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CCL5 deficiency rescues pulmonary vascular dysfunction, and reverses pulmonary hypertension via caveolin-1-dependent BMPR2 activation

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a devastating cardiopulmonary disorder characterized by pulmonary arterial remodeling mainly due to excess cellular proliferation and apoptosis resistance of pulmonary arterial smooth muscle cells (PASMCs). Reduced bone morphogenetic protein receptor 2 (BMPR2) expression in patients with PAH impairs pulmonary arterial endothelial cells (PAECs) function. This can adversely affect PAEC survival and promote PASMCs proliferation. We hypothesized that interventions to normalize the expression of genes that are targets of the BMPR2 signaling could restore PAECs function and prevent or reverse PAH. Here we characterized for the first time, in human PAECs, chemokine (C-C motif) ligand 5 (CCL5/RANTES) deficiency restore BMP-mediated PAECs function.

In the cell culture experiments, we found that CCL5 deficiency increased apoptosis and tube formation of PAECs, but suppressed proliferation and migration of PASMCs. Silencing CCL5 expression in PAH PAECs restored bone morphogenetic protein (BMP) signaling responses and promoted phosphorylation of SMADs and transcription of ID genes. Moreover, CCL5 deficiency inhibited angiogenesis by increasing pSMAD-dependent and-independent BMPR2 signaling. This was linked mechanistically to enhanced interaction of BMPR2 with caveolin-1 via CCL5 deficiency-mediated stabilization of endothelial surface caveolin-1. Consistent with these functions, deletion of CCL5 significantly attenuated development of Sugen5416/hypoxia-induced PAH by restoring BMPR2 signaling in mice. Taken together, our findings suggest that CCL5 deficiency could reverse obliterative changes in pulmonary arteries via caveolin-1-dependent amplification of BMPR2 signaling. Our results shed light on better understanding of the disease pathobiology and provide a possible novel target for the treatment of PAH.

1. Introduction

Pulmonary arterial hypertension (PAH), a rare but often fatal disorder characterized by progressive obliteration of small pulmonary arteries (PAs) that leads to elevated pulmonary arterial pressure and right heart failure [1]. Most patients with severe pulmonary arterial hypertension (PAH) demonstrate persistent structural alterations in small PAs, including marked proliferation of pulmonary artery endothelial cells (PAECs), smooth muscle cells (PASMCs) and fibroblasts [2]. Current treatment strategies for PAH mainly include drugs with vasodilatory properties that improve cardiopulmonary function [3]. However, the obliterative vascular pathology usually continues to progress, leaving lung transplantation as the only option for many patients. Therefore, new approaches are required to reverse vascular remodeling. One therapeutic strategy could be to improve function of the bone morphogenetic protein receptor-2 (BMPR2) signaling.

Abbreviations: PAH, pulmonary arterial hypertension; CCL5, chemokine (C-C motif) ligand 5; CCR1, chemokine receptor 1; CCR3, chemokine receptor 3; CCR5, chemokine receptor 5; BMPR2, Bone morphogenetic protein receptor 2; RVSP, right ventricular systolic pressure; RVHI, right ventricularb hypertrophy index; SBP, systemic blood pressure; LVEDP, left ventricular end diastolic pressure; qRT-PCR, quantitative real-time polymerase chain reaction; PASMCs, pulmonary artery smooth muscle cells; PAECs, pulmonary artery endothelial cells; PAs, pulmonary arteries; WT, wild type; KO, knockout; H&E staining, Haematoxylin and Eosin staining; PCNA, proliferating cell nuclear antigen; DAPI, 4′6-diamidino-2-phenylindole; siRNA, small interfering RNA; NC, nontargeting control; siNC, nontargeting control siRNA; siBMPR2, knockdown of BMPR2 by small interfering RNA; DMEM, Dulbecco minimum essential medium; Cav1, caveolin-1; rhCCL5, recombinant human CCL5 protein; rhGremlin1, recombinant human Gremlin1 protein

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Altered BMPR2 signaling is an essential piece in the pathobiologic puzzle of PAH. Gremlin mutations causing loss of BMPR2 function are found in up to 75% of familial and approximately 25% of sporadic form of idiopathic PAH (IPAH) [4]. In addition, reduced BMPR2 expression and activation in PAs have also been reported in patients with PAH without a BMPR2 mutation or with PAH associated with other primary conditions [5]. Both PAECs and PASMCs dysfunction have been linked to dysfunctional BMPR2 signaling in the pathogenesis of PAH [6]. Loss of BMPR2 signaling promotes PAECs apoptosis, and reduced PAEC survival, which ultimately contribute to the loss of microvessels both in the clinical setting and in animal models of PAH [7]. Furthermore, decreased BMPR2 expression leads to attenuation of the angiogenic capacity of PAECs [8]. Restoring BMPR2 signaling improves PAECs survival and normal angiogenesis, and also represses proliferation and induces apoptosis of PASMCs [9,10]. Increased expression of Gremlin1, a secreted glycoprotein able to antagonize BMPR2 signaling through the binding of bone morphogenetic proteins (BMPs), was recently reported to contribute to the development of experimental pulmonary hypertension [11].

The BMPR2 signal is transduced via phosphorylation and nuclear translocation of SMAD1/5, and multiple other signaling pathways [12]. The interaction of BMP receptors with caveolin-1 (Cav1) has important implications for downstream signaling of BMPR2 [13]. Cav1 is highly expressed in vascular endothelial cells and is the major constitutive protein of caveolae, the flask-shaped plasma membrane invaginations. Progressive loss of Cav1 is observed with PAECs damage in rats with PAH and administration of Cav1 peptides reverses PAH. In patients with PAH, Cav1 is reduced in remodeled PAs and is barely detectable in plexiform lesions [14].

CCL5 (RANTES) is a member of the CC-chemokine family, a group of small proteins with chemoattractant activity that have a highly conserved tertiary structure [15]. CCL5 has a complex influence on the biology of a variety of cell types including T lymphocytes, monocytes, natural killer cells, dendritic cells, basophils, and eosinophils. In addition to these classical chemokine-activated responses, CCL5 also induces several biological effects that are unique to this chemokine [15]. Like all chemokines, CCL5 interacts with its receptors CCR1, CCR3, and CCR5 for binding and activation [16]. Several studies have explored the association of CCL5 levels with PAH [17]. CCL5 is strongly expressed on the principal cell types implicated in PAH progression, including vascular endothelial cells, smooth muscle cells, T cells, and macrophages [18,19]. Endothelial cells were the major source of CCL5 in lung tissue of patients with PAH. CCR5 pathway plays a critical role in atherosclerotic lesion formation and several studies have documented a protective effect of CCR5 inhibition in vascular lesions [20].

Our current studies describe convergence between the CCL5 and BMP signaling pathways through a novel interaction between CCL5 and BMPR2. CCL5 displays increased expression in PAECs of PAH patients with deficient BMPR2 expression. Notably, deletion of CCL5 attenuated Sugen5416/hypoxia -induced PAH and pulmonary vascular remodeling in mice through restoration of BMPR2 and activation of phosphorylation of BMP target proteins. Furthermore, siRNA-induced reduction of CCL5 levels in PAECs from patients with dysfunctional BMPR2 signaling improved PAEC survival and suppressed PASMC proliferation. The mechanism was linked to enhance BMPR2 signaling by promoting the interaction of BMPR2 with Cav1.

2. Materials and methods

2.1. Clinical samples collection

This study was approved by the Ethnic Committee for Use of Human Samples of the Nanjing Medical University (China) which was in accordance with The Code of Ethics of the Helsinki Declaration of World Medical Association for experiments involving humans. Each individual gave written informed consent prior to their participation. 10 preoperative lung tissue samples from patients with PAH (average age was 54 ± 2.3 years, mean pulmonary artery pressure was 68.2 ± 3.05 mmHg) and 10 matched healthy control samples were obtained from the Lung Transplant Group, Affiliated Wuxi People's Hospital of Nanjing Medical University (Wuxi, China) (Table 1). Paired healthy controls were collected from the donors not suitable for transplantation [21].

Table 1
Baseline clinical features of PAH patients and controls (unused donor lungs).

	Control (normal donors) (n = 10)	PAH patients (n = 10)	*P*
Age, y	50.7 ± 1.27	54 ± 2.33	0.22
Gender (%M/%F)	70/30	80/20	0.17
BMI (kg/cm^2)	20.95 ± 0.52	19.86 ± 0.49	0.14
Race	Yellow	Yellow	
mPAP (mmHg)	N/A	68.2 ± 3.05	

Definition of abbreviations: PAH, pulmonary arterial hypertension; BMI, body mass index; mPAP, mean pulmonary artery pressure (mmHg); M, male; F, femal; N/A, data not available. All values are given as mean ± SEM.

2.2. Animals and experimental protocols

BMP2-deficient ($BMP2^{-/-}$) and CCL5-deficient ($CCL5^{-/-}$) mice in background of C57/BL6 were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). $BMP2^{-/-}$ mice were crossed with $CCL5^{-/-}$ mice in order to generate $BMP2^{-/-} \times CCL5^{-/-}$ mice. For all experiments, age-matched male $BMP2^{-/-} \times CCL5^{-/-}$ mice were examined and compared to $BMP2^{-/-} \times CCL5^{+/+}$ mice as controls. The genotype was confirmed by polymerase chain reaction of tail snip DNA. All animal experimental procedures were approved by the Committee on the Ethics of Animal Experiments, Nanjing Medical University and complied with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). For the Sugen-hypoxia model of PAH, mice were given a single subcutaneous injection dose of semaxanib (Sugen5416, Sigma-Aldrich, St. Louis, MO, USA) (20 mg/kg) once weekly and then placed in chronic hypoxia (10% O_2) for 3 weeks [22]. After three weeks of hypoxia and weekly injections of Sugen5416, animals were returned to normoxia for an additional 3 weeks. Control mice were housed in room air and received vehicle (DMSO). The oxygen concentration in the chamber was maintained at 10% by controlling the flow rates of compressed air and N_2. Normoxic animals were kept at 21% O_2 adjacent to the hypoxic chamber in the same room.

2.3. Haemodynamic measurements

Assessments of right ventricular systolic pressure (RVSP) were determined by inserting the 1.4F Millar catheter (AD Instruments, Spechbach, Germany) into the right ventricle (RV) in anesthetized mice [23]. After data collection from the right ventricle, a catheter was inserted into the right carotid artery, and then advanced into the left ventricle (LV) where pressures were recorded [24]. Recordings were made a 3-min period and analyzed with Powerlab Pro Software (AD Instruments).

2.4. Assessment of right heart hypertrophy

Following haemodynamic measurements, mice were exsanguinated, lungs and hearts were perfused via the right ventricle with phosphate buffered saline (PBS). The heart was dissected free and kept on ice in PBS solution. RV hypertrophy was assessed by the ratio of the weight of right ventricle to that of the left ventricle and septum (S) [25].