Fibrosis of pulmonary vascular remodeling in carotid artery-jugular vein shunt pulmonary artery hypertension model of rats

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Abstract

OBJECTIVE: The aim of the present study was to observe the changes of hemodynamics, stereology in pulmonary vascular remodeling and messenger RNA (mRNA) expressions of transforming growth factor beta 1, and receptors in carotid artery-jugular vein (CA-JV) shunt pulmonary artery hypertension model of rats.

METHODS: Thirty-six Sprague-Dawley rats were randomized into three groups: CA-JV group, monocrotaline (MCT) administration group, and control group. Left CA-JV shunts were established in CA-JV group. Dorsal subcutaneous injections of MCT (60 mg kg−1) were received in MCT group. Ligations of left common carotid artery and external jugular vein were performed in control group. Right ventricular systolic pressure (RVSP) measurement, histological evaluation of the pulmonary tissue, and mRNA levels of transforming growth factor beta 1 (TGFß1), receptor 1 and receptor 2, were investigated after 6 weeks on MCT group, and after 12 weeks on both control and CA-JV groups.

RESULTS: Compared with control group, RVSP, percentage of fibrous tissue (F%) in pulmonary arterioles, mRNA levels of TGFß1, and receptors of CA-JV and MCT groups increased significantly. Severe hemodynamics change was found in MCT groups. On the other hand, CA-JV group demonstrated more obvious fibrogenesis and TGFß1 signals' upregulation in two pulmonary artery hypertension (PAH) models.

CONCLUSIONS: CA-JV shunt model of rats was a well-established PAH animal model simulating congenital heart disease with systemic-pulmonary shunt.

Keywords: Fibrosis hypertension • Pulmonary arterioles • Transforming growth factor beta 1 rat

INTRODUCTION

Pulmonary artery hypertension (PAH) is a syndrome characterized by proliferative and obstructive remodeling of pulmonary arterioles [1], together with progressive increase in pulmonary vascular resistance and, ultimately, right ventricular failure and death [2]. PAH is a multi-pathogenesis disease. Some are idiopathic; other etiological factors include hypoxia, pulmonary, and pulmonary vascular diseases; heart diseases; connective tissue diseases; and inflammation and drugs [3-6]. Congenital heart disease (CHD) with systemic-pulmonary shunt is common type in cardiac surgery.

The common animal models used in PAH research include monocrotaline (MCT)-induced and hypoxia-induced PAH rat models [7,8]. However, PAH formation mechanisms of these models are totally different to that of CHD. Carotid artery-jugular vein (CA-JV) shunt PAH model [9] is one model which induces PAH formation depending on increasing blood flow of pulmonary artery. The advantages of this model include simple operation, fewer traumas to animals, similar hemodynamics, and pathophysiological change with CHD.

Idiopathic and other types of PAH have very similar histopathologic changes, including vascular and interstitial lesions, the former of which is also called pulmonary vascular remodeling (PVR) [10]. It is commonly recognized that excessive vasoconstriction, PVR, and microvessels' thrombosis are pathophysiological basis of PAH formation [11]. In early researches, vasoconstriction is emphatically concerned. Recently, more and more researchers become aware of the importance of PVR [12]. The participation of cellular proliferation, including endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts, and extracellular matrix (ECM) oversynthesis in the mechanism of PVR was also studied [13].

Today, the widely adopted evaluation standard of PVR is Heath-Edwards six grades scale and its revisions [14,15]. However, the disadvantages of this scale are overly dependence of subjective assessment of pathologists and lacking quantitative basis of evaluation. With the help of the combination of
pathology and image analysis system, absolute value to describe the degrees of histopathological changes of PVR could be assessed. The aim of the present study was to observe the changes of hemodynamics, stereology in PVR and messenger RNA (mRNA) expressions of transforming growth factor beta 1 and receptors in CA-JV shunt PAH model of rats, to investigate the PAH formation mechanism and its differences to other models.

MATERIALS AND METHODS

Animal treatments and groups

Thirty-six male Sprague–Dawley rats weighing between 250 and 300 g were included in the present study. All animal care and experiments were performed in accordance with the guide for the care and use of animals of Laboratory Animals Center of Sun Yat-Sen University. Experiments were performed with permission of the local committee on animal experiment. All rats were equally randomly divided into three groups, including the control group, the operation (CA-JV) group, and the drug (MCT) group. Animals were housed three per cage. Food and water were freely available at all times, and the animals were maintained at a temperature-controlled room (21 °C; relative humidity 50–70%) on a normal light cycle.

Pulmonary hypertension animal models

Rats in the control group underwent ligations of left common carotid artery and external jugular vein. Left CA-JV shunts were established in the CA-JV group. Both artery and vein were skeletonized, ligated, cut distally, and occluded with vascular clips. The plastic coat of an artery indwelling catheter (20G, 1.1 mm χ 45 mm; BD, NJ, USA) was cut to an 8-mm-long tube and used as the shunt. The proximal artery end was everted after passing through the shunt, and then inserted into the proximal vein end. Clips were opened; blood flow and pulsation were observed. One dosage of 0.2 ml heparin (12,500 U/100 ml) was intraperitoneal injected for anticoagulation. A dose of 100,000 U penicillin was used before the operation for infection prevention. Rats of neal injected for anticoagulation. A dose of 100,000 U penicillin

Measurement of right ventricular systolic pressure (RVSP)

Rats were anesthetized with pentobarbital (40 mg/kg, intraperitoneal (i.p.) injection). At first oblique, incisions were made on the left neck; pulsation of artery-vein shunt was detected. Then the same incisions were made on the right neck; right common and external jugular veins were separated and ligated distally. A heparin-priming 3F catheter was inserted via right external jugular vein to right ventricle through superior vena cava and right atrium, to monitor RVSP.

Histological evaluation

After rats were sacrificed, the chests were opened via midline incisions. Marginal left lower pulmonary lobes were sampled for histological evaluation. Tissues were fixed in 10% formalin liquid and processed according to routine procedures. After dehydration and embedding in paraffin, serial transverse sections of lungs were obtained. Total fibrous tissue accumulation was determined by preparing tissue sections with the connective tissue-specific Masson stain. The analysis used an OLYMPUS BX51 microscopic system (Olympus Co., Tokyo, Japan). Pulmonary arterioles of external diameter between 30 and 100 mm were chosen for each section. At least eight random arterioles and five sections for each specimen were analyzed in image analysis system (Image-Pro Plus 6.0, Media Cybernetics Inc., Bethesda, MD, USA). Fibrosis of pulmonary arterioles was recorded in a quantitative way by stereologic analysis. Color segmentation was used to evaluate the percentage of fibrous tissue (F%). It was determined as follows: \( F% = \frac{\sum ai}{\sumAi} \times 100 \). \( \sum ai \) was the total area of fibrous tissue and \( \sum Ai \) was the total test area. All histological examinations were performed by two different pathologists without prior knowledge of the treatment given to the animals of which the specimens were taken.

Real-time PCR analysis

Lung tissue was used for total RNA isolation to study the changes in transcription of transforming growth factor beta 1 (TGFβ1), transforming growth factor beta receptor 1 (TGFβRI), and transforming growth factor beta receptor 2 (TGFβRII) involved in the initiation of the processes leading to fibrosis. Lungs were removed from rats, and left lower lobe tissues were dissected with thickness <0.5 cm, soaking in 1 – 2 ml RNAlater solution (Ambion Inc., Austin, TX, USA) and stored at 4°C until analysis. The total mRNA had been isolated from the tissue samples according to the manufacturer’s instructions (Omega Bio-Tek Inc., Norcross, GA, USA). Total RNA kit. mRNA concentrations in tissue samples were evaluated using the real-time polymer chain reaction (PCR) assay. The specific primers for the target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (synthesized by Biolink Bio-technologies Co., Guangzhou, China) used are described in Table 1. Totally 2 mg RNA sample of each group was reversely transcribed into cDNA. Then 5 ml cDNA was subjected to PCR reaction as the

<table>
<thead>
<tr>
<th>Table 1:</th>
<th>Primers for the target genes and GAPDH used in real-time PCR</th>
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<tbody>
<tr>
<td>Target genes</td>
<td>Primers</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>FP: 5'-CCGCACACACCCCATCTCA-3'</td>
</tr>
<tr>
<td></td>
<td>RP: 5'-TCGGTGCGTCGCCCTC-3'</td>
</tr>
<tr>
<td>TGFβRI</td>
<td>FP: 5'-CGGCTCTGGCTGCTGGG-3'</td>
</tr>
<tr>
<td></td>
<td>RP: 5'-AGGCTGCTGCTGCTGCTG-3'</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>FP: 5'-GCCAAGTCACCTACACCC-3'</td>
</tr>
<tr>
<td></td>
<td>RP: 5'-GGGAGAGGAGAGGAGGAG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FP: 5'-AGAAGGACACACCCATGTC-3'</td>
</tr>
<tr>
<td></td>
<td>RP: 5'-CTTCCCGTGCTGAGGTC-3'</td>
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</table>
template. PCR reaction condition was pre-denaturing at 94 °C for 3 min, 94 °C for 45 s, 52 °C (50 °C for TGFßRII) for 45 s, 72 °C for 1 min and 35 amplification cycles, then 72 °C for 10 min. The final products were identified by electrophoresis in 1.5% agarose gel. The calculation and analysis were performed by the computer detection system (GDS-8000 pc, UVP Inc., Upland, CA, USA).

Primers for the target genes were listed in Table 1.

Statistical methods

Data were analyzed using the SPSS 14.0 statistical package. Quantitative data were calculated as mean ± standard deviation. One way analysis of variance (ANOVA) was used to describe statistical differences and Bonferroni method was used for multiple comparison. A P < 0.05 was considered to be statistically significant.

RESULTS

The RVSP in rat models

RVSP increased significantly in CA-JV group (37.69 ± 3.00 mmHg) compared with control group (25.41 ± 2.08 mmHg, P < 0.0001). MCT administration (MCT group) also induced a more significant increase in RVSP (43.99 ± 4.60 mmHg, P < 0.0001 vs control group). There was also significant difference in RVSP between CA-JV and MCT groups (P = 0.004).

Histopathology

In the control group, walls of pulmonary arterioles were thin and even. Intima, media, and extima were difficult to be distinguished. Most tissues were in the Masson red staining. Fibrous tissues in blue staining could be observed rarely, dispersedly, and only extima distribution (Fig. 1). Conspicuous PVR could be observed in both CA-JV and MCT groups, characterized by pulmonary arterioles luminal stenosis and occlusion, media muscularization, intima and extima thickness, and fibrosis. Fibrous tissue hyperplasia in all layers demonstrated with the increase of the Masson blue staining. In some arterioles, intima and extima fibrosis manifested internal and external elastic lamina forming. Proliferative fibrous tissues in media distributed to concentric circles. Fibrous tissues also invaded in paravascular stroma (Figs. 2 and 3).

P% of pulmonary arterioles increased in two rat model groups (33.59 ± 7.58% in CA-JV group, 30.33 ± 7.24% in MCT group), which were significantly higher than control group (16.26 ± 7.27%, P < 0.0001 and P < 0.0001). There were significant differences in multiple comparisons of three groups (P = 0.002).

TGFß1 and receptors mRNA expressions

Compared with the control group, a sharp increase of TGFß1 mRNA level was already detected in CA-JV and MCT groups (1.17 ± 0.09 and 1.09 ± 0.07 vs 0.56 ± 0.06, P < 0.0001, P < 0.0001 vs the controls). There was significant difference between the CA-JV and MCT groups (P = 0.032).

The expression of TGFßRI mRNA level in CA-JV and MCT groups increased significantly compared with the control group (0.80 ± 0.06 and 0.93 ± 0.02 vs 0.20 ± 0.01, P < 0.0001, P < 0.0001 vs the controls). There was significant difference between the CA-JV and MCT groups (P = 0.002).
P < 0.0001). Difference between the CA-JV and MCT groups was also significant (P < 0.0001).

Significant increase of TGFβRII mRNA level in CA-JV and MCT groups was also detected (1.15 ± 0.05 and 1.17 ± 0.23, respectively, vs 0.46 ± 0.28 in the controls, P < 0.0001 and P < 0.0001, respectively). There was no significant difference between CA-JV and MCT groups (P = 1.000; Table 2).

### DISCUSSION

In the present study, we demonstrated that both CA-JV shunt and MCT administration could increase RVSP and induce PVR in model rats. These two models are both well-established PAH animal models. Numerous researchers prefer to use MCT-PAH model because of its easy establishment and short observation period (3-6 weeks). The mechanisms of MCT-PAH establishment include arterial endothelial cell injuries, vascular permeability increasing, and inflammatory materials releasing, which trigger severe and progressing PAH [16]. This process has something close to idiopathic PAH in human. However, in the clinical practice of cardiac surgery clinical practice, most PAH patients are CHD-PAHs, which base on the increasing of pulmonary blood flow. CA-JV shunt PAH model simulates these pathophysiologic changes of systolic-pulmonary shunt. The model needs more time to observe the formation of PVR in animals (12 weeks). This model supplies another optional and different research choice for, at least, cardiac surgery. Therefore, it should be necessary to investigate the relevant mechanism of PVR in CA-JV shunt model.

Differences could be found between two PAH rat models in this study. RVSP in MCT group is significant higher than that in CA-JV group. On the other hand, % of pulmonary arterioles in CA-JV group is significantly higher than that in MCT group. It is suggested that MCT could easily cause dynamic changes and CA-JV shunt led to more structural remodeling, especially fibrosis forming. MCT is a pyrrolidine alkaloid which is activated in liver and then transfers to lungs causing rapid and extensive inflammatory pulmonary artery injury and trauma. Pulmonary artery pressure (PAP) and RVSP increase dramatically followed by secondary amplified inflammatory responses subsequent to endothelial trauma. CA-JV shunt increases pulmonary circulation blood flow and involves in resistance pulmonary arteries at the beginning. The increasing blood flow causes constant, high shear stress to vessels. Shear stress can regulate function of endothelial nitric oxide synthetase (NOS) [17] and cytokines just as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [18,19]. This cytokines participate in the modulation of PVR. The results of the study suggested that the effect of constant high shear stress on inducing fibrous tissues proliferation, or fibrosis, was stronger than that of a single inflammatory trauma to endothelium. This would be the important characteristics for CA-JV shunt in the mechanism of PVR.

The definition of fibrosis is a condition in which fibrous connective tissue spreads over or replaces normal smooth muscle or other normal tissue. So the fibrotic proliferation of pulmonary arterioles could be defined as one kind of ‘fibrosis’, so-called vascular fibrosis. In the present study, the indicators of fibrosis activators, the mRNA level of TGFβ1, and receptors were detected in the models, indicating the existence of observed pulmonary arteriole fibrosis. TGFβ1 is one kind of multi-functional cytokines. It directly increases the synthesis, stimulates the formation of connective tissue, and inhibits degradation of extracellular matrix [20]. It is widely acknowledged as the most effective promoting factor for fibrogenesis and the key player in wound healing [21] and organ fibrosis, such as liver [22], kidney [23], and pancreas [24]. TGFβ1 performs its fibrosis effect through TGFβRs, and the TGFβRs related with the fibrosis effect are mainly via TGFβRI and TGFβRII. The present results showed that TGFβ1, TGFβRI, and TGFβRII mRNA levels in both two model groups were significantly increased compared with control group. The overexpression of TGFβ1 and TGFβRII mRNA in CA-JV group was much more significant than that in MCT group. The result suggested that there were distinct underlying mechanisms of PVR in CA-JV shunt and MCT administration PAH models.

Most previous reports have described structural and functional abnormalities of cells, such as ECs and SMCs, in PVR [25]. Seldom researchers concern about interstitial lesions, as fibrosis, though it is also important part of PVR. One of the reasons may be that it is difficult to be evaluated. Frequency and severity of the microscopic fibrosis lesions were quantified by a graded scale of 0-3: 0, normal and minimal lesions; 1, mild lesions; 2, moderate lesions; and 3, severe lesions. With the help of combination of pathology and image analysis system, it is possible to use a stereological indicator to objectively evaluate the severities of fibrosis. Exactly, compared with cellular proliferation, fibrosis would be much more threatening to the PAH patients. Because when the induction of apoptosis becomes more and more realistic in anti-proliferation treatment, degradation of fibrous tissue is still an unsolved problem. There is no any confirming efficient therapy for organ and tissue fibrosis in clinical practice currently. Therefore, TGFβ1 should be a therapeutic target in systemic-pulmonary shunt PAH.

### CONCLUSION

The present study demonstrated that CA-JV shunt PAH rat model was a well-established PAH animal model. The hemodynamics changes in this model were not as prominent as the widely used MCT model, but much significant increasing fibrogenesis of PVR and TGFβ1 signal mRNA overexpression were observed in CA-JV shunt PAH model than MCT model.

**Table 2: TGFβ1, TGFβRI and TGFβRII mRNA expressions in two pulmonary hypertension models of rats versus control group**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CA-JV</th>
<th>MCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>0.56 ± 0.06</td>
<td>1.17 ± 0.09</td>
<td>1.09 ± 0.07</td>
</tr>
<tr>
<td>TGFβRI</td>
<td>0.20 ± 0.01</td>
<td>0.80 ± 0.06</td>
<td>0.93 ± 0.02</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>0.46 ± 0.28</td>
<td>1.15 ± 0.05</td>
<td>1.17 ± 0.23</td>
</tr>
</tbody>
</table>

Bold and italic values mean three groups had significant difference. Only bold values mean CA-JV and MCT group had not significant difference, nevertheless had significant difference compared with control group.
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Conflict of interest: none declared.

REFERENCES


