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Review

Endothelial Progenitor Cell-Based Therapy for Pulmonary Arterial Hypertension

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A growing body of evidence in animal models and clinical studies supports the concept that endothelial progenitor cell (EPC)-mediated therapy ameliorates pulmonary arterial hypertension (PAH) and thus may represent a novel approach to treat it. Conversely, several experimental findings suggest that EPCs may be involved in PAH pathogenesis and disease progression. These discrepant results confuse the application of EPC transplantation as an effective treatment strategy for PAH. To improve the study of EPC transplantation in PAH therapy, it is high time that we resolve this dilemma. In this review, we examine the pathobiological changes of PAH, the characteristics of EPCs, and the underlying mechanisms of EPC effects on PAH.

Key words: Pulmonary arterial hypertension (PAH); Endothelial progenitor cells (EPCs); Transplantation; Mechanisms

INTRODUCTION

Pulmonary arterial hypertension (PAH) is characterized by a progressive increase in pulmonary vascular resistance (PVR) and vascular remodeling with ensuing right-heart failure and early death if untreated (30,59,61). Based on PAH pathogenesis, three main classes of drugs are currently used for the treatment of this disease: prostacyclins, endothelin-1 receptor antagonists, and phosphodiesterase-type 5 inhibitors (56). Although these drugs do delay the course of PAH, there still remains no cure for PAH, and disease progression is inevitable. The search for new therapeutic strategies is therefore desirable.

Endothelial dysfunction is thought to play an important part in the initiation and development of PAH (79) and ultimately represents an imbalance between the magnitude of injury and the capacity for repair (28). Endothelial progenitor cells (EPCs) constitute one aspect of the endothelial repair process and have been employed for the treatment of PAH in animal models and clinical studies over the past few years. Some of these studies have observed attenuated vascular remodeling, improved pulmonary hemodynamics, increased exercise capacity, and prolonged survival (47,52,68–70,84,88,92–94,96). In contrast with these promising results, other experimental findings have indicated that EPCs may be involved in the pathogenesis of PAH (4,12,27,77,94). This review examines the pathobiological changes of PAH, the characteristics of EPCs, and the underlying mechanisms of EPC effects on PAH to explain the discrepant results.

PATHOBIOLOGICAL CHANGES OF PAH

The characteristic pathobiological changes of PAH include intimal lesions, medial thickening, and adventitial remodeling (Fig. 1).

Intimal Lesions

Dysfunctional endothelial cells (ECs) play an important pathobiological role in PAH (6). Injured ECs in PAH may produce and release excessive amounts of paracrine factors that act as growth factors to induce the proliferation and migration of smooth muscle cells (SMCs) or as chemokines to recruit circulating inflammatory cells (62). These actions initiate or enhance pulmonary vascular remodeling and inflammation. There is evidence that EC apoptosis may

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trigger the development of PAH (7,90). A critical reduction in bone morphogenetic protein receptor type 2 (BMPR2) function in the endothelium may promote endothelial apoptosis in PAH (47,58,60,73). EC apoptosis could lead directly to microvascular obliteration due to degeneration of these fragile endothelial structures. Alternatively, repeated waves of EC loss may promote the emergence of hyperproliferative, apoptosis-resistant clones of ECs (86). The latter lead to the formation of plexiform lesions, which are a characteristic feature of intimal lesions (34).

ECs can transition into myofibroblasts (α-SM-actin$^+$) (1), which can generate long-lasting constriction (76). These myofibroblasts may appear to be different from SMCs, with a slower onset of contraction in response to vasoactive stimuli and with a failure to relax in response to vasodilating stimuli (13). Furthermore, it was found that ECs in plexiform lesions expressed abundant amounts of collagen IV, which is a component of basement membranes and a type of extracellular matrix (ECM) (80).

Medial Thickening

Thickening of the tunica media in pulmonary arteries (PAs) in PAH occurs by a combination of SMC hyperplasia and hypertrophy (80). As described above, SMC proliferation could be induced by EC dysfunction (62). Furthermore, a loss in integrity of the endothelial barrier allows the extravasation of factors that stimulate the release of vascular serine elastase from SMCs, which leads to the initiation of growth signals to medial SMCs (11). Also, SMC resistance to apoptosis might play an essential role in SMC-based pulmonary vascular lesions (41,81). BMPR2 has a regulatory role in limiting the survival and growth of SMCs, so the loss of BMPR2 activity could result in medial thickening and arteriolar narrowing in PAH (34). In addition, under increased luminal pressure, vascular smooth muscle may exhibit adaptive hypertrophy, which also contributes to medial thickening in PAH.

Vascular SMCs could transdifferentiate into smooth muscle-like cells expressing α-SM actin and accumulate in vascular lesions (47). Furthermore, the ECM protein tenascin-C was found to be expressed within the medial SMC layer of injured and remodeled PAs in PAH (32).

Adventitial Remodeling

Rather than just a structural support to pulmonary vessels, the adventitia may also regulate pulmonary vascular function from the "outside in" (68). The adventitia is primarily composed of fibroblasts, of which the most characteristic property is the powerful ability to undergo proliferation. Fibroblasts were found to undergo a marked

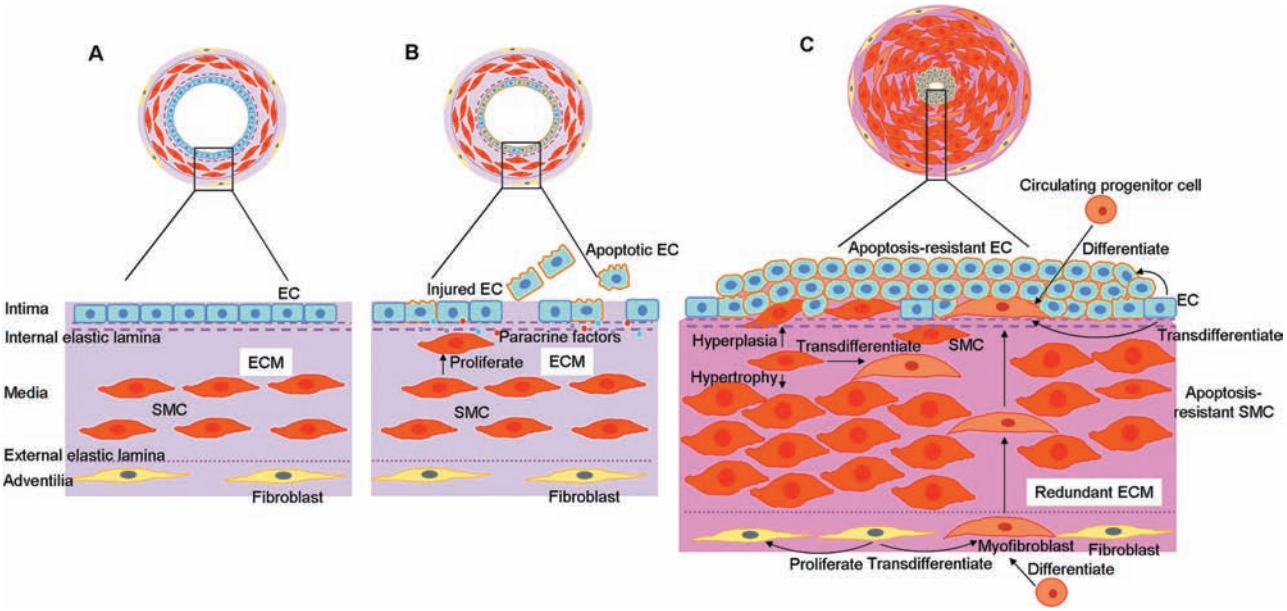

Figure 1. Pathobiological changes of PAH. Schema illustrating the pathobiological changes of early- and advanced-stage pulmonary arterial hypertension (PAH). (A) Pulmonary arteries (PAs) in the normal pulmonary circulation. (B) At early stages of PAH, injured endothelial cells (ECs) may produce and release excessive amounts of paracrine factors that act as growth factors to induce the proliferation and migration of smooth muscle cells (SMCs). (C) At advanced stages of PAH, repeated waves of EC loss may induce the emergence of hyperproliferative, apoptosis-resistant ECs which lead to the formation of plexiform lesions. SMCs also proliferate and migrate to the intima. Thickening of the tunica media in PAs occurs by a combination of hyperplasia and hypertrophy. "Apoptosis-resistance" also plays an essential part in SMC-based pulmonary vascular lesions. In the adventitia, fibroblasts undergo a marked increase in proliferation. Transition of other cells into myofibroblasts and deposition of the extracellular matrix (ECM) also occur in three layers of PAs.

increase in proliferation upon exposure to chronic hypoxia or monocrotaline (MCT) treatment (43,44).

The increased neovascularization of adventitial vasa vasorum microcirculation is considered to serve as a conduit for sustained delivery of progenitor cells to PAs (12). Recruited progenitor cells could induce a feed-forward loop of vasa neovascularization and assume myofibroblasts (19,69). Hypoxia-induced PAH is associated with early and dramatic increases in the transdifferentiation of fibroblasts into myofibroblasts (13,19,21). Activated fibroblasts and myofibroblasts also induce structural remodeling of the vessel by increasing the production of collagen and other ECM proteins, including fibronectin, tenascin, and elastin (13,19,21,68).

EPCs AND THEIR EFFECTS ON PAH

EPC Characteristics

EPCs are mainly identified in the bone marrow, cord, and peripheral blood (35,49,85,87,95,97). They can circulate, proliferate, and differentiate into mature ECs but have not acquired the characteristic mature endothelial markers or formed a lumen (2). EPCs could adhere to matrix molecules such as fibronectin and demonstrate dual positivity to acetylated low-density lipoprotein (acLDL) and *Ulex europaeus agglutinin I* (UEA-1) lectin (2). One method of identifing EPCs is based on the expression of various protein markers on the cell surface (45). To the best of our knowledge, there is no specific identifying marker for EPCs. Asahara et al. reported that circulating cluster of differentiation 34 positive ($CD34^+$) and fetal liver kinase positive ($Flk\text{-}1^+$) [also known as vascular endothelial growth factor receptor 2 (VEGFR2) or kinase insert domain receptor (KDR)] mononuclear blood cells may contribute to neoangiogenesis, and these cell surface markers were the first putative marker set for identifying the EPCs (2). Then CD34, KDR, and CD133 were chosen to characterize the functional characteristics of EPCs, and these biomarkers became the most commonly used molecules for defining an EPC population (51,83). However, Case et al. stated that $CD34^+KDR^+CD133^+$ cells expressed the hematopoietic lineage-specific antigen CD45 and were highly enriched in hematopoietic progenitor activity but did not form EPCs in vitro (8). The research team further demonstrated that $CD34^+CD45^+$ cells formed hematopoietic progenitor cells but not EPCs, while $CD34^+CD45^-$ cells formed EPCs but not hematopoietic progenitor cells (8).

EPCs have recently been shown to consist of two different subpopulations: early-outgrowth and late-outgrowth EPCs (52). It was reported that early-outgrowth EPCs are derived from $CD34^+CD45^+$ cells while late-outgrowth EPCs are only from a human cord blood or bone marrow $CD34^+CD45^-$ population of cells (75). Besides, the majority of early-outgrowth EPCs arise from a CD14-positive subpopulation of mononuclear cells, while late-outgrowth EPCs are developed exclusively from the CD14-negative subpopulation (24). Hristov et al. stated that early-outgrowth EPCs expressed CD34, CD133, VEGFR2, and CD31 markers, and late-outgrowth EPCs expressed CD34, VEGFR2, and CD31 markers but do not reveal CD133 (29). However, Timmermans et al. have showed that early-outgrowth EPCs express CD133 as expected, but not VEGFR2, whereas late-outgrowth EPCs express VEGFR2, but not CD133 (75). Published data also suggested that $CD34^-$ cells were able to take on an endothelial phenotype and that infusion of $CD34^-/CD14^+$ monocytes from bone marrow appeared to be also effective for reendothelialization after vessel injury (20,26). Gulati et al. demonstrated that early-outgrowth EPCs expressed endothelial nitric oxide synthase (eNOS) and caveolin-1, which are negative for late-outgrowth EPCs (24). Conversely, Hur et al. have reported that fms-related tyrosine kinase 1 (Flt-1 or vascular endothelial growth factor receptor 1), eNOS, and von Willebrand factor (vWF) can be expressed in both types of EPCs but at a higher level in late-outgrowth EPCs and that they also express higher levels of vascular endothelial cadherin and KDR (31). Miller-Kasprzak and Jagodzinski found that eNOS, vWF, vascular endothelial cadherin, and E-selectin were expressed in late-outgrowth EPCs while not expressed in early-outgrowth EPCs (45).

Beside disparity in phenotype, early-outgrowth EPCs have different morphologies, growth characteristics, and function from late-outgrowth EPCs. Early-outgrowth EPCs are spindle-shaped cells, die after ~4 weeks, and have a very low proliferative ability (31,55,65). They cannot form a vascular network in vitro, but can adhere to mature ECs, and improve network formation and repair injured or apoptotic ECs through a paracrine mechanism (65). Late-outgrowth EPCs have a cobblestone morphology, start to proliferate and differentiate into mature ECs after ~2–3 weeks, and incorporate directly into newly formed blood vessels (36,38).

Since the classification of EPCs into early-outgrowth and late-outgrowth EPCs was proposed, disagreement has persisted in the scientific community. As mentioned above, early-outgrowth EPCs are heterogeneous, containing mainly cells of lymphocyte ($CD45^+$) and monocyte ($CD14^+$) lineage that possess phagocytic properties. The cell surface phenotype of late-outgrowth EPCs is nearly indistinguishable from the pattern of expression displayed by vascular ECs (57). In fact, a recent comparison of the proteome of human late-outgrowth EPCs and dermal microvascular ECs revealed a 90% overlap (42). Late-outgrowth EPCs display remarkable similarities with "mature vessel wall ECs," so it is unknown whether the late-outgrowth EPCs "generated" or "expanded" ex vivo represent the differentiated progeny of an undifferentiated precursor or whether late-outgrowth EPCs are just

circulating mature ECs (74). Thus, only by excluding other potential "contaminating" cell types could we investigate the real properties of late-outgrowth EPCs. Besides, the function (such as incorporation and vasculogenic properties) and phenotype (such as surface marker profile) of in vitro propagated endothelial lineage cells may be changed compared to their in vivo counterparts (74). So we should be cautious that these cultured cells may not necessarily be representative of cell populations in vivo although they could reflect properties of these cell populations.

Hill et al. also identified another kind of EPCs (so-called colony forming unit-endothelial cells, CFU-ECs or CFU-Hill) based on their capacity to form colonies (28). These cells emerged from the cultured nonadherent human peripheral blood mononuclear cells after 48 h preplating on fibronectin-coated dishes. The number of CFU-ECs has been related to vascular function and cumulative cardiovascular risk (28). It is apparent that CFU-ECs contain various blood cells including monocytes, lymphocytes, and hematopoietic progenitor cells [see Richardson and Yoder (57) for a comprehensive overview].

EPC Status in PAH

EPCs could be used as a marker of vascular function and have been studied to assess the severity of cardiovascular diseases (16,38). The level of circulating EPCs predicts cardiovascular events (64,89). A low number of EPCs is associated with a worse prognosis in cardiovascular disease (28,84,89). It was found that the number of peripheral EPCs was reduced in patients with PAH compared with healthy control subjects (15,18,33). However, there were reports showed an increase in circulating EPCs in PAH (4,39,77) while Smadja et al. found no difference between PAH patients and normal controls (67). The reasons for these discrepant findings are unclear, but may reflect differences in the methods and surface markers used to identify and quantify cells as well as variation in patient selection and treatment (14). Changes in the numbers of peripheral EPCs before and after the onset of PAH were observed, and EPCs from PAH beagles experienced exhaustion and senescence after the acute stage of injury to pulmonary vessels induced by dehydromonocrotaline (92).

Besides change in the number of circulating EPCs, functional activities alteration also occurs in PAH. EPC migration to stromal cell-derived factor-1α, adhesion to fibronectin, incorporation into a vascular network, and nitric oxide production are impaired in chronic hypoxia-induced mice PAH (39). We found that EPC migratory ability and adhesive capacity were damaged in patients with PAH (33). Circulating angiogenic progenitor in PAH patient showed impaired ability to form vascular networks (77), while Asosingh et al. reported greater affinity for angiogenic tubes (4). As well, the differences in methods to identify cell may lead to this disparity.

Therapeutic Potential of EPCs in PAH in Animal Models and Humans

It is reasonable to believe that the transplantation of EPCs, which constitute one aspect of the endothelial repair process, has potential in the prevention and treatment of PAH (Table 1). In animal models, "prevention protocol" refers to infusion of EPCs before PAH has been induced, while "treatment protocol" refers to transplantation of cells after PAH has been established. Nagaya et al. reported that benefit could be obtained in rats in response to EPC therapy in MCT-induced PAH (48). Zhao et al. found that delivery of syngeneic bone marrow-derived EPCs nearly completely prevented the increase in pulmonary systolic pressure after MCT administration (97). Yip et al. also demonstrated that transplantation of autologous bone marrow-derived EPCs effectively ameliorated and reversed MCT-induced PAH (95). In experimental dogs with dehydromonocrotaline-induced PAH, EPC infusion also showed reversal effects. Transplantation of autologous EPCs from peripheral blood demonstrated significant improvements in hemodynamics and amelioration in the medial thickness of the small PAs (72). Our research team also found that EPCs cultured from human peripheral blood attenuated and reversed MCT-induced PAH in rat (91).

PAH is considered to be a multifactorial disease with genetic and environmental risk factors and associated conditions. It is therefore rational to suggest that a given therapeutic approach will not be efficacious for all patients (5). Recently, EPC transplantation combined with gene transfer has been used as a novel approach to treat PAH. The transplantation of adrenomedullin DNA-transduced EPCs markedly ameliorated pulmonary hypertension in MCT rats (48). Moreover, MCT rats transplanted with adrenomedullin-expressing EPCs had a significantly higher prevalence of survival than those given EPCs alone (48). In addition, treatment with eNOS-transduced EPCs is more effective than with EPCs alone in reversing pulmonary vascular disease in a MCT-induced model of PAH in rats (97). It was also found that infusion of prepro-calcitonin gene-related peptide-expressing EPCs may effectively attenuate established PAH and exert reversal effects on pulmonary vascular remodeling (96). Recently, Sun et al. have explored a new therapeutic strategy for the treatment of PAH. They reported that transplantation of autologous EPCs combined with cilostazol or sildenafil was superior to EPCs or cilostazol/sildenafil alone for preventing MCT-induced PAH (70,71).

The above-mentioned preclinical studies strongly suggested that EPC-based therapy is a potential important and effective approach for the treatment of PAH in patients.

Table 1. Effects of EPC Transplantation on the Treatment of PAH

EPC Phenotype	EPC Treatment	PAH Model	Time for Modeling (Days)[a]	Time of Infusion (Days)[b]	Outcomes	First Author, Year (Ref.)
$KDR^+/CD31^+/$ $VE\text{-}cadherin^+/CD14^+$	Human umbilical cord blood-derived EPCs or AM-EPCs (1×10^6)	MCT-induced in rats (60 mg/kg)	7	14	PAP↓, PVR↓, remodeling↓, survival↑	Nagaya et al. 2003 (48)
$CD34^+/CD31^+/vWF^+$	Dog PB-derived EPCs (1×10^6)	DHMC-induced in dogs (3 mg/kg)	42	28	PAP↓, PVR↓, CO↑	Takahashi et al. 2004 (72)
$Flk\text{-}1^+/vWF^+$	Rat BM-derived EPCs (1×10^6)	MCT-induced in rats (75 mg/kg)	3	18	RVSP↓, RVH↓, perfusion↑, survival↑	Zhao et al. 2005 (97)
	(1.5×10^6)		21	14		
	eNOS-EPCs (1.5×10^6)		21	14		
–	Mice BM-derived cells (5×10^6)	Hypoxia-induced in mice	28–56*	84–112*	Remodeling↑	Hayashida et al. 2005 (27)
–	Mice BM-derived cells (2.5×10^6)	MCT-induced in mice (5 mg/kg)	3	12	RVSP↓, RVH↓, remodeling↓	Raoul et al. 2007 (53)
		Hypoxia-induced in mice	3	12	RVSP-, RVH-remodeling-	
$CD34^+/KDR^+/AC133^+/$ VE-cadherin	Human PB-derived EPCs ($1.1\pm0.6\times10^7$)	Adult with PAH	–	84	PAP↓, PVR↓, CO↑, 6MWD↑	Wang et al. 2007 (87)
$CD34^+/VEGFR2^+/$ $AC133^+$	Human PB-derived EPCs or CGRP-EPCs (1×10^6)	Shunt-induced in rats	70	28	PAP↓, PVR↓, remodeling↓, survival↑	Zhao et al. 2007 (96)
$KDR^+/CD31^+/CD29^+/$ $vWF^+/CD90^+/VEGF^+$	Rat BM-derived EPCs ($1.0–1.2\times10^6$)	MCT-induced in rats (75 mg/kg)	28	62	RVSP↓, RVH↓, remodeling↓	Yip et al. 2008 (95)
$VEGFR\text{-}2^+/Sca\text{-}1^+/$ $CXCR\text{-}4^+/c\text{-}Kit^+$	Mice spleen-derived EPCs (5×10^5)	Hypoxia-induced in mice	7	14	RVSP-, RVH-	Marsboom et al. 2008 (39)
			14	7		
$CD34^+/KDR^+/AC133^+$	Human PB-derived EPCs ($0.6\pm0.33\times10^7$)	Children with PAH	–	84	PAP↓, PVR↓, CO↑, NYHA class↓, 6MWD↑	Zhu et al. 2008 (98)
$CD34^+/KDR^+/AC133^+/$ VE-cadherin	Human PB-derived EPCs (1×10^6)	MCT-induced in rats (60 mg/kg)	21	21	RVSP↓, RVH↓, remodeling↓	Xia et al. 2009 (91)
$CD34^+/KDR^+/CD31^+/$ $vWF^+/VEGF^+$	Rat BM-derived EPCs (2×10^6) ± cilostazol	MCT-induced in rats (70 mg/kg)	3	39	RVSP↓, RVH↓, remodeling↓	Sun et al. 2009 (70)
$KDR^+/CD31^+/CD14^+$	Human PB-derived early-outgrowth EPCs (1.5×10^6)	MCT-induced in rats (70 mg/kg)	3	21	RVSP↓, RVH↓, remodeling↓	Ormiston et al. 2010 (49)
$CD34^+/KDR^+/CD31^+$	Late-outgrowth EPCs (1.5×10^6)				RVSP-, RVH-remodeling-	
$CD34^+/KDR^+/CD133^+$	Human PB-derived EPCs (1.5×10^6)	MCT-induced in rats (75 mg/kg)	14	14 and 21	RVSP-, RVH-, remodeling-, survival-	Mirsky et al. 2011 (46)
			21	7 and 14		
$CD34^+/KDR^+/CD133^+/$ $CD31^+/c\text{-}kit^+$	Rat BM-derived EPCs (2×10^6) ± sildenafil	MCT-induced in rats (70 mg/kg)	3	32	RVSP↓, RVH↓, remodeling↓	Sun et al. 2012 (71)

AC133, cluster of differentiation 133 (CD133); AM, adrenomedullin; BM, bone marrow; CD31, cluster of differentiation 31; CGRP, prepro-calcitonin gene-related peptide; CO, cardiac output; CXCR4, chemokine (C-X-C motif) receptor 4; DHMC, dehydromonocrotaline; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; Flk-1, fetal liver kinase-1; KDR, kinase-insert domain-containing receptor; MCT, monocrotaline; NYHA, New York Heart Association; PAH, pulmonary arterial hypertension; PAP, pulmonary arterial pressure; PB, peripheral blood; PVR, pulmonary vascular resistance; RVH, right ventricular hypertrophy; RVSP, right ventricular systolic pressure; Sca-1, stem cell antigen-1; VE-cadherin, vascular endothelial-cadherin; VEGFR2, vascular endothelial growth factor receptor 2; vWF, von Willebrand factor; 6MWD, 6-minute walk distance. *EPCs were transplanted to mice, after 8 weeks the mice were exposed to consistent hypoxia for up to 4 or 8 weeks.
[a]Days for inducing animal model of PAH. [b]Days after cell infusion to end of study.

Our research team conducted a 12-week prospective randomized trial comparing the effects of intravenous infusion of autologous EPCs plus conventional therapy with conventional therapy alone in adults with PAH (87). Exercise capacity and hemodynamics were improved in EPC-treated patients compared with patients in the conventional therapy-alone group. Transplantation of autologous EPCs was also safe, feasible, and associated with significant improvements in exercise capacity and pulmonary hemodynamics in children with PAH (98). These clinical studies have demonstrated the feasibility and potential of EPC-based therapy for the treatment of PAH.

Possible Involvement of EPCs in the Vascular Remodeling of PAH

Recent studies suggested that EPCs may be involved in pulmonary vascular remodeling. It was reported that c-Kit$^+$ progenitor cells may participate in the thickening of vessel walls in hypoxia-induced PAH (12). Hayashida and colleagues transplanted the whole bone marrow of enhanced green fluorescent protein (GFP)-transgenic mice to the lethally irradiated syngeneic mice, and 8 weeks later, these mice were exposed to consistent hypoxia to induce PAH (27). These GFP$^+$ cells mobilized to hypertensive PAs, expressed α-smooth muscle actin, and contributed to the pulmonary vascular remodeling (27). The result was confirmed by another research team (53). The bone marrow-derived cells used in these two studies were heterogeneous and undefined. EPCs may be one constituent of these cells. However, we should not ignore the potential effects of other cell populations.

The role of EPCs in the vascular remodeling was also studied in patients with PAH. There was a remarkable upregulation of progenitor cell markers such as CD133 and c-Kit in remodeled arteries from patients with PAH, especially in plexiform lesions (77). In these patients, circulating levels of EPCs (CD34$^+$CD133$^+$VEGFR2$^+$) were increased while late-outgrowth EPCs' (CD34$^+$CD146$^+$ vWF$^+$CD133$^{+/-}$) ability to form vascular networks was impaired (77). Asosingh et al. also provided evidence that mobilization of high levels of proliferative CD34$^+$CD133$^+$ bone marrow-derived precursors is a characteristic of PAH and may participate in pulmonary vascular remodeling (4). In patients with chronic thromboembolic PAH, EPCs (CD34$^+$CD133$^+$Flk-1$^+$) were detected in the neointima of occluded vessels, and the microenvironment provided by thromboemboli promoted EPC differentiation into α-SM-actin$^+$ cells, which would enhance vascular remodeling (94). All of these studies suggested that dysfunction of circulating progenitors cells was involved in the vascular remodeling in PAH. One explanation for more proliferative progenitor cells in PAH may be that more primitive progenitors are mobilized into the circulation to fit ongoing pulmonary vascular shear stress and to repair endothelial injury (4). However, the hostile microenviroment of PAH may induce these mobilized progenitor cells differentiation into abberant cells, thus impairs their therapeutic efficacy.

The studies concerning the possible involvement of EPCs in PAH are mainly observational and focus on the role of endogenous progenitor cells mobilized from bone marrow. The bone marrow-derived cells were heterogeneous and contain stem/progenitor cells and many other cell populations, which makes it hard to reach a clear-out conclusion of EPC effects on PAH progression. However, the methods to identify EPCs were not unified in these studies. The exact effects of infused EPCs on the treatment of PAH and the underlying mechanisms remain unclear and need further investigation.

MECHANISMS OF EPC EFFECTS ON PAH

Role of EPC Differentiation in PAH

Late-outgrowth EPCs could differentiate into mature ECs and incorporate directly into newly formed blood vessels (38). Hence, EPCs may provide a circulating pool of cells that could form a cellular patch at the site of denuding injury or serve as a cellular reservoir that could replace injured endothelium (28).

In in vivo animal studies, EPCs contribute to vessel formation by differentiation into mature ECs and incorporation into the growing vessel wall (3). When spleen-derived EPCs were transplanted intravenously into splenectomized mice after wire-mediated vascular injury, systemically applied EPCs homed to the injured artery, resulting in improved reendothelialization associated with alleviative neointima formation (88). Two recent studies also found that intravenously implanted EPCs are incorporated into the distal PAs of MCT-injured lung and differentiate into mature ECs (48,97). Autologous infused bone marrow-derived EPCs are first trapped in the pulmonary arterioles and capillary networks and then incorporate into MCT-damaged pulmonary arterioles and capillaries (95).

Conversely, circulating EPCs could differentiate into hyperproliferative, apoptosis-resistant ECs, which may induce plexiform lesions at advanced stages of PAH (40). EPCs may also differentiate into myofibroblasts (12,94), which may contribute to the development of PAH.

Paracrine Role of EPCs in PAH

The injured tissue of the recipient may represent a hostile microenvironment for transplanted cells (54). This unfavorable microenvironment might impair the efficiency of cell transplantation. It is believed that a considerable number of EPCs die during and after transplantation (17).

A novel paradigm has recently emerged: the therapeutic mode of action of transplanted cells may be due to the release of protective paracrine factors (23). Evidence suggests that paracrine signals from therapeutic stem cells and

progenitor cells are primary players in various processes of tissue repair, integrating the mechanisms based on the differentiation and engraftment of cells (10,23). The secretory activity of EPCs has also been considered to be an important mechanism that exerts therapeutic and protective effects on vessels in vivo (63,82). It was found that early-outgrowth EPCs secrete several cytokines: vascular endothelial growth factor, hepatocyte growth factor, placental growth factor, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor (31,55). By transplanting human early-outgrowth EPCs into immunodeficient mice, Cho et al. showed that donor cells could express cytokines for up to 7 days (10). This transient expression of human cytokines facilitated enhanced recruitment of endogenous EPCs. The homing of early-outgrowth EPCs to injured tissue increases local concentrations of growth factors which exert anti-apoptotic and proangiogenic paracrine effects on the resident endothelium (10). Another study also suggested that infused EPCs might secrete several proangiogenic cytokines that promote the proliferation and migration of adjacent ECs in a paracrine manner (25). Recently, our research team found that conditioned medium from early-outgrowth EPCs not only represses the apoptosis of pulmonary microvascular ECs but also inhibits the augmented subsequent hyperproliferation (91). Moreover, it was found that the number of EPCs infused was eightfold higher than the number of cells required to produce the volume of EPC-conditioned medium needed to achieve the same therapeutic benefit (17). Considering EPC-conditioned medium significantly reduces the incidence of adverse immunological reactions, simplifies the procedure of production and thus increases the availability of the therapy, it is reasonable to support the concept that EPC-conditioned medium may represent a potent alternative for therapeutic use (17).

However, at advanced stages of PAH, EPCs may contribute to the formation of plexiform lesions by secreting growth factors such as vascular endothelial growth factor. Hence, extreme caution must be taken before EPC transplantation is used in the treatment of PAH.

A Hypothesis: Potential Differential Effects of EPCs at Different Stages of PAH

At early stages of PAH, EC dysfunction initiates pathobiological changes in PAs and contributes to increased PVR by three complementary pathways, (i) EC drop out and precapillary microvascular drop out; (ii) leading to endothelial dysfunction combined with increased SMC proliferation, which can result in arteriolar remodeling; (iii) creating the conditions that favor the emergence of hyperproliferative, apoptosis-resistant ECs (34). To repair and replace injured and apoptotic ECs at early stages of PAH, transplanted EPCs (if mainly as late-outgrowth EPCs) can differentiate into mature ECs and (if mainly as early-outgrowth EPCs) can secrete growth factors. The repair and replacement of injured and apoptotic ECs could avoid the initiation of the pathobiological changes of PAs and prevent further development of PAH by interrupting the three aforementioned complementary pathways. Hence, EPC transplantation could be beneficial in the treatment of early-stage PAH.

The pulmonary vascular remodeling of advanced-stage PAH is more complicated than that of early-stage PAH. The hostile microenviroment may induce EPCs differentiation into abberant cells, such as hyperproliferative, apoptosis-resistant ECs (40), and myofibroblasts (α-SM-actin$^+$) (12,94), thus promoting the progression of plexiform lesions (40). EPCs can secrete growth factors that are associated with the formation of plexiform lesions (78). Hence, in advanced-stage PAH, EPC infusion alone may be inefficient.

EPCs may also have various roles in different experimental models of PAH. In MCT-induced rat PAH, the transplantation of EPCs almost completely prevented the increase in right-ventricular systolic pressure. However, EPC infusion failed to prevent the progression of chronic hypoxia-induced PAH (39). The transplantation of bone marrow-derived cells into mice attenuated MCT-induced PAH but aggravated chronic hypoxia-induced PAH (53). Unlike MCT-induced reversible changes in PAs, chronic hypoxia induces fewer reversible changes, such as migration and proliferation of vascular SMCs and deposition of the ECM, which correlate with the remodeling events in advanced-stage PAH (9). These results suggested that EPCs play different parts at different stages of PAH from a different perspective. These data also showed that the effects of EPC transplantation on the treatment of PAH may be affected by the microenvironment of PAH. Although the above data implied the potential differential effects of EPCs at different stages of PAH, the direct evidence is still needed.

However, Ormiston et al. reported that early-outgrowth EPCs prevented MCT-induced PAH in rat when administered 3 days after MCT challenge, whereas late-outgrowth EPCs were ineffective (49). These early-outgrowth EPCs secrete interleukin (IL)-10, stimulate natural killer cells, thus prevent further development of MCT-treated PAH (49). It is reasonable to believe that transplantation of early-outgrowth EPCs may affect the course of PAH by exerting immunomodulatory effects (14). Mirsky et al. stated that treatment with human early-outgrowth EPCs either at the early (14 days post-MCT injection) or late (21 days post-MCT injection) time points did not lead to increased survival, and therapy did not prevent or reduce the severity of PAH (46). These transplanted cells lacked a unified phenotype and origin. In addition, the methods used to induce PAH were also different. The underlying mechanisms, however, remain to be determined in future studies.

OBSTACLES AND POSSIBLE SOLUTIONS

In general, there are two obstacles to EPC transplantation for PAH treatment, i.e., how to (i) standardize EPCs and the procedure of EPC transfusion and (ii) more efficiently alleviate or even reverse the pathobiological changes of advanced-stage PAH.

Many studies about EPC transplantation had limited analyses of the cell phenotype. The use of an often poorly defined label "progenitor cells" for heterogeneous therapeutic cell populations complicates comparison of studies and makes reaching definitive conclusions about their efficacy quite challenging (54). A cell type that exert beneficial effects in an experimental model could be harmful in a different setting (54). To make studies of EPC transplantation more comparable, defining and standardizing EPC surface markers is extremely important. Transplanting a certain number of cells to injured tissue is also essential to exert the therapeutic effects of cell-based therapy. The optimal cell delivery route has not been determined and several issues persist related to route of delivery of cells (50). It will require cell-based imaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET) to track the survival and distribution of EPCs (38). MRI-based tracking of cells labeled with iron oxide has been explored owing to high spatial resolution. Mai et al. and our research team have developed a simple protocol to label EPCs using superparamagnetic iron oxide nanoparticles at an optimized low dosage without significantly affecting their functional activity (37,93). PET can be used to monitor the homing and engraftment of transplanted cells by the use of suitable tracers, PET reporter genes, and probes (22). Moreover, how many cells are needed and how often cell infusion may be suitable for pulmonary vascular repair in patients with PAH remain unanswered questions.

EPC transplantation combined with gene transfer may exert more beneficial effects on alleviation or even reversal of the pathobiological changes of PAH. It was found that EPCs transfected with adrenomedullin, eNOS, and preprocalcitonin gene-related peptide gene had better effects on PAH treatment (48,96,97). The Pulmonary Hypertension and eNOS Cell Therapy (PHACeT) trial to assess the safety of autologous infusion of eNOS transfected-EPCs in patients with PAH has recently commenced (34). Since gene therapy remains ethically controversial in its clinical application, searching for a safer adjunctive therapeutic tool to enhance curative effects of EPCs on PAH may offer a novel clinical direction (71). EPC autologous transplantation combined with cilostazol and sildenafil has been shown to be superior to EPCs or cilostazol/sildenafil alone for preventing MCT-induced PAH (70,71).

In animal models, EPC administration is effective in PAH induced by MCT, dehydromonocrotaline (DHMC), and shunt, while it seems ineffective in hypoxia-induced PAH. Owing to differences in species and methods to induce PAH, the pathobiological changes of PAs in these animal models cannot fully duplicate the complex lesions in patients with PAH. So the data obtained in animal models could not be simply translated to clinical application. The clinical classification of PAH is complicated. The current classification system has been revised in 2008. Five major and many minor categories were recognized (66). So far, clinical studies of EPC therapy for PAH have just focused on idiopathic PAH patients. The effects of EPC therapy on other types of PAH are presently ill understood. An extremely important study in the field of EPC therapy for PAH, the PHACeT trial, is underway in Canada, under the leadership of Dr. Duncan Stewart. The initial enrolment of the trial was restricted to patients with idiopathic PAH; however, this together with other exclusion criteria made it difficult to identify suitable patients (34). Recently, the protocol has been amended to involve the enrolment of patients with PAH associated with systemic sclerosis (34). We are awaiting further data as this exciting trial evolves.

CONCLUSIONS

EPC transplantation represents a novel approach to treat PAH. Transplanted EPCs may repair and replace injured or apoptotic ECs, avoid the initiation of pathobiological changes of PAs, and prevent further development of PAH through differentiation into mature ECs and release of protective paracrine factors. Meanwhile, studies also suggest that EPCs may be involved in vascular remodeling. Considering the complex pathology of PAH, future studies should further explore the effects of infused EPCs on the treatment of PAH and the underlying mechanisms. Methods to identify EPCs were not unified in previous studies. Standardization of EPC surface markers is needed for future studies. EPCs combined with gene transfer or drug regimen was proved to be an efficient therapy by repairing injured endothelium and inhibiting or even reversing vascular remodeling. So cell transplantation in combination with gene transfer or drug regimen may be a promising strategy for the future EPC therapy for PAH.

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