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Circulating Endothelial Progenitor Cells: A Review of Definition, Characterization and Identification

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Review Article

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ABSTRACT

The healthy endothelium is a major player in the control of blood fluidity, platelet aggregation and vascular tone. Several risk factors present in some diseases promote endothelial dysfunction, which is characterized by reduction of bioavailability and impairment of vasodilator effect, as a consequence, the endothelium not only becomes dysfunctional, but endothelial cells can also lose integrity, progress to senescence, and detach into the circulation. A mechanism of repair is promoted by circulating endothelial progenitor cells (EPCs) recruited from the bone marrow. Circulating endothelial cells (CEC) may be an indicator of vascular damage, while circulating EPCs may be a biomarker for vascular repair. Actually, there is a variety of procedures that one can use to assist in the isolation and quantification of EPCs. This review describes the functions performed by EPCs at each stage of the repairment process, emphasizing the current methods for identifying or quantifying the endothelial lineage.

INTRODUCTION

The endothelium is the monolayer of endothelial cells mechanically and metabolically strategically located, lining the lumen of the vascular beds and separating the vascular wall from the circulation and the blood components [1]. The healthy endothelium is a major player in the control of blood fluidity, platelet aggregation and vascular tone, a major actor in the regulation of immunology, inflammation and angiogenesis, and an important metabolizing and an endocrine organ. This organ weighs approximately 1 kg and consists of 1 to-6x 1013 cells. Endothelial cells controls vascular tone, and thereby blood flow, by synthesizing and releasing relaxing and contracting factors such as nitric oxide (NO), metabolites of arachidonic acid via the cyclooxygenases, lipoxygenases and cytochrome P450 pathways, various peptides (endothelin, urotensin, nautriuretic peptide type C, adrenomedullin, etc.), angiotensins, prostaglandins, reactive oxygen species (ROS), among others. Normally these factors act in a coordinated manner so that the vasodilator and vasoconstrictor influences are locally balanced and regulate the resistance of the vascular tone to maintain steady tissue perfusion [2]. Additionally, these mediators have effects on other endothelial functions such as regulation of cell-cell adhesion, thrombosis and fibrinolysis.

Endothelial dysfunction and circulating endothelial cells (CECs)

Endothelial dysfunction is also referred as endothelial activation, by some authors, which represents a switch from a quiescent phenotype toward one that involves the host defense response [3]. In general, cardiovascular risk factors promote endothelial dysfunction, which is characterized by reduction of bioavailability and impairment of vasodilator effect of endothelium-derived relaxing factors, such as NO, prostacyclin or endothelium-derived hyperpolarizing factor [2]. In addition, can occur an increased production and biological activity of the potent vasoconstrictor and pro-inflammatory peptide endothelin (ET-1). The decrease of NO is an important factor in this process that results from reduced activity of eNOS, (as a result of endogenous or exogenous inhibitors) and to decreased bioavailability of NO [4].This results in an increase of ROS formation (in the presence

of superoxide dismutase), lead to generation of hydrogen peroxide, which, can diffuse rapidly throughout the cell and react with cysteine groups in proteins to alter their function. Moreover, hydrogen peroxide leads to degradation of the eNOS cofactor BH4, leading to "uncoupling" of eNOS, and results in superoxide formation. When ROS are generated at low concentrations can function as signaling molecules participating in the regulation of fundamental cell activities such as cell growth and cell adaptation responses, whereas at higher concentrations, results in very different consequences, such as phosphorylation of transcription factors, induction of nuclear chromatin remodeling and transcription genes, and protease activation [5]. In certain circumstances, when exposure to cardiovascular risk factors is prolonged and repeated, chronic production of ROS may exceed the capacity of cellular enzymatic and nonenzymatic anti-oxidants, as a consequence, the endothelium not only becomes dysfunctional, but endothelial cells can also lose integrity, progress to senescence, and detach into the circulation [3]. Therefore, the number of circulating endothelial cells (CECs) may reflect the state of endothelium dysfunction. Circulating endothelial cells have been recognized as a potential marker of endothelial damage in a variety of vascular disorders. A number of antigens have been used to identify cells of endothelial origin such as Muc-18 (CD146), Thrombomodulin (CD141), VE-cadherin (CD 144), vascular cell adhesion molecule 1 (CD106), Endoglin (CD105), E-selectin (CD62e), intercellular adhesion molecule 1 (CD54) and platelet endothelial cell adhesion molecule-1 (CD31) [6]. According to some authors, CECs have high expression for CD34 marker [7]. In addition mature endothelial cells may express endothelial-specific markers, including type 2 receptor of vascular endothelial growth factor (VEGFR-2), also designated CD309, however they lose this marker when beginning the senescence process [8]. In other hand, these cells have negative expression to leukocyte common antigen (CD45) and Prominin 1 (CD133) [9]. However, endothelial integrity depends not only on the extent of injury, but also on the endogenous capacity for repair. Over time two mechanisms of repair have been identified. One through the adjacent mature endothelial cells that can replicate locally, and replace the lost and damaged cells, another through the repairment by circulating endothelial progenitor cells (CEPCs) recruited from the bone marrow. These cells, once in circulation can differentiate into mature cells with endothelial characteristics [10].

ENDOTHELIAL PROGENITOR CELLS

Definition, characterization and identification

Endothelial progenitor cells (EPCs) are small, immature precursor and bone marrow derived cells that can be found in the peripheral and umbilical cord blood [11]. They are extremely rare events in normal peripheral blood, representing somewhere between 0.01% and 0.0001% of peripheral mononuclear cells [12]. These cells were first isolated from adult peripheral blood (PB) in 1997 by Asahara et al. using magnetic micro beads, on the basis of proteins in cell surface (also known as surface markers); in this case, they based on expression of hematopoietic progenitor cell antigen marker (CD34) [13]. With this discovery, the dogma that differentiation of mesodermal cells to angioblasts and subsequent endothelial differentiation exclusively occur in embryonic development was overturned, since EPCs from adults can differentiate *ex vivo* to an endothelial phenotype [14]. In addition to the aforementioned markers, EPCs show expression of various endothelial markers in their surface, such VE-cadherin (CD144), platelet endothelial cell adhesion molecule-1 (CD31), endothelial NO synthase, E-selectin (CD62E) and von Willebrand factor (CD41), show expression of stem cell markers such Prominin 1 (CD133), and also express surface markers of Hematopoietic System such c-kit (CD117) and Leukocyte Common Antigen (CD45) [11, 15]. However, no marker was identified as specific for EPCs. Moreover, the mentioned surface markers are dependent on the state and localization of the EPCs, because, the surface markers presented in early EPCs are different from the surface markers expressed by mature EPCs. The surface markers present in early EPCs are principally CD133, CD34 and VEGFR-2, termed also kinase insert domain receptor (KDR) or CD309. In the peripheral circulation of adults, more mature EPCs are found that obviously have lost CD133 but are positive for CD34 and VEGFR-2 [16]. It seems, therefore, that the loss of CD133 reflects the transformation of circulating EPCs into more mature endothelial-like cells. However, it is not clear at which time point the EPCs begin to lose CD133, either during their transmigration from the bone marrow into the systemic circulation or later during their mobilization. This indicates that are found two types of EPCs in the peripheral blood and that the cells change their progenitor properties in the circulation [14]. In the present review will be addressed in detail the both types of EPCs (early and late).

FUNCTIONS OF ENDOTHELIAL PROGENITOR CELLS

In response to endogenous or exogenous signals, EPCs are mobilized from bone marrow and home to peripheral tissue sites to participate in endothelial repair. These cells were identified by their functional capacity to form EC colonies and enhanced eNOS expression after shear-stress exposure [17]. Therefore, EPCs have a key role in the maintenance of vascular integrity and are involved in wound healing, tissue regeneration in ischemia, tissue remodeling, growth of tumors and physiological neovascularization [18].

Neovascularization is an essential mechanism determining the formation, but also the maintenance, of the cardiovascular system. It is thought to depend mainly on two processes, angiogenesis and vasculogenesis [19, 20]. Angiogenesis is the process by which new vessels are formed from pre-existing vessels by the activation, proliferation and migration of endothelial cells (ECs). Vasculogenesis is defined as the process by which new vessels are generated when there are no pre-existing vessels, by the migration and differentiation of vascular endothelial growth factor receptor 2 positive (VEGFR-2+) mesodermal precursors, into ECs that adhere to form a primary vascular plexus during embryonic development [21].

It has long been accepted that vasculogenesis is limited to early embryogenesis and was believed not to occur in adults, whereas angiogenesis occurs in both the developing embryo and postnatal life. However, accumulating evidence suggests that EPCs have been identified in peripheral blood in adults and have an important role in homeostasis of the vascular network, [15]. ECs responsible for the formation of primitive vascular structures differentiate from angioblasts in embryos and from EPCs, mesoangioblasts, and multipotent adult progenitor cells in adult bone marrow [22].

Therefore, EPCs might not only be involved in the formation of new vessels in ischemic tissues, but might also contribute to the repair of pre-existing vessels. These cells may influence angiogenesis by the release of paracrine factors or differentiate into mature endothelium and integrate into the vessel lumen [21]. Thus, EPCs might be interesting candidates for novel therapeutic approaches, such the repair of injured vessel wall, the neovascularization or regeneration of ischemic tissue, and the coating of vascular grafts [23].

Various studies have demonstrated that EPCs improve neovascularization, after critical ischemia in animal and human models of several diseases. According to Kawamoto, *ex vivo* expanded human EPCs incorporate into foci of myocardial neovascularization and have a favorable impact on the preservation of left ventricular function in rats [24], and Fan, demonstrated that transplantation of autologous expanded EPC in rabbits can promote neovascularization in ischemic hind limbs [25]. In addition to these studies, some authors demonstrated the effects of EPCs on ischemic brain injury in a mouse model and concluded that systemic delivery of EPCs protects the brain against ischemic injury, promotes neurovascular repair, and improves long-term neurobehavioral outcomes [26]. Relatively to diabetes disease, previous reports exhibiting the effectiveness of EPCs on the wound repair in experimental diabetic animal model [27]. Moreover according to Kirana et al. the EPCs transplantation improves microcirculation and complete wound healing in the transplant groups [28].

Taken together, EPCs serve as a highly promising and novel therapeutic option for ischemic tissue repair. Thus additional investigations related to optimize techniques for EPC isolation, expansion, mobilization, recruitment and survival strategies post transplantation in a ischemic tissue are needed to continue the advancement of this novel therapeutic modality [29].

MECHANISMS BY WHICH EPCS IMPROVE NEOVASCULARIZATION

In the past, the regeneration of injured endothelium has been attributed to the migration and proliferation of neighboring endothelial cells. More recent studies indicate that additional repair mechanisms may exist to replace denuded or injured arteries and EPCs are involved in this process [14]. The process from which EPCs mobilize from the bone marrow and circulate in peripheral circulation to ischemic tissues and tumors, comprises several steps that are recruitment, mobilization, differentiation, homing and regenerative potential of EPCs [16].

Recruitment

Recruitment and incorporation of EPCs requires a coordinated sequence of multistep adhesive and paracrine signals termed chemoattraction, which have utmost importance to allow for recruitment of reasonable numbers of progenitor cells to the ischemic or injured tissues [30]. Those paracrine signals (growth factors or cytokines) generated by ischemic tissue and tumor cells include vascular endothelial growth factor (VEGF) and stromal-cell derived factor (SDF-1) production. However, additional factors inducing mobilization of progenitor cells from the bone marrow, as granulocyte-colony stimulating factor (G-CSF), the granulocyte monocyte-colony stimulating factor (GM-CSF), erythropoietin (EPO) and chemokines such SDF-1 also increased the levels of EPCs [31].

Mobilization and differentiation

Physiologically, ischemia, caused by hypoxia, is believed to be the predominant signal to induce mobilization of EPCs from the bone marrow, because hypoxia, in tumors and ischemic tissues, mediate activation of hypoxia inducible factor gene (HIF-1) [32]. This gene is a heterodimeric transcription factor consisting of a β-subunit and an oxygen-regulated-α-subunit. The HIF-1α and HIF-1β proteins both contain basic helix-loop-helix motifs that bind DNA and cause subunit dimerization. This gene, whose activation is prompted by hypoxia conditions, can interact with enzymes and other transcription factors in other to control vascularization and tissue growth. Therefore, HIF-1 activation promotes an increase synthesis of a potent angiogenic factor, termed VEGF, which is a major regulator of angiogenesis, which promotes endothelial cell migration toward a hypoxic area. This happens, since during hypoxia, HIF-1 binds the regulatory region of the VEGF gene, inducing its transcription and initiating its expression [33]. In turn, the expression of VEGF will promote activation of matrix metalloproteinase-9 (MMP-9) in bone marrow, which will cleave the membrane-bound kit ligand (mKitL) and will induce the release of soluble Kit ligand (KitL, also known as stem cell factor, SCF). Subsequently, cKit-positive stem and progenitor cells, including also a common hematopoietic and angioblast precursor cells (Hemangioblast, HABL), moves to the vascular zone of the bone marrow microenvironment. This translocation activates the cells from a quiescent to a proliferative state. The signals, which initiate the diversion of the hemangioblast to either hematopoietic precursor cells or EPCs are largely unknown, but may include angiogenic growth factors from the periphery, as VEGF and SDF-1 [16]. However, a study reports that thrombin also promotes EPC differentiation and the effects of thrombin on EPCs were mediated via the thrombin receptor PAR-1 [34]. However, further studies are necessary to elucidate the coordinated interplay of transcription factors and other signaling molecules during EPC differentiation [14].

After differentiation, the immature EPCs are spindle-shaped and have a limited proliferation potential [35]. These cells can be characterized by the expression of CD133, CD34, CD31, CD45, vWF, VE-cadherin markers [16].

Homing and regenerative potential of endothelial progenitor cells

It is known that after their differentiation, EPCs leave the bone marrow and move through systemic circulation to the ischemic tissues and contact with injured endothelial cells, this process is known as homing. It is thought that EPCs mobilization from the bone marrow is mediated by integrins. This class of proteins is responsible for cellular tissue architecture and also functions as signal transducers regulating survival, proliferation, differentiation and migratory signaling pathways [21]. The main integrins that regulate the mobilization of EPCs from the bone marrow microenvironment are the α4 integrins, namely α4β1 and α4β7. The α4β1 integrin mediates cell adhesion to vascular cell adhesion molecule-1 (VCAM-1) the α4β7 integrin is important in lymphocyte homing and it also binds to VCAM-1 [36]. This data suggests that EPC mobilization is an active process involving direct interaction between molecular targets expressed on homing tissues and adhesion molecules, namely integrins, expressed by EPCs. Circulating EPCs, during homing, differentiate from immature to mature EPCs and CD133 expression is lost. The mature EPCs begin to express CD309 in addition to CD34, CD31, CD45, VE-cadherin, vWF markers and have a cobblestone appearance [35].

The next step of homing of progenitor cells to ischemic tissue involves adhesion of these cells to endothelial cells activated by cytokines, and the transmigration of the progenitor cells through the endothelial cell monolayer. It is known that adhesion of various cells, including hematopoietic stem cells and leukocytes to endothelial cells, is also mediated by integrins [10]. The integrins that are capable to mediate cell-cell interactions are the β2-integrins and the α4β1-integrin. The latter are expressed by several cell types including endothelial cells and hematopoietic cells, whereas β2-integrins are found preferentially on hematopoietic cells. β2-integrins not only mediate the adhesive interactions of EPCs to mature endothelial cells and to extracellular matrix proteins but are also critical for chemokine-induced transendothelial migration of EPCs. During firm adhesion of leukocytes to the endothelium, members of the β2-integrin family, interact with endothelial counter ligands such as ICAM-1, VCAM-1, and surface-associated fibrinogen [36]. After adhesion and insertion into the monolayer of surrounding mature vascular ECs, this process may be completed, and the injured monolayer is repaired [16].

METHODS FOR EPC'S ISOLATION

In 1997, Asahara et al.isolated CD34+ mononuclear blood cells (EPCs) from human peripheral blood by means of magnetic beads coated with antibody to CD34 [13]. Since this discovery of EPCs, significant steps forward have been taken to reach a better definition and a detailed functional characterization of these cells. However, the outcome and success of several studies have been limited by the lack of unambiguous and consistent definitions of EPCs [37]. Actually, there are a variety of procedures that one can use to assist in the isolation and quantification of EPCs, but these can be simplified into two approaches: *in vitro* adhesion and growth and selection by cell surface phenotype using fluorescent labeled antibodies or flow cytometry [38]. Of note, all current methods for isolating, identifying or quantifying the endothelial lineage potential of circulating cells have limitations in that none has been shown to reliably predict the behavior of the circulating cells in a relevant *in vivo* context [37].

***In vitro* culture of EPC's**

Most culture assays were used to obtain circulating EPCs from peripheral blood for identification of EPCs as biomarkers for cardiovascular disease, for analysis of intracellular signaling pathways, or for enriching cells for therapeutic angiogenesis [37]. After isolation of Peripheral Blood Mononuclear Cells, the cells are cultured in medium with specific growth factors (eg, VEGF, bovine brain extract, and epidermal growth factor), which facilitates the growth of endothelial-like cells. The incubation *in vitro* with a mixture of growth factors, the adhesion of specific substrates (eg. Fibronectin), and the contact with the extracellular matrix or the surrounding mature ECs *in vivo* will probably influence the proliferation or differentiation of bone marrow-derived EPCs [39]. The vast majority of studies used one of the following three culture media: Medium 199 (Gibco, Carlsbad, California, USA) has been used for the culture of coloning forming unit (CFU) assay with fetal bovine serum only, endothelial growth medium (EGM; Clonetics, San Diego, California, USA) supplemented with bovine brain extract and human epidermal growth factor, and EGM-2 (Clonetics, San Diego, California, USA) that contains defined concentrations of VEGF-2, human fibroblast growth factor 2, human epidermal growth factor, insulin-like growth factor 1, ascorbic acid, heparin and hydrocortisone. In addition to different culture media, different extracellular matrix proteins have been used for the coating of cell culture dishes, such cell culture dishes coated with collagen, fibronectin or gelatin, which might also influence the outcome [23]. Considering this, through time three major methods have been used for EPCs *in vitro* culture.

Because the proliferative capacity might be one criterion to define a progenitor cell, several groups established colony assays [37]. The most prominent assay, termed Culture of colony forming unit–Hill cells (CFU-Hill), which cells are plated and after 4-9 days, the nonadherent peripheral blood mononuclear cells give rise to the colony. In this method all cell populations of the PBMC fraction are cultured together, which implies the risk of contamination with mature circulating endothelial and monocytic cells. To minimize contamination, some authors included a pre-plating step, which the nonadherent cells are removed, based in principle that mature endothelial cells should adhere to the culture surface [38].

Another method the Circulating Angiogenic Cells (CAC) that consists in culture of adherent mononuclear cells along 4-7

days. In this case, colony formation not occurs. Finally, the another method commonly used is termed Endothelial colony forming cells (ECFC), which mononuclear cells are plated, and the nonadherent cells are discarded. The remaining cells, which are, the adherent cells are cultured along 7-21 days in endothelial conditions, and after this time colonies with cobblestone morphology appear. With this method high proliferation capacity was verified. The CFU-Hill and CAC methods present an early outgrowth, whereas ECFC method presents a late outgrowth [38]. In addition to the efforts made to improve the techniques of EPCs *in vitro* culture, there will always be a high risk of contamination, and also it has been demonstrated that the frequency of EPCs quantified by culture methods does not correlate with the number of EPCs quantified by flow cytometry [39].

EPCs identification by flow cytometry

Flow cytometry is a technique defined as the simultaneous measurement of multiple physical characteristics of a single cell as the cell flows in suspension through a measuring device [40]. This technique allows measurements on cells (prokaryotic and eukaryotic) or particles (cytokines, chromosomes and beads) that are in single cell liquid suspension.

A flow cytometer is a system consisting of five elements: a light source (mercury lamp or laser), a flow chamber, units of optical filters for selecting a range of specific wavelength, the spectral range from a more wide, photodiodes or photomultiplier for sensitive detection and signal processing with interest and a unit that processes data collected. In the most common scenario, one or more lasers cross each particle or cell, and the light scatter properties are recorded, namely the side scatter (indicator of the particle's complexity) and forward scatter (indicator of the particle's size) [31]. When the laser strikes the cell, the light is diffracted around the edges of the cell, producing a diffraction pattern along the path of the laser beam. This scattered light (forward scatter and side scatter) is approximately equivalent to the cell circumference and is the same wavelength as the exciting laser light. The sample cells in suspension can be labeled with specific antibodies linked to fluorochromes, which allows the identification and quantification of cells with specific features based on the fluorescence [41]. The fluorochromes emit light when excited by light of a shorter wavelength, and can be conjugated directly to the primary or secondary antibody, or to streptavidin.

This technique has several advantages, since it allows multiparameter analysis in a large number of cells and in short time, allowing the identification of a homogeneous population within a heterogeneous population. In addition, also allows the detection of extremely rare populations of events (frequencies less than 10^{-6}), such as stem cells, dendritic cells, endothelial cells, among others [40]. Flow cytometry is currently the best method to obtain pure quantitative data on putative EPCs. Being sensitive, specific and reproducible, should be considered the gold-standard when count of peripheral blood EPC is conceived as a disease biomarker [37]. In addition, it is a rapid and convenient way to measure rare events, thus, this method is clearly well suited for detection and quantitation of EPCs.

Nevertheless, the development of cytometric assays was constrained by the lack of reasonably specific monoclonal antibodies for this task [12]. However, several methodologies have been suggested for EPCs identification but there is not a consensual definition by cell surface antigen expression. In addition, according to Mund, some of the clinical trials published claiming to quantify EPCs actually quantified hematopoietic stem cells, because they did not use enough surface markers [41]. Delorme et al. proceed to distinction of CECs and EPCs, in samples of cord and peripheral blood, based essentially on the surface marker CD146, which is an adhesion molecule present in endothelial cells. According these authors, using 4-color flow cytometry analysis, they discriminate EPCs (CD146+ CD34+ CD45+CD133+ or CD117+) and CECs (CD146+ CD34+ CD45- CD133- or CD117-), in samples of peripheral blood collected from patients after myocardial infarction. As results of four color cytometry analysis, the authors obtained, according to CD45 expression, two distinct subpopulations of CD34+ cells, respectively CD34+CD45+ cells, representing more than 90% of the circulating CD34+ cells, and CD34+CD45- cells, representing less than 3% of the CD34+ cells. These two subpopulations were analyzed individually for the co-expression of CD146, CD117 and CD133, among the CD34+CD45+, a low percentage of cells co-expressed the referred surface markers, and this subpopulation was defined by authors as EPCs. In turn, the CD34+CD45- subpopulation, co-expressed CD146 in a higher percentage, but not co-expressed CD117 and CD133. This subpopulation defined mature CECs. According these authors, a 4-color cytometry analysis of selected CD34+ cells from cord and peripheral blood clearly discriminated between two subsets of circulating CD146+ cells [42]. On the other hand, Khan et al. in their review assume that CD31 and CD146 are present on CECs but not on EPCs or hematopoietic stem cells, and in addition assume that CD133 will help to identify EPCs because it is not present on CECs or any mature endothelial cells. However, CD133 provided a means for detecting primitive stem cells in the circulation without the use of CD34. In addition, according these authors, the CD34+CD309+ combination, is a potential combination of surface markers for EPCs identification. Concerning to CD45 expression of these cells, has been reported for various groups to be positive or negative, due to its Dim expression (slightly increased when compared to the negative control) in these cells [12]. Some studies suggest that particularly the fraction of CD45- cells may harbor the "true" circulating EPCs. For prove this, some authors such as Schmidt-Lucke et al., through samples of patients with coronary artery disease (CAD), considered, in flow cytometry analysis, the CD45- and CD45dim expression, and in their quantification using CD45, CD34 and CD309 surface markers and gating strategy, CD34+ cells were subdivided in CD45-, CD45dim and CD45bright. The data obtained with this protocol showed numbers of CD45dimCD34+CD309+ cells significantly higher in healthy controls compared to patients with CAD and according the authors, this study confirm that indeed only the fraction of CD45dim cells harbours the "true" circulating EPCs [43]. Another parallel analysis of CD45 expression has been also proposed to distinguish EPCs, and most (90%) CD34+ progenitor cells express CD45 at low

intensity (CD45dim), whereas less than 10% are CD45- [44]. Despite this controversy, EPCs were assessed by Hristov et al. in the peripheral venous blood of patients with stable coronary artery disease by 3-color flow cytometry, by CD34+CD309+CD45-/low combination. As result, in flow cytometry analysis, circulating EPCs were obtained in very low percentages, and to improve this, the authors recommended additional strategies in order to increase the sensitivity and accuracy of the method. These included the use of specific high-quality monoclonal antibodies, selection of high-intensity fluorochromes to reveal low-density markers, automatic compensation, exclusion of dead cells, and multiparameter gating [16].

In other study, EPCs and CECs were quantified in patients with venous thromboembolism and myeloproliferative neoplasms, by Torres et al. CECs and EPCs were quantified in peripheral blood samples by CD45-CD146+CD133- and CD45+lowCD146+CD133+ immunophenotyping, respectively. The chosen strategy used CD146 to recognize CEC, and also to identify EPC and CD133 to distinguish between CEC and EPC, since that CD133 is absent in CEC. The results of this study indicate that both patients groups had a significant increase in the CEC numbers, as compared to controls, and a decrease of EPC numbers (in both patient groups) relatively to controls, although differences were not statically significant [45]. In turn, Rustemeyer et al. through umbilical cord blood, bone marrow and whole blood samples, selected the CD309+CD34+ cells, because the EPCs should be contained in this fraction. In addition, to exclude a major portion of shredded cells from the vessel wall, they measured the number of CD133+CD34+ cells. This fraction showed more CD309+CD34+ cells than CD133+CD34+ [46]. In other hand, CD133 is expressed on more immature cells than CD34 and, for that reason, CD133+CD309+ cells are rarer than CD34+CD309+ cells in the circulation, in steady-state conditions [37]. Some authors, such as Distler et al. defended that EPCs identification requires a multicolor approach, that is, the use of several surface markers labeled with fluorochromes. They also recommend the use of CD34, CD133 and CD309 to increase the specificity of the analysis [22]. In addition, Mund et al. assume that in several studies of EPCs identification the contamination with false-positive events and nonspecific fluorescent event readings may occur. Specifically monocytes, red blood cells, and dead cells autofluorescence and nonspecifically bind antibodies. In his study, trough peripheral blood samples and cord blood samples, a population of cells containing endothelial colony-forming cell (ECFCs) and mature circulating endothelial cells was determined by varying expressions of CD34, CD31, and CD146, but not CD133 and CD45. The results show that if red blood cells, monocytes, and dead/apoptotic (LIVE/DEAD) cells are not excluded, it may lead to occurrence of false-positive events [41]. In review Fadini et al. based on the definition of EPCs, recommend that the minimal antigenic profile should include at least 1 marker of stemness/immaturity (usually CD34 and/or CD133), plus at least 1 marker of endothelial commitment (usually CD309) [44].

Beyond all controversy, some authors agree in several issues of quality in flow cytometry, such as, the use of blocking serum to inhibit nonspecific binding, the use of real-time viability stain, the establishment of a dump channel to exclude cells not of interest to analysis, the collection of a large number of events to identify adequate numbers of the rare event population, and clean the cytometer before data acquisition, to remove amounts of cellular debris that have the potential to contaminate the sample of interest. In CEC and EPC assays, at least 500,000 to 1 million list mode events should be collected [12]. In addition, is recommended the setting and monitoring of fluorescence detectors sensitivity and the use of a multicolor approach, because no markers are entirely specific for these cells [23].

Taking together, in comparison to *in vitro* culture assays, flow cytometry is a recent technique that allows a quantitative, more sensitive, rapid and accurate analysis, convenient to rare events. Moreover this technique avoid the high risk of contamination that occurs on *in vitro* assays [16].

SUMMARY

EPCs are strongly involved in, wound healing, tissue regeneration in ischemia, tissue remodeling and physiological neovascularization, which depends mainly on two processes, angiogenesis and vasculogenesis. In these cases, EPCs are recruited from bone marrow to peripheral circulation. Once in circulation, these cells can differentiate into mature cells with endothelial characteristics, and homing to ischemic tissues to initiate the repairment process. From a therapeutic point of view, these functional activities might be interesting for novel therapeutic approaches such the repair of injured vessel wall, the neovascularization or regeneration of ischemic tissue. The mechanisms of EPC recruitment, mobilization, differentiation and homing are regulating for a variety of chemokines and cytokines, and several physiological and pathological conditions. Taking in account the potential of these cells and their rare nature in the blood circulation, is important the knowledge of the more appropriate methods for their rapid, sensitive and accurate identification and quantification. Flow cytometry is currently the best method to obtain pure quantitative data on putative EPCs.

This review article summarized the research progress of EPCs in recent years, such as the functions performed by EPCs at each stage of the repairment process, emphasizing the current methods for identifying or quantifying the endothelial lineage.

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