

Review Article

Endothelial Progenitor Cells in Acute Ischemic Kidney Injury: Strategies for Increasing the Cells' Renoprotective Competence

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Acute ischemic kidney injury is the most frequent cause of acute renal failure in daily clinical practice. It has become increasingly recognized that microvascular endothelial cell dysfunction (ED) in peritubular capillaries inhibits the process of postischemic renal reperfusion. ED can serve as therapeutic target in the management of acute ischemic kidney injury. Postischemic reflow can be restored by systemic administration of either mature endothelial cells or of endothelial progenitor cells. Endothelial progenitor cells EPCs can be cultured from the peripheral circulation of humans and different animals. The cells act vasoprotectively by direct and indirect mechanisms. The protective effects of EPCs in acute ischemic kidney injury can be stimulated by preincubating the cells with different agonistic mediators. This paper summarizes the currently available data on strategies to improve the renoprotective activity of EPCs in acute ischemic kidney injury.

1. Endothelial Dysfunction in Acute Ischemic Kidney Injury

Acute ischemic kidney injury is the most frequent cause of acute renal failure in daily clinical practice [1]. Despite the significant progress that has been made in the fields of intensive care medicine and renal replacement therapy over the past 30 years, the mortality of acute renal failure in hospitalized patients still varies from 30 to 70% [1–3]. Although hypoperfusion of the kidney, resulting from various pathologic conditions, primarily affects the function and structure of tubular epithelial cells, it has become increasingly recognized that microvascular endothelial cell dysfunction (ED) in peritubular capillaries inhibits the process of postischemic renal reperfusion and thereby prolongs kidney malfunction [4, 5]. First studies came from Flores et al. [6]: rats that underwent transient renal artery clamping showed intense swelling of all cellular elements in the kidney, leading to persistent renal hypoperfusion after the end of ischemia. Such no-reflow, which was in part also attributable to endothelial cell swelling, could effectively be treated by the injection of *hypertonic* mannitol solution, but remained unaffected by an equivalent expansion of the extracellular

fluid volume with either isotonic saline or isotonic mannitol [7]. Further studies, published in 2001, showed that ischemia associated endothelial cell dysfunction in addition can result in *permanent* damage to peritubular capillaries. This damage worsens long-term outcome of kidney function [8, 9]. These data suggested that postischemic renal ED could potentially serve as new therapeutic target in the management of acute ischemic kidney injury. Therefore, newer investigations performed by Yamamoto and Brodsky [4, 5] focused on the treatment of postischemic ED by the administration of mature endothelial cells (HUVECs—human umbilical vein endothelial cells). In vivo microscopic analyses confirmed the aforementioned significant postischemic endothelial cell swelling within the peritubular capillary network, and in addition showed that complete normalization of microvascular tissue perfusion occurs as late as 24 hours after ischemia. In this setting, systemic administration of HUVECs markedly inhibited endothelial cell swelling and promoted a faster functional and structural recovery of the organ. Histologically, injected cells had partly been incorporated into the endothelial layer of small blood vessels surrounding the tubular integrity [4, 5]. These studies showed for the first time that targeting postischemic ED by

the administration of cells of the endothelial lineage is a true option in the treatment of acute ischemic kidney injury.

2. Endothelial Progenitor Cells

For many years it had been assumed that new blood vessels in the adult vertebrate organism exclusively develop as the result of sprouting of preexisting, mature vessels. This process, named “angiogenesis”, occurs as a result of diminished oxygen and nutrient supply to tissues [10–12]. The second process by which blood vessels can be generated, “vasculogenesis”, is typically found during embryogenesis [13]. The human organs and tissues are formed during the first eight weeks after conception. Less differentiated progenitor or precursor cells develop into numerous specialized cell types that build the whole organism. Blood vessel wall cells are derived from the so-called hemangioblast [14, 15]. The concept of neovascularization has dramatically been convulsed by the landmark studies of Asahara and colleagues, published in 1997 [16]. First, CD34⁺ cells were isolated from human umbilical vein blood and cultured under defined conditions. After several days, the cells displayed phenotypical and functional characteristics of endothelial cells. Systemic injection of the cells into immunoincompetent animals with hindlimb ischemia significantly improved postischemic function and structure of the reperfused tissue. Microscopic analysis showed that injected cells had partly been incorporated into the vessels’ intima. This was the first time the term “endothelial progenitor cells” or EPCs was introduced. It was also the first time that vasculogenesis had been documented to occur in an adult vertebrate organism. Meanwhile, numerous studies on the subject have been published [11–13, 17–29], but, as a matter of fact, the exact nature of EPCs or, if spoken more provocatively, the question if EPCs really exist, is still intensively debated [27, 28]. According to newer concepts of EPC biology, at least two major populations of “EPCs” can be differentiated [27, 28, 30, 31]. The first and by far more in detail analyzed population is represented by cells that primarily develop from pluripotent hematopoietic stem cells in the bone marrow. These cells can be isolated from the circulation of humans and different animals. They are cultured on fibronectin-coated dishes in EBM-2 media [32], they bind certain types of lectins (BS-1-lectin [mouse] or UE-lectin [humans]), and they ingest LDL. In culture, the cells appear after 5–7 days which led to their definition as “early outgrowth” EPCs [33–35]. They do express, on one hand different immature or “stem” cell marker molecules such as CD133, c-Kit, and CD34 [30], and, on the other hand markers of the endothelial lineage (KDR or Flk-1, CD31, eNOS). They are most likely identical to the so-called “colony forming unit endothelial cells” (CFU-ECs). CFU-ECs can also be cultured from mononuclear blood cells of various origin. Culturing the cells is a two-step procedure, in which the cells are first plated on fibronectin for two days. Nonadherent cells are then replated on new dishes where they give rise to colonies. The colonies consist of rounded cells in the center, surrounded by spindle-shaped cells in the periphery [28, 32]. The ongoing controversy about the true

endothelial nature of the cells results from the fact that after systemic cell treatment of recipient animals, direct cell incorporation into the endothelial layer is rarely seen [27, 28]. There is no doubt that “early outgrowth” EPCs can act proangiogenically/antiischemically, since numerous experimental studies proved a pathophysiological role/therapeutic value in ischemic heart disease, peripheral artery disease, cerebrovascular disease, and uremia, respectively [19, 36–46]. Nevertheless, vasoprotection mediated by the cells is probably more attributable to indirect effects [33]. Rehman and colleagues showed secretion of vasoprotective substances by the cells (VEGF, HGF, IGF-1) [47], and proteomic analysis performed by Pula et al. showed that the enzyme thymidine phosphorylase plays a major role in this process [48]. It has been concluded that such factors mediate recovery from endothelial cell dysfunction. Following this concept, “early outgrowth” EPCs travel to sites of tissue ischemia where they act in a paracrine manner. In a newer manuscript, the authors therefore suggest the term “proangiogenic hematopoietic cells” as opposed to “endothelial progenitor cells” [27, 28].

The second EPC population is represented by cells that share more characteristics with mature endothelial cells [49]. They can also be cultured from blood mononuclear cells [50]. Culturing is performed on collagen type 2 coated dishes in EBM-2 media [50, 51]. In contrast to “early outgrowth” EPCs, these cells appear in culture after a period of 2–3 weeks (“late outgrowth” EPCs [28, 49–52]). “Late outgrowth” EPCs do not express hematopoietic but endothelial cell marker molecules. After systemic cell injection, direct cell incorporation into the endothelial layer does substantially occur [28, 53]. The only difference between mature endothelial cells and “late outgrowth” EPCs is the much more pronounced *in vitro* proliferation of the latter. It has recently been discussed that “late outgrowth” EPCs possibly are mature endothelial cells, derived from bone marrow residing vessels, from which they were shed into the circulation [28]. Nevertheless, both EPC populations, “early outgrowth” EPCs and “late outgrowth” EPCs have been documented to be involved in neovascularization under both physiological and pathological conditions [24, 25, 50, 54]. Direct as well as indirect mechanisms are involved in vessel repair. At the moment it seems that “early outgrowth” EPCs mostly act by indirect mechanisms, whereas “late outgrowth” EPCs predominantly mediate direct endothelial regeneration by incorporating into the vessels’ walls. Table 1 summarizes the characteristics of “early outgrowth” EPCs in comparison to “late outgrowth” EPCs.

3. “Early Outgrowth” EPCs in the Treatment of Acute Ischemic Kidney Injury

The earlier mentioned studies by Yamamoto and Brodsky [4, 5] pointed for the first time towards a new therapeutic strategy for treating acute ischemic kidney injury (iAKI). That is, to target postischemic renal ED in order to optimize renal reperfusion. Still today, only limited data are available on the pathophysiological role of EPCs in iAKI. The first study was published by Patschan et al. [24]. FVB/NJ mice,

TABLE 1: Biological properties of “early outgrowth” as opposed to “late outgrowth” EPCs (see text).

“Early outgrowth” EPCs	“Late outgrowth” EPCs
Proliferate in culture after 5–7 days (fibronectin coated dishes)	Proliferate in culture after 21 days (collagen type 2 coated dishes)
Express endothelial and hematopoietic cell marker molecules (CD133, CD45 and CD14)	Do not express hematopoietic but endothelial cell marker molecules
Capable of differentiating into hematopoietic cells	Do not differentiate into hematopoietic cells
Low proliferative activity	High proliferative activity
Minimal endothelial tube formation in vitro	Significant endothelial tube formation in vitro
Proangiogenic activity in vivo	Proangiogenic activity in vivo
⇒ Proangiogenic hematopoietic cells	⇒ “True” (?) progenitors of endothelial cells

subjected to transient unilateral renal ischemia showed a rapid EPC mobilization, characterized by substantial splenic accumulation of the cells. Ischemic preconditioning of the animals mitigated these effects but promoted direct homing of mobilized cells into the postischemic kidney, where they foremost accumulated within the medullary borderzone. EPC-enriched mononuclear cells, isolated from the kidneys of preconditioned animals protected mice from acute ischemic renal failure if injected right after reperfusion. This manuscript was the proof-of-principle that EPCs (according to the marker molecules that were used to identify the cells: “early outgrowth” EPCs) can serve as therapeutic option in the treatment of iAKN. Further studies revealed that acute renal ischemia mobilizes endogenous “early outgrowth” EPCs by increasing blood levels of uric acid [55]. The mechanisms of uric acid-induced EPC mobilization were further elucidated by Kuo et al. [56]. It was demonstrated that monosodium urate (MSU) in vitro and in vivo induced exocytosis of Weibel-Palade bodies with subsequent release of IL-8, von Willebrand factor, and angiopoietin-2 into the culture medium or into the circulation, respectively. In Toll-like receptor 4 null mice, acute elevation of uric acid levels by injection of uric acid did not result in the release of vasomodulatory factors into the blood. These data suggested that uric acid-induced exocytosis of Weibel-Palade bodies is mediated through this receptor. Two years ago, ex vivo expanded syngeneic mouse-derived “early outgrowth” EPCs were for the first time successfully administered in murine iAKI [25]. Injections of either 0.5 or 1×10^6 untreated cells after a 30 or 35 minutes period of ischemia protected recipient animals from acute renal failure. This was no longer possible if ischemia lasted for 40 minutes. Histological analysis showed accumulation of injected cells within the interstitial area, in close proximity to peritubular vessels. Since with longer periods of ischemia (40 minutes), a certain number (0.5×10^6) of untreated “early outgrowth” EPCs did not substantially protect mice from acute renal failure, the question arose whether therapeutic strategies could be established in order to increase the renoprotective capacity or competence of the cells.

4. Increasing Renoprotective Competence of “Early Outgrowth” EPCs in iAKI

The idea of modifying/increasing the competence of EPCs in the process of neovascularization arose soon after their first

description by Asahara et al. [16]. In an attempt to enhance inhibition of neointimal hyperplasia, EPCs were genetically manipulated in order to overexpress the enzymes endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1) [57]. Murasawa et al. increased mitogenic activity, migratory activity, and cell survival of human EPCs by inducing overexpression of telomerase reverse transcriptase [58]. Increased differentiation of EPCs into mature endothelial cells (ECs) was achieved by constitutively overexpressing protein kinase A. As a result, cellular Flk-1 and neuropilin 1 were significantly stimulated [59]. Regardless of the exact mechanisms by which EPCs (“early outgrowth” or “late outgrowth” EPCs) mediate vasoprotection in ischemia, it could be assumed that by increasing the numbers of cells in postischemic tissues, antiischemic effects must be more pronounced. According to this concept, we sought for a strategy to stimulate EPC homing (in our studies “early outgrowth” EPCs) into the reperfused kidney. Integrin molecules represent one of four major populations of molecules responsible for mediating cell-cell and cell-matrix adhesion. The other three families of cell adhesion molecules (CAMs) are represented by cadherins, selectins, and members of the immunoglobulin superfamily [60, 61]. Each integrin molecule is composed of one α - and one β -chain, respectively [62]. The binding specificity of integrins varies depending on the structure of these individual subunits [63]. Beta1-integrins are expressed on the surface of certain subpopulations of leukocytes [64]. Mature endothelial cells on the other hand do express different members of the immunoglobulin superfamily (e.g., VCAM-1, PECAM), from which some act as binding partners of integrins on leukocytes [65]. This interaction is the prerequisite for transvascular leukocyte trafficking into the perivascular space [64]. In theory, stimulating integrin-mediated transvascular cell migration would increase the numbers of cells leaving the blood stream. Recently, the substance 8-pCPT-2'-O-Me-cAMP (8-O-cAMP) has been documented to increase migratory activity of EPCs by agonizing integrins [66]. 8-O-cAMP activates the so-called cAMP-Epac-Rap-1 signal transduction cascade, which has been shown to be critically involved in regulating cell adhesion events [67]. In order to analyze whether 8-O-cAMP would increase the cells' renoprotective activity through stimulating the integrin system, we pretreated syngeneic murine EPCs with the substance [25]. Systemically, injection of pretreated cells into postischemic C57Bl/6N mice completely protected animals from acute ischemic

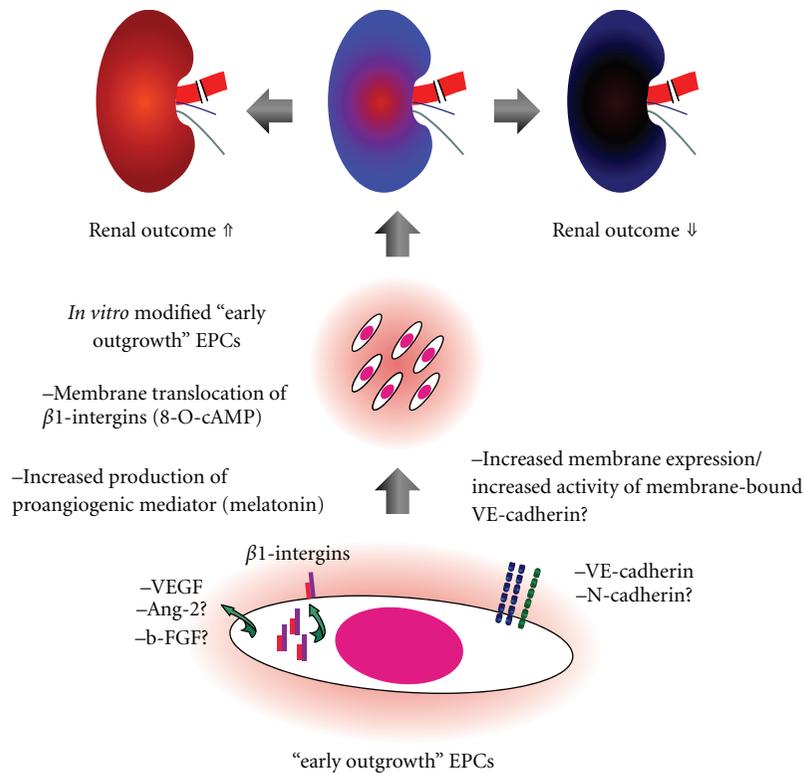


FIGURE 1: Mechanisms that are potentially involved in the stimulation or inhibition of EPC-mediated renoprotection after acute renal ischemia. Agonistic effects on $\beta 1$ -integrins, as they are induced by the Epac-1 activator 8-O-cAMP, increase renoprotective effects of the cells by stimulating EPC homing to postischemic tissue sites. Another agonistic mechanism is increased production of vasostabilizing substances (e.g., VEGF) by the cells. Such increased production can be induced by cell pretreatment with the hormone melatonin. In contrast, inhibition of the VE- and N-cadherin system most likely results in decreased renoprotective competence of EPCs (for further explanation see text).

renal failure. To further confirm this data, we then pretreated the cells with combined 8-O-cAMP and the integrin receptor blocking peptide cyclic arginine-glycine-D-aspartic acid (cRGD) [68]. Administration of combined pretreated cells partly abrogated the former effects. Histologically, the numbers of cells present in the kidneys did not differ between animals that received 8-O-cAMP and animals that received 8-O-cAMP/cRGD pretreated cells. It was concluded that binding of cRGD to integrins, while not significantly decreasing homing of EPCs per se, alters the functional competence of the cells, which is reflected by a reduction of their renoprotective activity. Single cell analysis of 8-O-cAMP prestimulated cells revealed cellular redistribution of $\beta 1$ -integrins towards the cellular surface, the total amount of $\beta 1$ -integrin expression, which was measured by Western-Blot analysis, remained stable. Taken together, agonizing $\beta 1$ -integrins in “early outgrowth” EPCs was identified as a first pharmacological strategy to increase the renoprotective competence of the cells in the setting of acute ischemic kidney injury [25]. Since then, our studies focused (and still focus) on further cell-cell/cell-matrix adhesion processes that could potentially be modified by the cAMP-Epac-Rap-1 pathway as well. Newer, yet unpublished data indicate that VE-cadherin inhibition possibly plays a role in *reducing* EPC-mediated renoprotective activity. Angiotensin-1 and -2 are endogenous mediators, involved in regulating endothelial

cell growth [69]. They compete with each other in the interaction with endothelial Tie-2 [70]. Angiotensin-1 acts as Tie-2 agonist, thereby stimulating expression of VE-cadherin in endothelial cells. Endothelial VE-cadherin expression in fact is also stimulated by cAMP-Epac-Rap pathway activation [71]. Mice that are injected with Angiotensin-1 pretreated EPCs show significantly higher postischemic serum creatinine levels than animals injected with untreated cells if VE-cadherin is blocked by specific peptides. The mechanisms responsible for such worsening of renal function can only be speculated at the moment. In another, quite different approach, “early outgrowth” EPCs were preincubated with the hormone melatonin (unpublished data). N-acetyl-5-methoxytryptamine (melatonin), a product of the tryptophan metabolism is highly preserved throughout phylogeny. It is synthesized in the pineal gland and released into systemic circulation in order to exert regulatory roles on circadian rhythms [72]. It had once been suggested that melatonin functions as *the* key regulatory molecule in sleep wake rhythm. Meanwhile, this concept has been modified [73, 74]. Nevertheless, it has been documented that the hormone is involved in numerous physiological events including the detoxification of free radicals and their related oxygen derivatives [72]. Melatonin mediates its effects exclusively via interacting with MT-1 and -2 receptors expressed by cells of various phenotype [75]. The hormone has been shown to

improve proangiogenic activity of mesenchymal stem cells in vitro [76]. If 0.5×10^6 melatonin pretreated EPCs were injected into mice after a 40 minutes period of bilateral renal ischemia, renal function did not differ from untreated (nonischemic) animals. While melatonin did not have an influence on TGF-beta-induced EPC apoptosis or necrosis, production and secretion of proangiogenic VEGF by the cells were enhanced after incubation with the hormone. In contrast, cellular production/release of Angiopoietin-2 and fibroblast growth factor-2 (FGF-2) remained unaffected. In addition, migratory activity of the cells was stimulated. These data are currently under review for publication.

5. Summary

Although acute renal ischemia primarily affects the function and structure of tubular epithelial cells, postischemic ED within perivascular vessels is an important perpetuating factor of prolonged postischemic kidney dysfunction. ED-based therapeutic strategies (e.g., systemic administration of mature endothelial cells or endothelial progenitor cells) allow to initiate a faster postischemic reperfusion. "Early outgrowth" EPCs are a promising tool for treating acute ischemic kidney injury as the most common cause of acute renal failure in clinical medicine. It is possible to increase the cells' renoprotective activity in this setting by either increasing their homing to sites of tissue ischemia and/or by stimulating production/secretion of vasostabilizing mediators such as VEGF (Figure 1). Nevertheless, additional strategies still need to be established in order to enforce endogenous EPCs to travel into the postischemic kidney.

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