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Review

Technical notes on endothelial progenitor cells: Ways to escape from the knowledge plateau

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Abstract

In the last 10 years an increasing interest has been devoted to the study of endothelial progenitor cells (EPCs), a subtype of immature cells involved in endothelial repair and neoangiogenesis. EPCs have been discovered as a novel integrated part of the cardiovascular system, which plays a comprehensive role in tissue homeostasis. Consistently, alterations and/or reduction of the circulating EPC pool have been associated with different manifestations of cardiovascular disorders and atherosclerosis. This is why, the extent of the EPC pool is now considered a mirror of vascular health, while EPC reduction has become a surrogate biomarker of cardiovascular risk and of the ongoing vascular damage. Unfortunately, the methods used to study EPCs still lack standardization, and this is significantly decelerating progress in the field. In this review, we focus on some aspects related to the two methods used to assess circulating EPCs: flow cytometry and cell culture. We uncover the many traps hidden in the choice of the right protocol, and suggest the best solutions on the basis of evidence and background theories.

Keywords: Endothelial progenitor cells; Endothelium; Cell culture; Flow cytometry

Contents

1. Introduction

Since 1997 [1], a huge amount of literature has described the involvement of endothelial progenitor cells (EPCs) in cardiovascular disease. EPCs were originally thought to derive exclusively from the bone marrow, but recent studies have indicated alternative sources of EPCs, including parenchymatous organs and blood vessels [2,3]. We have learned from animal studies that EPCs participate in endothelial homeostasis and stimulate the formation of new blood vessels [4,5]. Clinically, EPCs have been found to be reduced in peripheral blood of subjects with cardiovascular risk factors and/or established atherosclerosis [6–12]: this is why depletion of circulating EPCs is considered a marker of the ongoing vascular damage [13]. Additionally, EPC reduction

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is an independent predictor of future cardiovascular events [14,15], a notion that strengthens the concepts that EPCs are at the same time markers and actors in the entire atherogenetic process. Most importantly, cell therapies that restore the EPC pool have proven beneficial in patients with coronary and peripheral artery disease [16,17]. Really, this has been a revolution, as EPCs are now considered an integrated part of the cardiovascular system.

Unfortunately, expanding interest and lack of a consensus have multiplied the number of different methodologies to study EPCs. A myriad of small studies are now available, each using slightly or profoundly different methods. Likely, this will ingenerate a sort of "knowledge plateau": because of mutual inconsistencies, new studies often raise more questions than they answer. Herein, we would like to focus attention on a few simple technical aspects that all researches should consider when approaching the study of EPCs.

To start, let us define what an EPC is: a cell that derives from the bone marrow and probably from other sources [18], circulates in the bloodstream, proliferates, and can differentiate into a mature endothelial cell [19]. Basically, there are two techniques to study EPCs: (i) flow cytometry of fresh samples and (ii) cell culture.

2. Flow cytometry

Cell count by flow cytometry is based on immunolabelling cells with antibodies directed against surface or intracellular antigens. This method has two important limitations: first, we do not know the precise antigenic phenotype of EPCs, mainly because it overlaps with that of other cell lineages (this is why we should always refer to as "putative EPCs"). Second, definition of EPCs by flow cytometry implies a conceptual abstraction, because a presumed function is attributed to a relatively simple antigenic phenotype. In fact, when performing flow cytometry on fresh peripheral blood, the rarity of circulating EPCs imposes the use of a very limited number of surface antigens. Therefore, assuring that a phenotype based on 2–3 antigens definitively identifies a cell population with a complex function is virtually impossible. Despite these important limitations, flow cytometry is currently the best method to obtain pure quantitative data on putative EPCs. Being sensitive, specific and reproducible, flow cytometry should be considered the gold-standard when count of peripheral blood EPC is conceived as a disease biomarker [20].

To define the antigenic phenotype of EPCs in accordance with the term "endothelial progenitors" we should use at least one marker of immaturity/stemness plus at least one marker of the endothelial lineage. The most common stemness markers used in humans are CD34 and CD133. CD34 is the prototypical stem cell antigen. This 110 kD sialomucin is expressed on haematopoietic stem cells and on the activated endothelium of certain microvasculature, especially in the tumour stroma, but not on large size vessels [21]. CD34 likely functions as an adhesion molecule for the interactions between endothelial cells and haematopoietic precursors, and its deletion induces both vascular and haematopoietic defects [22]. CD133 (also known as AC133 in humans, and prominin-1 in mice) is a 120 kD cholesterol-binding glycoprotein selectively expressed on haematopoietic stem and progenitor cells [23]. The exact function of this molecule is still unknown, but it has been shown that $CD133^+$ cells retain the ability to differentiate into multiple phenotypes, including endothelium [24]. Typical endothelial antigens include KDR/Flk-1 (Kinase-insert Domain Receptor in humans, and Fetal Liver Kinase-1 in mice, representing type 2 vascular endothelial growth factor receptor (VEGFR)-2, CD31, also known as platelet-endothelial cells adhesion molecule (PECAM)-1, and von Willebrand factor (vWf). KDR is the primary receptor transmitting VEGF signals in endothelial cells, and mediating proliferation, sprouting, migration and tube formation. KDR is also expressed on other cell types besides endothelial cells, including early non-committed stem cells [25,26]. Thus, it appears that both CD34 and KDR display an overlapping expression on stem cells and endothelial cells, and they are expressed on primary haemangioblast islets in the yolk sac mesoderm during early embryionic vasculogenesis. $CD34^+KDR^+$ cells could be immature cells with endothelial priming and, therefore represent putative EPCs, or post-natal haemangioblasts [27]. This antigenic definition is the most consistent with the original description by Asahara et al. [1], which showed that human peripheral blood cells sorted according to the expression of CD34 *or* KDR, fully differentiated into mature endothelial cells and formed new vessels *in vivo*. On the contrary, total $CD34^+$ cells should be considered as generic progenitor cells (mostly haematopoietic) rather than EPCs, because a minority of these circulating cells express endothelial lineage antigens. It has been criticized that the $CD34^+KDR^+$ phenotype may overlap in part with that of mature endothelial cells, because CD34 is known to be expressed also on some microvascular endothelia. However, original studies reporting *in situ* expression of CD34 on activated endothelium did not take into account the hypothesis that these vessels contained $CD34^+$ EPCs. The degree of phenotypic overlap between EPCs and circulating mature endothelial cells (CECs) is presently unclear, because CD146, that was thought to be a very specific antigen to identify mature endothelial cells [28], is expressed also on activated lymphocytes [29].

Unlike CD34, CD133 is never expressed on mature endothelial cells and, therefore, $CD133^+KDR^+$ cells may better correspond to EPCs. Unluckily, CD133 is expressed on more immature cells than CD34 and, for this reason, $CD133^+KDR^+$ cells are rarer than $CD34^+KDR^+$ cells in the circulation, in steady-state conditions [30]. Despite these limitations, both $CD34^+KDR^+$ and $CD133^+KDR^+$ can be included among putative antigenic phenotypes of EPCs. The intersection between these two (i.e. $CD34^+CD133^+KDR^+$) could be used as a restrictive EPC phenotype, but these cells are so rare in the circulation that, in some subjects,