Clinical Application of Endothelial Progenitor Cell: Are We Ready?

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The discovery of circulating endothelial progenitor cells (EPCs) opened up a new era of EPC-based therapies for cardiovascular diseases. While researchers are enthusiastic about applying EPCs to clinical therapy, progress has been substantially limited due to the lack of a thorough characterization and understanding of early and late outgrowth EPCs (also called endothelial colony-forming cell, ECFCs) biology. As a means of facilitating the understanding of how late EPCs can most effectively be applied to clinical therapeutics, this article reviews the recent progress covering 5 important issues: (1) The best passages of ex vivo-cultivated EPCs for cell therapy; (2) inflammatory activation of late EPCs: a real world consideration; (3) late EPC is not an endothelial cell: an issue of cell contamination; (4) ways to improve EPC function and differentiation; and (5) how to separate and delete smooth muscle progenitor cells (SPCs).

Key Words: Cardiovascular disease • Cell therapy • Endothelial progenitor cell • Smooth muscle progenitor cell

ENDOTHELIAL PROGENITOR CELL (EPC)

The discovery of circulating EPCs in 1997 opened up a new era of EPC-based therapies for angiogenesis in critical ischemic tissues,[1-3] post-injury vascular endothelial regeneration,[4-6] and ex vivo tissue engineering.[7] Previous studies demonstrated that 2 major types of circulating EPCs, early and late EPCs, can be derived and identified from peripheral blood.[8] The early EPCs appear after 3-5 days of culture, are spindle-shaped, have peak growth at approximately 2 weeks and die by 4 weeks. These have been variously termed 'early EPCs' by Gulati et al.[9] and Hur et al.,[10] 'attaching cells' by Asahara et al.[1] and CACs (circulating angiogenic cells) by Rehman et al.[11]

The second type of EPCs appears only after longer culture of approximately 2-3 weeks, forming a cobblestone monolayer with near-complete confluence, and can show exponential population growth without senescence over 4-8 weeks and live for up to 12 weeks. These were termed 'late EPCs' by Hur et al.[10] or OECs by Lin et al.,[12] also called endothelial colony-forming cells (ECFCs). Early and late EPCs (ECFCs) share some endothelial phenotype but show different morphology, proliferation rate, survival features, and functions in neovascularization.

Although early EPCs enhance angiogenesis by providing a variety of cytokines, cell therapy with highly proliferative ECFCs is an emerging therapeutic option to promote endothelial regeneration for a variety of cardiovascular diseases. While researchers are enthusiastic about applying ECFCs to clinical therapy, progress has been substantially limited due to the lack of a thorough characterization and understanding of ECFC biology.

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Similar to endothelial cells, ECFCs may become activated in response to proinflammatory stimuli.[13] This activation potential can be estimated for each patient's own ECFCs, and may be relevant to disease outcomes and possibly used to tailor individualized therapeutic strategies. Moreover, tissue-engineering cardiovascular structures with potentially atherogenic ECFCs may even be detrimental.[13] So far, it has yet to be established as to when ex vivo expanded ECFCs switch from being healthy to atherogenic, and whether ECFCs from patients with different risk factors behave differently in their response to proinflammatory stimuli.

Before ECFCs can be extensively applied to clinical therapeutics, we need to be more familiar with ECFCs. This article reviews recent progress made in the process to better understand ECFCs, which are outlines as follows:

(1) The best passages of ex vivo-cultivated ECFCs for cell therapy.
(2) Inflammatory activation of ECFCs: a real world consideration.
(3) ECFC is not an endothelial cell: an issue of cell contamination.
(4) Ways to improve EPC function and differentiation.
(5) How to separate and delete smooth muscle progenitor cells (SPCs).

THE BEST PASSAGES OF EX VIVO-CULTIVATED ECFCS FOR CELL THERAPY

Understanding ECFCs heralds an era of applying autologous endothelial cell therapy to cardiovascular diseases. However, currently there is no consensus agreement as to the best passage of ex vivo-cultivated ECFC for cell therapy because bone marrow- and peripheral blood-derived cells are used clinically in ischemic heart disease. Our own investigation, and the research of others have recently demonstrated that ex vivo-cultured ECFCs can be substantially contaminated by other cell types in peripheral blood, such as circulating mesenchymal stem cells,[14] SPCs,[15] and inflammatory cells. Our data revealed that the purity of ECFCs was acceptable for their use in early passages (i.e., from passage 3 to 5).[16] The later passages are associated with remarkably lower cell purity. On the other hand, low expression levels of these endothelial phenotypes were also found in subjects with diabetes mellitus (DM), male gender, and a family history of or the presence of coronary artery disease (CAD); this may have been due to impaired differentiation and proliferation capacities, and increased heterogeneous populations of circulating cells.[17]

Biological functions of ECFCs

Nitric oxide (NO) is the key endothelium-derived relaxing factor that plays pivotal roles in maintaining vascular function in a healthy state.[18] ECFCs exhibit well-preserved biological functions in early passages, which is manifested by having good NO-secreting and angiogenesis capacity.[16] The NO-secreting capacity is remarkably decreased at later passages. Furthermore, the biological function of ECFCs is also significantly impaired in subjects with CAD or risk factors, which is caused by a lower ECFC purity and is related to the ECFC dysfunction. The impaired tubule-forming ability of ECFCs in DM patients mirrored the clinical obstacles in tackling angiogenesis dysfunction in patients with DM. On the other hand, the relations between coronary risk factors to eNOS expression, NO secretion and the tubular formation capacity of ECFCs support the hypothesis that functional assays of ex vivo-cultured ECFCs in early passages can potentially be used to assess individualized endothelial function, which has been implicated in the pathogenesis of various cardiovascular diseases and is associated with a risk of cardiovascular events.[19,20]

Inflammatory activation of ECFCs: a real world consideration

Over approximately the last decade, the concept has emerged that vascular inflammation plays a key role in endothelial dysfunction, and a variety of soluble forms of inflammatory markers were evaluated as predictors of atherosclerotic lesion formation and outcomes.[21] The upregulated expression of adhesion molecules on activated endothelial cells has been used as an indicator of atherogenesis.[22] Our data support the use of ECFCs in early passages again based on their lower adhesion molecule expression levels and activation potential.[16] The higher activation potential in late passages might have been due to aging or endothelial dysfunction in the ex vivo environment, although the exact

underlying mechanisms still need to be elucidated.

Relations linking risk factors to either endothelial dysfunction in experimental studies, or to clinical cardiovascular events, were also demonstrated in ex vivo-cultured ECFCs.[20,23-25] Surprisingly, ECFCs were already activated before inflammatory stimulation in subjects with CAD or various risk factors. This significantly deviates from previous reports and the concept that endothelial activation is only induced upon inflammatory stimulation. The deviation can be explained by the enrollment of CAD patients and controls with various risk factors in our study. Most of the previous studies recruited subjects much younger than those in our study, or healthy volunteers.[15,26] Our findings raised the notion, for the first time, that ECFCs are already activated in an in vivo pathological environment. The crosstalk between coronary risk factors and the activation potential of ECFCs supports the activation profile of ECFCs being capable of representing the in vivo health of ECFC, and potentially being used for cell-based risk stratification. This notion discloses the impaired self-repair ability of ECFCs in patients with cardiovascular diseases and is a likely explanation for the divergent results reported in recent clinical trials.[27-30] Modulating the effects of risk factors on ECFCs either in vivo or ex vivo may ameliorate the biological behavior of ECFCs.[13]

In line with the effect of hypertension, hyperlipidemia, DM, and aging on ECFCs, previous clinical studies reported very similar associations of circulating adhesion molecule levels to these risk factors, suggesting activation on endothelial progenitors.[31,32] Previous reports demonstrated that increased oxidative stress and insulin resistance play a mechanistic role in linking risk factors to endothelial activation.[33] Managing oxidative stress and insulin resistance both in vivo and ex vivo appears to be seminal for ECFCs. Moreover, many factors and drugs can influence EPC function and differentiation. Pioglitazone,[34] statins,[35] high-density lipoprotein,[36] hyperglycemia,[37] shear stress,[38] sex hormone,[39,40] and adrenomedullin[41] are reported to influence EPC function.

ECFC IS NOT AN ENDOTHELIAL CELL: AN ISSUE OF CELL CONTAMINATION

Although it was shown that the number of late EPC colonies is higher in patients with CAD than in control subjects,[42] our data apparently demonstrated a higher number of other contaminating cells in subjects with coronary risk factors. Cytokines released by ischemic stress probably mobilize progenitors other than ECFCs. Previously, we showed that ECFCs contain a subpopulation of cells co-expressing smooth muscle phenotypes.[43] Thus, specific purification or pharmaceutical manipulation processes should be conducted before cell implantation.[13,43]

Actually, circulating vascular progenitor cells (VPCs) are a heterogeneous population, containing EPCs, SPCs, and a subpopulation co-expressing both endothelial and smooth muscle phenotypes (Figures 1 and 2). We found that the level of VE-Cad^{low}α-SMA^{+} VPCs was associated with the severity of coronary atherosclerosis as quantified by the coronary Gensini score.[44] As this relates to vascular intervention, in-stent restenosis is largely due to intimal hyperplasia. The number of VPCs mobilized at the acute phase after stenting is associated with intimal hyperplasia. We also found that the amount of VE-Cad^{low}α-SMA^{+} VPCs is related to the development of post-stent restenosis. Manipulating this subpopulation may provide a way of attenuating atherosclerosis and the incidence of post-stent restenosis in the future.[45]

Speaking of pure SPC, SPCs were shown to expedite lesion formation during restenosis, and also serve to stabilize atherosclerotic plaques by producing extracellular matrix proteins.[15,44,46-48] SPCs were intriguingly shown to act as a double-edged sword in the pathogenesis of atherosclerosis. To fully clarify the roles of SPCs in atherosclerosis, it is important that a distinct panel of SPC surface markers be developed. We found that surface markers of heterogeneous SPCs exhibit various functions associated with atherosclerotic pathophysiology. Consequently, quantification of surface marker-defined SPCs provides a platform for studying SPCs in cardiovascular diseases.

WAYS TO IMPROVE EPC FUNCTION AND DIFFERENTIATION

To improve biological function and attenuate ECFC activation

Ex vivo pretreatment with atorvastatin improved

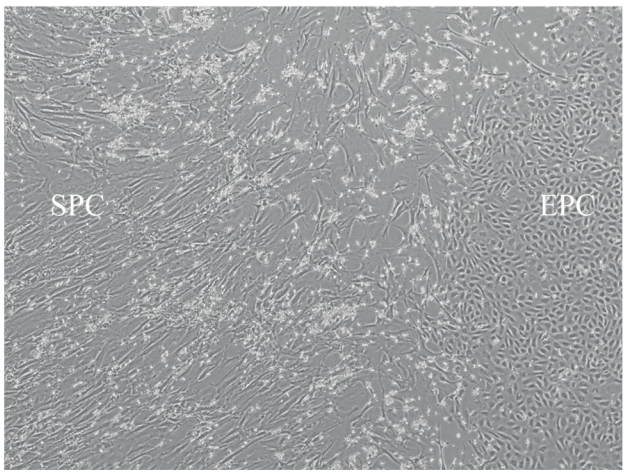

Figure 1. *Cultivation of peripheral blood mononuclear cells gives rise to heterogeneous populations of vascular progenitor cells, including smooth muscle progenitor cells (SPCs), and endothelial progenitor cells (EPCs).*

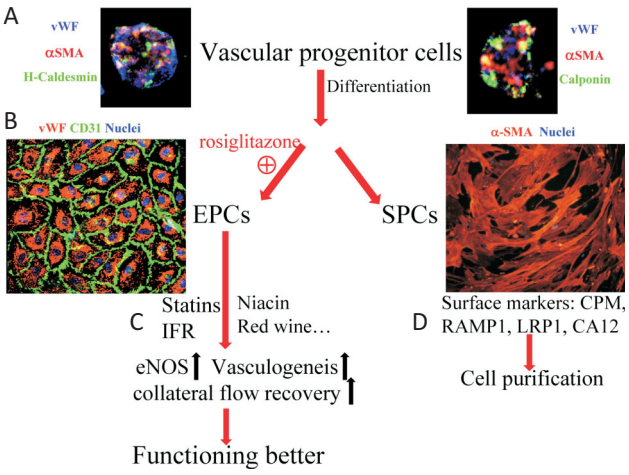

Figure 2. *A summary of this review article. (A) Vascular progenitor cells (VPCs) are cells expressing both endothelial and smooth muscle markers. VPCs have the potential to differentiate into endothelial (EPCs) and smooth muscle progenitor cells (SPCs). (B) Rosiglitazone helps VPC differentiate towards endothelial lineage. (C) Statins, niacin, infra-red, and red wine, etc. improve endothelial nitric oxide synthase (eNOS) function, vasculogenesis and collateral flow recovery after tissue injury. (D) Surface markers of smooth muscle progenitor cell help cell purification between EPCs and SPCs.*

the eNOS expression of ECFCs in CAD patients (although not the same as controls as demonstrated in our study),[16] and attenuated the activation levels of ECFCs as demonstrated by others (Figure 2).[13] Although statins were shown to attenuate oxidative stress and improve insulin resistance as shown in our previous report,[49] the exact mechanisms through which statins exert these beneficial effects still need to be elucidated.

Although far infra-red (IFR) therapy has been shown to exert beneficial effects in the cardiovascular system, the effects of IFR on EPC and EPC-related vasculogenesis remain unclear. We used the model of streptozotocine-induced diabetic mice, and found that administration of IFR therapy promoted collateral flow recovery and new vessel formation (Figure 2). In addition to the thermal effect, increasing evidence suggests that the nonthermal effects of IFR therapy exert beneficial effects in the cardiovascular system through a NO-related pathway.[50] These beneficial effects may derive from enhancement of EPC functions and the homing process itself.[51] On the other hand, niacin was shown to inhibit acute vascular inflammation and improved endothelial dysfunction independent of changes in plasma lipids. We also demonstrated that niacin increased blood flow recovery after tissue ischemia in diabetic mice through enhancing EPC mobilization and functions via NO-related pathways.[52] In addition, we reported that red wine consumption enhanced blood flow recovery after tissue ischemia in diabetic mice. These effects may partly derive from enhanced EPC functions by upregulation of eNOS activity.[53]

Guide the differentiation to ECFC

Previously we demonstrated that VPCs are at least bipotential and able to differentiate into endothelial and smooth muscle lineages (Figure 2). The peroxisome proliferators activated receptor-gamma agonist, rosiglitazone, promotes the differentiation of these VPCs toward the endothelial lineage and attenuates restenosis after angioplasty.[43] Clarifying the underlying mechanisms may help endothelial differentiation, and also avoid the possible cardiovascular side effect of rosiglitazone.

EPC transplantation in clinical trials

Numerous clinical trials are now ongoing, with the goal of elucidating the therapeutic effects of EPCs as seen in animal models on ischemic diseases, using cell populations which are all believed to consist of or enriched by EPCs. Bone marrow- and peripheral blood-derived cells are used clinically in ischemic heart disease. Although three independent meta-analysis studies of injection therapies using these cells showed the overall feasibility and safety, the improvements of cardiac

functions were modest [the increases of LV ejection fraction (EF) were 3.0%, 3.0%, or 3.7%, respectively].[54]

Additionally, chronic ischemia in the lower extremities is mainly caused by arterial obstruction/stenosis in the leg. The therapeutic concept of EPC transplantation for neovascularization has been established by a number of preclinical studies using animal models of hindlimb ischemia. In the past decade, many cell therapies for critical limb ischemia have been developed and reported.[55-74] The types of therapeutic cells used to date have been bone marrow mononuclear cells,[55-64] PBMNC,[55,65,66] G-CSF-mobilized (M)-PBMNC,[67-71] CD34-antigen-positive mononuclear cells and CD133-antigen positive cells.[72-74] These outcomes have suggested and confirmed the safety and feasibility of this cell-based therapy in patients with critical limb ischemia.

HOW TO SEPARATE AND DELETE SMOOTH MUSCLE PROGENITOR CELLS

Simper et al. first reported the presence of circulating human SPCs in 2002.[15] However, the identification and pathophysiological exploration of SPCs have encountered rigorous challenges. The main obstacle is primarily attributed to the difficulty in defining and tracking SPCs in vivo at different stages. Fully understanding the contributions of SPCs to vascular maintenance and repair in early and late stages depends on further elucidation of SPC-specific markers. Currently, surface-marker definitions of circulating SPCs are widely varied based on putative assumptions, including $CD34^+PDGFR\text{-}\beta^+$, $CD45^+CD34^+$, and $SM\text{-}MHC^+$ high side-scatter cells.[74-76] From our experience in performing flow cytometry, the expression intensity of $PDGFR\text{-}\beta^+$ on circulating mononuclear cells is low and not clearly discriminated from negative cells, and the enumeration of $CD34^+$ cells is highly experience- and technique-dependent. SM-MHC is a protein expressed in the late stage of full smooth muscle differentiation. Furthermore, identifying SM-MHC and other intracellular proteins specific to smooth muscle cells all require cell permeabilization, which limits subsequent applications of those cells.

The heterogeneous characteristics and divergent phenotypes of SPCs used in different studies fostered limited progress in understanding SPCs in various pathological environments. Recently, we adopted a microarray approach to develop a distinct panel of SMPC surface markers, including carboxypeptidase M (CPM), receptor activity-modifying protein 1 (RAMP-1), carbonic anhydrase 12 (CA12), and low-density lipoprotein receptor-related protein (LRP1) (Figures 1 and 2).[78] These markers explored the heterogeneity of peripheral blood-derived SPCs with different functional capacities in response to cues provided by the microenvironment after vascular injury.

Surface markers of heterogeneous SPCs exhibit various functions; in the literature, reported functions of SPCs are inconsistent, even contradictory.[15,44,47,59] Our data revealed that peripheral blood-derived SPCs actually contain heterogeneous populations with different panels of surface markers. Interestingly, in addition to PDGFR-β, all of these selected markers were linked to possible roles of SPCs in atherosclerosis.[78] CPM, a membrane-bound metallo-carboxypeptidase, can reportedly degrade or activate several extracellular peptides including bradykinin, epidermal growth factor, and some growth factors, and mediates vasodilatation.[79] Our data demonstrated that the expression of CPM on SPCs was related to a decreased migration capacity. RAMP1, a type I transmembrane protein, is required to transport the calcitonin-receptor-like receptor to plasma membranes to function as a CGRP receptor, which is associated with inhibiting bone resorption and inducing vasodilation.[80] Our study showed that RAMP1 was associated with the function of SPCs in ECM synthesis and anti-inflammation.

CA12 is an extracellular enzyme that catalyzes the reversible hydration of carbon dioxide, and is involved in regulating microenvironmental acidity. Acidification of the extracellular environment favors invasion and migration; however, alkalinization of the cytoplasm maintains cell proliferation and survival.[81,82] In addition, CA12 was demonstrated to be related to arterial calcification,[83] and increases the tolerance of cells to a hypoxic environment as shown in our study. LRP1, a member of the LDL receptor family, is able to recognize more than 30 distinct ligands, and plays diverse roles in various biological processes including lipoprotein metabolism, and protection against atherosclerosis.[84] Previous reports suggested that LRP1 is abundantly expressed in vascular smooth muscle cells, but is not as abundant in the en-

dothelium.[85,86] Based on the advance in defining the SPC markers, it is possible to get rid of SPCs before applying EPCs to clinical therapeutics.

PROSPECTIVES

Currently, researchers around the world are enthusiastic about studying powerful stem cells for tissue engineering and therapeutic resolutions, such as induced pluripotent stem cell, and embryonic stem cells. However, the applications of these cells are still limited due to risks related to using virus vector, and ethic issues. EPC is one of the cell types that are autologous and have the potential to be directly used in the human body. However, the therapeutic use of EPCs depends on a further realization of the issues associated with purity, biological function, individual variation, and ways to improve EPC functions before applying back to the body. On the other hand, a well-established concept on SPC functions and identification is definitely helpful for purifying EPCs or any possibilities of combining EPC and SPC for tissue regeneration.

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DISCLOSURES

Authors have no conflicts of interest to disclose.

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