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ENDOTHELIAL PROGENITOR CELLS: FROM SENESCENCE TO REJUVENATION

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Abstract

Discovered more than 15 years ago, endothelial progenitor cells attract both basic and translational researchers. It has become clear that they represent a heterogeneous population of endothelial colony forming cells, early or late outgrowth endothelial cells, or blood outgrowth endothelial cells, each characterized by differing proliferative and regenerative capacity. Scattered within the vascular wall, these cells participate in angiogenesis and vasculogenesis and support regeneration of epithelial cells. There is growing evidence that this cell population is impaired during the course of chronic cardiovascular and kidney disease when it undergoes premature senescence and loss of specialized functions. Senescence-associated secretory products released by such cells can affect the neighboring cells and further exacerbate their regenerative capacity. For those reasons adoptive transfer of endothelial progenitor cells is being used in more than 150 on-going clinical trials in diverse cardiovascular diseases. There is emergence of attempts to rejuvenate this cell population either *ex vivo* or *in situ*. The progress in this field is paramount to regenerate the injured kidney.

Keywords

stress-induced premature senescence (SIPS); senescence-associated secretory products (SASP); regeneration; cell therapy; rejuvenation therapy

Amid the on-going debates as to the validity of the term “endothelial progenitor cells”, its substantive meaning, heterogeneity of and perceived hierarchy within this cell population, even the very existence of such progenitors, the one irrevocable and indisputable aspect remains clear: adoptive transfer of this cell population affords therapeutic benefits in diverse diseases. It has been calculated that over 150 clinical trials involving these cells in one way or another and in various pathological situations have been registered (1). This alone provides a strong argument in favor of persistent search for the identity, differentiation

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potential, and functional properties of endothelial progenitor cells (EPC). This brief overview of the subject will focus on the development of premature senescence and dysfunction of endothelial progenitor cells in chronic kidney disease, adoptive transfer of intact cells and attempts to rejuvenate their endogenous pool.

EPC identity crisis

The cells in question were first described by Asahara et al (2), who isolated them (CD34+VEGFR2+ cells) from the circulation and coined the term endothelial progenitor cells (EPC). Similar populations of cells were later identified in the bone marrow and in multiple peripheral organs. As mentioned above, studies of EPC are complicated by uncertainty due to the lack of specific markers, diversity of protocols for their isolation, and differences in their experimental usage, not unlike the studies of other stem and progenitor cells. The collective term “EPC” is a convenient *nom de guerre* used to indicate endothelial colony forming cells (ECFC), early or late outgrowth endothelial cells (EOEC and LOEC), or blood outgrowth endothelial cells (BOEC), although more recent publications tend to specify the exact subpopulation. ECFCs have been characterized by their robust proliferative potential and *de novo* vessel formation *in vivo* (3). LOEC are capable of expanding more than 1000-fold in culture in contrast to the early outgrowth cells which can expand only 20-fold (rev in 4). It appears that there is a hierarchy of putative EPC which obscures individual subpopulations (5). Expression of the markers used for identification of EPC (CD31+CD34+VE-cadherin+VEGFR+Tie-2+ and in humans CD133+) overlaps with the markers of mature endothelial cells (except for CD-133). The distinguishing feature of EPC is their ability to form colonies in culture dishes – colony-forming units (6). Obviously the discovery of specific cell-surface markers and efficient isolation techniques will be central issues for the practical use of endothelial cell populations in tissue engineering and regenerative medicine. The subject has been comprehensively discussed elsewhere (7).

Ontogeny of EPC

During embryonic development, mesodermal cells aggregate in the extra-embryonic yolk sac to create hemangioblastic “blood islands”. The outer luminal layer of these islands contains endothelial precursors, angioblasts, whereas the inner mass consists of hematopoietic precursors. Subsequently, the aorto-gonado-mesonephric region (AGM), which harbors EPC, becomes the first embryonic hematopoietic organ due to the ability of EPC to give rise to HSC and MSC (8,9), which persists in adulthood, long after the disappearance of AGM. Genetic cell fate tracing studies have demonstrated in mice with an inducible VE-cadherin Cre that their progeny migrate to fetal liver and later to bone marrow. AGM mesenchyme, traced using myocardin Cre mice, is capable of generating endothelial cells, but incapable of hematopoiesis (8).

In adulthood, cells with EPC-like characteristics have been described in the bone marrow, circulation, and in the blood vessels (10–13). In the vascular wall, solitary cells or small clusters of EPC are present in all three layers: adventitial, medial and intimal. These c-Kit+/VEGFR2+/CD45- cells are clonogenic and can differentiate toward mature endothelial cells, SMC and fibroblasts (14). Subsets of ECs from umbilical cord, peripheral blood, or from adult vasculature also show clonogenic potential (15).

It has been demonstrated (14) that c-Kit⁺ adult vascular endothelial stem cell (VESC) reside in the coronary blood vessels. Fang and Salven described a small subpopulation of c-Kit-expressing endothelial cells (lin CD31⁺CD105⁺Sca1⁺CD117⁺/c-Kit⁺) that reside in the adult blood vessel endothelium and are capable of undergoing clonal expansion *in vitro* and *in vivo* (16). These c-Kit⁺ adult vascular endothelial stem cells (VESC) comprise 0.4% of all adult vessel wall lin-CD31⁺CD105⁺ ECs. Cell transplantation experiments confirmed that a single c-kit⁺ VESC can generate *in vivo* functional blood vessels that connect to host circulation. By performing repeated rounds of isolation and *in vivo* serial transplantation experiments, these investigators showed that VESC also display a long-term self-renewal capacity.

In addition to VESC and EPC, the vascular wall harbors MSC, HSC, and possibly smooth muscle progenitors (Figure 1). Hu et al (17) identified adventitial progenitor cells as Sca-1⁺Flk-1⁺c-Kit⁺ and showed that in ApoE^{-/-} mice they migrate towards the injured intima to participate in neointimal formation of atherosclerotic lesions and differentiate toward SMC. Originally thought to egress from the circulation, mast cells and macrophages found in the vasculogenic zone are currently proposed to originate from the resident precursors (18). The periadventitial zone represented by perivascular adipose tissue has recently become the focus of numerous investigations describing the release from this adipose tissue of vasorelaxing and vasoconstricting factors, which can potentially modulate the behavior of vascular wall stem cells (19). Hence, the vascular wall is endowed with various stem and progenitor cells which have the capacity to influence each other and, possibly, give rise to cells of different lineages.

In aging animals, aortic explants show 40% reduction in capillary sprouting induced by VEGF compared to young animals (20,21). A similar defect occurs in prematurely senescent Klotho-mice (22). Notably, caloric restriction rescues angiogenic competence of aortic rings obtained from 24-months-old rats by 45–63% (23). The downstream target of caloric restriction, sirtuin-1, is robustly expressed in EPC and EC, declines with age and following application of cardiovascular stressors (24). The stress-induced sirtuin-1 deficiency leading to premature senescence of EPC is driven by the loss of integrity of lysosomal membrane and leakage of cathepsins, which are capable of directly degrading this deacetylase. EPC isolated from the bone marrow of mice genetically engineered to lack endothelial sirtuin-1 exhibit premature senescence and higher rate of apoptosis even at young age (24). This may be explained in part by the fact that sirtuin-1 maintains telomere length (25). In aged humans, Williamson et al (26) demonstrated that EPC, specified as the outgrowth endothelial cells, exhibit impairment in cell migration in wound healing assay and this correlated with the alterations of heparan sulfate proteoglycans, components of the glycocalyx.

Endothelial cell turnover, under physiological conditions, is very slow and varies from 2 months to 3 years (rev in 27). The disposal of damaged endothelial cells occurs as a result of patrolling function of non-invading subset of Ly6C^{low} monocytes. Intravital microscopy revealed that these monocytes crawl on the luminal surface of glomerular and peritubular capillaries and scavenge microparticles (28). In response to “danger” signaling Ly6C^{low} monocytes exhibit prolonged dwell for longer periods of times in glomerular and peritubular

capillaries, exhibit more complex patrolling routes, attachment to damaged endothelial cells, and recruit neutrophils, which induce focal necrosis and disposal of cellular debris. Cells that escape this in situ disposal mechanism, detach from their basement membranes and appear in the circulation. EPC can indirectly support the recovery of vascular endothelial integrity (29).

Chronic kidney disease cause premature senescence of endothelial and endothelial progenitor cells: effects of senescence-associated secretory products

The idea that premature cell senescence plays a role in diseased kidney has been initially promoted by Halloran's group (30). These investigators described appearance of cells expressing senescence markers in young animals with kidney disease and in CKD and post-transplant patients exhibiting signs of tubular atrophy and interstitial fibrosis. Notably, no attrition of telomers was detected. Chen et al (31) showed that stress-induced premature senescence (SIPS) of endothelial cells occurs even in the presence of relatively unaffected telomeres. A diverse group of stressors, such as pro-oxidants, asymmetric dimethylarginine (ADMA), non-enzymatically glycation-modified proteins induce cell cycle arrest, SIPS and eventual apoptosis in low-passage cultured cells and in young mice (24). These studies emphasize the fact that SIPS can be reversed after the withdrawal of the offending stressor; however, if the stressor persists, SIPS becomes irreversible, endothelial cells may undergo apoptosis, which culminates in microvascular rarefaction. What determines this point-of-no-return remains to be established.

Senescent endothelial cells, when present in sufficient numbers, could theoretically compromise the function of the vascular endothelial lining. Indeed, this has been demonstrated by our group in mice with the Tie-2-driven excision of the exon 4 of sirtuin-1 gene, thus disabling sirtuin-1 function in endothelial cells and EPC (24). Accelerated senescence of endothelial cells and EPC is accompanied in these mice by the impaired endothelium-dependent vasorelaxation in response to acetylcholine and reduced ability to form capillary-like structures. The phenotype of these mice, however, is mild. The existing defect in endothelial sirtuin-1 is unmasked by the imposition of the "second hit", namely induction of chronic tubulointerstitial fibrosis as it occurs in folic acid-induced nephropathy. In this model of endothelial SIPS, mice exhibit an exaggerated fibrotic response to the stressor. This data demonstrate that, while under basal conditions sirtuin-1 is dispensable for endothelial and EPC function, it is a necessary cofactor for structural and functional recovery of the damaged kidney, when pro-regenerative and pro-fibrotic signals collide. Most recently, the decline in sirtuin-1 activity was shown to stabilize hypoxia-inducible factor-1 α leading to the metabolic switch to Warburg-type glucose metabolism even in the face of unimpaired oxygenation (pseudohypoxia) (32). A similar switch to Warburg-type energy metabolism with enhanced normoxic glycolysis and suppressed oxidative phosphorylation was detected using untargeted proteomic analysis by our group in cultured endothelial cells and in renal microvasculature after induction of endothelial dysfunction by chronic administration of inhibitors of endothelial NO synthase (33.)

A number of microRNA regulators of sirtuin-1 has been identified. One potent regulator of sirtuin-1 expression in EPC is represented by microRNA-34a which promotes SIPS by suppressive effect on sirtuin-1 mRNA (34). Similar effect is exerted by microRNA-217. In contrast, an endothelial-specific microRNA-126, which may be enriched in endothelial microparticles, promotes angiogenesis by suppressing anti-angiogenic signaling (rev in 35). MicroRNAs 221 and 222 negatively correlate with the number of circulating EPC and have been implicated in EPC dysfunction in patients with coronary artery disease, while their levels are reduced by statin therapy (36).

Equally important is the fact that SIPS cells can affect the neighboring cells by their secretome collectively referred to as senescence-associated secretory products (SASP), as illustrated in Figure 2. SASP contain IL-6, IL-8, TGF- α , galectin-3, IGFBP-3,-4, and -6, and MIC-1 (rev in 37). Dysfunctional senescent endothelial cells release collagen XVIII and its C-terminal antiangiogenic fragment, endostatin (38). High-resolution mass spectrometry analysis of the secretome of endothelial progenitor cells disclosed 133 membrane-bound or secreted proteins (39). In particular, soluble forms of VEGF receptors, adhesion molecules, semaphorin 3F and TGF- β , CD109, members of roundabout (robo) family, as well as endothelial markers were detected. Non-targeted mass spectrometry of the secretome of the entire hierarchy of endothelial cells, namely colony-forming units, precursors, or mature endothelial cells identified 272 non-redundant proteins of which 124 were also found in cultured EPC (40). Secretory products included MMP-9, IL-8, MIF, various cathepsins and protease inhibitors, S100 proteins A11, A8 and A4, PAI-2 and apolipoprotein E, as well as a pro-angiogenic and pro-survival factor, thymidine phosphorylase. Collectively, these studies emphasize the role of chronic kidney disease in developing SIPS of EPC and other cells (Figure 3), influence of senescent cells on their neighbors, failure of senescent EPC and mature endothelial cells to maintain vascular functions and support epithelial functions, and generation of proinflammatory microenvironment. They characterize SIPS as the state of accumulation of dysfunctional organelles and misfolded proteins due to the failed autophagy, mitophagy and pexophagy.

Microvascular and tissue regeneration: role of stem and progenitor cells

Identification of endothelial progenitor cells (2) and their potential to regenerate blood vessels has unleashed a large number of studies confirming their role in diverse diseases. Specifically, adoptive transfer of EPC has been shown to improve the course of several cardiovascular and renal diseases, as reviewed elsewhere (41–45). Therapeutic utility of EPC has been comprehensively reviewed by Khakoo and Finkel (3).

Recent studies, however, have unsettled the idea of direct involvement of bone-marrow-derived or circulating stem cells in general and EPC in particular in regenerative processes. Using an adult mouse model of regeneration of a distal phalanx and genetic fate-tracing technology, Rinkevich et al (46) documented that bone marrow-derived or circulating stem cells do not directly contribute to tissue regeneration, rather the germ-layer and lineage-restricted stem/progenitor cells are responsible for the restoration of tissue architecture. It has been concluded that endothelial stem/progenitor cells involved in adult angiogenesis are represented by tissue-resident cells.

As noticed by Zovein et al (7), EPC have the ability to undergo reversible transitions to MSC or HSC. By employing nestin-driven green fluorescence protein expressing mouse model Amoh et al (47) showed that hair follicle stem cells participate in development of skin blood vessels and wounding the skin enhances their participation. Moreover, new vessels still carrying the GFP tag expressed characteristic endothelial cell markers, CD31 and von Willebrand factor. On the other hand, Udani et al (48) used bone marrow transplantation from GFP-expressing mice to lethally irradiated mice and examined brain tumor angiogenesis. Investigators detected vascular incorporation of GFP+, CD34+, Flk1+, and CD45+, but could not detect expression of von Willebrand factor by these cells with characteristics of HSC and endothelia.

The importance of the vascular endothelium for tissue regeneration has been illustrated by Rafii's group (49). The investigators provided evidence for angiocrine signals generated during vascular regeneration through production of EGF-like laminin fragments. The latter fosters growth of the pulmonary epithelia in a pneumonectomy model. Similarly, endothelial progenitors have been shown to support regeneration of liver parenchyma after partial hepatectomy (50). Another mechanism whereby endothelial cells can participate in tissue regeneration consists in the expression of SDF-1 by these cells at the site of injury, thus attracting CXCR4- and CXCR7- (both receptors for SDF-1) positive cells assisting in restoring tissue architecture (51). How relevant are these scenarios to the regeneration of the kidney remains to be established.

In addition to their role in supporting homeostasis and regeneration of epithelial cells, endothelial cells have the capacity to support hematopoietic stem cells (52), undergo reprogramming to generate multipotent stem-like cells (53). A small population of cells expressing *Zfp423*, a gene controlling preadipocyte determination, is capable of giving rise to pericytes and preadipocytes (54,55). On the other hand, members of E-twenty six family of transcription factors regulating vascular development and blockade of TGF β signaling can efficiently reprogram non-vascular cells, like mature amniotic cells, into induced vascular endothelial cells with the remarkable longevity (56). All these recent findings indicate the role of microenvironmental and transcription factors in endothelial cell plasticity.

***Ex vivo and in situ* rejuvenation of EPC**

Along the long line of investigations demonstrating therapeutic efficacy of exogenous, adoptively transferred EPC in various diseases, a handful of studies shifted the focus to microenvironment of stem cells in their niches as potential contributors to the failure of endogenous resources. Inflammatory cytokines, reactive oxygen species, and overdriving Wnt signals are among the most common extrinsic causes of functional decline of endogenous stem cells (57). Using heterochronic parabiosis model of shared circulation between old and young mice, Conboy et al (58) have demonstrated rejuvenation of progenitor cells. Chemokine eotaxin (CCL11) has been implicated in induction of premature aging of stem cells (59). Thus induced intrinsic changes in stem cells may be reversible, when they occur via epigenomic alterations, such as dysregulated acetylation or methylation of histones, and the erasure of these marks is possible. This route represents one of the

strategies to reprogram dysfunctional stem cells. For these reasons, correcting functions of enzymes controlling the balance of acetylation and methylation of histones opens remarkable therapeutic opportunities (60).

Another potentially reversible common pathogenetic mechanism of stem/progenitor cell dysfunction is represented by the impairment of autophagy and accumulation of misfolded proteins. Hyper-activation of mTOR or suppression of AMP-activated protein kinase (AMPK) results in the inhibition of autophagy and metabolic switch from oxidative to glycolytic metabolism (61), which is essential for stem/progenitor cell maturation (62). This pathway can involve lysosomal dysfunction with the impaired phagocytosis and leakage of lysosomal enzymes into the cytoplasm (24), as depicted in Figure 4. By suppressing mTOR activity or activating AMPK it is possible to reverse these intrinsic alterations in stem/progenitor cells. Similarly, polyamines, such as spermidine, through activation of autophagy improve epithelial stem cell functions (63).

Notably, stem cells in general and EPC in particular either reside in a low partial oxygen pressure compartments or utilize oxygen sparingly, thus protecting themselves from excessive production of reactive oxygen species (64). Not surprisingly, these cells express lower numbers of mitochondria and usually supply their metabolic demands by glycolysis. Nonetheless, the enzymatic machinery for the switch to oxidative phosphorylation is present to account for the switch to metabolize glucose through Krebs cycle. It is important to note that in dysfunctional senescent endothelial cells two key mitochondrial enzymes regulating the entry of glucose to Krebs cycle, aconitase-2 and enoyl-coA-hydratase-1, are downregulated (33), thus committing these cells to invariant glycolysis. It is likely that the similar enzymatic defect occurs in EPC. This could explain their inadequate recruitment and functional incompetence. By supplying animals with endothelial dysfunction or dysfunctional cultured cells with an intermediate of Krebs cycle, α -ketoglutarate or its precursor glutamine, which bypasses the enzymatic bottleneck, it is possible to dramatically improve endothelial functions and reverse vasculopathy (33,65).

As mentioned above, EPC, as well as other stem cells, reside in specialized compartments, niches, which shield them from shifts in environmental composition, provide them with structural support, means for quiescence, self-renewal, and sufficient routes of communication with the mobilizing stimuli (66). The precise location and composition of the niche for EPC is unknown at present. Artificial niches can be produced using hyaluronic acid hydrogels precoated with pronectin (67), which preserves EPC quiescence, protects from the noxious stimuli, and allows for their on-demand mobilization by the injection of hyaluronidase. We believe that this is the preferred route for therapeutic delivery of EPC. It appears that EPC require structural support. Mobilized EPC or circulating exogenous EPC are short-lived (minutes-to-hours) and tend to home to the spleen or damaged organs expressing stromal-derived factor-1 (68). This area of research into EPC niches may offer many clues as to the improvement of cell function in chronic kidney disease by restoring the composition of the niche; however, it remains in its infancy.

EPC express endothelial nitric oxide synthase (eNOS), a prerequisite to their ability to mobilize, migrate, participate in angiogenesis, home to injured tissues and alleviate

endothelial dysfunction (69). Most relevant to chronic kidney and cardiovascular disease, EPC dysfunction is a direct consequence of elevated ADMA, as well as oxidized LDL levels, which impair their clonogenicity, participation in angiogenesis, and ability to restore endothelial functions (rev in 69). Similarly, EPC obtained from eNOS knockout mice have a dramatic reduction in functional properties and viability. Therapeutic interventions leading to enhancement of eNOS function, such as administration of eNOS enhancer AVE9488 or gene therapy, improve EPC therapeutic effects on neovascularisation and viability after adoptive transfer to ischemic tissues (70,71).

Two related factors with a broad base among diverse pathological conditions, reactive oxygen species and oxidative stress, have a profound effect on stem cells, including EPC (69). Preventing or reducing oxidative stress and its consequences could potentially reverse stem cell dysfunction. In a model of type 2 diabetes mellitus, Chen et al (72) demonstrated enhanced oxidative stress associated with reduced resistance to stress and viability of bone marrow-derived EPC. Ex vivo treatment of EPC with a seleno-organic peroxynitrite scavenger and antioxidant, Ebselen, significantly improved therapeutic efficacy of adoptively transferred “diabetic” EPC over the non-treated cells. In the same study it was shown that in vivo therapy with ebselen also improved EPC and endothelial dysfunction associated with diabetes (Figure 5). Ebselen improved viability of stressed EPC, reduced proportion of senescent cells, increased EPC secretion of IL-10 and reduced secretion of IL-6, both impaired in untreated cells (unpublished data). These studies illustrate the strategy of *ex vivo* and *in vivo* therapy to improve functional properties of impaired stem/progenitor cells.

To recapitulate, despite continuing discourse as to the precise characterization of EPC, there are significant breakthrough studies illuminating functional and metabolic aspects of these cells, their role in the maintenance of neighboring cells, pathways leading to their premature senescence and dysfunction in chronic cardiovascular and renal disease, manifestations of SIPS, generation of SASP, and developing alterations in the composition of EPC microenvironment. The microenvironmental cues and their abnormalities may represent a *bona fide* therapeutic target. Overall, latest studies raise the question of strategic therapeutic superiority of “cell therapy” and promote an alternative or parallel approach of “cell-free therapy of the microenvironment”, as briefly discussed herein. Several clinical trials of cell and cell-free therapy in patients with cardiovascular diseases are underway. It would be expected that similar clinical trials in patients with chronic kidney disease should soon emerge.

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Figure 1. Distribution of stem and progenitor cells in all layers of the vascular wall intima, media, adventitia and perivascular adipose tissue.

Figure 2.

Targets of senescence-associated secretory products (SASP) of senescent cells. In contrast to the normal secretome of EPC and endothelial cells, SASP is enriched in IL-6, IL-8, TGF- α , galectin-3, IGFBP-3,-4, and -6, and MIC-1, which compromise functions of all neighboring cells in the paracrine fashion.

Figure 3. EPC and VESC are engaged in the maintenance of vascular and epithelial homeostasis. Development of SIPS in the course of chronic kidney disease (CKD) perturbs both of these functions, thus disrupting homeostatic regulation and producing a pro-inflammatory microenvironment, which in turn further compromises functions of other stem and mature cells, potentially aggravating the primary disease process and derailing regeneration. Thick arrows indicate the presumed inhibitory functions of prematurely senescent cells. Boxed area introduces “cell therapy” and “cell-free therapy”.

Figure 4.

Pathways responsible for the development of SIPS. Initiating noxious stressors induce permeabilization of the lysosomal membrane, leakage of lysosomal enzymes and degradation of sirtuin-1, which in turn results in the activation of mTORC and inhibition of autophagic degradation of misfolded proteins, dysfunctional mitochondria and peroxisomes. Developing endothelial dysfunction is associated with glutamine depletion, which, depending on the biological context, may activate or inhibit mTORC. Importantly, supplemental glutamine reverses endothelial dysfunction and restores metabolism in mice with experimental model of chronic inhibition of endothelial NO synthase.

Figure 5.

Examples of “cell-therapy” and “cell-free rejuvenating therapy” in mice with the model of type 2 diabetes (db/db mouse). General schema of the experiments (a). Db/db mice adoptively transferred with the bone marrow EPC obtained from control db/m mice (dbTxm) exhibited significant improvement of macro- and microvasculopathy, as opposed to db/db mice adoptively transferred with EPC obtained from db/db mice (b and c). The similar effect was observed after chronic therapy with the seleno-organic antioxidant and peroxynitrite scavenger ebselen. Macrovasculopathy was also improved by adoptive transfer of db/db EPC pretreated in culture with ebselen (Txdb-Ebs-ex vivo). Modified from ref 72.