetylsalicylic acid (ASA, 10 µM).

2.2. Blood samples

After clean puncture of an antecubital vein with a 21 gauge needle, venous blood was drawn (first 3 mL not kept) without applying venostasis and was anticoagulated with sodium citrate 3.2%, the protocol was authorized by the ethics committee of the Universidad de Talca in accordance with the "British Committee for Standards in Hematology" [23]. Blood from twenty healthy volunteers (range 20–30 years and the written informed consent was obtained to participate in this study) who had not taken any drugs affecting platelet function was collected into 10 mL syringes, containing ACD/Theophylline extraction buffer (theophylline, prostaglandin E1 20 mg/mL, 1 µM ACD, pH 7.4) in a 1:10 ratio. The last milliliter of each syringe was discarded and processed within 60 min. All samples obtained from each volunteer were processed independently for each assay.

2.3. Platelet preparation

After 5 min, the sample was centrifuged at 250*g* for 10 min at room temperature (Eppendorf 5804 centrifuge) to obtain platelet-rich plasma (PRP). The plasma was separated into 1.5 mL conical base eppendorf tubes; it was re-centrifuged at 650*g* for 10 min at 4 °C (Eppendorf 5424R centrifuge). The supernatant was transferred to a 1.5 mL conical base eppendorf tube; this will be considered as baseline PDMPs count.

The pellet was washed with HEPES-Tyrode's buffer (134 mM NaCL, 2.9 mM KCL, 12 mM NaHCO3, 0.34 mM Na2HPO4, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 5 mM glucose, and 1 mM MgCL2, pH 7.3) in presence of PGE1 (120 nmol/L). Washed platelets were prepared in HEPES-Tyrode's buffer at a concentration 4×10^6 platelets/µL (BC-2800 Auto Hematology Analyzer). All eppendorf tubes were kept on ice for later use.

2.4. PDMPs preparation and labeling

The generation of microparticles was adapted from Suades et al. [7]. Briefly, 400 µL of washed platelets were dispensed in siliconized glass cuvettes at 37 °C with constant stirring at 1000 r.p.m. and stimulated with thrombin 0.5 IU/mL for 15, 30, 60 and 120 min. Then centrifuged at 16,000*g* for 60 min at 4 °C and the supernatants were analyzed on Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA).

Posterity, the PDMPs were identified according to their positivity against specific markers, and characteristics based on size and complexity (Forward scatter/side scatter; FSC/SSC).

For cytometry they were used unlabelled samples (autofluorescence control) and labelled samples, those that were incubated for 30 min in the dark with 5 µL of different conjugated monoclonal antibodies (FITC, Fluorescein isothiocyanate and PE, Phycoerythrin): a) FITC Mouse Anti-Human PAC-1 (recognizes an epitope on the glycoprotein IIb/IIIa complex of activated platelets; BD Pharmingen), b) PE Mouse Anti-Human CD62-P (CD62P is a type I transmembrane glycoprotein that is also known as P-Selectin, is stored in the α -granules of platelets and is rapidly transported to the plasma membrane upon activation; BD Pharmingen) and c) PE Mouse Anti-Human CD61 (CD61 is a transmembrane glycoprotein that is also known as platelet glycoprotein IIIa; BD Pharmingen). The PE markers were performed in separate tubes.

2.5. Flow cytometric analysis

Analyses were performed on Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA). The size of PDMPs was evaluated by comparison with calibration beads. An analysis was performed until the count of 1000 total events [24]. The FSC, SSC and fluorescence data were obtained with logarithmic scale configuration. All buffers were double-filtered by a 0.22 µm filter. In all graphs, we show the representative image.

2.6. Statistical analysis

For statistical analysis, we used GraphPad Prism 6 software for Windows. All results were expressed as mean ± standard error. Student's *t*-test was used to analyze the statistical significance between the control and the values obtained. Values $p \leq 0.05$ were considered as statistically significant and all determinations were run in triplicate.

3. Results

3.1. Standardization, detection and generation of PDMPs

The PDMPs were characterized from calibrated commercial beads. These represented the sizes of 0.5, 1.0 and 3.0 µm (Fig. 1A). In order to establish the region belonging to the PDMPs and to carry out the detection and generation tests of PDMPs in this research, a segment determination was performed referring to the beads, considering to the reported by Abrams et al. [5], we selected as upper limit the gate of 1.0 µm bead and the lower limit the gate of 0.5 µm bead, we excluding in future analysis, any sample outside of this region (Fig. 1B). In summary, the identification of PDMPs was normalized through calibrated beads, generating a specific region to recognize them.

The principal source of the plasma microparticles was the platelets for approximately 70–90% [8]). In this context, for standardizing the detection of PDMPs, centrifuged plasma from Healthy Volunteers and for the analysis, we use three of most important platelet antigens using antibodies that bind to GPIIIa (Anti-Human CD61-PE), P-selectin (Anti-Human CD62P-PE) and GPIIb/IIIa (FITC Anti-Human PAC1). First, we selected the same area obtained by beads calibrations in this sample in a graph of forward and side scatter (Fig. 2A), then this area was analyzed for the positivity of the three antigens, we found that CD61 presents the highest percentage of positive PDMPs (82.41 ± 2.37%; Fig. 2B), being selected as a propitious antibody to carry out the following tests. On the other hand, a lower percentage of reactivity to markers is observed like CD62P and PAC1 (1.39 ± 0.11% and 1.58 ± 0.15% respectively; Fig. 2B).

Then we wanted to standardize the generation of PDMPs, for this first, we determined of the basal line that corresponding without platelet agonist stimulation (3.40 ± 0.1%; Fig. 3A and F). In Fig. 3A–E, we show representative graphs of all determinations for generating PDMPs.

Then for determining the best time for generation of PDMPs, we stimulated washed platelet at 37 °C with constant stirring, with thrombin 0.5 IU/mL for 15, 30, 60 and 120 min and centrifuged at