Cell Therapy for Pulmonary Arterial Hypertension: Potential Efficacy of Endothelial Progenitor Cells and Mesenchymal Stem Cells

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Pulmonary arterial hypertension (PAH) presents a challenging problem for health care providers, as effective long-term therapies have been elusive. An emerging paradigm for the pathogenesis of PAH is that endothelial cell injury and apoptosis at the level of the precapillary arteriole could be the initiating event in the pathogenesis of this disease. This hypothesis has spurred research on novel regenerative approaches using stem and progenitor cells. In this review, we compare findings from the latest preclinical and clinical studies using endothelial progenitor cell (EPC) and mesenchymal stem cell (MSC) therapy to treat PAH. Additionally, we highlight recent advances in gene-enhanced cell therapy, an approach that promises to augment the therapeutic potential of EPCs and MSCs especially for the reversal of established PAH. These new regenerative approaches have shown great promise in preclinical studies; however, large, rigorously designed clinical studies will be necessary to establish clinical efficacy.

Pulmonary arterial hypertension is a challenging disease, and our understanding of the underlying mechanisms and pathophysiology remains incomplete. However, there have been a number of important advances, which have greatly enriched our knowledge about the key mechanisms that contribute to the functional and pathological abnormalities of PAH. With each new wave of understanding come new opportunities for the rational design of therapies. Thus, in the 1980s, increased awareness of the importance of endothelial dysfunction in PAH fuelled the development of "vasodilator" therapies, which now form the accepted standard for clinical treatment. First and foremost among these was the introduction of prostacyclin, which remains the gold standard, particularly for severe and progressive disease. However, the need for chronic parenteral therapy and all of the attendant complications of an indwelling intravenous line spurred the development of the first effective oral therapy, an endothelin receptor antagonist. This class of therapy was based on the discovery of the endothelin system and its role in mediating pulmonary vasoconstriction and arterial remodelling in these patients.[1,2] Other agents were then introduced to increase the activity of the nitric oxide pathways (ie, phosphodiesterase-5 inhibitors), and even today new agents that can address these cellular pathways in a novel manner, such as prostacyclin receptor agonists or direct guanylate cyclase stimulators, are being tested in clinical trials.

Nevertheless, none of these therapies are curative and while they can improve function, the vast majority of patients with idiopathic or associated PAH will progress. Indeed, it has been questioned whether there has been any significant improvement in survival in PAH since the introduction of specific therapies,[3] and while this can be disputed the prognosis remains poor with a survival of 3-5 years after diagnosis.[4] The next generation of PAH therapies will be inspired by the recent developments in the understanding of the cellular, molecular, and genetic mechanisms of PAH. These may include the development of treatments targeted to control dysregulated vascular cell growth, which appears to bear some similarity to neoplastic cell growth.[5] Thus, a number of receptor tyrosine kinase inhibitors that were initially developed to treat malignant diseases have been repurposed for PAH, including imatinib, which is now in clinical trials.[6] Moreover, the emerging role of adult stem and progenitor cells in the maintenance of vascular cell homeostasis and repair of injury has stimulated interest in the potential for cell therapies in the therapy of PAH.

EPC THERAPY IN PAH—FROM BENCH TO BEDSIDE

Endothelial progenitor cells are bone marrow-derived mononuclear cells that circulate in the blood and are believed to repair blood vessel damage by migrating to sites of vascular injury and differentiating into endothelial cells.[7] EPC therapy is now emerging as a potential regenerative approach to PAH. The integrity of the pulmonary endothelium is crucial to maintaining the balance of vasodilators, such as prostacyclin[8] and nitric oxide,[9] and vasoconstrictors such as thromboxane[10] and endothelin-1 (ET-1).[2] In PAH, there is an imbalance of these factors, contributing to vasoconstriction and vas-

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Table 1: Summary of Preclinical and Clinical Studies of EPC Treatment for PAH

Donor	Recipient	Model	Dose (cell #)	Citation/Key Findings
Preclinical studies using EPCs				
Dog	Dog	MCT	1×10^6	**Takahashi et al, 2004[29]** o Prevention of PAH
Rat BM-MNC	Rat	MCT	1×10^6	**Zhao et al, 2005[20]** o Prevention of PAH
Rat BM-MNC	Rat	MCT	1.2×10^6	**Yip et al, 2008[30]** o Prevented progression of PAH
Human PB-MNC	Rat	MCT	1×10^6	**Xia et al, 2009[22]** o Prevented progression of PAH
Human PB-MNC	Rat (nude)	MCT	1.5×10^6	**Ormiston et al, 2010[28]** o Prevention of PAH with early but not late outgrowth EPCs
Human PB-MNC	Rat (nude)	MCT	1.5×10^6	**Mirsky et al, 2011[27]** o No rescue of PAH
Preclinical Studies Using EPC-Based Gene Therapy				
Human UC-MNC	Rat	MCT	1×10^6	**Nagaya et al, 2003[19]** o Adrenomedullin-enhanced EPCs superior to EPCs alone
Rat	Rat	MCT	1×10^6	**Zhao et al, 2005[20]** o eNOS-enhanced EPC therapy reversed PAH in rats
Human PB-MNC	Rat	Shunt	1×10^6	**Zhao et al, 2007[33]** o CGRP-enhanced EPCs decreased mPAP and PVR
Clinical Studies Using EPCs				
Human PB-MNC	Human	PAH Patients	1.1×10^7	**Wang et al, 2007[31]** o Significant improvement in 6-minute walk distance, mPAP, PVR, and cardiac output

BM = bone marrow, PB = peripheral blood, UC = umbilical cord, MNC = mononuclear cell, mPAP = mean pulmonary arterial pressure, PVR = pulmonary vascular resistance

cular remodelling with narrowing of pulmonary arterioles.[11-13] However, arteriolar narrowing may only be part of the mechanism of increased pulmonary vascular resistance, and recent findings have implicated endothelial cell (EC) injury and apoptosis as critical triggers for the development of PAH.[14,15] So far, conventional therapy is aimed at restoring the balance of vasoactive substances but does not target repair of the endothelium, creating an unmet need for treatments such as EPC therapy.

The intra-alveolar pulmonary arteriole is a fragile structure that consists of an endothelial tube surrounded by a sparse matrix and few or no supporting medial cells (ie, pericytes or smooth muscle cells). Moreover, by virtue of its location directly adjacent to the distal airway, it is exposed to potentially damaging factors in the air. Given the delicate nature of these microvessels, endothelial injury and apoptosis could lead directly to degeneration and "dropout" at the level of the precapillary arteriole, resulting in loss of the efficient, low-pressure connection with the distal capillary bed. EPCs are thought to aid in the repair process of the endothelium; however, it has been reported that circulating EPC levels are altered[16,17] and EPCs are dysfunctional in PAH patients.[18] Studies attempting to replace decreased or dysfunctional EPCs via transplantation have shed light on the role of EPC-mediated repair of pulmonary vasculature. Experimentally, EPCs transplanted *in vivo* are able to "home" to and incorporate into the damaged endothelial lining of distal pulmonary arteries and limit the progression of disease in animal models of PAH.[19,20] However, the frequency of these events is low and likely cannot explain the full therapeutic effects of EPCs.[21] Alternatively, EPCs probably exert a paracrine effect by secreting proangiogenic growth factors such as VEGF, SDF-1 (stromal derived factor), IGF-1 (insulin-like growth factor 1), HGF (hepatocyte growth factor), and NO (nitric oxide) that can stimulate proliferation, migration, and survival of nearby endothelial cells.[21,22]

In recent years, EPC therapy for the treatment of PAH has gained considerable momentum. A number of preclinical studies have demonstrated the efficacy of EPC transplantation as a therapy for PAH (Table 1). There are a number of differing "definitions" of EPCs, which refer to overlapping but distinct populations of cells. Originally, Asahara et al identified endothelial progenitor as CD34 positive mononuclear cells (MNCs), having the capacity to differentiate into both hematopoietic and endothelial lineages (ie, hemangioblast).[7] However, since this seminal report, a number of other markers have been suggested to identify the putative circulating EPC population, including CD133, VEGFR2, Tie2, and eNOS[7,23,24]; to date there is no consensus on what the

specific EPC markers are. Moreover, selection based on surface determinants is inefficient since only a very small proportion of the circulating MNC pool expresses CD34 (1%-2%), and this problem is compounded if combinations of markers are utilized. Alternatively, a highly angiogenic MNC population can be derived by plating unselected MNCs on an appropriate matrix (ie, fibronectin) in the presence of a cocktail of endothelial growth factors.[25,26] After three days the attached cells become rod-shaped and begin to express some endothelial markers (ie, CD31, VEGFR2), but still retain a MNC phenotype with CD14 and CD45 expression. These so called early outgrowth ECs, or circulating angiogenic cells (CACs), are highly active in inducing neovascularisation and vascular repair in a number of experimental models.

In animal studies, PAH is commonly modeled in rats by administering monocrotaline (MCT), an endothelial toxin that causes pulmonary vascular injury and inflammation resulting in elevated pulmonary arterial pressures and right ventricular hypertrophy within weeks of exposure.[14] Experimental EPC therapy involves the isolation of mononuclear cells from the bone marrow, which are then cultured under defined conditions as described above to produce early outgrowth EPCs before transplantation into the host. Transplantation of 1-1.5 x 10^6 EPCs can prevent the development of PAH when given 3 days after MCT administration (Table 1).[20,22,27-30] The benefits of experimental EPC therapy include improved pulmonary hemodynamics, reduced right ventricular remodelling, and reduced muscularization of pulmonary arterioles. Additionally, our group demonstrated that EPC *treatment* could limit the progression of PAH and improve survival in rats with established PAH in a "therapy" protocol designed to mimic the clinically relevant scenario.[20] These encouraging preclinical results led to the initiation of several clinical trials. In a pilot study, Wang et al administered blood-derived, autologous early outgrowth EPCs to patients with idiopathic PAH. EPC treatment resulted in modest but significant improvements in hemodynamic and functional endpoints, such as 6-minute walk test (6MWT) compared to patients receiving conventional therapy (48 m vs 6 m, respectively).[31] However, this study was not blinded and participants received variable and relatively modest numbers of cells ($1.1 \pm 0.6 \times 10^7$) derived from a simple blood draw. Larger and more rigorously designed studies will be required to establish the efficacy and safety of EPCs for clinical use.

EPC-BASED GENE THERAPY

A major potential limitation with the use of autologous EPCs to treat PAH is that the patient-derived EPCs themselves may be dysfunctional. This is supported by evidence of functional deficits in cell proliferation, migration, and angiogenesis in EPCs isolated from PAH patients with the *BMPR2* mutation.[32] Moreover, even healthy EPCs may have limited ability to restore normal pulmonary vascular structure and function in the context of severe PAH, as supported by the preclinical studies outlined in Table 1. Thus, strategies to enhance the function of EPCs, for example, using genetic modification, may be required to increase the regenerative activity of the cells themselves or by augmenting the production of a paracrine mediator. The benefits of this approach may be 2-fold. First, EPCs may restore the integrity of the pulmonary vasculature by direct or indirect mechanisms. Secondly, the cells can be used as "vectors" to deliver therapeutic genes specifically to the site of disease. Nagaya et al utilized human-derived EPCs engineered to overexpress adrenomedullin, a potent vasodilator peptide, in immunodeficient rats.[19] After 21 weeks, they demonstrated that adrenomedullin-expressing EPCs administered 7 days after MCT injury significantly decreased mean pulmonary arterials pressure (mPAP) (-29%), reduced pulmonary vascular resistance (PVR, -39%), and improved pulmonary arteriolar remodeling and survival. Zhao et al employed human EPCs overexpressing calcitonin gene-related peptide (CGRP), a potent inhibitor of smooth muscle proliferation and vasoconstriction, to treat PH induced by abdominal aorta to inferior vena cava (left-to-right) shunt operation in rats.[33] Cell transplantation was performed at 10 weeks after shunt operation. Four weeks after treatment, total PVR and pulmonary artery wall thickness was significantly decreased in the EPC treatment group, while PVR and mPAP were decreased further by CGRP-EPC treatment. The studies above did not explore efficacy in treatment protocols designed to limit or reverse established experimental PAH. In both studies, a xenotransplantation treatment protocol was used to introduce human EPCs into nude athymic rats. One limitation of this protocol is that these rats are only partially immunodeficient, and residual natural killer (NK) cell activity in nude athymic rats may have limited the persistence of xenogeneically administered EPCs in a rat PAH model.[28]

In contrast, our group has explored using syngeneic (same species) bone marrow-derived early outgrowth EPCs engineered to overexpress endothelial nitric oxide synthase (eNOS),[20] which produces nitric oxide, a critical factor for the maintenance of normal vascular structure and function. Moreover, eNOS is endogenously expressed at low levels in EPCs and is believed to play a key role in cell homing and in their angiogenic activity.[34] Indeed, we demonstrated that when delivered 3 weeks after MCT, eNOS-overexpressing EPCs were not only able to prevent progression of PAH, but were able to reverse established disease, reducing right ventricular systolic pressure (RVSP) at 5 weeks to levels that were not different from that of the control animals.[20] The ability for eNOS cell-based gene therapy to reverse established rat PAH yields great promise for the treatment of human PAH. Based on the success of these preclinical studies, the first clinical trial using autologous EPC-based eNOS gene therapy for PAH, the Pulmonary Hypertension and eNOS Cell Therapy Trial (PHACeT; Clinicaltrials.gov NCT00469027), has been initiated in Toronto and Montreal to establish safety and appropriate dosing.[14]

MSCs, ADVANTAGE OVER OTHER CELL TYPES

Over the past decade, MSCs have become dominant in the field of cell therapy, es-

pecially in the context of pulmonary vascular diseases.[35,36] Also known as marrow stromal cells or mesenchymal stromal cells, MSCs are adult stem cells that can be isolated from bone marrow and expanded extensively in culture.[37] In addition to MSCs' utilization in autologous cell therapies, MSCs have also been used in several clinical trials in an allogeneic fashion (transplantation of MSCs from donors to unrelated recipients).[38] In autologous cell transplantation, stem or progenitor cells have to be isolated from the patient's own tissue (such as in the case of EPC); this process is clearly cumbersome with added complexity and cost. Moreover, the activity of autologous cells may be influenced by host factors to yield a cell product that is dysfunctional due to the patient's existing disease state and therefore limited in therapeutic potential.[16,17,32] One of the primary advantages of MSC therapy over EPC therapy is the prospect of utilizing allogeneic cells that can be prepared in batch lots and be immediately available when needed, much like a pharmaceutical product. Studies have shown that MSCs may escape alloreactive recognition by a patient's own immune system due to their lack of major histocompatibility complex (MHC) class I and low expression of costimulatory molecules.[39] Soluble factors secreted by MSCs themselves, or by MSC-stimulated immune cells have also been implicated to be involved in the MSCs-mediated immunomodulatory and immunosuppressive effect.[40] Considering the emerging understanding of immune function and inflammation may contribute to the progression of PAH, MSC treatment may offer a unique approach to treat PAH patients.

MSC THERAPY IN PAH—PRECLINICAL EVIDENCE

Therapy employing MSCs for PAH was first described by Kanki-Horimoto et al in 2006.[41] In this pioneering study, bone marrow-derived MSCs (1×10^6 cells), injected intravenously to MCT rats, were able to prevent progression of PAH by significantly reducing MCT-induced increases in RVSP and RV hypertrophy by 28% and 22%, respectively. However, MSCs alone in this particular study were unable to reverse PAH or reduce mortality in animals with more advanced PAH. Nonetheless, other groups have subsequently shown that MSCs alone can be sufficient in reducing PAH in the same animal model, although a very high cell dose (ie, $3\sim5 \times 10^6$ cells) was required to achieve the observed beneficial effect.[42,43] Baber et al injected 3 x 10^6 cells through the trachea to achieve targeted delivery of MSCs to the pulmonary airway.[42] Delivered 2 weeks after MCT, they found that intratracheal administration of MSCs was able to attenuate MCT-induced PAH and improve pulmonary endothelial function. In a similar study, He et al delivered 5×10^6 MSCs intravenously to MCT rats 22 days after injury, and found MSC therapy increased survival of PAH rats from 50% to 90% by the end of 49 days.[43] Mean pulmonary artery pressure was reduced from 43 to 25 mm Hg in MSC-treated rats, in addition to a reduction in RV hypertrophy. To provide evidence that autologous MSCs isolated from PAH patients may still be therapeutic in a treatment scenario, Umar et al isolated MSCs from rats with established PAH (4 weeks after MCT) then treated PAH rats with these cells.[44] Of note, MSCs from PAH rats did exhibit lower proliferation potential and secreted a higher level of vascular endothelial growth factor compared to MSCs isolated from normal rats. Nevertheless, Umar et al were still able to show that transplantation of MSCs isolated from PAH rats provided a benefit. Treated animals exhibited reduced pulmonary arteriolar narrowing, reduced alveolar septum thickening, decreased RVSP, and improved RV function. Overall, the majority of preclinical evidence evaluating MSC therapy in PAH is positive, further supporting the potential use of MSC as a therapy for this devastating disease (Table 2).

MSC-BASED GENE THERAPY IN PAH

While the studies mentioned above suggested that MSCs by themselves may prevent or even reverse PAH, others suggest genetic modification or cotreatment may be necessary to reverse advanced PAH in animals[41,45] (Table 2). Similar to the approach employed by our group using EPCs,[20] Kanki-Horimoto et al overexpressed eNOS in MSCs. In their study, 1 x 10^6 MSCs alone did not reduce MCT-induced PAH mortality, whereas MSCs overexpressing eNOS, even at half the dose of unmodified MSCs (5×10^5), significantly improved pulmonary hemodynamics in an early treatment scenario (prevention study) in which treatment was given 1 week after MCT. These same cells increased survival in a rescue treatment scenario (therapy study, in which treatment was given to rats with established PAH 3 weeks after MCT).[41] In a study by Liang et al, MSCs isolated from normal mice modestly reduced RV hypertrophy but had no effect on RVSP in a model of hypoxia-induced PAH.[46] However, injection of MSCs isolated from mice overexpressing heme oxygenase-1 (HO-1), an enzyme known to play a crucial role in restoring homeostasis in many disease states,[47] significantly reduced PAH. Though the exact mechanism of protection by HO-1 has not been fully elucidated, authors in this study speculated the observed therapeutic benefit may be due to the release of carbon monoxide as a byproduct of HO-1, with its attendant vasodilatory and antiproliferative effects on pulmonary vessels. Finally, Takemiya et al showed that administration of MSCs overexpressing prostacyclin synthase (PGIS), which is well known to regulate pulmonary vascular tone, was superior in attenuating PAH and cardiac remodeling compared to MSCs alone.[45]

CONCLUSION

Cellular therapies are showing promise in preclinical studies as well as in very early phase clinical studies. However, based on the evidence in experimental models, it would appear that cell therapy alone, either with EPCs or MSCs, is only partially effective, especially in true treatment models of animals with established disease. The experiments testing cells as therapy in animals with established disease (as opposed to prevention of disease) are clearly more relevant for the preclinical assessment of any potential therapeutic benefits. A variety of cell enhancement

Table 2: Summary of Preclinical Studies of MSC Treatment for PAH

MSC Source	Recipient	Model	Dose (cell #)	Citation/Key Findings
Preclinical Studies Using MSCs				
Rat BM	Rat	MCT	1×10^6	**Kanki-Horimoto et al, 2006[41]** o Prevention but not reversal of PAH
Rat BM	Rat	MCT	3×10^6	**Baber et al, 2007[42]** o Reversal of PAH
Rat BM (from rats with established PAH)	Rat	MCT	1×10^6	**Umar et al, 2009[44]** o Reversal of PAH
Rat BM	Rat	MCT	5×10^6	**He et al, 2009[43]** o Reversal of PAH
Rat BM MNC	Rat	MCT	5×10^5	**Takemiya et al, 2010[45]** o MSCs alone did not reverse PAH
Mouse BM	Mouse	Hypoxia	1×10^6	**Liang et al, 2011[46]** o Reversed RV hypertrophy but not RVSP
Rat BM	Rat	MCT	1×10^5	**Luan et al, 2011[48]** o Prevention of PAH
Preclinical Studies Using MSC-Based Gene Therapy				
Rat BM	Rat	MCT	5×10^5	**Kanki-Horimoto et al, 2006[41]** o eNOS-enhanced MSCs superior to MSCs alone
Rat BM	Rat	MCT	1×10^6	**Takemiya et al, 2010[45]** o PGIS-enhanced MSCs reversal of PAH
Mouse BM (HO-1 overexpressor mice)	Mouse	Hypoxia	1×10^6	**Liang et al., 2011[46]** o MSCs isolated from HO-1 overexpressor mice reversal of PAH

BM = bone marrow, MNC = mononuclear cell

strategies, mainly using genetic engineering to over express potentially therapeutic transgenes, have shown real promise in augmenting the benefit of both EPCs and MSCs. This is particularly evident in the treatment models, suggesting that similar strategies will be required to achieve the full benefit when these approaches are translated into human clinical trials. In the future, cell therapies should be evaluated using the most relevant preclinical models, of which the Sugen/hypoxia model may be the most appropriate as it reproduces more of the relevant pathological features of the clinical disease.[49] This model is well suited to delayed therapy of established PAH, since it avoids the off-target toxicities associated with MCT. Moreover, because it exhibits angioproliferative, plexiform-like lesions, it also allows a full evaluation of the safety profile of cell therapy for PAH, in particular whether some circulating progenitor or stem cells might contribute to the development of these lesions. Finally, the further preclinical evaluation of innovative new therapies should be conducted with the same degree of rigor and care that is now routine for clinical studies, including incorporating procedures to ensure blinding and random allocation of animals to therapeutic groups.

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