Vasohibin attenuates bleomycin induced pulmonary fibrosis via inhibition of angiogenesis in mice

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Summary

Aims: Much evidence suggests that vascular remodelling in the lung plays a crucial role in the development of pulmonary fibrosis. Therefore, anti-angiogenesis therapy may be a promising treatment for pulmonary fibrosis. Recently, a new inhibitor called vasohibin has been discovered to operate as an intrinsic and highly specific feedback inhibitor in the process of angiogenesis. However, to date, the effect of vasohibin on anti-angiogenesis of pulmonary fibrosis has not been examined.

Methods: In this study, we utilised vasohibin to test the potential of pulmonary fibrosis therapy. We examined the role of vasohibin in the pathophysiology of bleomycin-induced pneumopathy in mice by transfection of the vasohibin gene.

Results: The results demonstrated that transfection of the vasohibin gene could attenuate pulmonary fibrosis via inhibition of angiogenesis, which markedly decreased lymphocyte infiltration, cytokine secretion and fibroblast proliferation.

Conclusions: This method may be beneficial for treating lung fibrosis and may provide a novel strategy for clinical application in the future.

Key words: Vasohibin, pulmonary fibrosis, angiogenesis.

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive disease characterised by fibrosis and remodelling of the lung parenchyma.1–5 The median survival of patients with the disease is about 3 years following diagnosis or 5 years following the onset of symptoms.6–8 The traditional therapeutic strategy is the administration of a cytotoxic agent, such as azathioprine.9–11 However, there is little evidence that these agents alter the natural history of the disease and there is no strong evidence supporting the clinical effectiveness of these treatments in patients with IPF. Therefore, novel therapeutic approaches are greatly needed.

Angiogenesis is considered an important factor in the development of fibrotic lung disease.12–14 Prior studies have strongly suggested a role for angiogenic vascular remodelling in pulmonary fibrosis, and emerging evidence indicates that new vessel formation is critical in airway fibrosis.15,16 For example, studies have shown that fibroblast specific expression of vascular endothelial growth factor (VEGF)-C promotes collagen constriction by fibroblasts and enhances microvascular endothelial cell migration, branching and capillary sprouting, and the overexpression of VEGF-C enhances this fibroblast mediated vasogenic effect.17,18

Recently, vasohibin was firstly identified to be an endothelium derived negative feedback regulator of angiogenesis. It was proved that the novel angiogenesis inhibitor was induced in endothelial cells stimulated by angiogenic factors and inhibits angiogenesis in an autocrine manner.20,21 The recombinant protein inhibited migration, proliferation, and network formation by endothelial cells as well as angiogenesis in vivo.22,23 However, to date, whether vasohibin can inhibit angiogenesis in fibrotic lung disease is still unknown. In the present study, we tested the possibility of vasohibin to inhibit angiogenesis and attenuate bleomycin induced pulmonary fibrosis. The results demonstrated that transfection of the vasohibin gene could attenuate pulmonary fibrosis via inhibition of angiogenesis, which markedly decreased lymphocyte infiltration, cytokine secretion and fibroblast proliferation. Thus, this approach may provide a promising way of treating lung fibrosis and a novel strategy for clinical application in the future.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from the Laboratory Animal Center (Third Military Medical University, Chongqing, China) and were used at ages 8–10 weeks. Animals were bred in the Laboratory Animal Center and the studies were performed in agreement with the local ethics committee of the Third Military Medical University.

Construction of recombinant adenovirus encoding vasohibin

The recombinant adenovirus vector encoding vasohibin was constructed using the Adeno-XTM Expression System (Clontech, USA) according to the manufacturer’s instructions. Briefly, the vasohibin cDNA was cloned into the shuttle vector pDC315 and sequenced. The desired replication-deficient adenovirus containing the full length cDNA of vasohibin was generated by homologous recombination through co-transfection of plasmids pDC315-vasohibin and pBHGI0XE1, 3Cre in HEK 293 cells using the DOTAP liposome reagent (Roche, Germany). After several rounds of plaque purification, the adenovirus containing the vasohibin gene was amplified and purified from cell lysates by banding twice in CsCl density gradients. Viral products were desalted and stored at −80°C in phosphate-buffered saline (PBS) containing 10% glycerol (v/v). The infectious titer was determined by a standard plaque assay. A second recombinant, E1-,E3-deleted adenovirus carrying the LacZ protein under the control of CMV promoter (Ad-LacZ), was used as a control vector.
Adenovirus mediated gene transfer and Western blot assay
Transduction of COS-7 cells with Ad-vasohibin was conducted in six-well plates, six with $1 \times 10^6$ COS-7 cells/well in 3 mL of RPMI1640 medium containing 10% fetal bovine serum (FBS). Virus was added to the wells at a multiplicity of infection (MOI) of 200 and the COS-7 cells were harvested after 24 h of incubation. For Western blot assay, proteins of the cell extracts were separated by sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. The membrane was incubated with 5% non-fat milk in PBS and then with anti-vasohibin antibody (Santa Cruz Biotechnology, USA) for 2 h at room temperature. After washing, the membranes were incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Amersham Biosciences, UK) for 1 h at room temperature. Immunoreactive bands were detected using the ECL Western blotting analysis system (Amersham Biosciences).

Bleomycin induced pulmonary fibrosis model
C57BL/6 mice were anaesthetised with an i.p. injection of pentobarbital sodium. The anaesthetised mice received 50 μL of bleomycin hydrochloride solution containing 1.5 U of bleomycin/kg body weight in sterile saline intratracheally. The mice were sacrificed at 14 days after the bleomycin instillation, and the lung tissues were fixed in 10% buffered formalin.

Systemic delivery of vasohibin
Mice received an intravenous tail vein injection of Ad-vasohibin [$1 \times 10^6$ plaque-forming units (PFu)] or Ad-LacZ ($1 \times 10^7$ PFu). The control mice received 100 μL PBS. The mice were sacrificed at 14 days after the bleomycin instillation. To assess the effect of the vasohibin gene transfection on the early inflammatory phase and the late fibrotic phase in bleomycin-induced pneumopathy, Ad-vasohibin or Ad-LacZ to the controls were administered 7 days before or 3 days after the bleomycin instillation.

Western blotting analysis of VEGF level
Frozen lungs were homogenised in hypotonic buffer containing 25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, 1 mg/mL leupeptin, and 1 mg/mL aprotinin using a polytron homogeniser. The homogenates were centrifuged at 15,000 g × g for 30 min at 4°C, and the supernatants were assayed for the VEGF by Western blotting analysis. The protein concentrations of the supernatants prepared above were determined using the Bio-Rad protein assay. In accordance with the protocols mentioned above, COS-7 cells were transfected with Ad-vasohibin or Ad-LacZ at MOI 200 for 24 h. As seen in Fig. 1, the expression of the vasohibin protein was detected after Ad-vasohibin transfection. However, there was no expression of vasohibin protein after Ad-LacZ transfection and in non-treated COS-7 cells.

Histopathological examination
After thoracotomy, the pulmonary circulation was flushed with saline and the lungs were explored. The lung samples were fixed with 10% formalin overnight and embedded in paraffin. Paraffin sections were adhered to slides and stained with haematoxylin and eosin (H&E). The pathological grade of inflammation and fibrosis was evaluated and determined according to the following criteria: 0, no lung abnormality; 1, presence of inflammation and fibrosis involving <25% of the lung parenchyma; 2, lesions involving 25-50% of the lung; and 3, lesions involving >50% of the lung.

Immunohistochemistry analysis of CD31 cells
Following deparaflinisation, immunohistochemistry was performed by a modified streptavidin-biotinylated peroxidase technique. Briefly, non-specific protein staining was blocked with rabbit serum for 30 min at room temperature. The sections were incubated with an anti-CD31 Ab (Santa Cruz Biotechnology, USA) at 37°C overnight. The sections were then incubated with a biotinylated secondary Ab for 30 min, before treatment with 0.3% hydrogen peroxide in methanol for 30 min to inhibit any endogenous peroxide activity. The slides were incubated with streptavidin-biotin-peroxidase complexes for 30 min and observed. In addition, microvessel density (MVD) analysis was determined by counting the number of microvessels per high power field in the sections.

Hydroxyproline assay
Samples of the lung tissue were frozen in liquid nitrogen, weighed and minced into a fine homogeneous mixture, and hydrolysed with 6 N HCl for 16 h at 120°C. The hydroxyproline content of each sample was determined according to the protocol of Woessner. A tracheotomy was performed in sacrificed mice. After insertion of a tracheal tube, the trachea was lavaged five times with 1 mL volumes of sterile saline at room temperature. The recovered fluids were filtered through a single layer of gauze to remove the mucus. The cells present in the lavage fluid were counted using a haemocytometer. The protein concentrations were determined using the Bio-Rad protein assay.

Enzyme linked immunosorbent assay (ELISA) of BALF
BALF was centrifuged, and supernatant was analysed with ELISA assay. MIP-2 level in BALF was measured with a cytokine specific ELISA (eBioscience, USA), and TNF-α level was measured with ELISA (eBioscience).

Statistics
The statistical significance of different findings between experimental groups and controls was determined by t-test and considered significant if $p < 0.05$.

RESULTS
Gene transfer and vasohibin protein expression
Protein expression of vasohibin was demonstrated by transient transfection of adenovirus into COS-7 cells and was detected by Western blot assay. In accordance with protocols mentioned above, COS-7 cells were transfected with Ad-vasohibin or Ad-LacZ at MOI 200 for 24 h. As seen in Fig. 1, the expression of the vasohibin protein was detected after Ad-vasohibin transfection. However, there was no expression of vasohibin protein after Ad-LacZ transfection and in non-treated COS-7 cells.

VEGF expression in lung tissue after bleomycin administration
Ad-vasohibin or Ad-LacZ to the controls were administered 7 days before or 3 days after the bleomycin instillation. The mice were sacrificed 14 days after the treatment.
bleomycin instillation. The VEGF concentration of the lung tissue homogenate was detected. As Fig. 2 shows, VEGF expression of the lung tissue was up-regulated 14 days after the bleomycin instillation. Ad-vasohibin could decrease the VEGF expression; however, the Ad-LacZ control had no effect. Three repeated experiments showed consistent results.

**Histopathology of lung tissue after bleomycin administration**

Ad-vasohibin or Ad-LacZ to the controls were administered 7 days before or 3 days after the bleomycin instillation. The mice were sacrificed 14 days after the bleomycin instillation. The lungs were explored after thoracotomy. The lung samples were fixed with 10% formalin overnight and embedded in paraffin. Paraffin sections were adhered to slides and stained with H&E. As Fig. 3 shows, a large number of lymphocytes had infiltrated into the lung interstitium, and a proliferation of fibroblasts was observed after the bleomycin instillation. The Ad-vasohibin markedly decreased the lymphocyte infiltration and fibroblast proliferation; however, the Ad-LacZ control did not affect the histopathological findings. In addition, semiquantification of the histological analysis showed that Ad-vasohibin significantly decreased the pathological grade compared with control groups. Three repeated experiments showed consistent results.

**Immunohistochemical analysis of CD31**

To explore whether the Ad-vasohibin could inhibit angiogenesis of bleomycin induced pulmonary fibrosis, Ad-vasohibin or Ad-LacZ to the controls were administered 7 days before or 3 days after the bleomycin instillation. The mice were sacrificed 14 days after the bleomycin instillation. The lung samples were fixed with 10% formalin overnight and embedded in paraffin. The paraffin sections were incubated with an anti-CD31 Ab for immunohistochemical analysis. As Fig. 4 shows, increased angiogenesis was observed after the bleomycin instillation, and the Ad-vasohibin markedly decreased the angiogenesis; however, the Ad-LacZ control did not affect the histopathological findings. Three repeated experiments showed consistent results.

**Effect of Ad-vasohibin on lung fibrosis**

To assess whether Ad-vasohibin could decrease the lung fibrosis, hydroxyproline in lung tissue was compared. Ad-vasohibin or Ad-LacZ to the controls were administered 7 days before or 3 days after the bleomycin instillation. The mice were sacrificed 14 days after the bleomycin instillation. Samples of the lung tissue were frozen in liquid nitrogen, weighed and minced into a fine homogeneous mixture, and hydrolysed at 120°C. The hydroxyproline content of each sample was determined according to the protocol of Woessner. As Fig. 5 demonstrates, Ad-vasohibin significantly reduced the content of hydroxyproline in lung tissues compared with control groups 14 days after the bleomycin instillation. Three repeated experiments showed consistent results.

**Effect of Ad-vasohibin on BALF**

To assess whether Ad-vasohibin could also decrease cell numbers and protein concentrations of BALF, Ad-vasohibin or Ad-LacZ to the controls were administered 7 days before or 3 days after the bleomycin instillation. The mice were sacrificed 14 days after the bleomycin instillation. The cells present in the lavage fluid were counted using a haemocytometer and the protein concentrations were determined using the Bio-Rad protein assay. In addition, BALF was centrifuged, and supernatant was analysed with
ELISA assay. The MIP-2 and TNF-α levels in BALF were measured with a cytokine specific ELISA. As Fig. 6 and 7 show, Ad-vasohibin significantly reduced the cell numbers, protein concentrations, and MIP-2 and TNF-α levels of BALF compared with control groups 14 days after bleomycin instillation. Three repeated experiments showed consistent results.

**DISCUSSION**

In this experiment, we found evidence that vasohibin attenuated bleomycin induced pulmonary fibrosis via inhibition of angiogenesis in mice, which had not been studied by other research groups.

Previous studies directed at understanding the pathogenesis of IPF have focused primarily on mechanisms related to fibroplasia and deposition of extracellular matrix. However, these investigations have often been performed at end-stage fibrosis when the extracellular matrix and the non-viable scar have replaced the cellular phase of IPF. Few studies have addressed the importance of angiogenesis in the lung during injury and subsequent fibrosis. Although angiogenesis has been shown to play a role in the evolution of tissue repair and fibroplasia associated with acute lung injury and sarcoidosis, the contribution of neovascularisation to the pathogenesis of fibrosis in IPF has been largely ignored until recently. The existence of morphological neovascularisation in IPF was originally identified by Turner-Warwick, who performed post-mortem studies on the lungs of patients with widespread IPF and demonstrated neovascularisation/vascular remodelling that was often associated with anastomoses between the systemic and pulmonary microvasculatures. In addition, further evidence also demonstrated that the balance between...
Angiogenic and angiostatic factors is central to the pathogenesis of pulmonary fibrosis. Angiogenic factors include VEGF and its family members FGF-2 and HGF, and inflammatory cytokines such as TNF-α and IL-8. VEGF is the principal angiogenic factor; it is induced by hypoxia and stimulates the migration and proliferation of endothelial cells. In addition, negative feedback regulation is one of the most important physiological mechanisms with which bodies are endowed, and has been demonstrated to control a wide range of phenomena. However, no such regulators have been established for the regulation of angiogenesis to date.

Recently, a novel angiogenesis inhibitor was identified that is induced in endothelial cells by angiogenic factors and inhibits angiogenesis in an autocrine manner; this was designated as vasohibin. Further study demonstrated that the recombinant vasohibin protein inhibited migration, proliferation, and network formation by endothelial cells as well as angiogenesis in vivo. Therefore, vasohibin might theoretically act as a potential target for anti-angiogenesis therapy. In this experiment, we constructed a recombinant adenovirus encoding vasohibin and assessed its anti-angiogenesis efficiency in the bleomycin induced pulmonary fibrosis model.

In this study, we used an E1,E3-deleted adenoviral vector containing the full length vasohibin gene under the CMV promoter. The results demonstrated that this is an adenovirus vector strategy that provides a highly efficient reproducible method of gene transfer. Western blot assay demonstrated that expression of the vasohibin protein in Ad-vasohibin transduced Cos-7 cells was significantly increased compared with that of untreated Cos-7 cells or Ad-LacZ transected Cos-7 cells.

To assess the effect of Ad-vasohibin on VEGF and angiogenesis after bleomycin instillation, the determination of VEGF concentration as well as immunohistochemical analysis of the lung tissue were performed. The results demonstrated that Ad-vasohibin decreased VEGF expression and lung angiogenesis, while the Ad-LacZ control had no effect.

Furthermore, to explore the effect of Ad-vasohibin on lung fibrosis after bleomycin instillation, histopathological examination, hydroxyproline assays and BALF were performed. All data suggested that Ad-vasohibin could markedly decrease lymphocyte infiltration, cytokine secretion and fibroblast proliferation; however, the Ad-LacZ control had no effect on the histopathological findings.

In summary, our findings support the idea that administration of Ad-vasohibin could effectively attenuate bleomycin induced pulmonary fibrosis via inhibition of angiogenesis. Accordingly, these results suggest that recombinant adenovirus encoding vasohibin targeting the regulation of angiogenesis may represent a viable therapeutic option for the treatment of pulmonary fibrosis in the clinic.

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