Angiotensin II-pretreated hucMSCs attenuate inflammation and reverse pulmonary fibrosis in SD rat lung

Wei Wang  (✉️ 330739406@qq.com )
Central South University

Aishi Song
Central South University

Weiyue Deng
Central South University

Yuying Zhang
Central South University

Omar Mukama
Guangzhou Institutes of Biomedicine and Health

Jean de Dieu Habimana
Guangzhou Institutes of Biomedicine and Health

Wei Xie
Central South University

Peng Zhou
Central South University

Amy L. Li
Guangzhou Institutes of Biomedicine and Health

Jiaxing Li
Central South University

Sihao Deng
Central South University

Bin Ni
Maternal and Child Health Access

Kexin Peng
Maternal and Child Health Access

Shusheng Zhang
Changsha Stomatological Hospital

Xiaoxin Yan
Central South University

Jufang Huang
Abstract

Background: Pulmonary fibrosis (PF) is an irreversible disease with a poor prognosis and a serious impact on patients' health. PF is also associated with COVID-19, especially in immunocompromised people. Herein, efforts have been made to treat PF using pretreatment of human umbilical cord mesenchymal stem cells (hucMSCs) with angiotensin II (Ang II) as a novel therapeutic method.

Methods: PF model of Sprague Dawley (SD) rats were established by tracheal injection of bleomycin (BLM) (5U/Kg). On day 15 after modeling, PBS, hucMSCs or hucMSCs-Ang II were injected into tail vein. On the 23rd day after modeling, samples were taken and corresponding indexes were tested.

Results: Our results first showed that Ang II pretreatment induced more hucMSCs to reach the injured lung and alleviated pulmonary fibrosis. Transplantation of hucMSCs-Ang II reduced inflammatory infiltration, increased IL-10 expression and enhanced macrophage matrix-metallopeptidase-9 (MMP-9) expression for collagen degradation. Moreover, the Ang II-treated hucMSCs decreased hydroxyproline (HYP) and alpha-smooth muscle actin (α-SMA) expression in SD rats and promoted collagen and collagen fiber degradation.

Conclusions: Ang II pretreatment enhanced the homing ability of hucMSCs, and hucMSCs-Ang II transplantation reversed PF by inhibiting inflammation and promoting collagen and collagen fiber degradation, promising its clinical application in the treatment of post-inflammatory PF caused by various disorders, including COVID-19 and related pneumonia.

Introduction

Pulmonary fibrosis (PF) is characterized by excessive deposition of extracellular matrix components, which is dominated by type I collagen(1, 2). It is characterized by lung epithelial cell damage, fibroblast activation, and extracellular matrix deposition(3). During the pathological progression of pulmonary fibrosis, functional alveoli are reduced, and alveolar chambers are gradually replaced by fibrotic tissue(4). PF has a high incidence in the elderly, and there is no specific drug for its treatment(5). The survival time of patients after diagnosis is only 2 ~ 5 years, and the 5-year survival rate is only 20%~25%(3, 6). More importantly, the death rate from chronic obstructive pulmonary disease increases annually(4).

At present, bleomycin (BLM) has become the most commonly used drug for the experimental modeling of PF(7). Bleomycin produces alveolar epithelial injury/necrosis(8). BLM-induced PF is patchy and uneven, which is more similar to human pulmonary fibrosis(4). In addition to intraperitoneal, subcutaneous, and inhalation administration, and BLM tracheal administration has become a common route of administration(7, 9).

Mesenchymal stem cells (MSCs) are pluripotent stem cells that can differentiate into many cell types(10). MSCs can treat a variety of diseases, such as skin lesions, Parkinson's disease, Alzheimer's disease, and ocular hypertension(11–14). They regulate inflammation, repair damaged tissue, and differentiate into
different tissues(15). The anti-inflammatory function of MSCs is mediated by multiple factors, such as indoleamine 2, 3-dioxygenase (IDO), and TNF-stimulating gene 6 (TSG-6)(10, 16). MSCs reach the site of injury in direct or paracrine signaling, and certain factors can be released after injury to attract MSCs to the site of injury for action(17). Rombouts' study showed that freshly isolated MSCs had stronger homing ability than cultured cells. The homing efficiency of MSCs cultured for 24 h can be reduced from 55%-65–10%. However, the number of newly isolated MSCs is often rare, and a large number of culture and amplification in vitro is usually required to increase the number of stem cells(18). Therefore, it is very important to improve the homing rate of MSCs to the site of injury in stem cell therapy.

Angiotensin (Ang) is an oligopeptide hormone, that mainly exerts its function through binding with receptors AT1R and AT2R(19, 20). Ang hydrolyzes into angiotensin I (Ang I) and angiotensin II (Ang II), and is mediated by the angiotensin-converting enzyme. Ang II has different research progress in different animal models(21–24). Ang II is commonly used as an inducer and enhancer for the differentiation of MSCs in stem cell therapy(25, 26). Ang II induces a cardiomyocyte-like phenotype by upregulation of Connexin43 and an early cardiac transcription factor Nkx25 in human amniotic fluid derived mesenchymal stem cells (AF-MSCs)(27). It could promote the differentiation of MSCs into cardiac progenitor cells, osteocytes, and blood vessels(22, 28–30). Thus, it is of great significance to further harness the role of Ang II in stem cell therapy to treat various diseases.

Stem cells were and are used in a variety of lung diseases, including PF. However, the homing of stem cells which plays an important role in treatment remains largely elusive. At present, the therapeutic effect of Ang II combined with hucMSCs in PF has not been reported. Therefore, we hypothesized that preconditioning hucMSCs with Ang II could induce more hucMSCs to reach the injury site and alleviate PF in rats, making the clinical application of hucMSCs-Ang II more promising. We established animal models by tracheal injection of BLM, and then transplanted Ang II-pretreated hucMSCs into the tail vein to treat PF in rats. Our results showed that Ang II-pretreated hucMSCs attenuated inflammation and reversed pulmonary fibrosis in SD rat lung. Therefore, we provide a valuable drug for stem cell preconditioning in clinical trials.

**Materials And Methods**

**Animals**

Seventy-two male Sprague Dawley (SD) rats (Hunan Slack Jingda Experimental Animal Co., Ltd, Changsha, China) weighing 200–250 g at six-week-old were used in this experiment. Animals were raised in the Department of Laboratory Animal Science (Central South University, Changsha, Hunan, China), and were housed in separate cages (SPF + IVC) with free food, water, and 12h light-dark cycle for seven days before the experiment. The animal studies were all conducted according to the“Guide for the Care and Use of Laboratory Animals, 8th ed., 2010”(National Institutes of Health, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee of Central South University (Changsha, China; Permit Number: 2020sydw0P10).
hucMScs culture

hucMSCs were purchased from the Hui Yisen Cell Gene Engineering Company (Changsha, Hunan, China). The stem cells were cultured using F12-DMEM (DMEM/F-12, Gibco, Cat# 11330500, USA) containing 10% fetal bovine serum (FBS, BI, Cat# 04-001-1ACS, Israel) for the primary culture (37 °C, 5% CO2) and were passaged after reaching 80%-90% confluence. The 5–10 generations of hucMSCs were selected for the experiment and pretreated with different concentrations of Angiotensin II (Ang II Cat# MB1677, China) for 24 h.

Wound healing

P5 generation hucMSCs were seeded in six-well plates. The cells were then scratched with a tip at 100% growth, and subsequently treated with different concentrations of Ang II (0 M, 10 − 8 M, 10 − 7 M, and 10 − 6 M) and kept for 6 h after serum starvation. The scratch area was produced by scraping the cell monolayer with the pipette head. Photographs were taken at 0 h, 12 h and 24 h time points.

Flow cytometry

Trypsinized hucMSCs were re-suspended in 0.5 mL PBS and then conjugated with a hucMSCs assay Kit (BD Biosciences, USA). CD44, CD29 (BD Biosciences, US) and CD31 (BD Horizon, US). Subsequently, hucMSCs were washed with PBS and re-suspended with 4% PFA. Then, FACS Calibur flow cytometry was used for flow cytometry analysis.

RNA isolation and real-time quantitative RT-PCR

Cells were collected and total RNA was extracted from the cells by using Trizol (Invitrogen, USA) and quantified. RT-PCR was performed using a fluorescent quantitative PCR instrument, and a reverse transcription system was configured. Reverse transcription was performed at 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 30 s. The relative changes of gene expression were normalized to the expression level of actin and calculated by the 2 (−△△Ct) method. The following gene-specific primers were used (forward (F) and reverse sequence (R)): AT1R (F: 5’-ATGAGCAGCCTTTTCTACC-3’; R: 5’-CCTCAAAACATGGTGCGAGGC-3’), AT2R (F: 5’-CAGCGTCTGAGAGAACGAGT-3’; R: 5’-AAATCAGCTTGCTTAGTGCCT-3’), actin (F: 5’-ACCCTGAAGTACCCCATCGAG-3’; R: 5’-TTGTTGCTTACCTGCACTCCTC-3’).

Cell proliferation

hucMSCs (5 × 103) were inoculated into 96-well plates and incubated at 37 °C for 24 h and subsequently added 10 µL of CCK-8 (APExBIO, Cat# K1018, USA) to each well and finally incubated for 2 h. The absorbance was measured with a microplate analyzer at 450 nm.

Experimental animal model

Before surgery, the rats were fasted for 12 h, and anesthetized with intraperitoneal pentobarbital (40 mg/kg) (ZaoZhuang Water Tailan Chemical Co. Ltd., Cat# No. 57-33-0, China). After anesthesia, the rats were fixed in a supine position, disinfected with iodophor, and the neck skin was cut open to expose the
trachea. A needle was inserted between the cricoid cartilage, and bleomycin (BLM, Nippon Kayaku Co Ltd, Japan) solution was quickly injected. The dosage of BLM was 5 U/Kg (Fig. S1A).

Rats were randomly divided into four groups (Sham, BLM, BLM + hucMSCs, and BLM + hucMSCs-Ang II). In the Sham and BLM groups, the PF rats were injected with PBS through tail vein. The PF rats in the BLM + hucMSCs groups were injected with $1 \times 10^6$ hucMSCs through tail vein, while the BLM + hucMSCs-Ang II groups, the PF rats were injected with $1 \times 10^6$ hucMSCs-Ang II through tail vein. After 7 days of treatment, SD rats were euthanized, and lung tissues were removed for further analysis (Fig. S1B).

**Measurement of hydroxyproline levels**

The left lung tissue of SD rats (80 mg) was randomly hydrolyzed in a boiling water bath for 20 min (mixed once at 10 min), and the HYP level was determined according to the Hydroxyproline Detection Kit (HDK, Trevigen, Cat# A030-2-1, China). The tissue blocks were cut into 10 mg blocks for cracking. Then, the pH value was adjusted (pH 6.0-6.8) and diluted with double distilled water. The diluent was added with activated carbon (20–30 mg) at 3500 RPM/10 min to obtain the supernatant for detection. The absorbance value of the supernatant was measured at 550 nm.

**Protein extraction and western blot**

The superior lobe of the left lung was collected and protein was extracted by RIPA (CWBiO, Cat# CW2333S, China) and protease inhibitor (CWBiO, Cat# CW2200S, China). BCA Kit (Thermo Scientific, Cat# 23225, USA) was used to determine proteins concentration and western blot to detect the expression of target proteins. After electrophoresis, proteins were transferred to PVDF membrane (Millipore, Billerica, MA, USA), and sealed with 5% skimmed milk for 2.5 h. The protein strips were incubated with primary antibody overnight at 4 °C. Relative primary antibodies were rats anti-MMP-9 (1:500, proteintech, Cat# 10375-2-AP, China), rats anti-α-SMA (1:500, Wanleibio, Cat# WL02510, China), rats anti-GAPDH (1:4000, Proteintech, Cat# 10494-1-AP, China), and rats anti-AT1R (1:1000, Abcam, Cat# ab124734, Britain). Finally, the strips were incubated with a suitable secondary antibody for an hour at room temperature. Protein bands were detected by Enhanced Chemiluminescence Kit (ECL, advansta, Cat# K-12045-D10, USA) and quantitated by ImageJ software (National Institutes of Health, USA).

**Morphology staining**

The superior lobe of the left lung was taken out and fixed for 24 h at 4 °C in 4% paraformaldehyde solution (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China). Next, the tissues were dehydrated by graded ethanol, embedded in OTC (Sakura Finetek, USA), and cut into 20-µm-thick sections by slicer (Leica, Wetzlar, Germany). Tissue sections were stained with Hematoxylin-Eosin (H&E) staining Kit (Solarbio, Cat# G1102, Beijing, China), the Masson's Trichrome Stain Kit (Solarbio, Cat# G1340, Beijing, China), and Sirius Red Staining Kit (Abiowell, Cat# AWI0466a, Changsha, China). The images were captured on Nikon confocal microscope (Nikon Instruments, Inc., Japan).

**Immunohistochemistry**
For immunohistochemistry staining, the sections were incubated in 0.3% hydrogen peroxide/1% PBST (Solarbio, Cat# T8200, Beijing, China) for 30 min and blocked with normal horse serum (Beyotime, Cat# C0262, Shanghai, China) /PBST (1:200) for 2 h. Then, the sections were incubated with anti-Ly-6G antibody (1:500, Biolegend, Cat# RB6-8C5, USA), anti-MMP-9 antibody (1:500, proteintech, Cat# 10375-2-AP, China), and anti-α-SMA antibody (1:500, Wanleibio, Cat# WL02510, China) overnight at 4 °C. Slices were then incubated with secondary antibody (Vectorlabs, Cat# BA-1300-2.2, USA) at 37 °C for 2 h and incubated with three antibodies (A liquid + B liquid + PBST) (Vectorlabs, Cat# BA-1300-2.2, USA). Next, the slices were washed with PBS and sealed with the coverslip by neutral resin (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China). Finally, cell nuclei were stained with DAPI, and images were captured on Nikon confocal microscope (Nikon Instruments, Inc., Japan).

Results

Characterization of hucMSCs

In this study, P0 hucMSCs samples were obtained from Hui Yisen Cell Gene Engineering Company (Changsha, Hunan, China), and it exhibited a flattened and spindle shape as shown in (Fig. 1A). By using in vitro cell differentiation strategy, we have successfully differentiated hucMSCs into adipocytes or osteoblasts under the induction of a special induction medium (Fig. 1B, C). Cell surface markers were identified by flow cytometry. The results showed that CD29, CD44, CD90, CD105, and CD73 were positive, while CD31, CD34, CD45, and HLA-DR were negative (Fig. 1D). Flow cytometry results and stem cell differentiation ability determination results confirmed that the cells used in the experiment were hucMSCs.

Pretreatment with Ang II promotes the migration of hucMSCs

To determine the optimal treatment concentration of Ang II for hucMSCs treatment, hucMSCs were pretreated with different concentrations of Ang II (0 M, 10^{-8} M, 10^{-7} M and 10^{-6} M) and pretreated-hucMSCs were used for scratch healing assay. Cell scratch experiments showed that hucMSCs had the strongest migration ability at a concentration of 10^{-7} M (Fig. 2A, B).

Besides, wound healing could result from enhancement of cell migration or the increase in the number of hucMSCs or likely from both factors. Surprisingly, the CCK-8 proliferation experiment of hucMSCs showed that there was no significant difference in proliferation ability of hucMSCs treated with different concentrations of Ang II (Fig. 2C). Therefore, pretreatment with different concentrations of Ang II can enhance the migration ability of hucMSCs and has little effect on the proliferation of hucMSCs.

Ang II mediates homing of transplanted hucMSCs at the injury site through binding of its receptor target AT2R

Ang II acts mainly by binding to receptors such as AT1R and AT2R(31). Our RT-PCR results showed that AT1R and AT2R were significantly overexpressed at 10^{-7} M of Ang II with a 2.5 amplification index of
AT1R (Fig. 3A) and 60 amplification index of AT2R (Fig. 3B), which is significantly higher than that of AT1R. RT-PCR results showed that Ang II pretreatment could enhance the expression of AT2R in hucMSCs. Thus, Ang II mainly binds with the AT2R receptor at the optimal concentration to promote the migration of hucMSCs.

To determine the role of Ang II mediating homing of engrafted hucMSCs, on day 15, after BLM modeling, SD rats in different groups were injected with PBS, DiR Iodide (DiLC18 (7)) tagged-hucMSCs, or DiR Iodide (DiLC18 (7)) tagged-hucMSCs-Ang II cells, respectively (Fig. 3C). Tracking through a live imaging system revealed that a significant number of Ang II-treated hucMSCs were navigated through the tail vein and observed more numerous occupying surfaces at the injury site and the highest fluorescence intensity was observed in BLM + hucMSCs-Ang II treated SD rat group (Fig. 3D, E).

**Transplantation of hucMSCs-Ang II reduces the inflammatory response in SD rat lung**

Bleomycin in the airway burns the lungs and this leads to a loss of water and food intake by the rodents. Therefore, the weight of SD rats would be reduced after BLM injection. After treatment with hucMSCs, the body weight of BLM + hucMSCs-Ang II group increased faster than that of BLM + PBS group (Fig. 4A). To determine the role of hucMSCs-Ang II in reducing the inflammatory response in SD following injury, we performed pathological assays such as hematoxylin and eosin (H&E) and immunohistochemistry (IHC) stainings. H&E stained tissue sections revealed a significantly reduced inflammation in animals treated with hucMSCs or hucMSCs-Ang II compared with control-treated counterparts (Fig. 4B). Similarly, IHC determination of infiltrating neutrophils at fibrotic lung tissue revealed that a reduced number of cells were observed in SD rats treated with hucMSCs-Ang II group compared with control PBS-treated SD rats (Fig. 4C, D). In addition, the expression of anti-inflammatory factor IL-10 in the hucMSCs-Ang II group was up-regulated in BLM + PBS and hucMSCs groups compared with control-treated groups (Fig. 4E). Taken together, these results suggest that the transplantation of hucMSCs-Ang II reduced inflammatory responses in SD rat lung by decreasing infiltrating neutrophils at injury sites and up-regulating anti-inflammatory IL-10.

**Transplantation of hucMSCs-Ang II reduced the expression of α-SMA and HYP**

Hydroxyproline (HYP) is low in elastin and absent in other proteins. Therefore, the amount of HYP reflects the collagen metabolism of connective tissue diseases(32). Based on this notion, we next sought to determine the role of hucMSCs-Ang II in α-SMA and HYP expression in SD rats following transplantation. The results showed that the BLM + PBS treated group had more HYP than the Sham group, and less HYP content in the hucMSCs-Ang II group, which indicated that the collagen content in the hucMSCs-Ang II group was reduced (Fig. 5A). We next performed Western blot analysis to determine the accumulation of α-SMA protein level, a marker of pathological remodeling of pulmonary fibrosis. The result showed that the expression of α-SMA in the BLM + PBS group was significantly increased compared with control groups (Fig. 5B, C). After treatment with hucMSCs, reduced accumulation of α-SMA was detected in SD rats. More importantly, the transplantation of hucMSCs-Ang II has significantly improved the accumulation of α-SMA at the injury locus. Similarly, the results from IHC analysis revealed that the
number of α-SMA positive cells reduced at injury sites after hucMSCs treatment compared with the PBS-treated group (Fig. 5D). Likewise, treatment of SD rats with hucMSCs-Ang II reduced significantly the accumulation of α-SMA positive cells at injury sites (Fig. 5E). Therefore, the transplantation of hucMSCs and hucMSCs-Ang II cells into PF SD rats could reduce the expression of α-SMA and HYP.

Studies highlighted that the ACE-Ang II-AT1R axis can promote pulmonary fibrosis(33–35). Conversely, ACE2-Ang-(1–7)-Mas axis inhibits fibrosis(35–37). We next detected the expression of AT1R in SD rats after hucMSCs transplantation. Western blot analysis demonstrated that the expression of AT1R was up-regulated in the BLM + PBS group (Fig. 5F), and down-regulated after hucMSCs treatment (Fig. 5G). The results showed that the degree of pulmonary fibrosis in SD rats was alleviated after hucMSCs-Ang II treatment.

**Transplantation of hucMSCs-Ang II promotes the synthesis of MMP-9 and degradation of collagen fibers**

Next, we determined the deposition of collagen (the main ECM component of tissue fibrogenesis) and MMP-9 after hucMSCs-Ang II transplantation. Western blotting results showed that the expression of MMP-9 (the main collagen degrading enzyme) in the BLM + hucMSCs-Ang II group was significantly increased (Fig. 6A, B). In addition, IHC staining demonstrated that the expression of MMP-9 was significantly increased in the BLM + hucMSCs-Ang II group, compared with a single treatment of hucMSCs (Fig. 6C, D). It is believed that the synthesis of MMP-9 can promote the degradation of collagen and collagen fibers(38). Therefore, we next determined the deposition of collagen in PF SD rats after injury induction. For this purpose, we induced PF in SD rats by injection of BLM. The results from Massion and Sirius red staining revealed that the BLM + PBS group exhibited severe fibrosis, while Ang Ⅱ-hucMSCs treatment significantly reduced PF compared with sole hucMSCs application (Fig. 6E-G). Therefore, transplantation of hucMSCs-Ang II in BLM-induced pulmonary fibrotic SD rats could reduce collagen deposition at injury locus through increased synthesis of MMP-9.

**Discussion**

In this study, hucMSCs transplantation reversed PF in SD rats. Our study is the first to demonstrate that Ang II preconditioning enhances the therapeutic effect of hucMSCs in PF. PF is an irreversible disease that causes symptoms such as coughing, dyspnea, fatigue, weight loss(39, 40). PF is caused by a cycle of injury and repair that occurs in the lung as a result of long time exposure to certain environmental factors, drugs or radiotherapy, and some inflammatory diseases such as dermatomyositis, polymyositis, sarcoidosis, scleroderma, and pneumonia-causing pathogens(41). Patients with PF have a reduced quality of life with progressive and irreversible organ dysfunction(42). The disease can seriously affect the patient's daily life. However, current drugs for PF can only provide relief and cannot reverse fibrosis(43). Yet, a lung transplant may sometimes be an alternative intervention for the patient survival who experience idiopathic PF — a chronic and progressive lung disease from unknown exact cause(44). However, suitable lung sources are extremely difficult to obtain, and immune rejection may occur after lung transplantation. This makes lung transplant success rates low.
Stem cells have emerged in recent years as a new therapeutic approach that promise to provide better treatments for many types of diseases. MSCs from different sources have been used for the treatment of PF, including bone marrow mesenchymal stem cells, Distal airway stem cells (DASCs), and human embryonic stem cells (hESCs), etc[45–49]. hucMSCs have also been used in the treatment of PF, and the treatment effect in the transplanted hucMSCs group is reportedly better than that in the model group[50–52]. However, the treatment efficiency of PF with Ang II-pretreated hucMSCs has not been reported. We wondered whether Ang II-pretreated hucMSCs therapy could enhance the migration ability, reduce inflammation, and promote the degradation of collagen to reverse PF in SD rats. Intriguingly, Ang II-pretreated hucMSCs could promote the migration of hucMSCs, thereby reducing inflammation and reversing pulmonary fibrosis in SD rats.

Ang II has been found to function primarily by binding to receptors AT1R and AT2R(19, 20). Ang II has different effects when it binds to different receptors. Xiao's study found that Ang II promoted keratinization of bone marrow mesenchymal stem cells through binding receptor AT1R(53). Ang II can promote the proliferation of MSCs under hypoxia by binding receptor AT1R(54). AT1R stimulates bone progenitor cell proliferation through TGF-β-mediated signaling pathway(55). Ang II promotes adipogenic differentiation of human adipose mesenchymal stem cells (ADSCs) and regulates the differentiation of MSCs into insulin-producing cells (IPCs) by binding receptor AT2R(56–58). Our in vitro experiments also found that the high expression of receptor AT2R at an optimal concentration significantly promotes the migration of hucMSCs.

The inflammatory state in PF rats attracts MSCs to migrate to the injured lungs. Studies demonstrated that the migration and homing of transplanted hucMSCs towards the injury site is primarily mediated by growth factors, chemokines, adhesion molecules, and their receptor targets(59–61). For instance, basic fibroblast growth factor (bFGF) increase the migration activity of MSCs by activating the Akt pathway(62). In addition, integrin β1 and fibre-mucin play key roles in cell adhesion, migration, and chemotaxis(63–65). Our in vivo live imaging also showed that Ang II-pretreated hucMSCs could induce the migration of more hucMSCs cells at injured sites of PF lungs, proving that the homing ability of hucMSCs could be improved by Ang II pretreatment. Moreover, our results showed that Ang II-pretreated hucMSCs had better treatment effect, and significantly reduced lung inflammation and fibrosis in PF rats. Our findings are in accordance with Lan's research which reported that hypoxia-preconditioned MSCs could enhance the survival rate of transplanted MSCs with improved therapeutic efficacy on BLM-induced pulmonary fibrosis(66).

PF is a chronic inflammatory disease, and inflammation plays a complex role in the development of fibrosis(67). The early stage of PF is mainly an inflammatory reaction, and the inflammation gradually changes to fibrosis in the middle and late stage. In normal animals, collagen synthesis and degradation are in a balanced state. However, McKlerol et al reported that the synthesis and degradation of collagen in fibrotic rats were unbalanced, and the degradation of collagen could not keep up with the synthesis rate of collagen(68). Too much collagen synthesis in the body leads to the accumulation of extracellular fibrotic proteins. PF has been shown to be associated with the deposition of extracellular matrix (ECM)
components in the lung interstitium, and matrix metalloproteinase (MMP) is the major protease group known to regulate ECM remodeling(69). MMP is activated during repair. Proteolytic enzymes (including MMP) cleat collagen fibers and promote collagen degradation(68). Our experimental results also showed that MMP-9 expression was up-regulated in hucMSCs treatment, and MMP-9 was involved in collagen degradation.

Myofibroblasts are the core of pulmonary fibrosis. Myofibroblasts are effective mitotic factors of fibroblasts, and their proliferation is a marker of PF(70). Myofibroblasts are characterized by high expression of α-SMA and excessive ECM protein(71). We detected up-regulation of α-SMA expression in the BLM group by WB, indicating overproliferation of myofibroblasts. We showed that Ang II-pretreated hucMSCs reduced the expression of α-SMA, substantially alleviating lung fibrosis.

Moreover, the expression of AT1R was increased in lung tissue following hucMSCs-Ang II transplantation. Previous reports highlighted that the AT1R axis is a known promoter of tissue fibrogenesis(36). AT1R expression is elevated in animals with pulmonary fibrosis(34, 35, 72). Chang et al. showed that treatment of bleomycin-induced vitamin D deficiency mice with the AT1R antagonist Losartan significantly ameliorated pulmonary fibrosis(33). However, the role of receptor AT2R in PF has remained unclear. Accordingly, we investigated the role of AT2R in stem cell therapy to explore PF in SD rat lungs. Our experimental results showed that AT2R expression was increased in the lungs of PF rats.

To recap, the present study demonstrated the impact of Ang II pretreatment in enhancing the homing ability of hucMSCs, and the anti-inflammatory and anti-fibrosis abilities of hucMSCs in the pulmonary fibrosis rat model. However, the differentiation pathway of hucMSCs was not thoroughly studied. Moreover, there is not enough evidence about the role of AT2R in PF. In the future, we will continue to explore the role of AT2R in pulmonary fibrosis. Further studies are also being endorsed to explore the differentiation effect of hucMSCs in the treatment of PF.

In summary, the treatment of PF remains a clinical challenge. Our results showed that Ang II pretreatment could promote the migration of more hucMSCs to lung tissues, up-regulate the expression of MMP-9 and IL-10, promote the degradation of collagen and collagen fiber, and reduce lung inflammation. Here we first present a potential treatment approach that offers new hope and benefits for patients with PF caused by a variety of diseases, including COVID-19 and other pneumonia.

Conclusion

Our results showed that Ang II pretreatment could promote the migration of more hucMSCs to lung tissues, up-regulate the expression of MMP-9 and IL-10, promote the degradation of collagen and collagen fiber, and reduce lung inflammation. Here we first present a potential treatment approach that offers new hope and benefits for patients with PF caused by a variety of diseases, including COVID-19 and other pneumonia.
**Abbreviations**

DMEM/F12: Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12; FBS: Fetal bovine serum; MHC: Major histocompatibility complex; DAPI: 4’,6-Diamidino-2-phenylindole; ANOVA: Analysis of variance; IL-10: interleukin-10; PF: pulmonary fibrosis; BLM: bleomycin; MSCs: MSCs; IDO: indoleamine 2, 3-dioxygenase; TSG-6: TNF - stimulating gene 6; Ang: Angiotensin; Ang I: angiotensin I; Ang II: angiotensin II; AT1R: Anti-Angiotensin II Type 1 Receptor; AT2R: Anti-Angiotensin II Type 2 Receptor; hucMSCs: human umbilical cord mesenchymal stem cells; SD: Sprague Dawley; HYP: hydroxyproline; H & E: Hematoxylin and eosin; IHC: immunohistochemical staining; α-SMA: α-smooth muscle actin; MMP-9: matrix-metallopeptidase-9; SARS-COV-2: severe acute respiratory syndrome coronavirus-2.

**Declarations**

**Supplementary information**

Supplementary information is available at (Molecular Therapy)’s website.

Correspondence and requests for materials should be addressed to Zhiyuan Li.

**Acknowledgments**

We are grateful to all who contributed to this article, especially Hui Yisen Cell Gene Engineering Company (Changsha, Hunan, China) for donating human umbilical cord mesenchymal stem cells.

**Author contributions**

W. Wang and A. Song designed, performed the experiments, and wrote the manuscript with contributions from all authors. W. Deng, Y. Zhang, J. Li and S. Deng analyzed the data. W. Xie, J. Huang, X.Yan, A. L-Li. and P. Zhou participated in staining materials and tissue morphology experimentation. B. Ni, K. Peng, S. Zhang, and Z. Li. performed the analysis with constructive discussions and interpreted the results. O. Mukama and J. d-D- Habimana offered significant suggestions for revisions and polished the article. Z. Li. conceptualized the idea, provided essential suggestions, and guided the experiments. All authors read and approved the final manuscript.

**Funding**

This work was supported by the Frontier Research Programs of Guangzhou Regenerative Medicine and Health Guangdong Laboratory (No. 2018GZR110105020), Guangdong Provincial Natural Science Foundation (2021A1515010526), Stem Cell New Drug, Innovation Hunan Team (2019RS1088), 2020 Hunan Province Key R&D Plan (2020SK2137).

**Availability of data and materials**
All the data supporting the findings of this study are available from the corresponding author on reasonable request.

Declarations

**Ethics approval and consent to participate**

This study was conducted with approval from Institutional Animal Care and Use Committee of Central South University (Permit Number: 2020sydw0P10). Written informed consent was obtained from the participant included in the study. All procedures performed in studies involving animals were conducted in “Guide for the Care and Use of Laboratory Animals, 8th ed., 2010”.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

**References**


18. Rombouts WJ, Ploemacher RE. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. Leukemia. 2003;17(1):160-70.


52. Zhang H, Wang H, Xia Y, Qi N. Dual Effects of Hypoxia-Inducible Factors-1 Alpha in Bleomycin-Induced Pulmonary Fibrosis Treated by Human Umbilical Cord Mesenchymal Stem Cells. Stem Cells


58. Sadik NA, Metwally NS, Shaker OG, Soliman MS, Mohamed AA, Abdelmoaty MM. Local renin-angiotensin system regulates the differentiation of mesenchymal stem cells into insulin-producing cells through angiotensin type 2 receptor. Biochimie. 2017;137:132-8.


Figures

Typical differentiation characteristics of hucMSCs. A hucMSCs morphology was spindle-shaped. Bar = 50 µm. B hucMSCs’ osteogenesis differentiation. C hucMSCs’ adipogenic differentiation. Bar = 50 µm. D hucMSCs’ expression of CD44, CD90, CD105, CD29, CD73 were positive. However, CD34, CD45, CD31 and HLA-DR were negative.
Figure 2

Effects of Different concentrations of Ang II on the migration and proliferation of hucMSCs. **A** Migration ability of hucMSCs after stimulations with different concentrations of Ang II (0 M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) examined using the scratch assay. The wound sites were observed and photographed at 0, 12, and 24 h (4 X). **B** Quantitative results of wound healing. n=3. **C** Effect of Ang II on the proliferation of hucMSCs. hucMSCs were stimulated with different concentrations of Ang II (0 M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) for 24 h. Cells cultured under normal conditions served as the baseline. The proliferation rate of hucMSCs following stimulation was evaluated using the CCK-8 assay. n=6. Bar = 50 µm. The results were expressed as the Mean ± SD, *p < 0.05, **p < 0.01 versus 0 M groups.
Figure 3

Pretreatment with Ang II promoted the homing of hucMSCs at appropriate concentration. **A** AT1R mRNA expression was determined by RT-PCR after stimulations with different concentrations of Ang II (0 M, \(10^{-8}\) M, \(10^{-7}\) M and \(10^{-6}\) M) for 24 h. **B** AT2R mRNA expression was determined by RT-PCR after stimulations with different concentrations of Ang II (0 M, \(10^{-8}\) M, \(10^{-7}\) M and \(10^{-6}\) M) for 24 h. n = 6. n: number of independent experiments. **C** DiR Iodide (DiLC18(7)) deep red fluorescent probe stained hucMSCs. **D** After tail vein injection of labeled hucMSCs in SD rats for 48 h, in vivo imaging of small animals was performed. **E** Quantitative results of fluorescence intensity. Bar = 50 µm. The results were expressed as the Mean ± SD, *p < 0.05, **p <0.01 versus Sham groups. #p < 0.05, versus BLM+hucMSCs groups. n = 3.
Figure 4

Transplantation of Ang II pretreated hucMSCs and inflammatory response in SD rat lung. **A** The body weights of the rats in each group after BLM injection. **B** HE staining of lung tissues SD rats in four groups: Sham, BLM+PBS, BLM+ hucMSCs, and BLM+hucMSCs-Ang II. **C** ELISA was used to detect the concentration of the anti-inflammatory factor IL-10. **D** Immunolabelling of the neutrophils protein, lung tissues SD rats in four groups: Sham, BLM+PBS, BLM+ hucMSCs, and BLM+hucMSCs-Ang II. **E** Quantitative analysis of the protein levels of neutrophils by ImageJ. Bar = 1000 µm. Enlarged pictures after the black frame. Bar = 100 µm. The results were expressed as the Mean ± SD, *p < 0.05, **p <0.01 versus Sham groups. #p < 0.05, versus BLM+hucMSCs groups. n = 3.
Transplantation of Ang II pretreated hucMSCs decreased the expression levels of HYP and α-SMA. **A** HYP expression detection by the hydroxyproline detection kit. **B** The protein levels of α-SMA were determined by western blotting. **C** Quantitative analysis of the protein levels of western blotting. **D** Immunolabelling of α-SMA, lung tissues SD rats in four groups: Sham, BLM+PBS, BLM+ hucMSCs, and BLM+hucMSCs-Ang II. **E** Quantitative analysis of the protein levels of α-SMA by Image J. Bar = 1000 µm. Enlarged pictures after the black frame. **F** The protein levels of AT1R was determined by western blotting. **G** Quantitative analysis of the protein levels of AT1R using Image J. Bar = 100 µm. The results were expressed as the Mean ± SD, *p < 0.05, **p < 0.01 versus Sham groups. #p < 0.05, versus BLM+hucMSCs groups. n = 3.
Figure 6

Transplantation of Ang II pretreated hucMSCs promoted the degradation of collagen fibers. **A** The protein levels of MMP-9 were determined by western blotting. **B** Quantitative analysis of the protein levels of MMP-9 using Image J. **C** Immunolabelling of MMP-9, lung tissues SD rats in four groups: Sham, BLM+PBS, BLM+ hucMSCs, and BLM+hucMSCs-Ang II. **D** Quantitative analysis of the protein levels of MMP-9 by Image J. **E** Massion staining of lung tissues SD rats in four groups: Sham, BLM+PBS, BLM+ hucMSCs, and BLM+hucMSCs-Ang II. **F** Quantitative analysis of the fibrosis levels of Massion by Image J. **G** Sirian red staining of lung tissues SD rats in four groups: Sham, BLM+PBS, BLM+ hucMSCs, and BLM+hucMSCs-Ang II. Bar = 1000 µm. Enlarged pictures after the black frame. Bar = 100 µm. The results were expressed as the Mean ± SD, *p < 0.05, **p <0.01 versus Sham groups. #p < 0.05, versus BLM+hucMSCs groups. n = 3.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigure.tif