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Inhibition of endocan attenuates monocrotaline-induced connective tissue disease related pulmonary arterial hypertension

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ABSTRACT

Connective tissue disease related pulmonary arterial hypertension (CTD-PAH) is characterized by vascular remodeling, endothelial dysfunction and inflammation. Endocan is a novel endothelial dysfunction marker. The aim of the present study was to investigate the role of endocan in CTD-PAH. Monocrotaline (MCT)-induced PAH rats were used as the CTD-PAH model. Short hairpin RNA packed in a lentiviral vector used to inhibit endocan expression was intratracheally instilled in rats prior to the MCT injection. Endocan was found to be increased in the serum and lung of MCT-induced PAH rats. Short hairpin RNA mediated knockdown of endocan significantly decreased right ventricular systolic pressure, attenuated pulmonary remodeling and inflammatory responses in the lung. In the in vitro study, tumor necrosis factor-α (TNF-α) exposure caused increased endocan expression in the primary cultured rat pulmonary microvascular endothelial cells (RPMECs). Endocan knockdown inhibited the permeability increase and adhesion molecules secretion in RPMECs induced by TNF-α. In addition, TNF-α induced MAPK activation was blocked when endocan gene was knocked down. These data demonstrate that endocan may play an important role in the development of CTD-PAH. This study provides novel evidence to better understand the pathogenesis of CTD-PAH, which may be beneficial for the treatment of this disease.

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1. Introduction

Pulmonary arterial hypertension (PAH) is a syndrome characterized by increased mean pulmonary arterial pressure (mPAP). PAH develops pulmonary vessel remodeling and changes the features of hemodynamic, and finally leads to right heart failure. Connective tissue disease (CTD) is closely associated with PAH. Patients with CTD are at increased risk for developing PAH [1]. The prevalence of PAH in systemic sclerosis, a CTD that is most commonly associated with PAH, is 7.85–13% of patients [2–4]. Moreover, CTD-related PAH (CTD-PAH) has a poor prognosis. In China, the survival rates of patients with CTD-PAH are inferior to those of patients with idiopathic PAH [5]. However, the pathogenesis of CTD-PAH has not been fully revealed yet. To investigate the possible molecular mechanisms of CTD-PAH would be beneficial for the treatment of this disease.

Endothelial cell (EC) dysfunction is one of the pathological processes that contribute to the development of PAH. ECs are essential for vascular structure and functions. For example, ECs produce a number of cytokines that regulate the physical functions of pulmonary vessels, such as angiogenesis, vasoconstriction and vasodilatation. EC dysfunction results in imbalance of these cytokines, induces vasoconstriction and smooth muscle cells hypertrophy, and finally leads to vascular remodeling [6]. ECs also protect the smooth muscle cells and fibroblasts of the vessel from exposing to injurious factors. Damage of EC barrier integrity leads to vascular tissue cells expose to the excessive cytokines induced by stimulus and facilitates the injury.

Increasing evidence has accumulated indicating that inflammation plays an integral role in the pathogenesis of PAH [7]. Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine secreted by macrophages in response to inflammatory stimuli. Mice with overexpression of TNF-α showed right ventricle hypertrophy and pulmonary hypertension [8], and TNF-α antagonist etanercept attenuate monocrotaline (MCT)-induced PAH [9,10]. In addition, TNF-α also plays a key role in the progress of CTD. TNF-α antagonists are widely used in patients with various CTD such as arthritis, systemic sclerosis, and ankylosing spondylitis [11–14].

Endocan, previously known as endothelial cell specific molecule-1 (ESM-1), is a soluble dermatan sulfate proteoglycan which was first cloned from human umbilical vein endothelial cell (HUVEC) cDNA library in 1996 [15]. Endocan serves as a regulator of vascular cell processes such as proliferation, adhesion and migration [15,16]. Endocan is considered as a novel EC dysfunction marker because it is mainly secreted by EC and its alteration often occurs in conditions characterized

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by endothelial dysfunction. Till now, dysregulation of endocan has been found in cancers [17,18], sepsis [19], cardiovascular diseases [20,21] and diabetic retinopathy [22]. However, at best of our knowledge, no study has reported the role of endocan in CTD-PAH. In the present study, virus packed endocan- short hairpin RNA (shRNA) was injected into MCT-induced rats, a CTD-PAH model, to investigate the role of endocan in this disease. In addition, TNF-α-induced primary pulmonary microvascular endothelial cells were also used to investigate possible underlying mechanisms in vitro.

2. Materials and methods

2.1. Animals

All animal studies were approved by the ethics committee of the China Medical University. Male Sprague-Dawley (SD) rats weighing 180–220 g were obtained from Laboratory Animal Center of China Medical University, (Shenyang, China). The rats were maintained in a temperature and humidity-controlled room (21– 22 °C, 75– 80%) with a 12/12-h light/dark cycle and had free access to standard rat chow and water ad libitum. The animals were randomly divided into: 1) control group (Con, $n = 10$); 2) MCT-induced pulmonary arterial hypertension group (PAH, $n = 10$); 3) PAH and scramble shRNA group (PAH + scr shRNA, $n = 10$); and 4) PAH and endocan shRNA group (PAH + endo shRNA, $n = 10$). The *EGFP*-encoding lentiviral strain carrying the shRNA oligonucleotides that target 5′-GGTGACGAGTTTGGTGTC-3′ on endocan mRNA or a scramble shRNA with the sequence of 5′-TTCTCCGAACGTGTCACGT-3′ were obtained from Hanbio Co., Ltd. (Shanghai, China). Endocan shRNA or scramble shRNA was were administrated to the rats in the shRNA groups by intratracheal instillation through the mouth daily for 6 days (in total 1.5×10^8 transducing units in 300 μl PBS, 50 μl per day). Rats in the PAH groups received a single subcutaneous injection of 60 mg/kg MCT (Meilun, Dalian, China) which was dissolved in a mixed solution of ethanol and saline with the volume ratio of 2:8, at day 7. Rats in the control group received the same volume of vehicle. The rats were maintained for another 21 days. The blood was collected at day 1, 7, 14 and 21 after MCT injection.

2.2. Right ventricular systolic pressure (RVSP) measurement

At day 22, the rats were anesthetized intraperitoneally with 50 mg/kg of pentobarbital (i.p.). A heparin (0.3%) filled polyethylene catheter was introduced into the right ventricle through the right jugular vein. The catheter was connected to a BL-420F biological data acquisition and analysis system (Chengdu Techman Software Co., Ltd., Chengdu, China) using a pressure transducer. The digitalized RVSP was recorded. After the hemodynamic measurement, the rats were euthanized and the blood and lung tissue were collected for further analysis.

2.3. Histological analysis

For lentiviral transduction efficiency determination in the lung, the lungs were perfused with PBS and fixed in 4% paraformaldehyde for 2 h. Next, the fixed lung tissues were frozen and cut into 10-μm-thick sections. The nucleoli were stained using 4′, 6-diamidino-2-phenylindole (DAPI) and the intensity of EGFP was observed under a fluorescence microscope (BX53, Olympus, Tokyo, Japan).

For H&E staining, the lung tissues were fixed in 4% paraformaldehyde overnight and embedded in paraffin. The paraffin blocks were cut into 5-μm-thick sections and stained with hematoxylin and eosin (H&E, Solarbio Science & Technology, Co., Ltd., Beijing, China). The sections were examined under a light microscopy. The index of pulmonary arterial wall thickness was calculated as the following formula: (external diameter-internal diameter) / external diameter × 100%.

2.4. ELISA

Lung tissue and primary endothelial cells were homogenized in cool PBS and repeated freezing in liquid nitrogen and thawing. The homogenate was then centrifuged at 12,000 g for 10 min at 4 °C. Protein concentrations in the supernatants were determined using a BCA protein assay kit (Beyotime).

The levels of endocan and TNF-α in the serum, lung tissue or cell culture medium were analyzed using commercial enzyme-linked immunosorbent assay (ELISA) kits (Boster, Wuhan, China) following the manufactures' instructions.

2.5. Isolation and culture of RPMECs

Rat pulmonary microvascular endothelial cells (RPMECs) were isolated from pulmonary arteries as previously described [23]. Briefly, male SD rats (300–350 g) were anesthetized by an intraperitoneal injection of pentobarbital sodium. After thoracotomy, the pulmonary vasculature was perfused by injection of ice-cold PBS into the right ventricle. Lungs were then removed and placed in ice-cold serum-free Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA). Thin strips were removed from the outermost surface of the lung periphery, minced and digested using 3% type II collagenase (Sigma-Aldrich, St Louis, MO, USA). The mixture was filtered through a 100 μm mesh, centrifuged at 300 ×g, and washed twice with cold PBS. The cells were resuspended in RPMI 1640 supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin.

2.6. Inhibiting endocan expression in vitro

Primary cultured RPMECs were infected with either Lenti-EGFP-endocan-shRNA or Lenti-EGFP-scramble shRNA for 12, 24 and 48 h. The infection efficiency was examined by real time PCR and Western blotting.

2.7. Permeability assay in vitro

Permeability of RPMECs was quantitated spectrophotometrically by measuring the flux of Evans blue-bound albumin across RPMEC monolayers. RPMEC layer were incubated with indicated concentration of endocan in transwell chamber for 6 h. The cells were then washed with PBS for 3 times. Subsequently, fresh culture medium was added to the lower chamber, and the medium contain 0.67 mg/ml Evens blue and 4% BSA was added in the upper chamber. The optical density at 630 nm was measured in the lower chamber after 10 min incubation.

2.8. RNA isolation and quantitative real-time PCR

Total RNA was isolated from lung tissue or RPMECs using a RNAsimple Total RNA Kit (TIANGEN Biotech, Beijing, China) following the manufacture's protocol. Complementary DNA (cDNA) was generated from total RNA using oligo-dT and Super Moloney Murine Leukemia Virus Reverse Transcriptase (BioTeke, Beijing, China). Real-time PCR reactions were each performed in a total volume of 20 μl reaction mixture, containing 1 μl cDNA, 10 μl 2 × SYBR Green Master Mix (BioTeke, Beijing, China), and 0.5 μl of each primer on an Exicycler 96 fluorescence quantitative detector (Bioneer, Daejeon, Korea). The transcript number was calculated using a $2^{-\Delta\Delta Ct}$ method with β-actin as an internal reference. The primer sequences were listed as following: endocan: forward: 3′-TTCGGTGACGAGTTTGGTG-5′, reverse: 3′-TGTTTGGGAGGCAGAGGT-5′; β-actin: forward: 3′-GGAGATTACTGCCCTGGCTCCTAGC-5′, reverse: 3′-GGCCGGACTCATCGTACTCCTGCTT-5′.

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