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REGULAR ARTICLE

Isotype controls in phenotyping and quantification of microparticles: A major source of error and how to evade it

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Abstract

Background: The characterisation and quantification of cell-derived microparticles (MPs) using flow cytometry are often complicated by a low staining intensity and a non-discrete signal pattern of many cell surface antigens. Fluorescence-labelled isotype controls (ICs) are commonly used to set limits for the discrimination of antigen positive vs. negative events.
Objectives: The influence of different ICs on the characterisation and quantification of MPs was studied. Antigen negative MPs stained with an antibody of interest were evaluated as an alternative control.
Methods: MPs were prepared from platelets, endothelial cell lines and leucemic cell lines and stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) labelled antibodies or isotype controls. Results are given as the mean fluorescence intensity (MFI) or percentage of "false-positive" events above a fluorescence intensity >1.
Results: Using identical instrument settings, seven different ICs (FITC-conjugates $N=3$, PE-conjugates $N=4$) resulted in a wide range of MFI and percentage of positive events with a mean coefficient of variation (CV) of 0.77. Instead, NMPs showed less variability with a mean CV of 0.50 and allowed a reliable and reproducible quantification of MPs when set as controls with <2% false-positive events above an FI >1. As a result, the expression of certain antigens (e.g. CD62P) was lower compared to previous reports in the literature.
Conclusions: Diversity in the staining intensity of isotype controls is a potential source of error in the characterisation and quantification of MPs by flow cytometry. The use of antigen negative MPs to adjust instrument settings is suggested.

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Introduction

The physiological and pathological activities of cell-derived microparticles (MPs) have been extensively studied in the last decade and an astounding variability of effects on e.g. plasma coagulation, inflammation, angiogenesis, immunomodulation, stem cell engraftment, tumor metastasis among others has been attributed to them [1].

Commonly generic MP detection is accomplished by flow cytometric detection of phosphatidylserine (PS) with Annexin V [2] although it has been proven that not all MPs are positive for PS e.g. far more MPs from activated endothelial cells express CD62E than PS [3]. Thus, at the moment there is no single biological marker for the detection of all MPs in human blood and there is a wide range of antibodies used to determine MP origin.

While the amount of Annexin V positive signals can be easily distinguished from negative signals by using calcium-rich vs. calcium-depleted (e.g. EDTA) staining buffer, the discrimination between positive and negative MPs for most other phenotyping antibodies can represent a major obstacle as MP populations usually display a non-discrete fluorescence signal. This can be explained by size diversity (0.1–1.0 μm) and way of formation (shedding) which causes a random frequency of membrane antigens. Currently almost all groups quantifying MPs by flow cytometry are using commercially available isotype controls for setting a positive vs. negative discrimination limit in accordance to standard cell phenotyping. But due to the lower number of antigens, fluorescence intensity of MPs is usually lower than that of cells and thus even small variations in determining the antibody background binding by isotype controls may have significant consequences on the quantification of antigen positive MPs. Variation in staining of the same cell population by different isotype controls has been reported and thus even the use for routine clinical cell enumeration, like CD34+ stem cells, has been questioned and debated [4].

The variability of isotype controls regarding background binding can be mainly attributed to four relevant aspects: (a) non-compliance of isotype with antibody subgroup, (b) conjugation variations leading to differences in fluorochrome to protein ratio, (c) location of fluorochrome binding possibly interfering with "background binding site" and (d) isotype exactly matching antibody concentration.

While there is an abundance of papers measuring MPs in clinical situations, most of them based methodically on previously published protocols, there have been very few publications about "correct and reproducible" processing and quantification of MPs. Thus, a great heterogeneity remains at both pre-analytical and analytical stages. Although some issues have been investigated, e.g. influences of blood sampling site and technique [5], of anticoagulant in collection tube, of storage time at room temperature, the choice of quantification beads [6] and instrument settings of flow cytometer [7], other relevant aspects like loss of MPs by different centrifugation protocols or by freezing–thawing procedures as well as correct quantification in distinguishing positive from negative MPs remain open.

We therefore tested the background signals of 7 commercially available and often used FITC- or PE-conjugated isotype controls. As our results demonstrated great variations we furthermore established a new method of discrimination setting by using MPs, which were generated in vitro from whole blood, platelet-rich plasma or four other cell lines and lacked a certain antigen, as negative controls for the antibodies of interest.

Materials and methods

Reagents and monoclonal antibodies

The following antibodies were employed in this study. Suppliers (BD, Heidelberg, Germany; BC, Krefeld, Germany; AbD Serotec, Duesseldorf, Germany; DAKO, Hamburg, Germany; Abcam, Cambridge, UK; MBL, Woburn, MA, USA), clone and initial concentration are shown in parentheses.

Isotype controls (IC):
(F1) IgG1k-FITC (BD, X40, 50 μg/ml), (F2) IgG1-FITC (BC, 679.1Mc7, 50 μg/ml), (F3) IgG1-FITC (Serotec, W3/25, 100 μg/ml), (P1) IgG1k-PE (BD, X40, 50 μg/ml), (P2) IgG1k-PE (BD, MOPC-21, 50 μg/ml), (P3) IgG1-PE (BC, 679.1Mc7, 6.25 μg/ml), (P4) IgG1k-PE (DAKO, DAK-GO1, 100 μg/ml), IgG_{2b}k-FITC (BD, 27–35, 100 μg/ml after 1:10 dilution).

Antibodies:
Annexin V-Cy5 (Abcam), CD41a-PE (BD, clone HIP8), CD45-PE (BD, HI30), CD62P-FITC (Serotec, AK-6; BD, AK-4), CD62E-PE (BD, 68-5H11), CD142-FITC (AbD Serotec, I), CD146-FITC (Serotec, OJ79c), CD162-FITC (MBL, PL2), CD235a-FITC (BD, HIR2).

Other reagents included 1 μm standard microparticles (Sigma–Aldrich, Seelze, Germany), TRAP-6 (Bachem, Weil am Rhein, Germany), Calcimycin A23187 (Sigma–Aldrich), Annexin V binding buffer 10× (BD; final calcium concentration: 2.5 μM), Annexin V control buffer 1× (10 mM HEPES/NaOH, pH 7.4, NaCl 140 mM, EDTA 50 mM), TruCount Tubes (BD) and FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell culture conditions

The following cell lines were chosen according to their antigen profiles which have been extensively studied and can be viewed on the web-site of the "German Collection of Microorganisms and Cell Cultures" (www.dsmz.de). The antigen data for EA.Hy-926 were derived from proteome analysis [8]. U937 (Human hystiocytic lymphoma cell line, expressing markers and properties of monocytes), HL-60 (human acute myeloid leukaemia cell line, expressing myeloid markers), BV-173 (human lymphoblastic B cell precursor leukaemia, expressing lymphoid markers except for CD45) and EA.Hy-926 (immortalized endothelial cells derived from fusion of human umbilical vein endothelial cells (HUVECs) with lung carcinoma cell line A549) were long-term cultured in-