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Review

Circulating endothelial progenitor cells: Do they live up to their name?

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ABSTRACT

Preclinical and clinical studies have suggested that specific subsets of cells isolated from the bone marrow or peripheral blood, collectively named endothelial progenitor cells (EPCs), play an essential role in neovascularization and are biomarkers of atherosclerosis, inversely related to the presence and progression of the disease. Conclusive evidence for both the pathophysiological and the biomarker role of these cells is, however, missing, with lack of a unique and universally accepted interpretation for their role, and the absence of general agreement to prompt their use by the practicing clinician. In fact, the engraftment of EPCs after injection into ischemic areas is poor, their secretome is still largely unknown, and there are still many confounding factors—such as co-morbidities and medications—that limit their use as a faithful biomarker of disease. Here we briefly review the literature on EPCs and discuss their significance in cardiovascular disease both as mediators and as biomarkers, including current methods for their identification.

Contents

1. Introduction

Cardiovascular disease (CVD), with its manifestations, such as acute coronary syndromes, stable coronary heart disease, stroke, or peripheral arterial disease, represents the main cause of death worldwide. CVD is caused by the athero-thrombotic narrowing or occlusion of blood vessels, with the resulting tissue ischemic damage. Therapeutic attempts aimed at restoring tissue perfusion and inducing tissue repair are currently focusing on the regeneration of new vessels, a process termed therapeutic angiogenesis. Here a main role is played by endothelial cells (ECs), which are essential for the formation of new vessels. Since mature ECs possess limited proliferative and repair capacity, much interest in recent years has been directed toward progenitors of ECs, capable of differentiating into mature ECs and of contributing to the recovery and repair of ischemic tissues [1]. Endothelial progenitor cells (EPCs) were first described in 1997 by Asahara and colleagues [2]. These authors showed that purified $CD34^+$ hematopoietic progenitor cells from adults can differentiate into ECs. Later on, a number of experimental studies have shown that these cells can increase angiogenesis in ischemic tissues [3], while also being inversely related to the presence and progression of atherosclerosis. In particular, these studies have shown that EPCs do not home at sites of atherosclerotic lesions [4]. The complete or near complete absence of these cells in developing atherosclerotic lesions led to the conclusion that these cells do not contribute to atherogenesis [4]. Hence, EPCs were proposed as cellular biomarkers of disease and predictors of cardiovascular outcomes [5–7]. Since then, a number of clinical trials have tested the ability of bone marrow-derived as well as non-bone marrow derived EPCs to home at sites of vascular injury [8], showing however that the engraftment of these cells after injection into areas of infarcted myocardium is poor.

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The inconclusive results from clinical trials have raised doubts on whether EPCs play a pathophysiologic role in therapeutic angiogenesis, but broadly confirmed that they can be considered biomarkers of CVD. This has however led to the need of a redefinition of their role and of a better understanding of their biological properties in health and disease.

The randomized controlled trial by Fadini et al. published in this same issue of *Vascular Pharmacology* [9] highlights the role of a subset of EPCs, which should be better termed as circulating angiogenic cells (CACs), both as factors and biomarkers of disease. The study focused on the effects of a short time of statin discontinuation on the levels and functional activity of CACs in patients with type 2 diabetes. The authors show that statin discontinuation for 5 days in such diabetic patients induces an increase not only in circulating levels of CACs, but also in their functionality, favoring angiogenesis, without raising inflammatory markers. This effect occurs in parallel with the expected marked worsening in the lipid profile, with clinically relevant increases in low-density lipoprotein (LDL) cholesterol. Cholesterol metabolism, rather than cholesterol-independent "pleiotropic" effects of statins, such as the level of activation of endothelial nitric oxide synthase (eNOS), here appeared to be a major regulator of CAC trafficking and angiogenic activity. At the same time, the study shows that statin-sensitive pathways may be target for stimulating vascular repair in diabetes [9].

2. Identification and characterization of EPCs

It is rather surprising that, after a decade of research, there is no single marker or a universally accepted combination of markers that unambiguously identify true EPCs. There are two possible ways to identify and characterize these cells: flow cytometry, looking at cell surface antigenic markers, and cell culture methods. By flow cytometry, markers most commonly used for EPCs are the expression of CD34, vascular endothelial growth factor receptor-2 (VEGFR2, KDR), and CD133 [2,10,11] (Table 1). However, most of these markers are also expressed in other hematopoietic cells and mature ECs. KDR and CD34 are expressed in both hematopoietic stem and progenitor cells. Specifically, CD34 is expressed by microvascular endothelial cells, hematopoietic progenitors (HPCs) and hematopoietic stem cells (HSCs). Furthermore, cells isolated from the bone marrow and peripheral blood on the basis of the sole positivity for these markers were never demonstrated as being able to generate new endothelial cells in vitro or in vivo [11]. Recently, Case et al., by using hematopoietic and EC clonogenic assays, showed that these cells, or at least most of them, are not true EPCs, but HPCs that express the hematopoietic cell surface marker CD45. Instead, they identified a rare subpopulation of cells, which are $CD45^{-}$, that are devoid of hematopoietic activity and appear to be a real source of EPCs [12]. This observation pointed out to the need of primarily using cell culture methods for the true identification of EPCs.

3. EPC culture methods

EPCs can be isolated from the bone marrow or directly from the peripheral blood by density centrifugation [11]. By this method, peripheral blood mononuclear cells (PBMCs) are isolated in the buffy coat after centrifugation of the sample in a density gradient of Histopaque, Lymphoprep, or FicollPaque. This cell layer is then carefully washed, and is expected to yield a rare EPC population, amounting to ~0.5–1% of the original cell number. Hence, culture methods have been developed to expand this rare cell population. Currently, three main methods are used for the culture of three types of EPCs with different functions and angiogenic potential: Endothelial colony-forming cells (ECFCs, which are considered as genuine or true EPCs); colony-forming unit (CFU)-Hill cells; and CACs (Fig. 1). The first method developed is widely known as the CFU-Hill colony counting method. Here, PBMCs are preplated on fibronectin-coated dishes for two days; non-adherent cells are then re-plated in the presence of specific serum supplements, giving rise to colonies within 7 days of culture [13]. These cells are positive for CD45, CD34, CD31 and KDR, and have the capacity to uptake acetylated LDL labeled with 1,1′-dioctadecyl–3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiLDL) (Table 1). A second method, also known as early-outgrowth EPCs or CACs, was described by Vasa et al. in 2001 [5]. Briefly, PBMCs are plated on fibronectin-coated dishes in the presence of endothelial growth factors and serum. On day 4, non-adherent cells are removed from the culture, and adherent cells are tested for the expression of endothelial progenitor markers. These cells are spindle-shaped, show the same antigenic characteristics as CFU-Hill cells, and have the ability to secrete an array of angiogenic cytokines [5]. A third method is referred to as late-outgrowth colonies or ECFCs, in which a cell population emerging late in culture shows clear endothelial characteristics. These include the cobblestone phenotype, as well as the expression of mature EC markers, i.e., CD34, CD31 and KDR. In addition, these cells are CD45-negative. Rehman et al. have demonstrated that EPCs derived from CFU-Hill colonies do not proliferate, but release pro-angiogenic mediators such as VEGF, hepatocyte

Table 1
Key markers for human endothelial and endothelial progenitor cells.

CD	Alternative name	Ligands and associated molecules	Function	EPCs	ECs
CD31	PECAM-1, endoCAM	CD38, Glycosaminoglycans (GAGs), integrins	Cell-adhesion, activation, migration	Yes	Yes
CD34	GP105-120	L-selectin, MadCAM, CRKL	Cell-adhesion	Yes	No
CD45	Leukocyte Common Antigen (LCA)	p56lck, p59fyn, Src kinases	Regulator of T- and B-cell antigen receptor signaling; regulator of cell growth and differentiation	yes/no	no
CD54	ICAM-1	LFA-1, Mac-1, Rhinovirus	Cell adhesion, lymphocyte activation, and migration	No	Yes
CD62E	E-selectin, ELAM-1, LECAM-2	Sialyl Lewis x, a, CLA, CD162	Cell adhesion	No	Yes
CD62P	P-selectin, GMP-140, PADGEM	CD162, CD24	Cell-adhesion	No	yes
CD106	VCAM-1	Integrin $\alpha4\beta1$, VLA-4	Adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium	No	Yes
CD133	AC133, PROML1, Prominin 1 hematopoietic stem cell antigen		Suppression of cell differentiation	Yes/no	No
CD309	VEGFR2, KDR, Flk1	VEGF	Vascular development and regulation of vascular permeability	Yes	Yes
CD324	E-cadherin		A calcium dependent cell adhesion protein	Yes	Yes
Lectin	Carbohydrate-binding protein	Carbohydrate	Regulation of cell attachment, binding of bacteria and virus	Yes	Yes
DiLDL	Low density lipoproteins labeled with 1,1′-dioctadecyl–3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate	LDL receptor	Uptake acetylated low density lipoproteins	Yes	Yes

Legend: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; LDL, low density lipoprotein; EPCs, endothelial progenitor cells; ECs, endothelial cells.