The angiogenic related functions of bone marrow mesenchymal stem cells are promoted by CBDL rat serum via the Akt/Nrf2 pathway

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Abstract

Hepatopulmonary syndrome (HPS) is a complication of severe liver disease. It is characterized by an arterial oxygenation defect. Recent studies have demonstrated that pulmonary angiogenesis contributes to the abnormal gas exchange found in HPS. Additionally, mesenchymal stem cells (MSCs) are considered the stable source of VEGF-producing cells and have the potential to differentiate into multiple cell types. However, it has not been determined whether bone marrow mesenchymal stem cells (BM-MSCs) are mobilized and involved in the pulmonary angiogenesis in HPS. In this study, a CFU-F assay showed that the number of peripheral blood MSCs was increased in common bile duct ligation (CBDL) rats; however, there was no significant difference found in the number of BM-MSCs. *In vitro*, CBDL rat serum induced the overexpression of CXCR4 and PCNA in BM-MSCs. Consistently, the directional migration as well as the proliferation ability of BM-MSCs were enhanced by CBDL rat serum, as determined by a transwell migration and MTT assays. Moreover, the secretion of VEGF by BM-MSCs increased after treatment with CBDL rat serum. We also found that the expression of phospho-Akt, phospho-ERK, and Nrf2 in BM-MSCs was significantly up-regulated by CBDL rat serum in a time dependent manner, and the blockage of the Akt/Nrf2 signalling pathway with an Akt Inhibitor or Nrf2 siRNA, instead of an ERK inhibitor, attenuated the migration, proliferation and paracrine capacity of BM-MSCs. In conclusion, these findings indicated that the number of MSCs increased in the peripheral blood of CBDL rats, and the Akt/Nrf2 pathway plays a vital role in promoting the angiogenic related functions of BM-MSCs, which could be a potent contributor to pulmonary angiogenesis in HPS.

Key words: angiogenesis; common bile duct ligation; Hepatopulmonary syndrome; mesenchymal stem cells.

1. Introduction

Hepatopulmonary syndrome (HPS) is defined by arterial hypoxemia and an abnormal age-corrected alveolar–arterial oxygen gradient caused by the development of intrapulmonary vascular dilatation (IPVD) in the setting of liver disease and/or portal hypertension [1]. The prevalence of HPS in cirrhotic patients is assessed to range from 5 to 32% [2]. The occurrence of HPS significantly increases the mortality of patients, and liver transplantation is considered the only efficient therapy [3]. Common bile duct ligation (CBDL) rats are the only accepted animal model of HPS. Pulmonary angiogenesis occurs in CBDL animals as experimental HPS develops [4]. However, the mechanisms behind this process remain to be further investigated.

Mesenchymal stem cells (MSCs) have been considered a regulator of vascular growth because of their potential for transdifferentiation into endothelial cells (ECs) or their paracrine effect [5]. Over the years, there has been much focus on the therapeutic application of MSCs in different types of disease, especially trauma or ischaemia disease. However, there are relatively few studies on MSCs involved in the pathologic changes of angiogenesis-dependent illnesses. The mobilization of bone marrow MSCs (BM-MSCs) can occur in response to a variety of stimuli, including chronic hypoxia, inflammation and injury [6–8]. The liver and remote organs can be damaged by an increase in bilirubin, endotoxins and inflammatory mediators in the early stages of CBDL [9, 10]. Meanwhile, hypoxia is accompanied by the whole late phase of CBDL, which indicates that BM-MSCs may be mobilized in the rat experimental model of HPS. The SDF-1/CXCR4 axis, an important chemokine/chemokine receptor pair in BM-MSCs, not only plays a key role in the metastasis and infiltration of malignant tumours but is also critical in the directional migration of BM-MSCs [11]. Consistently, it is reported that the SDF-1/CXCR4 axis is important for stem cell homing during stem cell therapy to treat myocardial infarction [12]. Furthermore, it has been reported that hypoxia pretreated BM-MSCs facilitates angiogenesis in diabetic rats with lower ischaemia because of its enhanced paracrine ability [13]. Taken together, BM-MSCs may play a role in the pulmonary angiogenesis of HPS.

Nf-E2 related factor 2 (Nrf2) is a transcription factor that can activate various antioxidant responsive element (ARE)-dependent genes in response to oxidative stress [14, 15], and our previous study found that phospho-Akt and ERK1 levels are increased in lung tissues 3 weeks after CBDL, which has been shown to activate Nrf2 [10, 16, 17]. Nrf2 has been confirmed to control stem cell survival under oxidative damage [18–20], and the activity of Nrf2 declines during replicative senescence and ageing [21, 22]. Moreover, it also has been found that Nrf2 activation promotes the expression of CXCR4 in haematopoietic stem cells [23].

Therefore, in this study, we assessed the number of MSCs in the peripheral blood of CBDL rats and explored the effects of CBDL rat serum on the angiogenic related functions of BM-MSCs, including their directional migration, proliferation and paracrine ability.

2. Materials and methods

2.1. Animals

To build the HPS rat model, male Sprague-Dawley rats (200–250 g, Third Military Medical

University, Chongqing, China) were subjected to CBDL operation using an improved method compared to our previous study [24]. The sham animals underwent common bile duct exposure without ligation. Lung tissues and the peripheral blood were collected for further analysis. All rats were housed under a suitable temperature and had free access to food and water. All experiments were approved by the Ethical Committee of Southwest Hospital for Animal Research.

2.2. Isolation, culture and identification of BM-MSCs and PB-MSCs (Peripheral Blood-Mesenchymal Stem Cells)

SD rats were anaesthetized using sodium pentobarbitone (50 mg/kg); their femurs and tibias were isolated and removed under sterile conditions. The metaphysis was cut off, and the bone marrow cavity was flushed with MSC complete medium for SD rats (Cyagen Biosciences, Guangzhou, China). Large tissues were removed using a 200-mesh nylon filter, and the isolated BM-MSCs were inoculated in a 25 cm² cell culture bottle using MSC complete medium (Cyagen Biosciences, Guangzhou, China) and maintained at 37 °C in a 5% CO₂ incubator. The medium was first replaced 24 hours later to remove the non-adherent cells. After that, the medium was refreshed every 3 days, and the cells were passaged when cell confluency reached >80% and seeded at 10,000 cells per cm². The BM-MSCs after 3-5 passages were used for further experiments *in vitro*. Fresh peripheral blood samples were collected and added on the surface of Ficoll-Hypaque solution (Solarbio, Beijing, China) in a centrifuge tube. The low-density mononuclear cells (MNCs) were acquired to culture after density gradient centrifugation. The cell culture approach was as described for BM.

The surface markers were detected using a LSR II cytometer (BD Biosciences) and the FlowJo software was used for analysis. Cells suspended in PBS at a density of 10⁶/ml were incubated at room temperature for 1 h with the following antibodies: PE conjugated anti-rat CD29 (BD Biosciences), PE conjugated anti-rat CD44 (Santa Cruz Biotechnology, Santa Cruz, CA), FITC conjugated anti-rat CD34 (Santa Cruz Biotechnology, Santa Cruz, CA), and FITC conjugated anti-rat CD45 (Santa Cruz Biotechnology, Santa Cruz, CA). Isotype-matched antibodies were used as the controls.

2.3. The CFU-F assays for MSCs

In the colony-forming unit fibroblast (CFU-F) assays, all the isolated BM cells were plated on a 25 cm² flask at a density of 2X10⁶ cells and cultured in proliferation culture medium, whereas for PB-MSCs, a cell density of 10⁷ cells per 25 cm² flask was used. The culture medium was replaced 2 days later, and adherent colonies (>50 cells) derived from the CFU-Fs were counted on days 8. All samples were tested 3 times.

2.4. Nrf2 siRNA transfection

Nrf2 siRNA and control siRNA were purchased from RiboBio (RiboBio Co., Ltd, Guangzhou), and the transfection was performed according to the manufacturer's instructions. BM-MSCs were seeded at the same concentration (10⁶/cm²) and were divided into an Nrf2 siRNA (50 nM) transfected group, a non-transfected group and a control siRNA (50 nM) transfected group. Culture conditions were set at 37 °C and 5%

CO₂. The efficacy of Nrf2 silencing was assessed using western blotting 72 h after transfection.

2.5. Western blotting

The cultured cells were collected and lysed in RIPA buffer for extracting proteins (Sigma-Aldrich) according to the manufacturer's instructions. Subsequently, a BCA protein assay was used to determine the protein concentrations, and whole lysates were mixed with 5×SDS loading buffer at a ratio of 1:4. Equal quantities (40 mg of protein) of the whole lysates were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) after the samples were heated at 100 °C for 5 min. Then, the samples were transferred to PVDF membranes. The membranes were blocked for 2 h using 5% milk in Tris-Buffered Saline Tween-20 (TBST) and then incubated overnight with the primary antibody at 4 °C. After washing three times with TBST, the membrane was incubated with an IgG-HRP secondary antibody for 1 h at room temperature. Finally, the membranes were stained with an enhanced chemiluminescence reagent, scanned, and stored using a gel imaging system. The protein bands were analysed using the Image Lab software. Primary antibodies for Western blotting were acquired from Abcam, including anti-Akt, anti-phospho-Akt, anti-ERK, anti-phospho-ERK, anti-Nrf2, anti-CXCR4, anti-PCNA, anti-β-tubulin and anti-GAPDH. The secondary antibody used was HRP-conjugated anti-rabbit. The images shown in the figure are representative of 3 repeats.

2.6. Immunofluorescence

BM-MSCs were subcultured on 13 mm×13 mm coverslips in a 24-well plate, at a density of 5×10⁴ cells/well. After 48 h, BM-MSCs were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 for 30 min at room temperature. 10% normal goat serum was used to block non-specific interactions. Subsequently, the cells were incubated with 1:500 dilution of Nrf2 antibody overnight at 4 °C. Following washing with PBS, the coverslips were incubated with an Alexa Fluor 555-labeled secondary antibody for 30 min. DAPI was used to stain the cell nuclei at a concentration of 0.5 µg/ml.

2.7. MTT assay

BM-MSCs were harvested and seeded onto a 96-well plate at 10⁵ cells/well with different treatments for 48 h. The treatments included Sham serum, HPS serum, HPS serum + Akt inhibitor, HPS serum + ERK inhibitor and HPS serum + Nrf2 siRNA. The MTT solution was prepared at a concentration of 5 mg/ml, and 20 µl of the MTT solution was added to each well for continued incubation for 4 h at 37 °C, 5% CO₂. Finally, the medium was removed, and 150 µl DMSO was added to each well following 10 min of agitation to dissolve the crystals. The absorbance at a wavelength of 490 nm was measured using an ELISA reader.

2.8. Migration assays

Transwell plates with 6.4 mm diameter inserts with 8 µm pore filters were used in this migration assay. The cells were randomly divided into three groups according to their

various stimulations: the control group (10% CBDL rat serum), the HPS group (10% CBDL rat serum), the HPS + Akt inhibitor group, the HPS + ERK inhibitor group and the HPS + Nrf2 siRNA group. After preconditioning for 24 h, these cells were added to the upper chamber of the inserts into 200 μ l of DMEM containing 0.1% BSA, and the lower chambers were loaded with 500 μ l of DMEM containing 10% FBS and SDF-1 (100 ng/ml). Following incubation for 8 h, the cells in the upper chamber were wiped, and the cells that migrated to the bottom surface were stained and then counted in at least five random separate fields (100-fold magnification).

2.9. ELISA analysis

The conditioned medium was collected after BM-MSCs were cultured for 48 h. The amount of vascular endothelial growth factor (VEGF) in the culture supernatants was determined using an ELISA assay kit (eBioscience), according to the manufacturer's instructions.

2.10. Statistical analysis

All data were expressed as the mean $\pm$ SD. The Bonferroni-corrected analysis of variance was conducted for multiple comparisons between groups, and Student's t-test was used for the other data. Statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. MSCs mobilization is increased in the CBDL rat

To assess the mobilization of BM-MSCs, BM and PB samples from 4 wk sham and CBDL rats were used to perform CFU-F assays. The results demonstrated that the mean value of CFU-Fs was 10.2 ± 3.6 per 10^6 cells in the PB of CBDL rats ($n=4$) compared with 1.4 ± 0.8 per 10^6 cells in the PB of sham rats ($n=4$; Fig. 1A). However, there was no significant difference between the CFU-F frequency in the BM of CBDL rats ($n=4$) and in the BM of sham rats ($n=4$), with values of 35.2 ± 7.4 and 37.8 ± 5.8 CFU-Fs per 10^6 cells, respectively (Fig. 1C). These results indicated that the number of MSCs increased in the peripheral blood of CBDL rats compared with the control may be due to the enhanced mobilization or proliferation of BM-MSCs

3.2. Characterization and identification of BM-MSCs and PB-MSCs

As seen under a light microscope, a large number of cluster cells randomly distributed on the bottom of the plates after 24 h incubation. The primary cells showed fibroblast-like morphology on day 5. They reached an 80% fusion rate, and cell colonies were found on days 7-8. To identify these cells, we performed flow cytometry, and these cells were marked positive for CD29 and CD44 but negative for CD34 and CD45 (Fig. 1C, D). These results demonstrated that the characteristics of BM-MSCs and PB-MSCs are similar.

Fig. 1. The CFU-F assays and identification of BM-MSCs and PB-MSCs. (A, B): The CFU-F frequency (mean $\pm$ SD) of cultured PB-MSCs and BM-MSCs from 4 samples of 4-wk sham and CBDL rats, * $p < 0.05$. (C, D): The flow cytometry identification of PB-MSCs and BM-MSCs. The surface markers are positive for CD29 and CD44 but negative for CD34 and CD45.

3.3. The expression of CXCR4, PCNA in BM-MSCs and its paracrine activity of VEGF are promoted by CBDL rat serum

To determine the influence of CBDL rat serum on the angiogenic related functions of BM-MSCs, we evaluate the protein levels of CXCR4, PCNA in BM-MSCs stimulated by CBDL rat serum. The western blot results showed that the protein levels of CXCR4 and PCNA, which are an indicator of directional migration and proliferation, respectively, were significantly increased in a time dependent manner (Fig. 2A, B). Consistently, the MTT assay showed that the proliferation ability of MSCs was improved by CBDL rat serum (Fig. 2C). In addition, we also used an ELISA to detect the VEGF level in the conditioned medium of BM-MSCs with or without CBDL rat serum at various time points. We found that the VEGF level in the HPS group was 185% compared with the control group after 48 h treatment, and the amount of VEGF in the HPS medium without BM-MSCs is similar with that of the control medium, which is significantly lower than in the BM-MSCs conditioned medium (Fig. 2D). These results indicated that the directional migration, proliferation and paracrine activity of BM-MSCs were promoted by CBDL rat serum, which is reflected by the increased levels of CXCR4, PCNA and VEGF.

Fig. 2. The expression of CXCR4 and PCNA, the MTT OD value and the VEGF secretion of BM-MSCs increased following treatment with CBDL rat serum. (A): The changes in the expression levels of CXCR4 and PCNA in MSCs as determined by Western blotting after treatment with CBDL rat serum. (B): The protein expression levels of CXCR4 and PCNA are presented as the mean $\pm$ SD. $n=5$. * $P<0.05$ and # $P<0.05$ vs. 0 h. (C): The MTT assay showing the OD value of MSCs cultured for 12 h, 24 h and 48 h. (D): The VEGF secreted by BM-MSCs after treatment with CBDL rat serum was assessed via ELISA. * $P<0.05$ vs. control group and # $P<0.05$ vs. between the indicated groups.

3.4. The overexpression of phospho-Akt, -ERK and Nrf2 was induced by CBDL rat serum exposure

To define whether Akt, Erk or Nrf2 is activated in BM-MSCs under stimulation with CBDL rat serum, we detected the phosphorylation level of Akt and Erk and the protein level of Akt, Erk and Nrf2. We found that the protein level of Nrf2 increased in BM-MSCs treated with CBDL rat serum as the exposure time increased (12 h, 24 h, 48 h) (Fig. 3A). The results of western blots also demonstrated that the protein levels of Akt and Erk were not obviously altered after being treated with CBDL rat serum. However, the expression of phospho-Akt and phospho-ERK and the ratios of p-Akt/Akt and p-Erk/Erk were significantly increased following exposure to the CBDL rat serum (Fig. 3B, C). These findings show that Akt, Erk and Nrf2 were activated in BM-MSCs upon stimulation with CBDL rat serum.

Fig. 3. The expression of phospho-Akt, phospho-ERK, and Nrf2 in BM-MSCs was up-regulated by CBDL rat serum in a time dependent manner. (A): The changes in protein level of Nrf2 for each time point were determined in both the control and HPS groups. (B, C): The ratios of p-Akt/Akt and p-ERK/ERK were increased. Values are displayed as the mean $\pm$ SD. n=5. *P<0.05 vs. control group and #P<0.05 vs. 12h HPS group.

3.5. The Akt/Nrf2 signalling pathway mediated the enhanced proliferation, migration and paracrine effect of BM-MSCs induced by CBDL rat serum

Nrf2 activation is known to be regulated by several kinases, and in our previous study, we found Akt and ERK are significantly activated in lung tissues of CBDL rats. Thus, in this study, we examined the effects of Akt and ERK activation on the Nrf2 level in BM-MSCs upon stimulation with CBDL rat serum. We found that the protein level of Nrf2 and Nrf2 nuclear translocation in BM-MSCs induced by CBDL serum is obviously reduced by the Akt inhibitor LY294002, whereas the ERK inhibitor PD98059 caused no effect (Fig. 4A-C). We also found that the expression of CXCR4 and PCNA in the HPS group decreased after knocking down Nrf2; however, knocking down Nrf2 had a limited effect on the expressions of CXCR4 and PCNA in control group (Fig. 5A, B). In the MTT assay, the OD value of the HPS group was 195% of the control value and 220% of the value of the HPS serum + Nrf2 siRNA group, which demonstrated that CBDL rat serum promoted the proliferation of BM-MSCs. Additionally, the OD value of the HPS serum + Akt inhibitor group was 62% of the value of the HPS group, but there was no significant difference between the HPS + ERK inhibitor group and the HPS group (Fig. 6A). To explore the role of the Akt/Nrf2

signalling pathway in the improved paracrine effect of BM-MSCs, we detected the VEGF level. VEGF is an important cytokine for angiogenesis that could be secreted by BM-MSCs. The ELISA results revealed that the VEGF level in the HPS group was 173% compared with the control group and decreased when the activation of Nrf2 or Akt was inhibited. However, the amount of VEGF in the HPS + ERK group was similar to the HPS group (Fig. 6B). The transwell results also showed that BM-MSCs, when pretreated with CBDL rat serum, exhibited improved migration ability towards SDF-1 compared with the control group, and this enhanced migration could be attenuated by knocking down Nrf2 or inhibiting Akt instead of ERK (Fig. 6C, D). These results indicated that CBDL rat serum promoted the angiogenic related functions of BM-MSCs through the Akt/Nrf2 pathway.

Fig. 4. The increased expression and nuclear translocation of Nrf2 is associated with Akt activation (A, B): BM-MSCs were pretreated with 20 μ M LY294002 or PD98059 for 1 h and then exposed to CBDL rat serum for 48 h; the CBDL rat serum-induced overexpression of Nrf2 was significantly decreased by LY294002, whereas no effect was

found for the ERK inhibitor PD98059. (C): The levels of Nrf2 nuclear translocation was evaluated by immunofluorescence staining. Nrf2 are stained with Alexa Fluor 555 (red); The nuclei are stained with DAPI (blue). Values are displayed as the mean $\pm$ SD, $n=5$. * $P<0.05$ vs. LY294002 treatment group, # $P<0.05$ vs. the indicated groups. * $P<0.05$ vs. Group C + si Nrf2 and # $P<0.05$ vs. Group HPS + si Nrf2.

Fig. 5. The expression of Nrf2 is partially associated with the increased level of CXCR4 and PCNA induced by CDBL rat serum. (A, B): The levels of Nrf2 were evaluated by Western blot analysis. The CDBL rat serum increased the CXCR4 and PCNA protein levels, and the levels decreased when Nrf2 was knocked down in BM-MSCs. Values are displayed as the mean $\pm$ SD. * $P<0.05$ vs. Group C + si Nrf2 and # $P<0.05$ vs. Group HPS + si Nrf2.

Fig. 6. The Akt/Nrf2 signalling pathway mediated the enhanced directional migration, proliferation and paracrine ability of BM-MSCs induced by CDBL rat serum. (A): The MTT

assay showing the OD values (490 nm) of BM-MSCs cultured for 48 h in each group. (B): The VEGF concentrations in the conditioned media were determined by ELISA. (C): The lower chamber of the transwell plates under a light microscope for each group. (D): The numbers of BM-MSCs that migrated to the lower chamber with different treatments. All values are presented as the mean $\pm$ SD, n=4. *P<0.05 vs. control group, #P<0.05 vs. HPS group.

Discussion

Pulmonary angiogenesis is one of the important pathogenic features of HPS, and animal experiments have confirmed that anti-angiogenic agents could improve the oxygenation of HPS rats [3, 4]. It has also been reported that BM-MSCs could be mobilized in response to chronic hypoxia, tissue injury, and cytokines, including VEGF and HGF [7, 8, 25]. SDF-1 has been confirmed to mediate the VEGF-dependent mobilization of myeloid cells to the blood, and then the mobilized cells are attracted to angiogenic vessels by SDF-1 [26], which is mainly secreted by vascular endothelial cells and perivascular myofibroblasts [27, 28]. This could be up-regulated by hypoxia. Although the circulating progenitor cells which are recruited by the VEGF-SDF1 loop have not been completely identified, it is possible that MSCs are responsible for this process via the SDF-1 receptor CXCR4 because CXCR4 overexpression in MSCs strongly promoted their migration to injured lungs [29]. Furthermore, the surface expression of CXCR4 on MSCs increased in culture by adding a cocktail of cytokines, which leads to its enhanced homing ability [30]. There are also many reports documenting the contribution of MSCs to stromal architecture, blood- and lymph-vessel formation and the production of growth factors or chemokines [31, 32]. Interestingly, the improved migratory capacity of MSCs for SDF-1 augments myoangiogenesis in the infarcted myocardium [33]. Moreover, Liotta F et al. found that the MSC cell population is enriched in head and neck tumours when compared with control healthy tissue and the population correlates with tumour size [34]. Several studies have reported that MSCs could be isolated from adult PB in mammals using culture conditions that are the same as those used for BM-MSCs and the MSCs present in PB are similar to BM-MSCs [35-37]. In this study, a significant increase in the CFU-F frequency was observed in the PB of CBDL rats compared with sham rats; however, the CFU-F frequency in BM was unchanged. We suppose this may be due to two reasons. First, the microenvironment in the BM and PB may be different; second, both the proliferation and migration ability of MSCs were promoted in CBDL rats, which lead to proliferative cells entering into the peripheral blood quickly. We also observed that CBDL rat serum increased the surface expression of the homing CXCR4 receptor on BM-MSCs, resulting in the increased migration ability for SDF-1 *in vitro*. Moreover, we found that the proliferation and paracrine ability of MSCs is promoted by CBDL rat serum.

In our previous study, we found that the Akt and Erk pathways, which are widely studied in cell survival, are significantly activated in the lung tissues of CBDL rats [10]. A recent study reported that hypoxia pretreatment of BM-MSCs promoted angiogenesis in ischaemic muscle by enhancing its paracrine capability, and this effect may lead by the enhanced expression of p-Akt [13]. Therefore, we hypothesized that the promoted angiogenic functions of BM-MSCs, which are stimulated by CBDL rat serum, were associated with the Akt or the Erk pathway. We also focused on finding the key regulator downstream of these pathways that may responsible for this effect. The overexpression of Nrf2 in MSCs inhibits their apoptosis under oxidative stress [38], and heme oxygenase-1, which can be activated by Nrf2, is indispensable for the paracrine effect of MSCs in cisplatin-induced renal injury [39]. It also has been revealed that the PI3K/Akt/Nrf2 pathway plays an important role in the anti-oxidation of EA.hy926 endothelial cells [40]. However, whether the Akt/Nrf2 pathway is involved in the CBDL-rat serum induced alteration of the function

of MSCs has not been confirmed.

The data presented in this study showed that the directional migration and the proliferation ability of BM-MSCs were promoted by CBDL-rat serum *in vitro*, as determined by the transwell migration assay and the MTT assay. This effect was also reflected by the increased protein levels of CXCR4 and PCNA. In addition, the secretion of VEGF by BM-MSCs increased in response to the stimulation of CBDL-rat serum *in vitro*. We also observed the increased expression of phospho-Akt, phospho-ERK and Nrf2. We found that these promoted functions of BM-MSCs were attenuated by blocking the Akt/Nrf2 pathway, instead of the ERK/Nrf2 pathway. The results of the current study have two implications, which are as follows: First, BM-MSCs may be involved in the pulmonary angiogenesis of HPS because of their increased mobilization in the CBDL rat model and enhanced angiogenic functions induced by CBDL rat serum *in vitro*. Second, pulmonary angiogenesis of HPS may be an overreaction to oxidative or inflammation in HPS because of the overexpression of Nrf2. However, our research has some limitations. First, we did not provide direct evidence that BM-MSCs participate in the process of pulmonary angiogenesis in HPS. Thus, further *in vivo* testing needs to be performed in future studies. Second, our studies were carried out in a rat model, and the actual situation in clinical patients may be different.

Competing Interests

The authors declare no competing interests.

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