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Research article

Tumor necrosis factor-related apoptosis-inducing ligand regulate the accumulation of extracelluar matrix in pulmonary artery by activating the phosphorylation of Smad2/3

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Abstract: *Introduction:* Previous studies have found that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was involved in the progression of pulmonary hypertension (PH), and TRAIL knocking (KO) has an inhibitory effect on PH, but its mechanism is not completely clear. *Methods:* The effects of TRAIL on the accumulation of extracelluar matrix (ECM), which is one of the most important processes of vascular remodeling, were observed in mice and isolated pulmonary artery smooth muscle cells (PASMCs). In vivo, mice were divided into four groups: Control group (n = 5), hypoxia-induced PH mice group (n = 8), anti-TRAIL antibody (TRAIL-Ab) treatment group (n = 8) and IgG antibody (IgG) group (n = 8). The effects of TRAIL-Ab on ECM expression in hypoxic induced PH were researched; in vivo, PASMCs were divided into three groups: Control group, hypoxia-induced group, TRAIL-Ab group. Expressions of p-Smad2/3 and p-Smad1/5/8 were compared among the three groups. *Results*: Hypoxia-induced PH mice had significant increases in right ventricle systolic pressure (RVSP) ($P < 0.001$), right ventricular hypertrophy (RVH) ($P = 0.007$), vascular stenosis ($P < 0.001$) compared with controls. Mice with anti-TRAIL antibody had lower levels in RVSP ($P < 0.001$), RVH ($P < 0.001$), vascular stenosis ($P < 0.001$) than PH mice. Besides, the TRAIL-Ab significantly inhibited the phosphorylation of Smad2/3 compared with hypoxia-induced group. *Conclusion:* TRAIL regulates the accumulation of ECM in pulmonary artery by activating pSmad2/3.

Keywords: tumor necrosis factor-related apoptosis-inducing ligand; extracelluar matrix; pSmad2/3; pulmonary hypertension; mechanism

1. Introduction

Pulmonary hypertension (PH) is a progressive, refractory and extremely malignant vascular disease, which is characterized by small pulmonary arterial stenosis and obstruction induced by vascular remodeling. As we know, this disease ultimately leads to an elevation of pulmonary artery pressure (PAP), right heart failure and finally death [1].

Pathologically, PH is characterized by pulmonary vasoconstriction, vascular remodeling, thrombosis and inflammation [2,3]. Numerous evidences demonstrate that vascular remodeling, which was observed as accumulation of extracelluar matrix (ECM), proliferation and hypertrophy endothelial cell (EC), smooth muscle cell (SMC) and adventitial fibroblast (VAF), plays a central pathological role in the progress and prognosis of PH [4–6]. The ECM is mainly composed of collagen, fibronectin, tenascin and elastin produced by the vascular wall cells especially pulmonary artery smooth muscle cells (PASMCs) [7].

TNF is a cytokine that induces apoptosis and that the TNF receptor family has plenty of molecules involved in the regulation of apoptosis [8,9]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family of cytokines, abundantly expressed in many tissues such as lung [10]. It was reported that TRAIL protein and mRNA were significantly expressed in airway wall structural cells, including fibroblasts, endothelial cells and mainly located in the smooth muscle cells of the pulmonary artery and aorta [11,12]. The expressions of TRAIL protein and its receptors, especially R3, obviously increased in pulmonary smooth muscle cells of PH patients compared to control group [13]. There is evidence that the proliferation and migration of SMCs in vitro are TRAIL-R3-dependent [14,15]. In recent years, the role of TRAIL in PH fields has been studied gradually. We have previously demonstrated that human and rodents serum TRAIL concentrations are positively related to the severity of PH [16]. Hameed AG [17] has previously demonstrated that TRAIL-KO and administration of TRAIL inhibitors can obstruct the development of PH in mammals by reducing the expression and deposition of ECM. The activation of transforming growth factor-β (TGF-β) and BMP signaling pathways play major roles in the expression of ECM [12,13,18].

However, up till now, there is no report on which signal pathway TRAIL participates in the expression and deposition of ECM in PH. Whether TRAIL is involved in PH pathological process by affecting ECM with activation of BMP or TGF-β signaling pathways remains unknown. In our study, we hope to explore the role of TRAIL in ECM deposition and its downstream signaling pathway.

2. Methods

2.1 Animals

C57/BL6 WT male mice (6–8 weeks old, purchased from Jinan Pengyue Experimental Animal Co., Ltd. (Jinan, Shandong Province, China)) were stratified into four groups: Control group (n = 5), hypoxia-induced PH mice group (n = 8), TRAIL-Ab treatment group (n = 8) and IgG group (n = 8).

Control group: The mice were exposed to normoxia; hypoxia-induced PH mice group: Exposed to hypoxia (10% oxygen and 90% nitrogen) for 3 weeks; TRAIL-Ab and IgG group: TRAIL-Ab (Catalog: AF1121, R&D Systems, Minnesota, USA) and IgG antibody (Catalog: IC108P, R&D Systems, Minnesota, USA) were delivered at 0.8 ng/g/h for 4 weeks after hypoxia using osmotic mini-pumps for 3 weeks.

2.2 Measurements of RVSP and RVH

Four weeks after TRAIL-Ab and IgG antibody pumping, all mice were anesthetized with 10% chloral hydrate (3 mL/kg). Right ventricular catheterization was performed via the right external jugular vein as previously described. Right ventricular systolic pressure (RVSP) was measured by a Power-Lab data acquisition system (AD Instruments Shanghai Trading Co.). The hearts were rapidly separated after the measurement of RVSP. Segregating the left ventricle (LV), right ventricle (RV) and ventricular septum (S) with shears then weighing them to assess RVH. The index of RVH was determined by the ratio of weight of the RV to the LV plus S [RV/(LV+S)].

2.3 HE staining and Immunofluorescence

The lungs were immediately taken out after the measurement of RVSP. The lung were washed with ice saline then perfused with 4% paraformaldehyde, embedded in paraffin, sectioned (5 μm in thickness), and stained with hematoxylin and eosin for observing the vascular remodeling, which was calculated by the following formula: Outer diameter-lumen diameter/outer diameter. Ten-fifteen vessels with a diameter of 30–80 μm in each group were randomly selected for statistical analysis. Lung tissue sections were deparaffinized antigen retrieval. Tissue sections (5 μm) were blocked by diluted normal serum firstly. Then were incubated with anti-α-SMA (Catalog: 19245; Cell Signmling Technology, Danvers, USA) followed by Alexa Fluor 488-conjugated secondary goat anti-rabbit IgG H&L (Catalog: Ab150077; ABCAM, Cambridge, USA). The sections were sealed with anti-quenching agent.

2.4 PASMC culture and hypoxia stimulation

The PASMCs (GrowGn Biotechnology Co., Ltd.) in the experiment were cultured with F12K medium under normoxia (95% O_2, 5% CO_2) or hypoxia (1% O_2, 5% CO_2, 94% N_2) for 24 hours. TRAIL-ab was added to cells during hypoxic treatment.

2.5 Biochemical measurements

Plasma TRAIL was measured in duplicate by specific, commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer's instructions and analyzed with an ELISA reader at 450 nm.

2.6 Proteins isolation and Western Blotting

Cracking on ice for 30 min after adding cell lysate and PMSF (phenylmethanesulfonyl fluoride)

to PASMCs or isolated lungs from mice. Then the liquid were centrifuged at 12000 rpm for 30 minutes at 4 ℃. The supernate was taken as protein samples. Different concentrations of SDS-PAGE gels were prepared according to the molecular weight of target protein for each equal amounts of sample protein (50 μg) then transferred to PVDF membranes. The membranes were blocked for 1 h with a buffer containing 5% skim milk powder before incubated with the following primary antibodies: Collagen I (Catalog: Ab21286; ABCAM, Cambridge, USA), Smad2/3 (Catalog: 5678; Cell Signmling Technology, Danvers, USA), p-Smad2/3 (Catalog: Sab4504208; Sigma), Smad1/5/8 (21684;SAB), p-Smad1/5/8 (AB3848-1; millipore) over night at 4 ℃. Then the membranes were incubated with goat anti-rabbit IgG (Catalog: Ab6721; ABCAM, Cambridge, USA). Proteins were performed by chemiluminescence with an ECL kit. We usually quantize the grayscale of Western Blotting figures using the software ImageJ.

2.7 Statistical analyses

Continuous data were summarized as mean ± SEM. T-test was used for analysis of two sets of quantitative data while one-way analysis of variance (ANOVA) was used for more than two sets of quantitative data. A P-value of < 0.05 was deemed statistically significant (Graphpad 5, San Diego, CA, USA; SPSS, version 16.0; SPSS inc: Chicago, IL, USA).

3. Results

3.1 Treament of hypoxia-indcued PH with TRAIL-Ab prevents the development of disease

In our previous study, the level of TRAIL was increased in PH patients and hypoxia-indcued PH mice [18]. Mice in experiment were divided into four groups: Control, hypoxia-induced PH mice, TRAIL-Ab and IgG groups. Hypoxia-induced PH mice had significant increase in RVSP (29.48 ± 1.42 versus 21.97 ± 1.29 mmhg, $P < 0.05$), RVH (34.02 ± 0.26% versus 25.46 ± 0.68%, $P < 0.05$), vascular stenosis (47.56 ± 3.34% versus 26.56 ± 3.42%, $P < 0.05$) compared to controls. Mice with anti-TRAIL antibody had lower RVSP (23.11 ± 0.84 vs. 29.48 ± 1.42 mmhg, $P < 0.05$), RVH (24.45 ± 0.29% versus 34.02 ± 0.26%, $P < 0.05$), vascular stenosis (36.21 ± 5.21 versus 47.56 ± 3.34%, $P < 0.05$) than PH mice, whereas IgG group had no significant improvement in hemodynamics and morphology (Figure 1).

3.2 TRAIL influence vascular remodeling by regulating the expression of ECM

Vasoconstriction, thrombosis and vascular remodeling are key components in the pathogenesis of PH. Synthesis and deposition of ECM play an important role in vascular remodeling. We found that collagen (a component of the ECM) increased significantly in pulmonary artery after hypoxia treatment, and reduced after treatment of TRAIL-Ab (without expression in the article). In vitro experiments, we also found that TRAIL-Ab inhibit the expression of collagen (Figure 2).

3.3 TRAIL regulates the accumulation of ECM by activating TGF- β signaling pathway

So far, many studies have shown that TRAIL plays an important role in PH. Hameed AG [17]

have demonstrated that TRAIL block the development of PH by reducing the expression and deposition of ECM. All we know that TGF-β and BMP signaling pathway participate in the expression of ECM. In our experiment, we confirmed that TRAIL-Ab inhibited the expression of ECM in hypoxic-PH mice and PSMC. So we hope to do further research on whether TRAIL interfere with PH through TGF-β and BMP pathway or not. After 24 hours of hypoxic treatment in PASMC, we found that the TRAIL-Ab significantly inhibited the phosphorylation of Smad2/3, which is the downstream signaling of TGF-β (Figure 2).

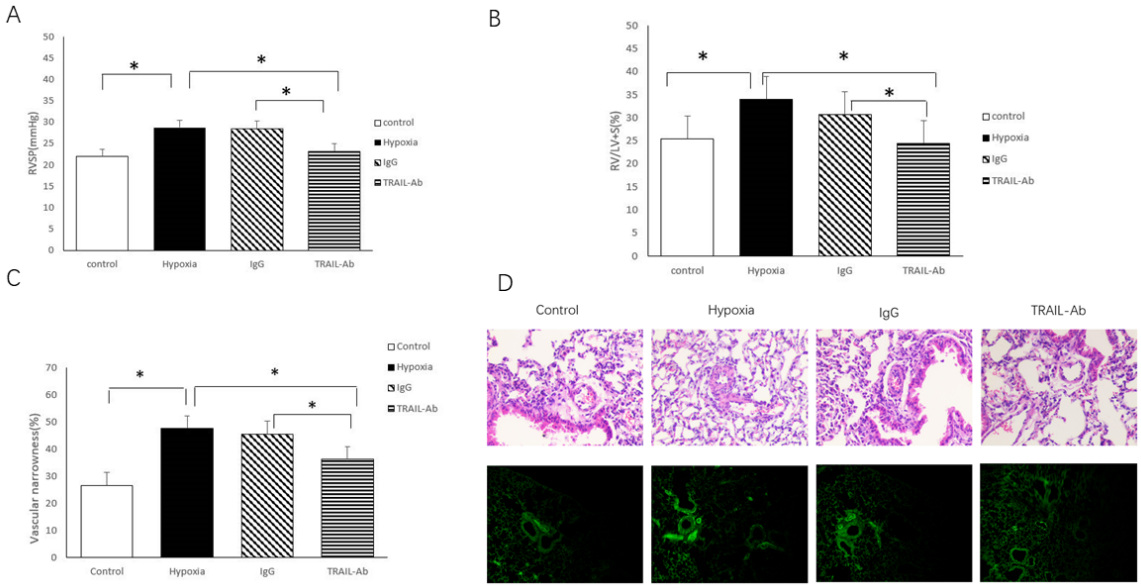

Figure 1. TRAIL-Ab prevents progression of Hypoxia-induced PH. Effect of TRAIL-Ab on RVSP (A) and RV/LV + S (B) in Hypoxia-induced PH. (C) Quantification of vascular narrowness. (D) H&E staining and immunofluorescence of lung section. Bar scale, 50 um. (*P < 0.05 was deemed statistically significant).

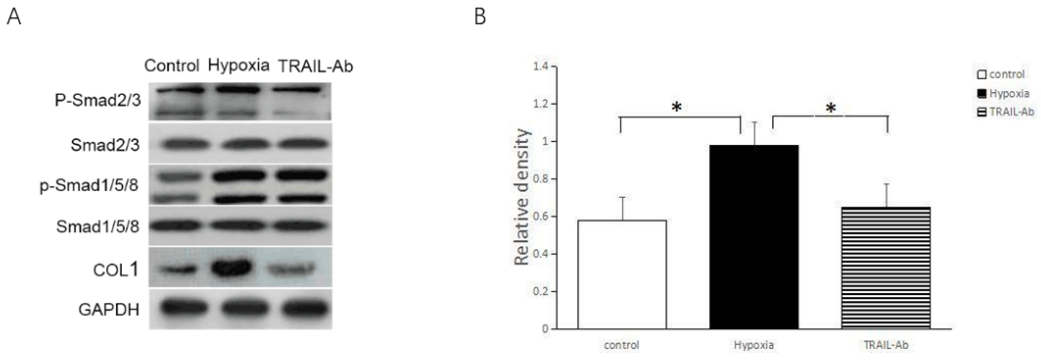

Figure 2. TRAIL mediates accumulation of ECM through activation of pSmad2/3 in PASMC response to hypoxia. (A) WB analysis of Smad collagen during normoxia, hypoxia and hypoxia with TRAIL-Ab. (B) Quantification of Smad. (*P < 0.05 was deemed statistically significant).

4. Discussion

In this experiment, we observed a significant decrease in RVSP and a decline in the expression of ECM during hypoxia-induced PH mice with the intervention of TRAIL-Ab. The pSmad2/3 proteins were highly expressive in the cultured hypoxia-stimulated PASMCs. Addition of 100 ng/ml TRAIL-Ab resulted in a significant decline in the expression of pSmad2/3.

TRAIL, one member of the tumor necrosis factor (TNF) superfamily [19] is known as Apo-2 ligand (Apo-2L)/TNFSF10 [20]. TRAIL is a type II transmembrane protein with extracellular C-terminal domain which can hydrolyze from the cell surface and transform into a soluble cytokine [21]. TRAIL interacts with the four transmembrane receptors TRAIL-R1, R2, R3, R4 and a soluble receptor OPG [22]. TRAIL-R1 (DR4 or TNFRSF10A) and TRAIL-R2 (DR5 or TNFRSF10B) contain cell death domains and induce apoptosis by interaction with FADD via binding caspase-8 and/or caspase-10 to their death domains [23]. Conversely, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) are considered as decoy receptors to protect normal cells from apoptosis for lack of the intracellular death domain and the apoptosis-inducing ability [24,25]. OPG inhibits TRAIL-mediated apoptosis by competing with the binding of TRAIL-R1 and TRAIL-R2 to TRAIL [26]. In our study, we found that collagen (a component of the ECM) increased significantly in pulmonary artery after hypoxia treatment, and reduced after treatment of TRAIL-Ab, which means TRAIL influence vascular remodeling by regulating the expression of ECM. Similarly, Hassoun [5] demonstrated that ECM, with deposition of collagen and elastin, contributing to the remodeling of the adventitia. Besides, matrix metalloproteinases (MMP), a kind of proteolytic enzymes, play an important role in collagen degradation and the regulation of ECM.

Due to the role of pro-apoptotic there are many researches in oncology over the past two decades [18,27]. Previous researches demonstrated that TRAIL can induce apoptosis in malignant cells [21]. It has recently been reported that TRAIL also plays an important role in various aspects such as inflammation and immune regulation. Importantly, TRAIL abundantly expressed in vascular smooth muscle cells and can be induced to promote apoptosis by autocrine or paracrine in diabetes, hyperlipidemia, chronic renal failure and atherosclerosis [12]. There is evidence that TRAIL considerably promotes the generation of atherosclerotic plaque by inducing apoptosis of macrophages and increasing the proliferation of VSMCs [28]. In addition, the expression of TRAIL and its receptors have previously been described in abdominal aortic aneurysm and promote the calcification of its vascular wall [29].

Angiogenesis, which includes the instability of blood vessels, the degradation of ECM, and the proliferation and migration of EC and SMC [30] can be stimulated by pro-angiogenic cytokines (VEGF, bFGF, PDGF), pleiotropic cytokines such as TGF-β and chemokines (MIP) [31]. Recent work has demonstrated that TRAIL is closely related to these cytokines. It was observed that the TGF-β1 and BMPs downregulated TRAIL mRNA [11]. TGF-β plays a key role in the pathogenesis of pulmonary fibrosis and vascular remodeling. TGF-β promotes the synthesis of collagens and fibronectin by down-regulated MMPs and up-regulates TIMPs, results in fibroblast transdifferentiation and excessive ECM accumulation [32–34]. In addition, some researchers pointed out that TGF-β1 gene polymorphisms were associated with PAH [35]. BMP pathway plays a major role in the regulation of tissue homeostasis and vasculogenesis which including cell migration and proliferation as well as ECs and SMCs apoptosis [36,37]. Activated TGF-β and BMP signaling pathways act on their phosphorylated receptors respectively located in the cell membrane, then exert

phosphorylation on the downstream Smad2/3 and Smad1/5/8, affecting the transcription of target gene and the expression of target protein after entering into the nucleus under the synergistic effect of Smad4 [38,39].

Hameed AG [17] has found that anti-TRAIL antibody resulted in the normalization of RVSP and improvement of pulmonary artery remodeling. In our study we demonstrated that anti-TRAIL antibody induced significant improvement of disease in mice with chronic hypoxia-indcued PH and suppressed hypoxia-stimulated ECM protein expression by interfering with Smad2/3 signaling.

One limitation of the mice studies was that the pathological characteristics of hypoxic-induced PH animal models could not represent all types of pulmonary hypertension. Furthermore, TRAIL is important in inducing apoptosis in a variety of tumors, using TRAIL inhibitors may increase the risk of lung cancer and respiratory infections [40]. Regardless of the fact that there are several limitations of our issue, substantial evidence shows that TRAIL is a key driver in the pulmonary vascular remodeling.

The median survival of patients with PH especially idiopathic pulmonary arterial hypertension (IPAH) are less than three years without treatment [41]. Current treatments have failed to significantly improve the prognosis. Considering the poor prognosis and limited treatment of PH, early effective therapy is very important. Although many deficiency of TRAIL, our study further demonstrated that TRAIL plays an essential pathogenic roles in hypoxia-induced PH mice model and provides a new idea to explore effective treatment of PH.

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Conflict of interest

The authors declare there is no conflict of interest.

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