Molecular and cellular characterization of cardiac overload-induced hypertrophy and failure

Umar, S.

Chapter 5

Novel approaches to treat pulmonary arterial hypertension

S. Umar
P. Steendijk
D.L. Ypey
D.E. Atsma
E.E. Van der Wall
M. J. Schalij
A. van der Laarse

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Abstract

Objective: Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by an increase in pulmonary artery pressure leading to right ventricular (RV) hypertrophy, RV failure and, ultimately, (sudden) cardiac death. Current treatments can improve symptoms and reduce the severity of the hemodynamic disorder in patients with PAH but frequently gradual deterioration in the patient's condition necessitates a lung transplant. In this review we discuss the treatment options tested so far in experimental models of PAH.

Data sources: PubMed.

Study selection: These treatment options include a spectrum of pharmacologic agents ranging from elastase inhibitors, endothelin receptor antagonists, phosphodiesterase inhibitors and phytoestrogens to Rho-kinase inhibitors, serotonin receptor antagonists and statins. In addition, we discuss the emerging trends of using gene and cell therapy for the treatment of PAH. Finally, we discuss the possible applications of experimentally tested interventions for therapeutic purposes in humans with PAH.

Conclusions: Several of these therapeutic options have been shown to be effective also in PAH patients leading to improved life expectations and a better quality of life. However, many patients remain symptomatic despite therapy. Cell therapy is a novel treatment option, but more animal data should be collected to investigate optimal cell type, *in vitro* cell transduction, route of cell administration, and number of cells to inject. Autologous MSC therapy is expected to be a safe and efficacious option to treat patients with PAH.

Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a progressive condition characterized by elevated pulmonary arterial pressures leading to RV failure. PAH is primarily a lung disorder which is associated with increased pulmonary vascular resistance, pulmonary vascular pathology, medial hypertrophy of arterioles, and inflammation of the lungs. Causes of PAH are many fold, including idiopathic PAH, familial PAH, chronic hypoxia-induced PAH, congenital heart disease associated PAH, infections and HIV-associated PAH, PAH due to inflammation and collagen vascular disease, and drugs- and toxins-induced PAH.
Current therapeutic strategies may improve symptoms and reduce the severity of the hemodynamic abnormality, but deterioration of pulmonary and cardiac functions in many cases ultimately necessitates a lung transplant. Novel approaches in treating PAH include reversing the advanced occlusive structural changes in the pulmonary circulation causing PAH and regeneration of damaged pulmonary tissue using stem cells. These approaches have been tested using (***i***) pharmacotherapy, (***ii***) gene therapy, and (***iii***) cell therapy.

Animal Models of Pulmonary Arterial Hypertension

Various experimental models have been proposed for induction of PAH in animals. These models include:
1. Monocrotaline-induced pulmonary hypertension with or without aortocaval shunt
2. Pulmonary artery banding-induced pulmonary hypertension
3. Chronic hypoxia-induced pulmonary hypertension
4. Chronic embolism-induced pulmonary hypertension
5. Ligation of ductus arteriosus
6. Genetically modified animal models
In the past 10 years most experimental studies on therapy of PAH have employed the monocrotaline-induced PAH model.

Monocrotaline-induced Pulmonary Arterial Hypertension

Mechanism and Pathology of Pulmonary Toxicity of Monocrotaline

Monocrotaline (MCT), a pyrrolizidine alkaloid derived from *Crotalaria spectabilis*, causes a pulmonary vascular syndrome in rats characterized by proliferative pulmonary vasculitis, pulmonary artery hypertension (PAH), and cor pulmonale. Current lines of evidence of the pathogenesis of MCT-induced pneumotoxicity indicate that MCT is activated to one or more reactive metabolites in the liver, particularly a MCT pyrrole called dehydromonocrotaline [1-3], and is then transported by red blood cells to the lung [4], where it initiates endothelial injury [5, 6]. The endothelial injury does not appear to be acute cell death but rather a delayed functional alteration that leads to smooth muscle cell (SMC) proliferation

in the media of pulmonary arteriolar walls by unknown mechanisms. The role of inflammation in the progression of MCT-induced pulmonary vascular disease is uncertain. Both perivascular inflammation and platelet activation have been proposed as processes contributing to the response of the vascular media [3]. MCT and dehydroMCT are known to be toxic to a variety of domestic and laboratory animals and to humans. Major pathological effects induced by MCT poisoning include hepatic cirrhosis and megalocytosis, venocclusive disease, PAH, and RV hypertrophy. There is a positive correlation between progressive PAH, thickening of the medial wall of small pulmonary arteries and arterioles, and RV hypertrophy as a function of time [7].

Treatment Options for Experimental Pulmonary Arterial Hypertension

1. Pharmacological interventions

Several pharmacologic agents have been used either alone or in combination to treat MCT-induced pulmonary hypertension. These agents are discussed below.

1.1 Serine elastase inhibitors

Progression of PAH is associated with increased serine elastase activity and the proteinase-dependent deposition of the extracellular matrix protein tenascin-C. Tenascin-C amplifies the response SMCs to growth factors, which are also liberated through matrix proteolysis. Recent organ culture studies using hypertrophied rat pulmonary arteries have shown that elastase inhibitors suppress tenascin-C and induce apoptosis of SMCs [8, 9]. This initiates complete regression of the hypertrophied vessel wall by a coordinated loss of cellularity and extracellular matrix. Elastase inhibitors can reverse advanced pulmonary vascular disease produced in rats by injecting MCT. If the peptidyl trifluoromethylketone serine elastase inhibitors M249314 or ZD0892 were orally administered from 21 days after injection of MCT onwards, survival after 1 week was 92%, compared with only 39% survival in untreated or vehicle-treated rats. Pulmonary artery pressure and muscularization were reduced by cardiomyocyte apoptosis and loss of extracellular matrix, specifically elastin and tenascin-C. After 2 weeks, pulmonary artery pressure and structure normalized, and survival was 86%, compared with 0% in untreated or vehicle-treated rats [10].

1.2 Platelet-derived growth factor inhibition

Progression of PAH is associated with increased proliferation and migration of pulmonary vascular SMCs. Platelet-derived growth factor (PDGF) is a potent mitogen and is involved in this process. It was reported that the PDGF receptor antagonist STI571 (imatinib) reversed advanced pulmonary vascular disease in two animal models of PAH. If in rats with MCT-induced PAH daily administration of STI571 was started 28 days after induction of the disease, there was 100% survival in the first 2 weeks, compared with only 50% in sham-treated rats. PAH and RV hypertrophy were reversed to near-normal levels. Similar results were

obtained in chronically hypoxic mice, which were treated with STI571 after full establishment of PAH. Moreover, expression of the PDGF receptor was found to be significantly increased in lung tissue from patients with PAH compared with healthy donor lung tissue. In conclusion, STI571 reversed vascular remodeling and cor pulmonale in severe experimental PAH regardless of the initiating stimulus. This regimen offers a unique novel approach for anti-remodeling therapy in progressed PAH [11].

1.3 Prostacyclin therapy

Male Wistar rats with flow-associated PAH due to an aortocaval shunt, in addition to MCT-induced PAH, were treated with low-dose aspirin (25 mg/kg/day) or iloprost (72 µg/kg/day), a prostacyclin analogue. Ninety % of the untreated rats with PAH developed dyspnea and pleural fluid, whereas this was seen in 50% and 10% of the aspirin and iloprost-treated rats, respectively. This could not be attributed to changes in pulmonary artery pressure, wall to lumen ratio of the pulmonary arterioles, or RV hypertrophy. However, both therapies restored the reduced RV capillary to cardiomyocyte ratio in rats with PAH (0.95±0.10 in MCT-treated rats vs. 1.38±0.18 in healthy rats, 1.32±0.11 in aspirin-treated MCT rats and 1.29±0.90 in iloprost-treated MCT rats) which was associated with improved RV contractility. Thus, interventions in the prostacyclin–thromboxane metabolism improve outcome in rats with flow-associated PAH. However, these effects may be attributed to effects on cardiac rather than on pulmonary vascular remodeling [12].

Subcutaneous administration of a novel prostacyclin agonist (ONO-1301) markedly attenuated MCT-induced PAH and improved survival in rats. The beneficial effects of ONO-1301 occurred through its long-lasting stimulation of cAMP and inhibition of thromboxane synthase [13].

Although prostacyclin is recognized as a therapeutic breakthrough for pulmonary hypertension, it needs continuous infusion because of its short half-life in plasma. Therefore, Obata *et al.* developed a new drug delivery system for prostacyclin by preparing ONO-1301MS, a novel sustained-release prostacyclin analogue polymerized with poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres. A single injection of ONO-1301MS resulted in sustained activity for 3 weeks, and attenuated PAH, partly through its antiproliferative effect on vascular SMCs via inhibition of ERK phosphorylation [14]. The inhalation of iloprost has been shown to reverse PAH and vascular structural remodeling in MCT-treated rats. This regimen demonstrated the feasibility of a reverse remodeling therapy in PAH [15].

1.4 Combined prostacyclin and phosphodiestersae inhibition

Combination therapy with oral sildenafil, a phosphodiesterase inhibitor, and beraprost, an oral prostacyclin analogue, attenuated the development of MCT-induced PAH compared with treatment with either drug alone [16]. The combined administration of iloprost, a long-acting prostacyclin analogue, and a dual-selective phosphodiesterase 3/4 inhibitor, tolafentrine, reversed the development of PAH and cor pulmonale in response to MCT in rats [17].

1.5 Rho-kinase inhibition

Long-term treatment with a Rho-kinase inhibitor fasudil improved the mortality rate of MCT-induced PAH in rats [18], as well as ameliorated hypoxia-induced PAH in mice, partially by activation of endothelial nitric oxide synthase (eNOS) [19]. Fasudil exerts effective and selective vasodilatation of pulmonary arteries in rats with MCT-induced PAH, which explains its usefulness for the treatment of this fatal disorder [20]. Nagaoka *et al.* found in chronically hypoxic rats that inhalation of Rho-kinase inhibitors nearly normalized PAH, but had no pulmonary vascular selectivity [21].

1.6 Combined Rho-kinase inhibitor and prostacyclin therapy

Combination therapy has been advocated based on the potential for additive or synergistic effects. Long-term inhibition of Rho-kinase, an effector of the small GTPase Rho, ameliorated MCT-induced PAH in rats and hypoxia-induced PAH in mice. It was reported that prostacyclin and its oral analogue beraprost may lack direct inhibitory effect on Rho-kinase *in vitro*, suggesting that combination therapy with a Rho-kinase inhibitor and beraprost is effective for the treatment of PAH. Male Sprague-Dawley rats were given a subcutaneous injection of MCT (60 mg/kg), and subsequently treated without or with a Rho-kinase inhibitor (fasudil, 30 mg/kg/day), beraprost (200 mg/kg/day), or a combination of both drugs for 3 weeks. The combination therapy, when compared with each monotherapy, showed significantly more improvement in PAH, RV hypertrophy, and pulmonary medial thickness without any adverse effects. Plasma concentrations of fasudil were not affected by beraprost. These results demonstrated that combination therapy with a Rho-kinase inhibitor and a prostacyclin analogue exerts further beneficial effects on PAH [22].

1.7 Endothelin receptor antagonists

Endothelin-1 (ET-1), a potent vasoactive and mitogenic peptide, has been implicated in the pathogenesis of several forms of PAH. Hill *et al.* have shown that a nonspecific endothelin-receptor antagonist, bosentan, blunts MCT-induced PAH in rats [23].

The antagonism of the ET_A receptor was shown to be essential for the protection from MCT-induced PAH, irrespective of the presence of the ET_B receptors, although a protective role of ET_B receptor-mediated action in the pathogenesis of this disease model could not be ruled out [24].

Furthermore, in another study from the same group, the functional roles of endothelin ET_A and ET_B receptors in the development of MCT-induced PAH were investigated using MCT-treated rats in the absence or presence of a daily administration of A-192621, a selective ET_B receptor antagonist, ABT-627, a selective ET_A receptor antagonist, or a combination of both drugs. The results demonstrated that ET_A receptor-mediated action contributed exclusively to the pathogenesis of MCT-induced PAH [25].

1.8 Combined endothelin-A receptor antagonist and prostacyclin therapy

Rats with MCT-induced PAH were treated with oral ET_A receptor antagonist TA-0201 and/or the oral prostacyclin analogue beraprost for 19 days. The PAH-associated RV systolic pressure elevation was significantly depressed by TA-0201 and beraprost, and most strongly by TA-0201+beraprost. The indexes of RV hypertrophy showed the same tendency as the increase in RV systolic pressure in the groups MCT, MCT+TA-0201, MCT+beraprost and MCT+TA-0201+beraprost. The expression of the "fetal" gene β-myosin heavy chain was markedly upregulated in the "MCT only" group, and less upregulated in the MCT+TA-0201 and MCT+beraprost groups, and least upregulated in the MCT+TA-0201+beraprost groups. The same was true for medial wall thickness of the pulmonary artery, being most pronounced in "MCT only" group and least increased in the MCT+TA-0201+beraprost group. The combination of an oral ET_A receptor antagonist and an oral prostacyclin analogue appeared to be superior to the use of each drug alone in inhibiting the progression of PAH and its consequences to RV myocardium [26].

1.9 Serotonin transporter inhibition

Progression of PAH is associated with increased pulmonary expression of the serotonin transporter (5-HTT), which leads to hyperplasia of the pulmonary artery smooth muscle cells (PA-SMCs). Given the fact that overexpression of the 5-HTT gene in PA-SMCs leads to PAH [27], it was investigated whether the highly selective 5-HTT inhibitor fluoxetine prevented and/or reversed PAH in MCT-treated rats. Selective antagonists to the $5\text{-}HT_{1B/1D}$ receptor, $5\text{-}HT_{2A}$ receptor and $5\text{-}HT_{2B}$ receptor were used for comparative testing. MCT injection (60 mg/kg s.c.) was followed by an early peak in lung 5-HTT expression on day 1, which preceded the onset of PAH. Established PAH on day 15 was associated with a sustained 5-HTT increase. Continued fluoxetine treatment completely prevented PA-SMC proliferation and PAH development, and also suppressed the late 5-HTT increase, without affecting the early peak. The 5-HT receptor antagonists did not affect PAH. Oral fluoxetine (10 mg/kg/day) started 3 weeks after MCT injection completely reversed established PAH, normalizing pulmonary artery pressure and structure. MCT-induced PAH was also associated with increased expression of various cytokines, but only interleukin-1β and monocyte chemotactic protein-1 increased at the early phase and stimulated 5-HTT expression by cultured PA-SMCs. Complete reversal of established PAH by inhibiting 5-HTT provides a rationale for new therapeutic strategies in human PAH [28].

Furthermore, a selective serotonin re-uptake inhibitor (SSRI), sertraline, protects against MCT-induced PAH by decreasing pulmonary artery pressure, RV index, and pulmonary artery remodeling, probably related to a reduction in serotonin transporter mRNA [29].

A serotonin receptor antagonist, MCI-9042, attenuated the development of MCT-induced PAH, suggesting a pivotal role of serotonin in the development of PAH induced by MCT [30].

Specific $5\text{-}HT_{2A}$ receptor blockade with sarpogrelate, a $5\text{-}HT_{2A}$ receptor antagonist, immediately after MCT administration, inhibited PAH and prolonged survival in rats. These effects were accompanied by anti-inflammatory and anti-

proliferative effects in the lung tissue and marked improvement of pulmonary vascular endothelial dysfunction and activation [31].

1.10 Phosphodiesterase inhibition

Phosphodiesterase (PDE) inhibitors for treatment of PAH have been studied extensively. Oral sildenafil prevented (in a study in which sildenafil was given 1 day after MCT) and reversed (in a study in which sildenafil was given 3 weeks after MCT) the development of PAH in MCT-treated rats, associated with a reduction in the ET_A-receptor density in SMCs of pulmonary small arteries (diameter < 100 μm) [32]. The effects of oral pumafentrine, a mixed-selective PDE3/4 inhibitor, have been investigated in rats with MCT-induced PAH. When chronically administered in weeks 4 to 6 after a single injection of MCT (60 mg/kg), pumafentrine (10 mg/kg daily) partially reversed PAH and RV hypertrophy in rats [33]. In addition, small pulmonary arterial muscularization, medial hypertrophy and decrease in lumen area were largely reversed. Inhibition of PA-SMC proliferation by pumafentrine was demonstrated *in vivo*. Pumafentrine also had a pro-apoptotic effect on vascular cells *in vitro*. Moreover, pumafentrine dose-dependently increased cAMP levels and inhibited proliferation of cultured PA-SMCs. Thus, oral pumafentrine partially reverses MCT-induced PAH, pulmonary vascular remodeling, and RV hypertrophy in rats [33].
Schermuly *et al.* investigated chronic effects of sildenafil, a PDE5 inhibitor, in MCT-induced PAH in rats. Four weeks after a single subcutaneous injection of MCT, the animals displayed nearly threefold elevated pulmonary artery pressure and vascular resistance values, with a concomitant decline in central venous oxygen saturation and arterial oxygenation. Marked RV hypertrophy was evident, and massive thickening of the arteriolar SMC layer was histologically apparent. Sildenafil, administered from day 14 to day 28, significantly increased plasma cGMP and inhibited the development of PAH and RV hypertrophy, with improvement of central venous oxygen saturation and arterial oxygenation. A corresponding efficacy profile was also noted for treatment with sildenafil started at day 28 till day 42. Moreover, the death rate significantly decreased in those animals treated with sildenafil [34].
Pullamsetti *et al.* demonstrated for the first time that inhalation of combined PDE3/4 inhibitor tolafentrine reversed PAH that occurred in response to MCT in rats. This "reverse remodeling" effect included structural changes in the lung vascular wall and key molecular pathways of matrix regulation, concomitant with 60% normalization of hemodynamics [35].

1.11 Combination of phosphodiesterase and endothelin receptor inhibition

Clozel *et al.* evaluated the effects of bosentan, a nonspecific endothelin receptor antagonist, sildenafil, and their combination in rats with MCT-induced PAH [36]. A first group consisted of control rats without MCT injection. Four other groups of rats received MCT subcutaneously and were assigned to receive no treatment, 300 mg/kg/day bosentan in the food, 100 mg/kg/day sildenafil in drinking water, or their combination for 4 weeks. The doses of bosentan and sildenafil were the maximally effective doses based on a dose-range–finding study. Mortality was

0%, 53%, 11%, 11%, and 0%, respectively, in the five different groups. Bosentan and sildenafil significantly attenuated the increase in mean pulmonary arterial pressure, and the combination had an additional effect [36].

1.12 Caveolin-1 peptide

Caveolins, the principal structural proteins of caveolar microdomains, have been implicated in the development of PAH. Mice with homozygous deletion of the caveolin-1 (Cav-1) gene develop PAH and RV hypertrophy. In several animal models of PAH and in patients with severe PAH, reductions in pulmonary Cav-1 expression were apparent.

Whether in vivo modulation of Cav-1 expression could affect the development of PAH and RV hypertrophy was studied by Jasmin *et al.* [37].Thirty minutes after injection of saline or MCT, rats were assigned to receive a daily injection of saline, a peptide corresponding to the homeodomain of the Drosophila transcription factor antennapedia (AP; 2.5 mg/kg/day), or a peptide consisting of the Cav-1–scaffolding domain coupled to AP (AP-Cav; 2.5 mg/kg/day) for 2 weeks. MCT and MCT+AP rats developed PAH with RV systolic pressures of 40.2±1.5 and 39.6±1.5 mmHg, respectively. Administration of AP-Cav to MCT rats significantly reduced the RV systolic pressure to 30.1±1.3 mm Hg. MCT and MCT+AP rats also developed pulmonary artery medial hypertrophy and RV hypertrophy, which was normalized by administration of AP-Cav. Mechanistically, the development of PAH was associated with reduced expression of pulmonary Cav-1 and Cav-2, hyperactivation of the STAT3 signaling cascade, and upregulation of cyclin D1 and D3 protein levels, all of which were prevented by administration of AP-Cav. Thus, short-term administration of a Cav-based cell-permeable peptide to MCT rats prevented the development of pulmonary artery medial hypertrophy, PAH, and RV hypertrophy [37].

1.13 Estradiol

Daily supplementation of genistein, a phytoestrogen, potently attenuated MCT-induced PAH, RV hypertrophy, and pulmonary vascular remodeling in rats [38]. Also 2-methoxyestradiol (2ME), a non-estrogenic estradiol metabolite, prevented the development and retarded the progression of MCT-induced PAH [39]. 2ME significantly attenuated RV hypertrophy and pulmonary arterial medial hypertrophy, and reduced proliferative and inflammatory responses in the lungs. Furthermore, in diseased animals, 2ME (given from day 14 to 28) significantly decreased RV peak systolic pressure and RV hypertrophy, and reduced mortality. Thus, 2ME, a major non-estrogenic, non-carcinogenic metabolite of estradiol, prevented the development and retarded the progression of MCT-induced PAH [39, 40].

Recently, Tofovic *et al.* provided the first evidence that 2-ethoxyestradiol strongly inhibited vascular remodeling in PAH and suggested that anti-proliferative agents, including synthetic analogues of estradiol metabolites may be protective in PAH [41].

1.14 Statins

Various statins have been shown to be effective in treating experimental PAH. Simvastatin attenuated MCT-induced pulmonary vascular remodeling, PAH, and RV hypertrophy in rats [42]. Pravastatin reduced the development of MCT-induced PAH and improved endothelium-dependent pulmonary artery relaxation through reduced apoptosis and a restored eNOS expression of endothelial cells [43]. Rosuvastatin provided protection against the development of PAH and RV hypertrophy [44]. These studies demonstrate that the targeted preservation of coronary endothelial function and vasoactivity provides a novel approach to protect against cardiac remodeling.

1.15 Miscellaneous therapeutic agents

1.15.1 Amlodipine, a third-generation calcium channel blocker, inhibited the development of PAH and improved survival in rats independent of its effect on lowering blood pressure. These effects were associated with marked inhibition of the downregulation of eNOS and improvement of pulmonary vascular endothelial activation, as well as anti-inflammatory, antiproliferative and antifibrotic effects in the lung tissue. However, amlodipine failed to reverse established PAH [45].

1.15.2 Nicorandil, an ATP-sensitive potassium (K_{ATP}) channel opener with a nitrate-like action, inhibited development of MCT-induced PAH but failed to reverse it. These effects were associated with marked up-regulation of diminished lung eNOS concentration along with improvement of pulmonary vascular endothelial activation and anti-inflammatory and anti-proliferative effects in the lung tissue [46].

1.15.3 Infusion of **C-type natriuretic peptide (CNP)**, the third member of the natriuretic peptide family, attenuated MCT-induced PAH and improved survival. The beneficial effects were mediated by regeneration of pulmonary endothelium, inhibition of endothelial cell apoptosis, prevention of monocyte/macrophage infiltration, and restoration of fibrinolytic activity [47].

1.15.4 Repeated inhalation of **adrenomedullin**, a potent vasodilator peptide that was originally isolated from human pheochromocytoma, has been shown to inhibit MCT-induced PAH without systemic hypotension, and thereby improving survival in MCT rats [48].

1.15.5 Zhou *et al.* have shown that heme oxygenase-1 was critical for the antiproliferative and vascular protective effects of **rapamycin**, an immunosuppressive agent with antiproliferative activity not only against lymphocytes but also against vascular cells, *in vitro* and *in vivo* in MCT-induced PAH [49].

1.15.6 Granulocyte colony-stimulating factor (G-CSF) inhibited the progression of PAH in a rat model, possibly by stimulating pulmonary endothelial cells to proliferate at sites of impaired lung vasculature [50].

1.15.7 The NF-κB nuclear localization and vascular cell adhesion molecule (VCAM-1) expression are temporally and spatially associated with the development of MCT-induced PAH in rats. Administration of a **NF-κB inhibitor**, pyrrolidine dithiocarbamate (PDTC), reversed the MCT-induced development of PAH in rats [51].

1.15.8 In rats with MCT-induced PAH treatment with an **interleukin-1 receptor antagonist** started at the same time as MCT administration and continued for the first 2 weeks prevented the development of PAH and RV hypertrophy as assessed 3 weeks after MCT injection [52].

1.15.9 In rats with MCT-induced PAH treatment with **antibodies to monocyte chemotactic and activating factor/monocyte chemoattractant protein-1** started at the same time as MCT administration, or at day 3.5, 7 or 14 after MCT administration. Antibody therapy started together with MCT administration was the most effective in alleviating RV hypertrophy, as compared to later starts [53]. Antibody therapy started together with MCT administration did not prevent development of pulmonary hypertension, but right ventricular peak systolic pressure was significantly lower than observed in rats treated with MCT only (≈50 mmHg and ≈70 mmHg, respectively).

1.15.10 Rats were, 1 day after MCT administration (60 mg/kg), treated without or with a specific **inhibitor of p38 mitogen-activated protein kinase**, FR167653, for 27 days. Four weeks after MCT administration pulmonary artery pressure and RV weight had hardly increased, compared to rats that received only MCT. The beneficial effects of FR167653 were ascribed to attenuated expression of inflammatory cytokines, thereby preventing the progression of pulmonary hypertension [54].

1.15.11 PAH is associated with endothelial injury [55] and with NO-dependent endothelial dysfunction [56]. Rats treated with daily i.p. doses of **L-arginine** (500 mg/kg), started 3 days before MCT administration, and continuing till sacrifice of the animals at day 17 after MCT injection, prevented the development of PAH, RVH, and pulmonary vascular disease [57].

1.15.12 Rats were, 2 day before MCT administration (60 mg/kg), treated without or with an **inhibitor of lipoxygenase pathways**, diethylcarbamazine, for 23 days. Three weeks after MCT administration, therapy with diethylcarbamazine appeared to have blocked the development of pulmonary hypertension and RVH, associated with inhibition of influx of polymorphonuclear cells and alveolar macrophages into the alveoli and the activation of these cells by lipoxygenase-related products [58].

2. Gene therapy

2.1 There is ample evidence that oxidative stress contributes to the pathogenesis and/or development of PAH. Intratracheal gene transfer of **human extracellular superoxide dismutase (EC-SOD)** was studied in rats with MCT–induced PAH [59]. MCT-injected rats were intratracheally administered either vehicle (MCT group) or an adenovirus encoding β-galactosidase (Adβgal group), or human EC-SOD (AdEC-SOD group). After intratracheal gene transfer, EC-SOD was successfully expressed in lung tissue, bronchoalveolar lavage fluid, and plasma. Twenty-eight days after MCT injection, RV systolic pressure and the weight ratio of the RV to the LV plus septum were significantly lower in the AdEC-SOD group (42.5 ± 1.4 mmHg and 0.45 ± 0.02, respectively) than in the MCT group (59.8 ± 1.6 mmHg and 0.63 ± 0.02, respectively) and the Adβgal group (61.5 ± 2.1mmHg and 0.65 ± 0.03, respectively). Moreover, vascular remodeling and proliferation of vascular smooth muscle cells in pulmonary arteries were markedly suppressed in the AdEC-SOD group [59]. Thus EC-SOD acts as an antioxidant in PAH, and apparently increased oxidative stress plays an important role in the pathogenesis of MCT-induced PAH.

2.2 Ikeda *et al.* have suggested that **monocyte/macrophage chemoattractant protein-1 (MCP-1)**, a potent chemoattractant chemokine and an activator for mononuclear cells, may play a role in the initiation and/or progression of PAH [60]. They found that anti-MCP-1 gene therapy attenuated PAH in rats. Hence an anti-inflammatory strategy via blockade of the MCP-1 signal pathway may be an alternative approach to treat subjects with PAH.

2.3 Prostacyclin is a potent vasodilator that also inhibits platelet adhesion and cell growth. Intratracheal transfer of the **human prostacyclin synthase (PGIS)** gene to rats with MCT-induced PAH augmented pulmonary prostacyclin synthesis, ameliorated MCT-induced PAH, and improved survival in MCT rats [61]. An intramuscular injection of adeno-associated virus (AAV) vector harboring the PGIS gene (AAV-PGIS) also prevented MCT-induced PAH in rats. This approach provides an attractive therapeutic alternative for preventing PAH in humans [62].

2.4 Cell-based gene transfer with **angiopoietin-1 (Ang-1)**, a newly discovered ligand of the endothelial-specific tyrosine kinase receptor Tie-2, has been shown to improve survival and pulmonary hemodynamics in experimental PAH by a mechanism involving the inhibition of apoptosis and protection of the pulmonary microvasculature [63].

2.5 In another study, Zhao *et al.* demonstrated that cell-based **endothelial NO synthase (eNOS)** gene transfer was more effective than vascular endothelial growth factor A (VEGF) in reversing established PAH, associated with evidence of regeneration of pulmonary microcirculation [64].

2.6 Interleukin (IL)-10 is a pleiotropic anti-inflammatory cytokine with vasculoprotective properties. After rats were injected intramuscularly with an AAV serotype 1 vector expressing IL-10, followed by MCT injection, it was demonstrated that IL-10 expression prevented MCT-induced PAH in rats [65].

3. Cell therapy

3.1 Intratracheal mesenchymal stem cell therapy

Baber *et al.* studied the effect of intratracheal administration of rat bone marrow derived mesenchymal stem cells (rMSCs) on MCT-induced PAH and impaired endothelium-dependent responses in the rat [66]. rMSCs had been transfected with the lacZ gene before intratracheal administration. The intratracheal administration of 3 x 10^6 rMSCs 2 weeks after administration of MCT attenuated the rise in pulmonary arterial pressure and pulmonary vascular resistance, and restored pulmonary responses to acetylcholine toward values measured in control rats. Treatment with rMSCs decreased RV hypertrophy induced by PAH. Immunohistochemical studies showed widespread distribution of lacZ-labeled rMSCs in lung parenchym surrounding airways in MCT-treated rats. These rMSCs retained expression of von Willebrand factor and α-smooth muscle-actin, being markers specific for endothelial cell and SMC phenotypes, respectively. However, lacZ expressing rMSCs were not detected in the wall of pulmonary vessels. These data suggest that the decrease in pulmonary vascular resistance and improved responses to acetylcholine in PAH rats treated with MSCs were the result of paracrine effects of transplanted rMSCs in lung parenchym on vascular endothelial function in the injured lungs [66].

3.2 Intravenous administration of pulmonary artery smooth muscle cells

Primary cultures of PA-SMCs from Fisher 344 rats were labeled with a fluorescent, membrane-impermeable dye chloromethyl-trimethyl-rhodamine or transfected with the β-galactosidase (βGal) reporter gene under the control of the cytomegalovirus (CMV) promoter (pCMV-β) [67]. Transfected or labeled SMCs (5 x 10^5 cells/animal) were delivered to syngeneic recipient rats by injection into the jugular vein. The animals were killed at intervals between 15 min and 2 weeks, and the lungs, spleen, kidneys, and skeletal muscle were excised and examined. At 15 min after transplantation, injected cells were detected mainly in the lumen of small pulmonary arteries and arterioles, often in groups of three or more cells. After 24 h, labeled SMCs were found incorporated into the vascular wall of pulmonary arterioles, and transgene expression persisted *in situ* for 14 d with no evidence of any immune response. Approximately 57±5% of the labeled cells injected into the venous circulation were recovered in the lungs after 15 min, 34±7% at 48 h, 16±3% at 1 week, and 15±5% at 2 weeks. Similar results were observed using cells transfected with the LacZ gene. To determine whether this method of gene transfer is effective in inhibiting the development of pulmonary vascular disease, PA-SMCs were transfected *in vitro* with either the full-length coding sequence of the eNOS gene or with the control vector (pcDNA3.1) and injected simultaneously with MCT. At 28 d after injection the RV systolic pressure was 50±4 mmHg in animals injected with the null-transfected SMCs, and 33±3 mmHg in animals injected with the eNOS-transfected SMCs ($p<0.01$). These results indicate that a cell-based strategy of *ex vivo* transfection provides an effective nonviral approach for the selective delivery of foreign transgenes to pulmonary microvessels in the treatment of pulmonary vascular disease [67].

Campbell *et al.* prepared primary cultures of PA-SMCs and transfected these cells with vascular endothelial growth factor (VEGF)-A [68]. These cells were administered i.v. into Fisher 344 rats with MCT-induced PAH. Four weeks after MCT and i.v. SMC administration, PAH, RV hypertrophy and medial hypertrophy of pulmonary arterioles were significantly less in the VEGF-treated animals compared to MCT-treated animals that did not receive cell therapy. Four weeks after gene transfer, the VEGF mRNA was still detectable in the pulmonary tissue of animals injected with VEGF-transfected cells, demonstrating survival of transfected cells and persistent transgene expression. If cell-based gene transfer using VEGF-expressing PA-SMCs was delayed till PAH had developed, also a significant decrease in the progression of PAH and RV hypertrophy was documented [68]. These results indicate that cell-based VEGF gene transfer is effective in preventing the development and progression of PAH in the MCT model. Thus, a therapeutic role for angiogenic factors in the therapy of PAH is very likely.

3.3 Intravenous administration of endothelial progenitor cells

PAH is characterized by a progressive increase in pulmonary vascular resistance caused by narrowing and loss of pulmonary microvasculature, which in its late stages becomes refractory to traditional therapies. Zhao *et al.* isolated bone marrow–derived endothelial progenitor cells (EPCs) from Fisher-344 rats, cultured them for 7 to 10 days in endothelial growth medium and injected them intravenously in syngeneic MCT-treated rats [69]. The EPCs engrafted at the level of the distal pulmonary arterioles and incorporated into the endothelial lining of the MCT-injured lung. The administration of EPCs 3 days after MCT administration nearly completely prevented the increase in RV systolic pressure observed at 3 weeks with MCT alone (31±1 versus 48±3 mmHg, respectively; $p<0.001$), whereas i.v. administration of skin fibroblasts had no protective effect (51±5 mmHg). Delayed administration of EPCs 3 weeks after MCT prevented the further progression of PAH 2 weeks later, whereas animals receiving EPCs transfected with the human eNOS gene exhibited significant regression of established disease at day 35 (31±2 mmHg, $p<0.005$) compared with day 21 (50±3 mmHg). Fluorescent microangiography revealed widespread occlusion of pulmonary arterioles 3 weeks after MCT, whereas arteriolar-capillary continuity and microvascular architecture were preserved if syngeneic EPCs had been administered. Moreover, the delivery of EPCs to rats with established PAH resulted in marked improvement in survival, which was greatest in the group receiving eNOS-transduced EPCs [69]. Thus bone marrow–derived EPCs can engraft and repair the MCT-damaged lung, restoring structure and function of pulmonary microvasculature. Therefore, the regeneration of lung vascular endothelium by injection of EPCs, and in particular eNOS-transduced EPCs, may represent a novel treatment for patients with PAH.

3.4 Intravenous administration of bone marrow-derived cells

Recent evidence suggests that bone marrow–derived cells may differentiate into vascular cells that participate in arterial repair and/or lesion formation [70]. However, it remains uncertain whether bone marrow-derived cells can also participate in vascular remodeling associated with PAH. The bone marrow of

Sprague-Dawley rats was reconstituted with that of green fluorescent protein (GFP)–transgenic rats. The bone marrow-chimeric rats were injected intraperitoneally with 60 mg/kg MCT after unilateral pneumonectomy, and concurrently underwent wire-mediated endovascular injury in one femoral artery. After 28 days, they had elevated RV systolic pressure (58.8±5.4 versus 20.4±2.4 mmHg in sham-control; $p<0.01$) [71]. The pulmonary arterioles were markedly thickened, with an infiltration of GFP–positive macrophages into the perivascular areas. The endothelium of pulmonary arterioles contained only a few GFP–positive cells, and GFP–positive cells were seldomly detected in the media of thickened pulmonary arterioles. In contrast, bone marrow-derived smooth muscle–like cells could be readily detected in the thickened neointima and media of the wire-injured femoral artery. Moreover, intravenous injection of 10^8 unfractionated bone marrow-derived cells from young rats had no beneficial effects on PAH, pulmonary arterial remodeling, and survival in the aged rats treated with MCT plus unilateral pneumonectomy. No injected bone marrow-derived cell was identified as an endothelial cell or a vascular SMC [71]. These results suggest that bone marrow-derived cells can participate in arterial neointimal formation after mechanical injury, whereas they do not contribute substantially to pulmonary arterial remodeling associated with MCT-induced PAH in pneumonectomized rats.

Raoul *et al.* investigated the effect of bone marrow-derived cells on PAH induced by either MCT or exposure to chronic hypoxia in mice [72]. Intravenous administration of the active MCT metabolite (monocrotaline pyrrole, MCTp) to C57BL/6 mice induced PAH within 15 days, due to remodeling of small pulmonary arterioles. Three days after MCTp injection, the mice were injected with bone marrow-derived cells harvested from femurs and tibias of donor mice treated with 5-fluorouracil (3.5 mg i.p./animal) to deplete mature cells and to allow proliferation of progenitor cells. Bone marrow-derived cells significantly attenuated PAH as assessed by reductions in RV systolic pressure (20 ± 1 mmHg vs. 27 ± 1 mmHg, $p\leq0.01$), weight ratio of RV to the LV plus septum (0.29 ± 0.02 vs. 0.36 ± 0.01, $p\leq0.03$), and percentage of muscularized pulmonary arterioles (26.4% vs. 33.5%, $p\leq0.05$), compared to irradiated bone marrow-derived cells administered to MCTp-treated animals. Tracking cells from constitutive GFP-expressing male donor mice with anti-GFP antibodies or chromosome Y quantification by real-time PCR demonstrated the presence of bone marrow-derived cells in the lung. In contrast, chronically hypoxic mice subjected to the same procedure failed to show improvement in PAH [72]. These results demonstrate bone marrow-derived cells limit pulmonary vascular remodeling induced by vascular injury but not pulmonary vascular remodeling induced by hypoxia.

3.5 Intravenous administration of mesenchymal stem cells overexpressing endothelial nitric oxide synthase (eNOS)

Bone marrow-derived cell transplantation is reported to reduce the development of PAH by increasing or repairing vascular beds in the pulmonary circulation, and upregulated eNOS expression enforces this therapeutic effect. Kanki-Horimoto *et al.* have studied the efficacy of intravenous administration of eNOS-transfected, bone marrow-derived MSCs (MSCs/eNOS) in rats with MCT-induced PAH [73]. One week after MCT administration, the rats received 3 different treatments:

MSCs (MSC group), MSCs/eNOS, (MSC/eNOS group), or no treatment (PAH group). As the negative control, rats received saline instead of MCT (control group). RV systolic pressures in the MSC and MSC/eNOS groups were significantly lower than in the PAH group, and RV systolic pressure in the MSC/eNOS group was significantly lower than in the MSC group. Similar results were obtained with regard to RV hypertrophy in the 3 groups. The survival time of rats receiving MSCs/eNOS was significantly longer than survival time of PAH rats without treatment [73]. Hence, intravenous administration of MSCs/eNOS offers therapeutic effects on MCT-induced PAH, RV hypertrophy, and mortality.

3.6 Transplantation of endothelial progenitor cells into the lung

EPCs have been shown to promote neovascularization. Takahashi *et al.* have examined the effects of EPC transplantation into the lungs of dogs with dehydromonocrotaline-induced PAH [74]. The lung parenchym of dogs with PAH was injected with *ex vivo*-expanded, autologous EPCs using a bronchoscope. EPC transplantation resulted in significant improvements in mean pulmonary artery pressure, pulmonary vascular resistance and cardiac output. Histological evaluation revealed improvement in the medial thickness of the small pulmonary arteries and neovascularization of lung tissue [74]. These results indicate that EPC transplantation into the lung is effective at preventing the progression of dehydromonocrotaline-induced PAH in dogs, and may provide a new therapeutic option for patients with PAH.

3.7 Intravenous administration of MSCs from rats suffering with PAH

Recently, we completed a study in which rats with MCT-induced PAH were, 14 days after MCT injection, treated with a single i.v. MSC injection (10^6 cells/rat) obtained from from bone marrow of rats with MCT-induced PAH, or a single injection with PBS. Another 2 weeks later RV pressures were measured, the rats were sacrificed and heart and lungs were dissected. The PBS-treated MCT rats developed PAH as expected. In the MSC-treated MCT rats the RV pressures were significantly lower compared to the PBS-treated MCT rats. Accordingly, RV hypertrophy in the MSC-treated MCT rats was significantly lower compared to the PBS-treated MCT rats. In this study we have demonstrated that bone marrow-derived MSCs obtained from donor rats suffering from PAH when administered to acceptor rats with PAH reduce RV pressure overload and RV hypertrophy (unpublished results).

Cell Therapy for PAH: from Experimental Models to Clinical Disease

Cell therapy is a promising novel therapeutic option, and several cell types have been tested in experimental models of PAH. Endothelial progenitor cells have recently been explored as a potential source for neovascularization of the diseased pulmonary circulation in patients with PAH. A randomized trial by Wang *et al.* has indicated that intravenous infusion of autologous EPCs to patients with idiopathic PAH appears to be feasible and safe, and has beneficial effects on

exercise capacity and pulmonary hemodynamics in patients with idiopathic PAH [75].

Also MSC is a cell type that can improve the pulmonary pathology of PAH by either differentiating into other cell types leading to regeneration of the diseased vasculature or secreting an array of substances, the “pro-survival factors” including various growth factors and cytokines, leading to improvement in lung pathology by paracrine mechanisms. Autologous bone marrow-derived MSC therapy has a practical advantage over other types of cell therapies, as the mode of administration is rather simple and has proven to be safe.

Conclusions

In conclusion, a wealth of therapeutic modalities including pharmacotherapy, gene therapy and cell therapy has been tested in several animal models of PAH including MCT-induced PAH. Several of these therapeutic options have been shown to be effective also in PAH patients leading to improved life expectations and a better quality of life. However, many patients remain symptomatic despite therapy. Cell therapy is a novel treatment option, but more animal data should be collected to investigate optimal cell type, *in vitro* cell transduction, route of cell administration, and number of cells to inject. Autologous MSC therapy is expected to be a safe and efficacious option to treat patients with PAH.

Table. Classification of published therapies of experimental pulmonary hypertension

Anti-mitogenic (particularly towards PA-SMCs)	**(ref)**
PDGF inhibitors	(11)
Endothelin receptor blockers	(23-26,36)
Prostacyclin	(12-17,22,26)
Serotonin transporter inhibitors	(28)
Serotonin receptor blockers	(30,31)
Serotonic re-uptake inhibitor	(29)
PDE 4/5 and 3/4 inhibitors	(16,17,32-36)
Caveolin-1 blockers	(37)
Estradiol (derivatives)	(38-41)
Amlodipine	(45)
Rapamycin	(49)
Endothelial NO synthase gene	(64)
Prostacyclin synthase gene	(61,62)
NF-κB inhibitor	(51)
Pro-endothelial function, vasodilatation and pro-angiogenesis	
Rho-kinase inhibitors	(18-22)
Statins	(43,44)
Nicorandil	(46)
Granulocyte-colony stimulating factor	(50)
L-arginine	(57)
Prostacyclin	(12-17,22,26)
Endothelin receptor blockers	(23-26,36)
PDE 4/5 and 3/4 inhibitors	(16,17,32-36)
Adrenomedullin	(48)
Prostacyclin synthase gene	(61,62)
Angiopoietin-1 gene	(63)
Cell therapy using EPCs and MSCs	(66,69,73-75)
Cell therapy using differentiated cells transduced with eNOS gene	(67,69,73)
Cell therapy using differentiated cells transduced with VEGF gene	(68)
Anti-inflammatory and anti-oxidative	
Serotonin receptor blockers	(30,31)
Amlodipine	(45)
Nicorandil	(46)
Statins	(43,44)
C-type natriuretic factor	(47)
Interleukin-1 receptor antagonist	(52)
Antibodies to monocyte chemotactic and activating factor/MCP1	(53)
Inhibitor of p38 mitogen-activated protein kinase	(54)
Inhibitor of lipoxygenase pathways	(58)
Anti-monocyte chemoattractant protein-1 gene	(60)
Interleukin-10 gene	(65)
Extracellular superoxide dismutase gene	(59)
Pro-apoptosis of PA-SMCs	
Serine-elastase inhibitors	(10)
PDE 4/5 and 3/4 inhibitors	(16,17,32-36)

Abbreviations: PA-SMCs, pulmonary artery-derived smooth muscle cells; PDGF, platelet-derived growth factor; PDE, phosphodiesterase; NO, nitric oxide; EPC, endothelial progenitor cells; MSC, mesenchymal stem cells; eNOS, endothelial NO-synthase; VEGF, vascular endothelial growth factor; MCP1, monocyte chemoattractant protein-1.

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