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Research paper

A novel flow cytometric approach to distinguish circulating endothelial cells from endothelial microparticles: Relevance for the evaluation of endothelial dysfunction

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ABSTRACT

Circulating endothelial cells (CEC) and endothelial microparticles (EMP) are emerging as markers of endothelial repair and activation/apoptosis. Although significant changes in the number of CEC and EMP in pathological conditions have been reported, their reliable identification and quantification still remain a technical challenge. Here, we present a novel methodology for the identification and quantitation of CEC and EMP based on multicolor flow cytometry. Using a lyse/no wash protocol, we observed that in 50 μl of peripheral blood, the large majority of events expressing an endothelial phenotype (CD45−/CD146+/CD34+) are due to non-nucleated particles (DRAQ5−) carrying mitochondrial activity (MitoTracker+) and, therefore, classified as EMP. We enumerated circulating EMP by single platform absolute count in a lyse/no wash four-color flow-cytometric procedure, which allowed the distinction, within the whole endothelial compartment, of EMP derived from endothelial progenitors (CD45−/CD146+/CD34+/CD117+) and from mature endothelial cells (CD45−/CD146+/CD34+/CD117−). A significant increase in both subsets was observed in patients with diabetes mellitus. Thus, this simple and highly reproducible method may be useful for monitoring endothelial dysfunction in clinical settings.

1. Introduction

Novel biomarkers of endothelial dysfunction are needed to improve diagnostic accuracy and monitor therapeutic outcomes in diseases associated with endothelial damage, such as pre-eclampsia, diabetes, systemic lupus erythematosus, atherosclerosis, and inflammation. Anti-angiogenic cancer treatment could be also improved if reliable surrogate markers of drug activity were available. Accumulating evidence points to circulating endothelial cells (CEC) and endothelial microparticles (EMP) as suitable novel markers of endothelial dysfunction (Smadja et al., 2010). Unfortunately, consensus on methodology to distinguish and characterize CEC and EMP has not yet been reached and different techniques may give inconsistent outcomes. CEC represent a rare subset of peripheral blood cells (about 1 in 10^8 cells), which contribute to tissue revascularization and regeneration (Shaffer et al., 2006). CEC populations include mature cells exhibiting endothelial phenotype and circulating endothelial

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progenitor cells (CEPC), expressing markers of the mature endothelium as well as of endothelial progenitors (Masouleh et al., 2010; Peichev et al., 2000; Rafii, 2000). To date, no single marker specific for CEC has been found, since endothelial antigens are also present on other circulating cells. Therefore, a combination of markers is needed to recognize CEC. In this respect, flow cytometry has opened interesting perspectives for CEC detection and enumeration in clinical settings. A lyse/no wash procedure, in which CEC were defined as events expressing both CD144 and CD146 has been recently proposed (Ozdogu et al., 2007). This technique is faster and easier to standardize compared to enrichment-based methods, but it gives higher absolute CEC counts (Blann et al., 2005; Khan et al., 2005).

Microparticles (MP) are fragments of virtually all cell types (endothelium, platelets, leukocytes) released during cell apoptosis or activation and characterized by an integral plasma membrane expressing the phenotype of the cell from which they originated (Sabatier et al., 2002). MP may represent indices of cell activation and/or tissue degeneration occurring during pathophysiological events in vivo (Morel et al., 2004). They are distinct from exosomes and apoptotic bodies and carry relevant bioactions (György et al., 2011). EMP play key roles in coagulation, inflammation and angiogenesis (Dignat-George and Boulanger, 2011) and can be enumerated by flow cytometry using endothelial markers, i.e. CD144 and/or CD146 (Bardin et al., 1996; Petzelbauer et al., 2000). Thus, the CEC and EMP phenotypes overlap to a large extent, except that CEC, differently from EMP, are nucleated. Therefore, CD144+/CD146+ CEC can be confounded with EMP and overestimated if nuclear stain is not used (Bertolini et al., 2006). In the present study, we employed lyse/no wash procedures in combination with single platform flow cytometry, including absolute counting, to distinguish and enumerate CEC and EMP in peripheral blood.

2. Materials and methods

2.1. Subjects

Twenty patients with diabetes mellitus (DM) (15 males and 5 females) and fourteen healthy subjects (7 males and 7 females) were recruited for this study at the Diabetes Clinic of Chieti University Hospital (Supplemental Table 1). Type 2 DM was defined according to the criteria of the American Diabetes Association and all patients were on chronic low-dose aspirin in agreement with the Position Statement of the American Diabetes Association (American Diabetes Association, 2006). The study was approved by the local ethics committee and was carried out according to the declaration of Helsinki and subsequent revisions (World Medical Association declaration of Helsinki, 1997). All participants gave written informed consent. To minimize confounding factors, we selected a homogeneous patient population with respect to the degree of metabolic control (hemoglobin A_{1c}<8%). Patients on chronic treatment with statins were excluded, since these drugs are known to affect CEC number (Pignone et al., 2010).

2.2. Cells and antibodies

Peripheral blood was collected in ethylendiaminictetraacetic acid-containing tubes. Primary human umbilical vein endothelial cells (HUVEC), used as control for the endothelial phenotype, were obtained as previously described (Jaumdally et al., 2009). Briefly, after perfusion of umbilical cords with 0.1% collagenase at 37 °C, HUVEC were grown on 0.2% gelatine-coated plates in M199 endothelial growth medium (BioWhittaker) supplemented with 10% fetal bovine serum, 10 μg/ml heparin and 50 μg/ml of endothelial cell growth factor (Sigma, St. Louis, MO).

Fluorescein isothiocyanate-conjugated anti-CD45 was purchased from Ancell (MN, USA); FITC-conjugated anti-CD144 (CD144-FITC) was obtained from Acris Antibodies GmbH (Herforfd, Germany); phycoerythrin-conjugated anti-CD146 (CD146-PE), peridinin chlorophyl protein-conjugated anti-CD34 (CD34-PerCP) and allophycocyanin-conjugated anti-CD117 (CD117-APC) were purchased from Becton Dickinson (San Jose, CA); CD34-APC and CD45-APC were purchased from Immunotools (Friesoythe, Germany); DRAQ5 was obtained from Biostatus Limited (UK). MitoTracker Green FM was purchased from Molecular Probes—Invitrogen (Milan, Italy). The reagent list is summarized in Supplemental Table 2.

2.3. Flow cytometry: cell staining and analysis

For each test, 50 μl of whole blood was placed into a Trucount tube (BD) by reverse pipetting. The antibody/probe mix (Supplemental Table 3) was added to each tube and incubated for 30 min at room temperature (RT) in the dark. Samples were then incubated with 1 ml of FACS lysing solution (BD) for 10 min at RT in the dark. All antibodies and probes were titrated to obtain optimal dilution for the experimental settings. In each analysis 1,000,000 of total events was recorded. No threshold on morphological parameters was applied. Quality control included regular check-up with Rainbow Calibration Particles (6 peaks, BD Biosciences). Because rare events were analyzed, extensive rinsing was performed, to reduce "contamination" between samples. Comp-Beads (BD) as well as the antibodies used in the assay, single stained DRAQ5 or MitoTracker tubes were utilized to manually assess fluorescence compensation. To evaluate non-specific fluorescence, when defining positive events, we used Fluorescence Minus One (FMO) controls, instead of isotype controls. FMO controls represent the best control for any given marker of interest in a multicolor staining combination, since they contain all reagents except that of interest (Baumgarth and Roederer, 2000; Maecker and Trotter, 2006; Prontera et al., 2007; Roederer, 2001). Data were acquired using a FACScalibur flow cytometer (two-lasers, four-color configuration), equipped with the CellQuest 3.2.1.f1 (BD) software and data were analyzed using the FlowJo™ software (TreeStar, Ashland, OR).

2.4. Statistics

Data are reported as mean ± SD or as median and interquartile range. Comparisons between diabetic patients and healthy controls were performed using the *t*-test for independent samples or the Mann–Whitney *U* test, as appropriate. All tests were two-tailed and analyses were performed using SPSS17.0 (SPSS Inc., Chicago).