Exosomes and Microvesicles

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ARTICLE

Protocol Standardization Reveals MV Correlation to Healthy Donor BMI

Original Research Article

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Abstract Microvesicles (MVs) are cell-derived vesicles which are of interest in a clinical setting, as they may be predictive of early signs of disease and/or of treatment progression. However, there are growing concerns about using conventional flow cytometry (cFMC) for the detection and quantification of microvesicles. These concerns range from error-sources in collection through to the physical limitations of detection. Here we present a standardized method for collection and analysis which shows that the MV numbers detected by cFCM correlate to donor Body Mass Index (BMI). Although unlikely to be comprehensive, we also demonstrate how cFCM is a useful and valid tool in the analysis of MVs.

Keywords Quantification, BMI, Flow Cytometry

1. Introduction

Extracellular vesicles (EVs) are small membranous vesicles ranging from 0.05 to three microns in diameter, released from cells during apoptosis and cell activation (1). They have been subcategorized and defined in multiple ways by numerous groups (2, 3, 4). Although the nomenclature is used somewhat ambiguously, an excellent review article by György et al. (2011) recently outlined the broad classification we are following. One category of EVs is that of microvesicles (MVs), which are formed by activation or apoptosis (4). MVs range from 0.1 to one micron in diameter. In blood, circulating MVs have been found to range more specifically from 0.1 to 0.5 microns in diameter (5). It is these circulating plasma MVs which are the focus of this article.

For decades, FCM has been used as a cellular analysis technique due to its rapid, multiparametric capabilities at the single cell level (6, 7, 8). The development of a greater understanding of the importance of microvesicle biology is a growing issue, both in basic research and in a clinical setting (9, 10, 11). As a result of the cellular origin of MVs, as well as the fact that the requirements for analysing MVs are the same as those for cells, FCM appears to be a logical extension of cellular analysis for investigating MVs.

A conceptual concern is the fact that cells and MVs are not one and the same thing; mainly due to the small size of MVs, it is slowly becoming accepted that FCM is not a straightforward technique for the analysis of MVs (12, 13,

14, 15). There are many theoretical concerns in FCM which make this a suboptimal technique for the analysis of MVs. The use of polymer beads as a standardization measure is one of these concerns, as it is understood that a bead may scatter up to 100 times more light than an MV (16). Consequently, if we can detect a 100 nm diameter polymer bead by light scatter, this does not mean that we can detect a 100 nm diameter MV by the same means. This leads to the conclusion that conventional FCM (cFCM) is not a comprehensive technique for MV analysis; it in fact only analyses a proportion of the larger MVs as well as, potentially, the coincidence of smaller MVs. Another concern relates to coincidence event detection, where no individual MV would be detected above the background but, due to their close proximity when passing the detector, multiple MVs are registered as a single event (17, 18). Finally, in FCM we conventionally identify an event when light scatter is detected above the threshold in the forward scatter parameter. In small particle flow cytometry and microbial flow cytometry, there are physical reasons for identifying events when light scatter is detected above the threshold in the 90^{o} angle (side scatter) parameter (19, 20). However, if such small particles do not create light scatter that can be detected above the threshold, then the option to identify events by fluorescence is also available. Some literature suggests that fluorescence is actually a better parameter for identification (21). However, it is yet to be determined whether fluorescence or light scatter is a better method for detecting MV events by FCM, as there is now evidence in the literature that not all MVs are Annexin V positive (4, 22). Currently, there is no all-encompassing MV-stain by which to identify events by fluorescence, although there are several potential stains that appear to be suitable (23). As yet, however, this has not been confirmed. Therefore, while some MV analysis would be better suited to fluorescence detection, there would be sub-populations which may not be detected using this method. It may be that, depending on the application, this factor will determine whether fluorescence or scatter detection is more appropriate.

Currently, there are no well-developed answers to these analysis issues. Some suggestions have, however, been made, such as coincidence event determination by monitoring fluorescence and by serial dilutions of a sample. In the latter, a sample with high coincidence would be expected to show a reduced fluorescence signal in the dilutions. There still remains, however, the issue that only sub-populations of MVs can be identified and the fact that to date there is no agreed standard in MV analysis.

In addition to the concerns over analysis, there are mounting concerns over protocol standardization and over how to define the pre-analytical and analytical variables which impact the analysis. These variables include those relating to sample collection: phlebotomy technique, needle gauge and position of tourniquet (24); those relating to sample handling: vortexing and centrifugation conditions (25); those relating to detection protocols including instrument set-up (26); and those relating to potential false positives caused by confounding reagent particulates and antibody aggregates (27). These concerns are of intense importance as the manner in which these protocol steps, which may appear trivial, are carried out can lead to a significant variation in MV numbers.

In addition to the need for standardization of sample collection, handling and detection, there are also patient factors which need to be taken into account. These factors need to be considered so that analytical variables are understood and so that MVs can be reliably quantified in patients and other clinical settings. While there many studies which investigate the relationship between pathological states and physiological variables, to date few studies have examined variation among healthy individuals in relation to MV quantification. The possibility that donor parameters such as Body Mass Index (BMI) could function as a confounding factor on absolute baseline MV quantification has not been extensively examined.

To further complicate the already challenging area of MV study, it has recently been shown there is a cellular inflammatory response to fatty meals (28) which has been linked to an increase in MV numbers (29, 30). Another recent study suggests that strenuous exercise increases relative MV counts (31). In this study, we examine baseline MV numbers, as MV numbers are affected by many factors. Here, we have defined baseline MV numbers, after minimal disruption during collection and optimization of protocol. This allows us to measure a healthy individual's circulating plasma MV numbers from normal cellular turnover.

2. Materials and Methods

2.1 Prerequisites

This study was performed under an approved Institutional Review Board protocol. The inclusion criteria for the study included a minimum of four hours fasting as well as no meal over 50 g in fat (30) and no strenuous physical exercise (31) for 12 hours prior to sample collection.

2.2 Donor Demographics

All donors gave self-declarations of good health and were Caucasian males and females except for one East Asian female. The mean age was ± SD, 40.2 ± 15.7 years; age range 21 – 64 years.

2.3 Sample Collection

A stock of anticoagulant citrate dextrose (ACD) solution was made by dissolving 22.0 g trisodium citrate (Sigma-Aldrich, MO), 8.0 g citric acid (Fisher scientific, PA) and 24.5 g dextrose (Sigma-Aldrich) in one L deionized water (Millipore, MA), which was then passed through a 0.22 micron filter (Corning, NY). Two mL of the ADC solution was filtered with a 0.2 micron filter (Pall, MI) immediately prior to use. Two hundred μL of ACD solution was pre-loaded into a 10 mL syringe (BD Biosciences, NJ) to prevent clotting when drawing blood. All peripheral blood was drawn by a standard procedure from the arm, where the tourniquet was placed approximately four inches above the selected puncture site, using a 21 gauge butterfly needle (BD Biosciences). At this stage, a 100 μL aliquot was taken for analysis on a Hemavet 1700FS (Drew Scientific, CT), following the manufacturer's recommendations. Immediately after, the blood was put into a 15 mL Falcon tube (BD Biosciences) containing 1.8 mL of ACD solution, giving a total volume of 12 mL with a 5:1 ratio of blood to ACD solution.

2.4 Sample Handling

The 100 μL aliquot for acquisition on the Hemavet 1700FS (Drew Scientific) was used to analyse the total white blood cell counts, neutrophil counts, lymphocyte count, monocyte count, eosinophil count, basophil count, platelet count, red blood cell count and percent haematocrit blood parameters, all of which were recorded (Table 1). The 15 mL conical centrifuge tube (BD Biosciences) containing the ACD-blood solution (5:1 blood/ADC) was gently inverted to mix the sample. The sample was then centrifuged at 1500 x g for 30 minutes at room temperature. The plasma supernatant was removed and transferred to an ultra-centrifuge tube (Beckman Coulter, IN), leaving at least 300 μL supernatant undisturbed above the cell phase (3). The plasma was then centrifuged at 11,000 g for 30 minutes at room temperature to remove platelets. The platelet-free plasma supernatant was removed and placed into a 15 mL conical centrifuge tube, leaving at least 300 μL supernatant undisturbed above the pellet. From 10 mL of blood we obtained approximately four mL of platelet-free plasma.

2.5 Flow Cytometry

Sample data were acquired on an Epics XL (Beckman Coulter) flow cytometer, equipped with a 15 mW, 488 nm argon-ion laser and using a Nano Fluorescent Particle Size Standard Kit, NFPPS-52-4K (Spherotech, IL), for instrument standardization. Using the EPICS XL volumetric acquisition setting, 20 μL of plasma was acquired by identifying events above threshold on the side scatter parameter, displayed in logarithmic scale. Side scatter was set to 556 volts with a gain of five and a threshold value of three, as identified by a dashed line across Figure 1, plots A to D. Gates were set on a forward angle and a side scatter angle dual parameter plot, based on a sample population falling below the 1.33 micron bead population on side scatter. All reagents were diluted in 0.2 micron filtered FACSFlow (BD Biosciences) and used as background controls. Post-confirmation of MV presence in the sample was carried out via systematic triton lysis (26, 32) using 0.1% Triton X-100™ (Mallinckrodt, MO), where the MV population would dissolve from the MV gate, as seen in Figure 1.

2.6 Dynamic Light Scatter

Sample data were acquired on a Coulter N4 Plus instrument, acquiring the data at a 90° angle. Experiments were carried out at 22°C to determine the size distribution of the microvesicles.

2.7 Data Analysis

Hemavet blood parameters were recorded. The flow cytometric circulating plasma MV numbers were recorded within the MV gate, Counts within the MV gate from post-Triton X100™ lysis were then subtracted and this value was corrected for the 5:1 blood-to-ACD dilution to give absolute counts in 20 μL. This number was used to calculate the circulating plasma MV baseline number per mL of plasma. The baseline number was compared to the total BMI, age, sex and cellular blood parameters of the donor, using Statistical Analysis Software (SAS Institute, Inc., Cary, NC 27511) by means of a curve-linear coefficient of correlation where a $P>0.05$* was considered significant and graphically displayed using Microsoft Excel 2010.

3. Results

Volumetric FCM analysis of plasma samples quantified all subjects' absolute baseline circulating plasma MV numbers. A background sample was acquired for each patient immediately prior to the plasma sample being taken (Figure 1A). Twenty μL was acquired for each sample and the MV population was determined from a bead standard defining the upper limit of the MV population (Figure 1B). Gating was confirmed post-acquisition by systematic Triton X-100 lysis of the MV population (Figure 1C). The absolute baseline circulating plasma MV number was derived by subtracting the gated background number from the plasma sample gated number and then converting the result to numbers of MVs per mL of plasma.

Donor experimental data from a Hemavet 1700SF was acquired immediately after the sample was drawn. The haematocrit, white blood cell, red blood cell and platelet count were in the normal range, as shown in Table 1.There was found to be no correlation between the baseline circulating plasma MV number and any of the cellular blood parameters measured.

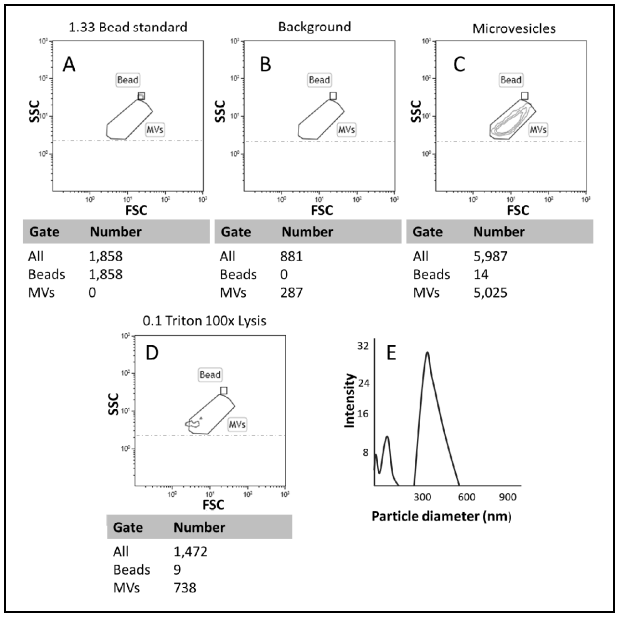

Figure 1. Typical EPICS XL FCM plot showing contour plots FS/SS for (A) 1.33µ diameter bead standard, (B) triton control background, (C) MVs, (D) immediately after 0.1% Triton X-100 lysis and (E) DLS size distribution data of representative circulating plasma MVs.

The baseline circulating plasma MV numbers were plotted against donor BMI using SAS by means of a curve-linear coefficient of correlation, as seen in Figure 2. There is significance ($P<0.05$) and a strong coefficient of correlation ($R^2=0.8867$) in the relation between baseline circulating plasma MV number and donor BMI.

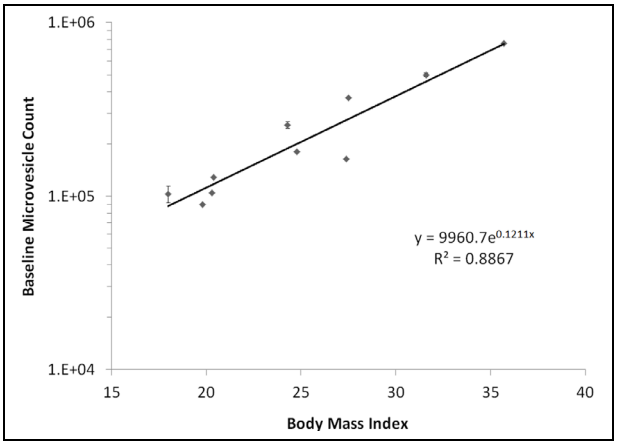

Figure 2. Chart showing the relationship between BMI and baseline circulating plasma MV numbers per mL, with derived equation and correlation values.

There are many areas for discussion, but as to the biological relevance of this correlation between quantitative MV count and BMI, we show that, when strict protocol standardization and rigorous control steps are taken, there is a strong correlation ($R^2=0.8867$) between baseline circulating plasma MV numbers and healthy donor BMI when analysed by FCM. The relevance of this is amplified by the lack of correlation in other measured cell-derived blood parameters and BMI from the Hemavet results (Table 1). No significant differences between circulating plasma MV numbers between male and female samples were observed, nor was there any relationship between baseline MV number and the age of the donor (Table 1).

Donor ID	Age (years)	Sex	Weight (lbs)	WBC count (10^6/ml)	Neutrophil count (10^6/ml)	Lymphocyte count (10^6/ml)	Monocyte count (10^6/ml)	Eosinophil count (10^6/ml)	Basophil count (10^6/ml)	Platelet count (10^6/ml)	RBC count (10^9/ml)	Haematocrit %	Baseline MV/mL
1*	28	M	265	6.32	3.10	1.69	0.65	0.62	0.25	223	5.64	55.17	499,960
2*	41	M	190	5.09	2.27	1.87	0.26	0.54	0.15	147	4.57	47.83	257,748
3*	56	M	125	3.44	2.04	0.79	0.18	0.33	0.10	166	4.48	46.80	103,020
4	31	F	145	6.68	4.72	0.85	0.79	0.16	0.16	184	5.13	49.90	128,460
5	52	F	155	4.28	2.35	0.73	0.54	0.53	0.13	153	4.93	50.40	180,000
6	56	F	250	6.62	3.78	1.90	0.50	0.27	0.18	111	5.07	51.10	756,000
7	64	M	202	3.14	1.68	0.76	0.26	0.41	0.03	96	4.30	43.70	368,520
8	31	F	115	5.38	2.89	1.79	0.39	0.19	0.12	140	4.60	42.10	104,400
9	22	F	120	5.80	3.13	1.73	0.29	0.55	0.09	293	6.20	39.20	89,520
10	21	M	185	6.18	2.47	2.04	0.95	0.57	0.15	60	5.27	45.90	164,640

Table 1. Table showing donor age, sex, BMI, circulating plasma MV numbers and Hemavet 1700FS (Drew Scientific) cellular blood parameters. Each sample was analysed by a curve linear coefficient of correlation ($P>0.05$). * Where available, averaged patient data from replicated samples taken on different days with Standard Error of the Mean (SEM) are shown.

4. Discussion

Previous studies investigating similar relationships between MV quantification and either patient BMI (33) or waist-to-hip ratio (34) did not report as strict a standardization of patient variables, nor acknowledge the rigorous protocol standardization necessary to minimize the confounding factors when quantifying MVs. Furthermore, these studies did not report the rationale for the analysis. Therefore, in light of issues raised recently, both here and in numerous previous publications (4, 10, 12, 15, 25, 29, 31), any further interpretation of the data in these respective publications is hard to justify.

It is known that MVs are released from cells during apoptosis and upon cell activation (2, 3, 4). Increased weight is intrinsically related to a state of chronic oxidative and inflammatory stress (35); it has also recently been implicated as a cause of endothelial damage and increased C-reactive protein levels, leading to apoptosis (36, 37). As MVs have been shown to increase oxidative activity (38) and been implicated in inflammatory stress (39), it is evident that there is significant overlap of associative effects between increased weight and increased MV activity. Most importantly, it has been demonstrated that increased weight increases cell activation (40), which we know to be a process of MV formation. This strongly supports our findings that BMI and MV number are inherently related, indicating that weight-related factors may be mechanistic in MV formation.

Irrespective of the concerns, there is a distinct correlation between baseline circulating plasma MV numbers and BMI (Figure 2). These findings suggest that circulating plasma MV numbers may have important and unique implications as a clinical cell-derived blood parameter and therefore warrant continued investigation.

It should be noted, however, that we acknowledge that we did not consider racial difference, which has recently been recognized as a factor affecting MV numbers (1). As there only limited donors of different racial backgrounds were used in this study, intra-variance due to this factor is cannot be determined by it.

Although cFCM has limitations in MV analysis, including in relation to detection and polymer beads for standardization (16) and coincidence events as a source of error (17, 18), as well as whether scatter or fluorescence is the best parameter to identify an event (41), we are able to show here that, with strict protocol standardization, cFCM does have a place in MVs research. Even with the acknowledged potential pitfalls of this study, we have seen a strong correlation of MV number and BMI. If the global MV population could be analysed and coincidence events reduced, then the results would be more accurate. The accuracy error cannot currently be defined by either cFCM or DLS alone, but the techniques used in tandem give greater assurance in the interpretation of data.

It has been suggested that dielectric measurements or modified FCM and DLS would be more accurate in determining the count and size distribution of MVs than conventional light scatter measurements alone (42). There are a number of potential instruments which would overcome these sources of error: the qNano by iZod (17, 43); the Invitrox Surface Antigen Detection and Enumeration (ISADE) by Invitrox (44); the DelsaMax Pro by Beckman Coulter; and the Nanoparticle Tracking Analysis (NTA) by NanoSight (45, 46). These are just a few which appear very promising in their ability to compliment cFCM analysis of MVs.

Although there are many concerns remaining with regard to best practices in FCM analysis of MVs [47], using FCM to analyse MVs to address biologically relevant questions should and no doubt will continue. At the same time, however, there is a need to better acknowledge and address the issues with small particle FCM through standardization and complimentary analyses.

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