Journal of Immunological Methods 406 (2014) 117–123

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Technical note

An analysis of endothelial microparticles as a function of cell surface antibodies and centrifugation techniques ☆

Adam S. Venable [a], Randall R. Williams [a], David L. Haviland [c], Brian K. McFarlin [a,b,*]

[a] University of North Texas, Applied Physiology Laboratory, Denton, TX, United States
[b] University of North Texas, Dept. of Biology, Denton, TX, United States
[c] Methodist Research Institute, Core Flow Cytometry Laboratory, Houston, TX, United States

ARTICLE INFO

Article history:
Received 2 December 2013
Received in revised form 1 February 2014
Accepted 24 February 2014
Available online 4 March 2014

Keywords:
Endothelial microparticles
Imaging flow cytometry
Platelet poor plasma
Atherosclerosis

ABSTRACT

Chronic vascular disease is partially characterized by the presence of lesions along the vascular endothelial wall. Current FDA-approved clinical techniques lack the ability to measure very early changes in endothelial cell health. When endothelial cells are damaged, they release endothelial microparticles (EMPs) into circulation. Thus, blood EMP concentration may represent a useful cardiovascular disease biomarker. Despite the potential value of EMPs, current flow cytometry techniques may not consistently distinguish EMPs from other small cell particles. The purpose of this study was to use imaging flow cytometry to modify existing methods of identifying EMPs based on cell-surface receptor expression and visual morphology. Platelet poor plasma (PPP) was isolated using four different techniques, each utilizing a two-step serial centrifugation process. The cell-surface markers used in this study were selected based on those that are commonly reported in the literature. PPP (100 μL) was labeled with CD31, CD42a, CD45, CD51, CD66b, and CD144 for 30-min in dark on ice. Based on replicated experiments, EMPs were best identified by cell-surface CD144 expression relative to other commonly reported EMP markers (CD31 & CD51). It is important to note that contaminating LMPs, GMPs, and PMPs were thought to be removed in the preparation of PPP. However, upon analysis of prepared samples staining CD31 against CD51 revealed a double-positive population that was less than 1% EMPs. In contrast, when using CD144 to identify EMPs, ~87% of observed particles were free of contaminating microparticles. Using a counterstain of CD42a, this purity can be improved to over 99%. More research is needed to understand how our improved EMP measurement method can be used in experimental models measuring acute vascular responses or chronic vascular diseases.

1. Introduction

The human leukocyte differentiation antigen (HLDA) workshop previously identified nine CD markers for the enumeration of endothelial microparticles (EMPs) in peripheral circulation. The proper identification of EMPs is essential for ascertaining their role in vascular homeostasis and various disease processes. Of these CD markers, CD31 and CD51 were reported to be highly expressed on human umbilical vein endothelial cells (Mutin et al., 1997). Given these findings, CD31, CD51, or a dual stain of CD31/51 are the most commonly reported markers for the identification of EMPs (Mutin et al., 1997; Jimenez et al., 2003; Carp et al., 2004; Bernal-Mizrachi et al., 2003; Minagar et al., 2001; Thomashow et al., 2013; Strohacker et al., 2012). One potential experimental problem

☆ Credits for Support: Partial funding for this project was provided by a Research Initiation Grant (RIG; Grant No. 6A9307) from the University of North Texas to Dr. McFarlin.

* Corresponding author at: University of North Texas, Dept. of Kinesiology, Health Promotion, and Recreation, Dept. of Biology, Applied Physiology Laboratory, 1921 W. Chesnut St., Denton, TX 76203. Tel.: +1 940 565 3165.
E-mail address: brian.mcfarlin@unt.edu (B.K. McFarlin).

http://dx.doi.org/10.1016/j.jim.2014.02.010
0022-1759/

with these markers is that while they are abundantly expressed on EMPs, recent research has also identified their presence on other types of microparticles. For example, CD31 is also expressed on platelets, macrophages, granulocytes, and lymphocytes. CD51 is also expressed on platelets, osteoblasts, and megakaryocytes. This presents a problem when considering the relative concentrations in venous blood of the above-mentioned cells (and their microparticles) compared to EMP concentrations. Specifically, EMP concentration represents only 9% of the circulating microparticles, while leukocytes, platelets, and granulocytes constitute 67% (Leroyer et al., 2007).

Contamination of EMP fractions with other cell types is often overlooked as a potential problem because the process of generating platelet poor plasma (PPP) is presumed to remove contaminating platelets, leukocytes, and cell fragments. According to the literature, there is a wide variety of techniques used to generate PPP. When coupled with the use of CD31/CD51 and the failure to account for the presence of contaminating cells, under or over reporting of EMP concentrations may occur. A secondary technique is the use of citrated whole blood, rather than PPP, as the source from which microparticle analysis is carried out (Awad et al., 2013). However, a review of the technical literature revealed that this method is an underwhelming minority and thus was not considered for the present technical note.

The key objective of this technical note is to evaluate the effectiveness of a more selective EMP marker (i.e. CD144) relative to commonly reported methods using CD31 and CD51. CD144 serves as a more specific cell-surface marker for EMPs. One explanation for this finding may be the entirely specific nature of the CD144 molecule on the surface of endothelial cells and EMPs. While CD144 also indicates the presence of the stem cell subset, it is highly unlikely that these would be present in PPP, as they are not found in the blood stream. A secondary objective was to compare previously reported methods of generating PPP in terms of their ability to remove contaminating platelets and granulocyte (GMPs)-, leukocyte (LMPs)-, and platelet-derived microparticles (PMPs). Previously defined methods have either left too many contaminants (in the case of two low serial centrifugation steps) or have been spun at too high a speed in a bench top centrifuge to leave a substantial EMP fragment in the resultant supernatant. As recommended by previous research, it is important to correctly define a centrifuge-protocol specific to the microparticle of interest (Orozco and Lewis, 2010). Therefore, it is the aim of the authors to outline a procedure specific to circulating EMPs.

2. Methods

2.1. Sample source

All sample collection procedures described in this report were conducted in accordance with the Declaration of Helsinki and approved by the University of North Texas Institutional Review Board. Subjects gave written consent for collection of their blood. After providing consent, subjects were screened to ensure that they were apparently healthy and did not have any active, diagnosed form of cardiovascular disease. After an overnight fast (>8-h), subjects reported to that laboratory and sat quietly in a chair for 30-min prior to blood collection. Venous blood was collected from a peripheral arm vein into an evacuated tube containing 0.35 mL 3.2% sodium citrate (Greiner Bio-One; Monroe, NC). Two vacutainers were drawn and discarded before the sodium citrate tube to minimize presence of endothelial cells that resulted from the initial venipuncture. Blood samples were held at room temperature and processed within 1-h of collection.

2.2. Reagent preparation

Prior to testing, all antibodies were serially diluted in PBS and incubated individually in PPP to determine an optimal working dilution that provided the highest signal-to-noise ratio (Table 1). In the literature, EMPs are traditionally defined using CD31-PECy7 (clone#WM59; eBioscience; San Diego, CA; DF = 1:10), CD51-Biotin (clone#NKI-M9; Biolegend; San Diego, CA; DF = 1:5), and Streptavidin-Brilliant Violet 421 (B164644; Biolegend; DF = 1:5). Counterstaining of PPP was accomplished using: CD66b-PerCPCy5.5 (GMPs; clone#G10F5; Biolegend; DF = 1:5), CD45-APCeFluor780 (LMPs; clone#HI30; eBioscience; DF = 1:5), and CD42a-FITC (PMPs; clone#GR-P; eBioscience; DF = 1:10). We also tested a less commonly used marker of EMPs relative to the markers that are traditionally used (CD144-PE; clone#16B1; eBioscience; DF = 1:5).

2.3. Experimental treatment

In order to generate PPP, sodium citrate treated blood underwent two separate centrifugation steps. Four different methods were used in order to generate PPP and test the efficacy of its ability to produce PPP with a high EMP content: 1) 10-min at 160 ×*g*, then 6-min at 1500 ×*g*; 2) 10-min at 1500 ×*g*, then 5-min at 13,000 ×*g*; 3) 10-min at 160 ×*g*, then 30-min at 15,000 ×*g*; and 4) 10-min at 160 ×*g*, then 6-min at 1000 ×*g*. For each of the four scenarios, the first centrifugation step was completed in sodium citrate tubes and using a swinging bucket rotor (TX-400; ThermoScientific; Waltham, MA) with no brake. In all cases, after the first centrifugation, the upper 800 μL of the platelet-rich plasma (PRP) was transferred to a second tube and underwent the second centrifugation step in a fixed angle rotor (Microliter 30; ThermoScientific) with no brake. Then, the upper half

Table 1
Dye combination used for the analysis of EMP.

Dye	Dilution factor	Excitation	Emission
CD42a-FITC	1:10	488 nm	Channel 2 (505–560 nm)
CD144-PE	1:5	488 nm	Channel 3 (560–595 nm)
CD66b-PerCPCy5.5	1:5	488 nm	Channel 5 (642–740 nm)
CD31-PECy7	1:10	488 nm	Channel 6 (740–800 nm)
CD51-BV421	1:5	405 nm	Channel 7 (430–505 nm)
CD45-APCeFluor780	1:5	642 nm	Channel 12 (740–780 nm)

Antibody and fluorochrome combinations with dilution factors used in the presented study. Sample acquisition was carried out on an Amnis FlowSight flow cytometer with 12 channels (2 dedicated to brightfield images).