Dysfunctional Endothelial Progenitor Cells in Chronic Kidney Disease

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ABSTRACT
Putative endothelial progenitor cells play a role in organ regeneration, and their incompetence may be important in the development of chronic kidney disease. The mechanisms of this incompetence are broad and range from poor mobilization, viability, and engraftment to impaired differentiation into mature endothelial cells. By contrasting the role of endothelial progenitor cells in tissue regeneration with their developing incompetence in chronic kidney disease, we emphasize the importance of designing rational pharmacologic strategies to tackle such incompetence in the broader search for therapies to attenuate chronic disease.


A landmark publication identifying circulating endothelial progenitor cells (EPCs) provides new impetus for their use as cell therapy in postnatal vasculogenesis.

In this seminal study, human circulating CD34+ cells were instructed to differentiate in culture into cells with endothelial-like properties. Moreover, administration of these cells to athymic nude mice with hindlimb ischemia resulted in the integration of transplanted cells into capillary vessels, improving collateral circulation. The idea that such cells derive from hematopoietic stem cells (HSCs) in bone marrow is also supported in mice, in which transplantation of a single HSC repopulated the bone marrow and the endothelium of retinal blood vessels after experimental retinal ischemia.

Comparable results have been published by others.

Embedded in a broad view of EPCs is the notion that angiogenic cells are capable of maturing into endothelial cells (ECs). These cells are mostly angioblastic or myelocytic descendants. Problems with the definition of EPCs reflect the changing landscape of surface markers as they traffic from the bone marrow to the circulation and tissues, gradually losing monocytic and pan-leukocytic markers as they mature into ECs (Table 1). A subset of angiogenic monocytes is characterized as CD14+,CD16+,Tie2+, whereas circulating bone marrow–derived EPCs are CD34+,VEGFR2 (Flk-1)+,CD45low. The consensus combination of markers and processes, although individually not unique to EPCs, is that EPCs express CD34, vascular endothelial growth factor receptor 2 (VEGFR2), Tie-2, CD133, and Ulex Europeus lectin binding; take up acetylated LDL; and have the ability to form colonies (colony-forming unit cells). The problems in identifying EPCs and distinguishing them from circulating ECs or mature ECs remain insurmountable and await future recognition of more specific markers; hence, limited definitions constrain all studies describing these cells as putative.

Transplantation of EPCs augments neovascularization of ischemic or infarcted myocardium, ischemic limbs, and brain. EPCs play a critical role in maintaining the integrity of vascular endothelia and serve in their repair after injury or inflammation. Comparatively fewer data have been published on EPCs in kidney regeneration. Rookmaaker et al. observed a more than four-fold increase in the number of bone marrow–derived glomerular ECs after anti–Thy-1.1 injection into rats that had received a bone marrow transplant. The participation of donor-derived cells in glomerular EC turnover was also observed in rats that had received a bone marrow transplant, undergone unilateral nephrectomy, and received anti–Thy-1.1 injection.

In a pig model of renovascular disease, Chade et al. demonstrated that intrarenal infusion of autologous EPCs attenuates vascular remodeling and fibrosis, improving renal function by stimulating angiogenesis. In patients with chronic renal insufficiency, numbers of circulating EPCs were almost 30% lower than in healthy control subjects. Collectively, these studies set the stage for in-depth exploration of EPC participation in regenerative processes.

Our knowledge regarding the pool of EPCs in postnatal life is growing. EPCs are present in the bone marrow, where CD34+ cells constitute approximately

Published online ahead of print. Publication date available at www.jasn.org.

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2% and AC133/CD133^+ cells 0.5% of cells. They also exist in the circulation (0.01% of mononuclear cells) and in the vasculogenic zone between the adventitial and medial layers of large and medium-sized blood vessels. This latter niche contributes to the residence of EPCs in many organs, including liver, kidney, heart, and lung. EPCs derived from all of these niches participate in postnatal angiogenesis, vasculogenesis, and tissue regeneration. For instance, liver-derived EPCs (c-kit^+ CD45^-) contribute to neovascularization of ischemic hindlimb. Relations between various EPC pools and their contribution to regenerative and angiogenic processes are depicted in Figure 1. Not only EPCs but also HSCs and mesenchymal stem cells (MSCs) reside in this vascular niche, and it is possible to envisage that transformation from one cell type to another takes place. Refining the local cues guiding these processes is an important task for future studies.

The idea that EPCs become dysfunctional during the course of a disease is predicated on the demonstration of their defective regenerative capacity or compromised ability to form colonies or migrate and form capillary-like structures. Aging is the most common cause of EPC dysfunction. Similar to all somatic cells, EPCs are subject to various environmental and endogenous stressors that impair their competence. Hyperglycemia reduces survival and impairs the function of circulating EPCs. In addition, EPC dysfunction is documented in types 1 and 2 diabetes, coronary artery disease (CAD), atherosclerosis, vasculitis with kidney involvement, and ESRD. There is emerging evidence that senescence may serve as an important mechanism mediating EPC dysfunction. Decreasing numbers and increasing proportion of senescent EPCs has been reported in patients with pre-eclampsia or hypertension, and angiotensin II induces EPC senescence through the induction of oxidative stress and its influence on telomerase activity, as does oxidized LDL. Our recent studies showed that bone marrow-derived cells (BMDCs) and bone marrow-derived EPCs from db/db mice with metabolic syndrome and diabetes were functionally incompetent, whereas adoptive transfer of BMDCs from syngeneic nondiabetic controls significantly improved vasculopathy, insulin sensitivity, and renal function in db/db recipients. Specifically, the development of type 2 diabetes and vasculopathy associated with reduced stress tolerance for EPCs, increased frequency of apoptosis, and premature senescence.

There is no clearcut definition of stem cell incompetence. On the basis of the known trafficking patterns of stem cells in general and EPCs in particular, mobilization, recruitment and engraftment, cell-dependent paracrine signaling, asymmetric proliferative capacity, or viability and resistance to existing stressors, one would extrapolate that stem cell incompetence is characterized by at least one of the following features: Know where, know when, and know how. Figure 2 illustrates this cycle of injury to regeneration involving all of these functions of stem cells and sites where these functions can be perturbed.

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Table 1. Expression of surface markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>BMH</th>
<th>HPP</th>
<th>BMEPC</th>
<th>CEPC</th>
<th>CEC</th>
<th>Mature EC</th>
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<td>Scl</td>
<td>+</td>
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<tr>
<td>CD133</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+(early)</td>
<td>-</td>
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<td>+ or -</td>
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<td>+</td>
<td>+</td>
<td>low</td>
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<tr>
<td>VE-cad</td>
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<tr>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vWF</td>
<td>-</td>
<td>+</td>
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BMH, bone marrow hemangioblast; HPP, hematopoietic progenitor; BMEPC, bone marrow EPC; CEPC, circulating EPC; CEC, circulating EC; VE-cad, VE cadherin; vWF, von Willebrand factor.
**KNOW WHEN TO ENGAGE, OR IMPAIRED MOBILIZATION**

Impaired EPC mobilization can occur as a result of inadequacy of mobilizing signals or their shielding or inefficient detachment of stem cells from their niches. A classical mobilization agent, G-CSF, executes dislodgment of stem cells from the bone marrow niche by activating neutrophil elastase, which in turn degrades stromal cell–derived factor 1 (SDF-1). Caveolin 1 deficiency blocks efficient dimerization of CXCR4, whereas caveolin 1 overexpression results in the enhanced SDF-1/CXCR4 binding. Impaired EPC mobilization can occur as a result of inadequacy of mobilizing signals or their shielding or inefficient detachment of stem cells from their niches. A classical mobilization agent, G-CSF, executes dislodgment of stem cells from the bone marrow niche by activating neutrophil elastase, which in turn degrades stromal cell–derived factor 1 (SDF-1). Caveolin 1 deficiency blocks efficient dimerization of CXCR4, whereas caveolin 1 overexpression results in the enhanced SDF-1/CXCR4 binding.

Mobilization of EPCs from their respective niches is accomplished by mechanical injury and ischemic stress through generation of hypoxia-inducible factor 1–regulated release of VEGF, erythropoietin, and SDF-1, as well as by placental growth factor and granulocyte-macrophage colony-stimulating factors. Some proinflammatory cytokines, such as IL-8, are potent stimulators of EPC mobilization, thus linking this response to stressors with proinflammatory conditions. An additional mechanism for EPC growth and differentiation has been attributed to apoptotic endothelial microparticles generated after injury to mature ECs, a mechanism that creates a feedback loop to enhance number and initiate the differentiation of EPCs when they are required by the demand for repair.

A number of investigators have examined whether EPCs are efficiently delivered to areas of tissue ischemia to preserve or restore end organ function by participating in vasculogenesis. In a model of myocardial infarction in mice that received a bone marrow transplant, histologic analysis showed donor-derived ECs in areas of neovascularization at the border zone of the infarct. These experimental data are strongly supported by clinical observations. Adams et al. found increased levels of circulating EPCs in patients with CAD after exercise-induced myocardial ischemia. Lamiøse et al. showed an inverse correlation between the density of coronary collaterals and numbers of peripheral EPCs in CAD. Such an inverse correlation between the number and function of circulating EPCs and severity of various chronic cardiovascular and renal diseases seems to be a general rule. (eNOS). Considering this enzyme is usually uncoupled in many diseases, it should come as no surprise that mobilization of EPCs is dependent on functional eNOS. Specifically, mice deficient in the enzyme showed reduced EPC mobilization.

Ischemia is one of the potent signals to mobilize EPCs; this has been unequivocally documented in humans and in experimental animals with myocardial ischemia, ischemic stroke, and renal ischemia. Despite the seeming universality of this response to ischemic insult, the precise molecular mechanisms responsible for it remain uncertain.

One of the mechanistic uncertainties is related to the question, “What are stress-signaling molecules, produced by and discharged from the ischemic tissue, that are capable of downstream mobilization and recruitment of stem cells and EPCs?” Uric acid, one of the prototypical alarm signals activating the innate immune system, exhibits a short-lived surge after ischemia-reperfusion. Previsstudies demonstrated that exogenous uric acid leads to a rapid mobilization of EPCs and HSCs, respectively, and protection of the kidney against ischemic injury. Kuo et al. demonstrated that monosodium urate (MSU) in vitro and in vivo results in exocytosis of Weibel-Palade bodies with the release of IL-8, von Willebrand factor, and angiopoietin 2 into culture medium or the circulation, respectively. In Toll-like receptor 4 null mice, acute elevation of uric acid level by injection of MSU does not result in the release of von Willebrand factor or angiopoietin 2 to the circulation, suggesting that the effect of uric acid on exocytosis of Weibel-Palade bodies is mediated through this receptor. The release of IL-8 in response to elevated uric acid level requires both Toll-like receptors 2 and 4. These findings outline a novel paradigm linking posts ischemic repair and inflammation by the release of constituents of Weibel-Palade bodies and further broaden the spectrum of alarm signaling that establishes constituents of Weibel-Palade bodies as potential second messengers not only for proinflammatory responses but also for mobilization of stem cells. It has to be emphasized that it is only a brief transient surge of uric acid that has this signaling function. In contrast, chronic elevation of uric acid is completely devoid of this type of alarm signaling; moreover, chronicity inhibits ischemia-induced mobilization.
Interactions between HSCs and stromal cells are mediated in part through α4β1 (VLA4)/VCAM-1. Accordingly, inducible ablation of α4β1 (VLA4) and conditional ablation of VCAM-1 both associate with the enhancement of G-CSF–induced mobilization of HSCs. An additional mechanism of leptin-induced upregulation of αVβ5 and α4 integrins may explain enhanced adhesion to neointimal lesions, but the same phenomenon seems to inhibit EPC mobilization, thus requiring further analysis. There are multiple remaining questions related to the efficacy of EPC mobilization by intrinsic or pharmacologic means in chronic kidney disease (CKD) that need to be addressed in future studies.

**KNOW WHERE TO GO, OR IMPAIRED ENGRAFTMENT**

Reduced CXCR4/SDF-1 expression or function impairs engraftment. Homing of HSCs, EPCs, or a subpopulation of MSCs that express CXCR4 is governed mainly by the α-chemokine SDF-1. Other potential ligands engaged in homing of stem cells, albeit significantly less explored, include chemokines MCP-3 (homes MSC) and GRO-1 (homes bone marrow–derived EPCs, especially in tumors). Factors affecting binding of SDF-1 to its cognate G-protein–coupled seven-transmembrane domain receptor CXCR4 are many, as summarized in Figure 3, illustrating a plethora of proinflammatory and other factors that displace integrins. Pretreatment of EPCs with TNF-α before their use in adhesion assays reduces their adhesion, whereas hypoxic pretreatment or exposure to elevated concentration of MSU results in a dramatic enhancement of EPC adhesion to ischemic kidney sections (unpublished observations). These differences in adhesion allude to possible changes in the capacity of EPC niches as affected by tissue ischemia or hypoxic preconditioning of EPCs.

**KNOW WHAT TO DO, OR IMPAIRED SIGNALING AND MATURATION**

Impaired signaling and transformation of EPCs into a mature EC occurs as a result of either perturbed paracrine secretion or differentiation. Ability of circulating progenitors to differentiate toward endothelial or smooth muscle cell lineages has been furthered by studies of parabiosis in which a wild-type mouse and a transgenic mouse expressing green fluorescence protein are conjoined subcutaneously by anastomosing circulations. Tanaka et al. demonstrated that mechanical injury to femoral arteries of wild-type mice result within 4 weeks in a chimerism of cells comprising developing neointima: 15 and 31% of parabiotic wild-type mice result within 4 weeks in a chimerism of cells comprising developing neointima: 15 and 31% of parabiotic wild-type mice result.
EPCs and 50% increase of macrophages, all changes that are correctable by statins. EPC differentiation is inhibited by vascular endothelial growth inhibitor TNFSF15, which decreases the expression of EC markers and induces apoptosis. It is obvious that the differentiation potential of EPCs and their impairment remain poorly investigated.

**KNOW HOW TO SURVIVE, OR IMPAIRED SELF-PROTECTION**

The loss of EPC viability and resistance to ongoing stress occurs in various disease states. Ito et al. convincingly demonstrated that chronic states of oxidative stress induced either by an inhibitor of glutathione synthesis or by deletion of the Atm (ataxia telangiectasia mutated) gene shorten the lifespan of HSCs through increases in proliferation, stem cell exhaustion, and bone marrow failure, expressed as defective bone marrow reconstitution. This is an example of induction of an accelerated type of replicative senescence by reactive oxygen species. Our own data demonstrate that oxidative stress in diabetic db/db mice associates with an increased proportion of apoptotic EPCs under basal conditions and reduced viability under conditions of stress. Similar findings were described for patients with type 2 diabetes: EPCs exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Pharmacologic restoration of stem cell competence, both ex vivo and in vivo using a selenoorganic antioxidant and peroxynitrite scavenger, is possible.

To draw a preliminary conclusion, stem or progenitor cells are differentially affected in different models of CKD. For instance, in adriamycin nephropathy, the prevailing defect traces to the inability of stem or progenitor cell homing to the kidney as a result of the cytotoxic effect of this anthracycline antibiotic; in diabetic nephropathy, the defect is explained predominantly by oxidative stress and reduced viability of stem or progenitor cells; in unilateral ureteral obstruction, pathology of stem or progenitor cell predilection toward fibroblastic differentiation is likely responsible for the ensuing tubulointerstitial fibrosis and scarring. These hypothetical pathogenic mechanisms require in-depth investigation, because the delineation of the causes of stem or progenitor cell incompetence is a prerequisite to designing effective therapies.

**THERAPIES TO IMPROVE EPC COMPETENCE**

Attempts to improve EPC viability and function are on the way. Genetic engineering for therapy of EPCs ex vivo involving overexpression of eNOS or heme- oxygenase 1 resulted in improvement of their function and inhibition of neo-intimal hyperplasia. Overexpression of telomerase reverse-transcriptase in EPCs led to improved neovascularization in ischemic limbs. EPCs stably overexpressing an anti-inflammatory protein, A20, showed resistance to TNF-α and IL-β. Another example of EPC therapy is ex vivo or in vivo treatment with a peroxynitrite scavenger and glutathione peroxidase mimetic, ebselein, which resulted in a striking improvement in vascular and renal function of db/db mice. Di Stefano et al. used a p66ShcA deletion to reduce glucose-induced oxidative stress and restore viability to EPCs. Downregulation of the Ets1 transcription factor in vasculogenic progenitor cells increased their numbers and differentiation to ECs in a mouse model of metabolic syndrome and type 2 diabetes, the lepr(db) mouse. Improved differentiation of EPCs into ECs was achieved by overexpression of a constitutively active form of protein kinase A, which led to induction of Flk-1 and neuropilin 1. These as yet tentative mechanistic attempts at correcting EPC incompetence and improving their survival await future refinement and verification.

**POTENTIAL MECHANISMS OF EPC-INDUCED ORGAN REPAIR**

EPC participation in reparative processes has been the subject of several excellent reviews and therefore is discussed here only briefly.

**EPC Incorporation**

Direct incorporation of EPCs into a presumably defective intimal layer was demonstrated by Asahara et al. EPC transplantation into diabetic mice resulted in vascular engraftment and restoration of blood flow in hindlimb ischemia. Cross-grafting aortic segments between Balb/c and Tie-2/LacZ mice demonstrated chimerism of ECs in the intimal layer, thus arguing in favor of EPC incorporation into vessels. This process is facilitated in part by platelet adhesion at the site of vascular injury, resulting in adhesion and maturation of circulating EPCs to ECs. Usually, direct EPC engraftment varies within 1 to 25%. There is growing concern whether BMDCs or EPCs actually repair endothelia, with the emphasis shifting toward progenitors within the vascular wall.

**Paracrine Effects of EPCs**

Cultured peripheral cells or BMDCs give rise to at least two populations of EPCs: Early VEGFR2+ and VE-cadherin+ cells coexpressing myeloid CD14 and pan-leukocytic CD45 markers after 4 to 7 days and late outgrowth cells emerging after 2 to 3 weeks from CD14− nonmyeloid populations expressing CD34, VEGFR2, and AC133. Both populations are capable of inducing neovascularization, but the mode of action differs. Whereas early outgrowth EPCs have limited capacity for population doubling and induce transient angiogenesis, late outgrowth EPCs expand to more than 100 population doublings. Cell therapy with both populations resulted in the enhanced engraftment and neovascularization during hindlimb ischemia. Early outgrowth EPCs exert angiogenic effects mainly by secretory products, whereas late outgrowth cells do this by direct engraftment. The secretome of EPCs using a combination of proteomic techniques reveal angiogenic factors such as thymidine phosphorylase as a major agonist, matrix metalloproteinase 9, IL-8, pre-B cell enhancing factor, and macrophage migration inhibitory factor, among others.
Cell–Cell Fusion
Fusion events have been elegantly documented between BMDCs and tubular epithelial cells after ischemic injury.79 Notably, this mechanism of repair occurs on a one-to-one basis. The ability of EPCs to fuse with renal resident cells remains to be established.

Tunneling Nanotubes
The formation of tunneling nanotubules between cultured cells is a viable mechanism of organellar exchange between the partners (Figure 4).80 This mechanism accounts for mitochondrial transfer between adult stem cells and somatic cells to rescue respiration.81 This mechanism, although difficult to demonstrate in vivo and therefore studied only in cultured cells, plays a significant role in intercellular communication.82 K.Y. et al. (unpublished observations) co-cultured human umbilical vein ECs (HUVECs) with EPCs, each cell type labeled with differentially emitting fluorophores, and observed that exchange occurred under basal conditions. EPC-to-HUVEC flux, however, increased threefold after exposure of HUVECs to a cytotoxic dose of adriamycin (unpublished data). Tunneling nanotuble mechanisms of organellar exchange may provide the means for the single EPCs to exchange organelles with multiple HUVECs. Disease processes, resulting in aberrant regeneration even when other functions of EPCs remain intact, may target each of these mechanisms.

USE OF ARTIFICIAL NICHES TO STORE AND DELIVER EPCs
One of the stumbling blocks in stem cell therapy is the problem of low percentages of transplanted cells that survive in the circulation or after tissue engraftment. Delivering cell therapy in synthetic scaffolds may circumvent this problem. The cornerstone idea behind artificial stem cell niches investigated thus far is in the generation of scaffolds with properties of extracellular matrix.83 Creation of artificial stem cell niches represents an attempt to mimic the natural niche by providing cells with a low-oxygen environment within these avascular scaffolds but ensuring their ability to preserve phenotype, quiescence, recruitability, and protection from noxious stimuli. Hyaluronic acid hydrogels are capable of storing, propagating, and differentiating stem cells.84 We have initiated studies of EPCs encapsulated in an artificial stem cell niche manufactured from polymeric hyaluronic acid and demonstrates that it is a dynamic storage compartment that not only preserves cells and improves resistance of EPCs to cytotoxic and genotoxic insults but also allows the recruitment of stem or progenitor cells on demand and improves their engraftment to affected organs and preserves their regenerative potential.

CONCLUSIONS
Two lines of evidence collide in this brief essay: On the one hand, the growing evidence of participation of EPCs in regenerative processes and, on the other, the development of EPC incompetence in various disease states. The prognostic value of circulating EPCs has been used to monitor endothelial dysfunction in critically ill patients.85 Evidence of EPC dysfunction in CKD continues to accrue.86,87 It is an urgent task for future studies to characterize the mechanisms of EPC dysfunction and design rational therapeutic strategies to restore their competence.

ACKNOWLEDGMENTS
These studies were supported in part by National Institutes of Health grants DK54602, DK052783, and DK45462 (M.S.G.) and Westchester Artificial Kidney Foundation.

DISCLOSURES
None.

REFERENCES

Figure 4. Architecture of tunneling nanotubules (TNTs) between cultured PC12 cells. Wheat germ agglutinin–stained PC12 cells are analyzed by three-dimensional (3-D) live-cell microscopy. (A and B) Cells are connected via one (A) or several (B) TNTs with surrounding cells. (C) Rarely, branched TNTs are observed (arrow). (D) A selected (x–z) section obtained from a confocal 3-D reconstruction. (E) TNTs contain actin but no microtubules. Fixed PC12 cells were immunostained with an antibody against α-tubulin (green), phalloidin-FITC (red), and DAPI (blue). A single (x–y) section of a deconvolved 3-D reconstruction. The inset depicts the corresponding (x–z) section through the marked TNT (arrow). (F and G) Ultrastructure of TNTs. PC12 cells analyzed by scanning electron microscopy (F) or transmission electron microscopy (G) of consecutive 80-nm sections. Reprinted from Rustom et al., with permission. (Right) A schematic illustration of a possible EPC repair of damaged HUVECs by a repeated formation of TNT between a single EPC and several HUVECs.


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