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Flow cytometric assessment of circulating microparticles – towards a more objective analysis

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Abstract

Circulating microparticles (MPs) are vesicles derived from membranes of activated or apoptotic cells. They are regarded as a reservoir of physiological and pathological effectors. Lack of standardization and high variability in MPs investigation hamper efforts to translate MPs -related research into clinical applications. The goal of our study was to compare different methods of flow cytometry analysis of MPs that could facilitate standardization. We found that methods based entirely on gating protocols were more time consuming and less accurate. Combining gating with Overton subtraction and/or probability binning algorithms may provide faster, more reproducible and objective alternatives.

Keywords: microparticles, flow cytometry, semi-automatic processing, Overton subtraction, probability binning.

Introduction

Circulating microparticles (MPs) are vesicles derived from membranes of cell in apoptosis (programmed death) or in activated state. MPs are variable in size (but generally under 1 μm in diameter) and in composition (expressing on their surface a selection of molecules from the parent cell) and have been regarded for many years as "cell dust" [1]. The importance of these membrane fragments has been gradually reconsidered and today they are regarded as a reservoir of effectors involved in the pathophysiology of inflammatory and prothrombotic responses, and vascular remodelling [2].

The potential role of MPs as biomarkers was suggested in many studies, addressing a wide pathology - diabetes mellitus, cancer, hypertension and atherosclerosis, other inflammatory and thrombotic diseases, autoimmune diseases such as primary Sjögren syndrome, systemic lupus erythematosus and rheumatoid arthritis, as well as osteoarthritis, periodontitis, kidney chronic diseases, pulmonary hypertension, gastric ulcer and bacterial infections [3]. MPs in these studies are derived mainly from plasma cell populations such as platelets, leucocytes, endothelial cells, etc. A causative link is investigated between cancer metastasis and platelets activation, as MPs derived from platelets membrane (PMP) generate a series of pro-angiogenic elements, as well as vascular endothelial growth factor and the basic fibroblast growth factor. In hormone-refractory prostate cancer, MPs derived from platelets and their interactions have predictive value for the survival rate [4]. There also seems to be a cause-effect link between MPs of leukocyte origin and their presence and the extension of the atherosclerotic process, as seen in subjects in preclinical stages [5]. MPs with pro-coagulant

activity have been found in greater numbers in patients with prothrombotic afflictions. Those of monocytic origin may contribute to the initiation of the thrombus [6], via the functionally active tissue factor, which is taken up by platelets through circulating MPs [7].

MPs of endothelial origin (EMP) and PMP are significantly elevated in patients with severe hypertension. There is also a significant positive correlation between blood pressure, EMP and PMP, an observation which supports the hypothesis that endothelial cells and platelet activation may play a role in the pathophysiological mechanisms involved in the accelerated organ damage seen in patients with severe hypertension [8]. MPs are involved in the revascularization process following an ischemic attack, acting as triggers of atherosclerotic plaque neovascularisation and development, which in turn will lead to the new apoptosis-generated MPs and to EC proliferation, in a pathogenic vicious cycle [9]. MPs levels are significantly increased in primary Sjögren syndrome, systemic lupus erythematosus and rheumatoid arthritis, thus representing a potential biomarker for the systemic level cellular activation [10]. In thrombotic thrombocytopenic purpura the characteristic hypercoagulable state is explained partly by factor XI-dependent pro-coagulant properties of erythrocyte-derived MPs [11].

As a natural extension of the investigations concerning pathogenesis, there is a growing interest about means of evaluating the effectiveness of specific treatments using MPs. Dosage and duration of therapy with hydroxyurea or L-arginine, for instance, or modulation of transfusion protocols may be adapted according to the results of MPs analysis [12]. Patients with chronic bleeding may benefit from pro-coagulant properties of MPs, as techniques are developed and refined to induce selective MPs production and isolation. Research on MPs led also to patents concerning their therapeutic role, as in cardiovascular diseases [13].

Given that the importance of MPs is increasingly recognized, a series of techniques were employed for their analysis, with flow cytometry being the most widely used [14-17].

Classical methods of flow cytometry analysis

The usual procedure for comparing a test sample (from healthy subjects or from patients) to its paired negative control begins with data acquisition for the control and test samples. This is followed by the gating process – the computer-aided step of delineating the contour of regions which include events of different MPs populations that express on their surface just a particular antigen or a combination of antigens. The "negative" events gate includes only events of minimal (background and non-specific) fluorescence. The positive events gate includes MPs defined by the specific fluorescence of fluorochrome-conjugated antibodies bound to antigens on MPs' membranes. A threshold including an arbitrary percentage of total events is defined, so that highly fluorescent readings (if any) in the negative control which lie on a histogram above this threshold will be assimilated with false positives. Cytometric gates created in the analysis of the negative control are then used as analysis blueprints for the paired test sample. The true percentage of MPs that are positive for a particular antigen is determined by subtracting the percentage of positive MPs in the negative control population from the corresponding percentage of MPs in the test population. Consequently, in this relative analysis method the real positivity of test sample events will be defined according to the negative control fluorescence based on positive percentages.

Overton cumulative subtraction

The algorithm of this absolute method evaluates the statistical difference between two distributions by creating a virtual overlay of histograms generated for the control sample and the test sample, followed by the subtraction of the control fluorescence values from the test sample fluorescence counterparts, for each individual channel [18, 19]. The result is a histogram of differences between the sample and control across the entire range of fluorescence, and not only of differences of arbitrarily set percentages. Overton subtraction

provides the percentage of positive events in the sample, but does not provide the probability that the two distributions are different, nor a confidence interval.

Probability binning with the metric T(X)

This is a variant of chi-square test used for comparing univariate and multivariate distributions [20-22]. The control distribution (represented by fluorescence values of the negative control) is divided so that each division has an equal number of events. This division is then applied to the test samples (maintaining the number of divisions obtained in control) and normalized chi-square values are computed. These values are converted into T(X) units similar to the t-score, and are used to estimate the probability that the two distributions (control and sample) are statistically different. An important advantage of this method is the possibility of ordering multiple distributions, in accordance with their degree of differentiation.

Materials and methods

Samples data

Cytometric data used in this study was compiled from several sets of data acquired previously on a BD FACSCanto II cytometer, totalling 51 samples from 20 healthy controls and 31 patients with cardiovascular disease in different stages.

MPs isolation and staining

There is a plethora of ways of preparing MPs for cytometric analysis, lacking a standard differential centrifugation protocol [23]. In our study, for the isolation of circulating MPs a volume of 3 mL of peripheral venous blood was collected in 3.2% sodium citrate tubes and pre-analytical steps were performed within two hours. Following 10 minutes of low speed centrifugation at 1500 x g, 1 mL of the platelet rich plasma was collected from the supernatant in capped 1.5 mL tubes and subjected to centrifugation at 13000 x g for another 10 minutes. A volume of 800 µL of supernatant (platelet poor plasma) was collected and centrifuged at high speed (21250 x g) for 30 min, at room temperature, so that MPs pellets lodged to the bottom of the tubes. 700 µL of supernatant were then collected and discarded and the resulting pellet was suspended in 100 mL and divided in two 50 mL control and test tubes. MPs were stained for 30 minutes on ice, in the dark, with periodic mild shaking, with mouse anti-human fluorochrome-conjugated antibodies (FITC-CD31, PE-CD62E, PerCP-CD42b, Alexa Fluor 647-Annexin V) and mouse IgG1, kappa isotype control antibodies as negative controls (from BioLegend, San Diego, CA, US). A washing step was performed by high-speed centrifugation (30 minutes at 21250 x g, 20° C), 1 mL of supernatant was collected and discarded from each tube and stained MPs in pellets were re-suspended in 400 mL of Annexin buffer (BioLegend).

Flow Cytometry

TransFluoSpheres fluorescent microspheres of 1 µm in diameter (Molecular Probes (Invitrogen), Eugene, Oregon, US) were used to create a MPs gate in a side scatter (SSC) vs. forward scatter (FSC) dot-plot. Events in this gate were further plotted in forward scatter area vs. forward scatter height and a singlets population was defined and subjected to phenotype analysis. For sample acquisition a BD FACSCanto II cytometer running FACSDiva 6.5 (BD Biosciences, San Jose, California, US) was used at medium flow-rate setting.

Data processing

This involved the comparison of manual (relative) methods and semi-automatic (absolute), using FACSDiva 6.5 and FlowJo 7.6 (TreeStar) software.

1. For the *first group* the sequential Boolean gating approach was used. Four relative gating variants were compared for data processing:
- one variant employing visual gating, based solely on visual inspection and manual gating;

- four variants of percentage positive gating: the percentages of accepted positivity in the isotype control were 1%, 2%, 5% and 10% respectively.
Starting from the MPs/singlets gate already created as previously described, a series of sequential and Boolean gating steps were applied and several phenotypic MP, EMP and PMP subpopulations were defined, as follows: EMP (CD31+/CD42b-), EMP (CD31+/CD62E+), EMP (CD62E+/Annexin V+), EMP (CD31+/CD42b-/CD62E+), EMP (CD31+/CD42b-/CD62E+/Annexin V+), PMP (CD31+/CD42b+), PMP (CD31+/Annexin V+/CD42b+), MP CD31+/Annexin V+.

2. The *second group* of processing methods included a semi-automatic processing, while the subjective gating process was greatly reduced and simplified:
- Overton cumulative subtraction method
- Multivariate probability binning

This type of data processing needs less gating; only well defined populations were gated, such as MPs' gate and singles' gate, without drawing additional gates for various phenotypic populations of MPs.

Results

For manual gating methods it would have been be difficult or even impossible to analyze some samples (Figure 1). This was mainly due to the negative value of the difference between the test samples and their isotype controls in the segment of set positivity. In such cases, these samples could not be included in the final statistical analysis, without increasing uncertainty.

On the other hand, Overton cumulative subtraction method and univariate/multivariate probability binning allowed for the analysis of these "difficult" samples by enabling the processing of all events in the flow cytometry files, including the low-fluorescence events, which form a large majority. Inclusion of these cases into analysis led to a correction of the results by 10% for control samples and up to 25% for patients.

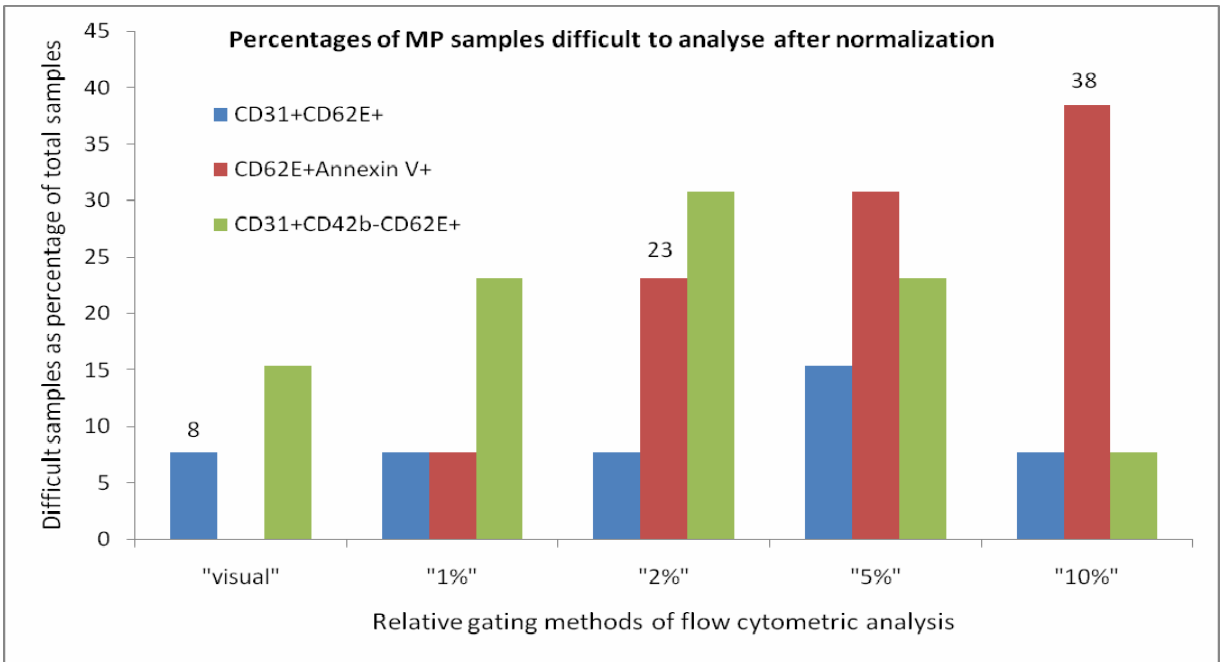

Figure 1. Comparison of five different methods of gating, showing high variability of the number of uninterpretable sample results, for which the negative control yields greater number of MPs in the "positive" gate than their test sample counterpart, leading to negative normalized values.

Discussion

Manual gating methods of analysis are relative, in the sense that they depend on the subjective choice of the cytometer operator. Even when choosing a fixed threshold of accepted false positivity (e.g. 1%, 2%, 5%, 10%), uncertainties derived from the cytometry software's graphical user interface, resolution of the screen and data input devices resolution etc. lead to results with high variability and low reproducibility, all the more important when dealing with MPs, rare cell subpopulations (low percentages of the parent population), or low specific fluorescence.

Inter-laboratory variability coefficient in cell analysis can reach 44% [24-27]), even when using standardized protocols and reagents. This variation is in good part attributed to differences in processing data and particularly to gating procedures, and it was reduced in some cases from 55% to 24% using centralized data processing and by applying automatic tracking algorithms and dynamic gates. Nonetheless, for well differentiated populations, intra- and inter-variability of the data processing flow are easier to manage and to correct. Acceptable ranges are presented in guidelines and algorithms of medical investigations, central to an accurate diagnosis and of great utility in research.

In contrast, in populations of particles which are low in numbers compared to the population of origin, as well as for those with relatively low fluorescence intensity, traditional processing methods may lead to significant variations that complicate or even render impossible the final biological interpretation. Moreover, there is a lack of standardization in both pre-analytical and analytical steps of MPs investigation [23], although efforts have being made in recent years in this respect [14, 28]). Options to improve separations between positive and negative populations are few and often difficult to apply in practice: lowering background fluorescence, increasing specific fluorescence, and improving the resolution of the cytometer. The gating process is hampered by a higher degree of variability, as the shape and size of the gates can vary significantly, depending on the cytometer, or even at different times for the same operator. MPs offer a much smaller surface available for antigen expression compared to the entire cellular surface, so they present a lower number of surface antigens. Consequently, MP fluorescence intensity is lower than that of the parental cell, so the distinction between positive and negative MPs is less clear. Univariate graphical representations of intensity distribution for CD31, CD42b-, CD62E and Annexin V frequencies were often rather single-mode histograms, with a peak which included both negative and (weakly) positive MPs populations.

Cytometry experiments are based on biological laws, with a significant stochastic component. Even if they are run under identical conditions, by the same staff and using the same equipment, each experiment will generate data that will be slightly different from the previous experiment and next one. Hence, any meaningful interpretation of its results cannot be purely statistical, and must be performed in a biological context. In MPs studies, test and control biological samples may show significant statistical differences that are not biologically relevant. As a result, a threshold was set, above which any statistical difference between control and test samples was considered as biologically significant as well. This threshold was determined through a comparison of samples with themselves, as they were biologically identical, but sensitive algorithms could have deemed them as statistically different.

Overton method added precision to univariate analysis of MPs, as they present frequency histograms which are not bimodal (they lack the classical pattern of two peaks representing negative and positive events). For such populations where there is a continuum of fluorescence levels, ranging smoothly from negative events to dim events and further to

medium and highly fluorescent events ("bright"), a rigorous analysis using conventional methods would not be always accurate, or sometimes even possible.

Of the two computational alternative methods tested, probability binning had an important advantage over the Overton subtraction, namely it could be applied to both single and multi-color fluorescence analysis, while Overton subtraction only could be a valid choice for single color comparison of different distributions.

Conclusions

Research on MPs may lead not only to a better understanding of their pathophysiological mechanisms, but to more applications in therapy. As a primary technique of investigating MPs, flow cytometry is in need of adopting better algorithms for data processing. The partly automatic variants discussed here may help achieving this goal and, ultimately, pave the way for moving to a fully automatic, entirely objective alternative of data analysis.

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