Transfer of human hepatocyte growth factor reduces inflammation and prevents pulmonary arterial remodeling in monocrotaline-induced pulmonary arterial hypertensive rats

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Abstract: Inflammation and endothelial dysfunction contribute to the pathogenesis and development of pulmonary arterial hypertension (PAH). This study was to investigate the therapeutic effect of human hepatocyte growth factor (HGF) gene transfer on monocrotaline (MCT) induced PAH rat models. PAH was induced by injecting MCT for 4 weeks. The rats were randomly assigned to phosphate buffered saline control group, MCT group, and HGF treatment group. After 2 weeks of induction, measures of mean pulmonary artery pressure (mPAP), weight ratio of the RV to the LV plus septum, percent wall thickness index (TI) and area index (AI) were significantly increased in MCT-group and HGF treatment-group compared with those in control group (all P<0.05). Those measurements in MCT-group were significantly higher than those in HGF treatment-group (all P<0.05). IL-6 significantly decreased in HGF treatment-group compared with MCT-group, but higher than that of control group (all P<0.05). IL-10 in HGF treatment-group significantly increased compared with MCT-group, but lower than that of control group (all P<0.05). Endothelial microparticles (EMP) started to decrease in the HGF treatment-group 3 days after treatment and was most significant after 1 and 2 weeks of treatment (all P<0.05). Our results showed that transfer of human HGF may attenuate the inflammatory cell infiltrate, reduce the expression of inflammatory factors, and those effects are possibly due to the inhibition of EMP production which may decrease pulmonary vascular wall damage in PAH.

Keywords: Human hepatocyte growth factor, pulmonary arterial hypertension, endothelial microparticles, monocrotaline

Introduction

Pulmonary arterial hypertension (PAH) is a progressive unruly disease with a poor forecast. PAH is characterized by deregulated proliferation of vascular endothelial and intimal smooth muscle cells, with an increased pulmonary arterial pressure [1]. Moreover, it is noteworthy that a variety of autoimmune disorders and infectious diseases have been reported to be associated with PAH [2]. Despite recent advances in therapies including endothelin-receptor antagonists and phosphodiesterase type-5 inhibitors [3-5], the effects of these drugs are not satisfied, especially in long-term effect.

Recent studies found that HGF as a specific vascular endothelial growth factor has the functions of stimulating endothelial cell proliferation and migration, promoting new vessels formation, and inhibiting cell apoptosis and tissue reconstruction [6]. Many investigations have reported that HGF gene transfer attenuates medial hyperplasia and matrix accumulation in PAH of rats [7, 8]. However, it’s not clear whether HGF was able to attenuate the inflammatory response in PAH.

Microparticles (MP) are shed membrane vesicles released during the apoptosis and activation of various cell types [9]. Moreover, circulat-
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Endothelial microparticles (EMPs) have been reported as a marker of endothelial injury and systemic vascular remodeling [10]. Vascular remodeling and endothelial dysfunction are involved in pulmonary hypertension, but treatment of HGF in regulating the release of EMPs and vascular remodeling is not reported.

The purpose of this study was to investigate changes of pulmonary hemodynamics, inflammatory response, and prevention pulmonary arterial remodeling in monocrotaline (MCT)-induced PAH after human HGF gene transfer.

**Materials and methods**

**Reagents**

Monocrotaline (MCT) (Sigma company); IL-6, IL-10 ELISA reagent kit (Excell Bio company); HGF ELISA reagent kit (BG company); Standard particles 0.8 μm, 3.0 μm (Sigma company); PE-CD31 antibody, FITC-CD42b antibody (eBio science company); HGF gene-recombined adenovirus (Ad-HGF), virus activity 6.3×10⁹ IU/ml (Benyuan Zhengyang genentech limited company).

**Animals and treatment protocols**

All procedures were approved by Institutional Animal Care and Use Committee, Affiliated Hospital of Guangdong Medical College (AIACUC-10-7-1). Thirty male SD rats with the weight ranges from 250 g to 300 g were purchased from the Animal Center of Guangdong Province. The rats were randomly divided into the following 3 groups: control group (n = 10), MCT group (n = 10), and HGF treatment group (n = 10). Rats in control groups were raised in normal condition. Pulmonary hypertension was induced by injection of MCT at 50 mg/kg for 3 weeks. In the meantime, rats were intratracheally given 1.3×10⁹ IU Ad-HGF (HGF treatment group) or 0.2 ml PBS (control group and MCT group).

**Pulmonary blood pressure measurements**

Two weeks after the treatment of Ad-HGF, pulmonary artery pressure was measured in anesthetized rats by intraperitoneal injection of 7% chloral hydrate. A polyethylene cannula filled with 2% heparin sodium was inserted into the right external jugular vein and forwarded to the right ventricular (RV). The data were recorded after stabilization of the tracing using a liquid pressure transducer, which was interfaced to a Med Lab electrophysiograph.

**Index of right ventricular hypertrophy measurements**

The extent of right ventricular hypertrophy was evaluated by the ratio of right ventricle to left ventricle plus septum weight (RV/LV+S).

**Histopathological analysis**

The lung was washed with 4°C 0.1% PBS and physiological saline repeatedly, fixed in 10%
formalin solutions overnight, and embedded in paraffin. For identification of vascularity and the thickness of pulmonary arteriolar, formalin-fixed paraffin-embedded lungs were sectioned, deparaffinized, and stained for elastic lamina by hematoxylin and eosin (HE). We calculated the thickness index (TI) and area index (AI) of pulmonary arterioles (diameter < 500 μm) wall by computer image analysis system (light microscopy, ×200, selected 5 cross-sections for each section).

**Inflammatory index**

Lung tissue (1 g) was cut into pieces, homogenized at 4°C, and resuspended with 5 ml physiological saline. After centrifugation at 4°C to get rid of debris, the content of HGF in the lung tissue was determined by ELISA.

Blood samples were obtained before, 7 days, and 14 days after MCT injection through posterior orbital venous. After centrifugation, sera
were collected for the detection of IL-6 and IL-10 (ELISA).

Circulated endothelial microparticles (EMP) measurement

Blood samples were centrifuged at 1000 rpm for 10 min at room temperature to remove hemocyte and then centrifuged at 3400 rpm for 10 min to remove blood platelet. The surface marker phenotype of these EMP was analyzed by flow cytometry using PE-CD31 and FITC-CD42b.

Statistical analysis

All quantitative data were expressed as mean ± standard deviation (SD). To compare values among these groups, analysis of variance (ANOVA) was applied. The statistical significance level was defined as $P < 0.05$. Analyses were performed using SPASS 17.0.

Results

Hemodynamic and RV weight analysis

Two weeks after treatment, the mean pulmonary artery pressure (MPAP) and right ventricular hypertrophy (RVH) were significantly lower in the HGF-treatment PAH groups compared with MCT-induced PAH group ($P < 0.05$), but were significantly higher compared with the control group ($P < 0.05$; Figure 1A and 1B).

Pulmonary artery wall observation

There was a significant medial wall thickening in the pulmonary arterioles of MCT-injected rats compared with that of control group 2 weeks after MCT injection. In contrast, medial wall thickness of the pulmonary arterioles appeared to be less in the HGF treated group (Figure 2A).

The ratio of medial wall area index and thickness index in the MCT-injected group significantly increased compared with control group 2 weeks after MCT injection (Figure 2B, $P < 0.05$). In the HGF treatment group, the ratio of medial thickness index of the pulmonary artery was significantly lower than that of MCT group (Figure 2B, $P < 0.05$).

Inflammatory index analysis

The content of HGF in the lung tissue was significantly higher in HGF treatment group than that of MCT group (0.35 ± 0.02 vs. 0.12 ± 0.04, $P < 0.05$, Figure 3). IL-6 significantly decreased in HGF group compared with MCT group, but higher than that of control group (all $P < 0.05$, Table 1). In contrast, IL-10 significantly increased HGF group compared with MCT group, but lower than that of control group (all $P < 0.05$, Table 1).

Circulated microparticles (EMP)

There was no significant difference in initial EMP between the two groups (MCT-injected & HGF treatment groups, $P < 0.05$). However, EMP was significantly lower in HGF treatment group compared with MCT group after treatment ($P < 0.05$) (Table 1); The values decreased time-dependently until 2 weeks after HGF treatment (Table 1).

Discussion

In the rats with MCT-induced PAH, the elevation of pulmonary arterial pressure correlates with a thickening of the medial wall in small pulmonary arteries and arterioles due to the proliferation of vascular smooth muscle cells [11, 12]. Previous studies have reported that transduction of the lung with the HGF gene via the pulmonary artery reduces medial hyperplasia in lung arterioles and inhibits overgrowth of pulmonary arterial smooth muscle cells [7, 8]. Here we demonstrated human HGF transfer reduced pulmonary IL-6 expression and adventitial infiltration of IL-6-expressing inflammatory cells and prevented pulmonary arterial remodeling in MCT-induced PAH rat model.

Administration of MCT in rats, can cause endothelial cell injury and elicit massive mononucle-
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Table 1. Expression of IL-6 and IL-10 were after HGF transfusion

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>MCT-injected</th>
<th>HGF-treated</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>0</td>
<td>40.5 ± 20.1*</td>
<td>237.6 ± 53.2*</td>
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<td></td>
<td>7</td>
<td>36.2 ± 26.8*</td>
<td>327.7 ± 32.4*</td>
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<td></td>
<td>14</td>
<td>42.7 ± 28.4*</td>
<td>398.8 ± 54.9*</td>
</tr>
<tr>
<td>IL-10</td>
<td>0</td>
<td>102.4 ± 46.6*</td>
<td>56.4 ± 7.4*</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>98.7 ± 25.4*</td>
<td>41.9 ± 6.6*</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>99.7 ± 23.0*</td>
<td>25.3 ± 7.5*</td>
</tr>
<tr>
<td>EMP</td>
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<td></td>
<td>14</td>
<td>39.9 ± 10.2*</td>
<td>78.8 ± 3.0*</td>
</tr>
</tbody>
</table>

*P < 0.05 when compared control group; #P < 0.05 when compared MCT group.

Infiltration into perivascular regions of arterial and muscular arteries, leading to the development of severe PAH after MCT exposure. A growing number of evidence supported that inflammatory mediators including IL-6 and IL-10 play a significant role in PAH [13-18]. The combination of HGF and c-Met phosphorylates GSK3β, NF-κB p65 in cytoplasm to depress the expression of IL-6 and raise the expression of IL-10 [15, 16]. Our study showed that after two weeks of Ad-HGF transfection in PAH rats, the expression of IL-10 increased while IL-6 decreased, accompanied with ameliorated PAH symptoms.

Normal pulmonary vascular endothelium release bioactive substances towards part vessels to regulate vascular tone and the growth of smooth muscle cells, maintaining the stability of the whole vessel. More and more studies demonstrated that endothelial dysfunction is an important aspect of pathogenesis of pulmonary hypertension. EMP was released into the microenvironment by endothelium cells that were activated or undergoing apoptosis [19]. These particles carried surface proteins and biological information of their parent cells and could regard as potential biomarkers for monitoring endothelial cell functional status [20]. These results demonstrated that monitoring the levels of circulating EMP could evaluate the relationship between pulmonary hypertension and severe endothelial injury. In addition, in hypoxia-induced pulmonary hypertensive rats, EMP induces endothelial cell dysfunction by reducing the NO generation and increasing oxidative stress [21]. In our study, EMP was significantly higher in MCT-induced pulmonary hypertension. However, Ad-HGF transfer downregulated the level of EMP. It’s possible that HGF could protect and repair pulmonary vascular endothelium by decreasing EMP levels and promote NO generation, which may delay the development of pulmonary hypertension. However, further studies are required to explore the underlying mechanisms.

The production of EMP also has a close relationship with inflammation. EMP may promote inflammatory reaction. A large amount of studies demonstrated that EMP has the ability to activate neutrophilic granulocyte, facilitating the interaction of lymphomonocyte and endothelial cells (EC), and mediating chemotactic effect on neutrophils [22]. These effects may be mediated through oxidized phospholipids. The interaction between EMP and monocyte also makes the latter to release mediators of inflammation, causing endothelial cells and monocytes paracrine and anticrime [23]. The level of EMP is positively correlated with IL-6 level, suggesting a connection between EMP and inflammation.

In conclusion, our results showed that transfer human HGF may attenuate the inflammatory cell infiltration, reduce the expression of inflammation factors, and those effects are possibly due to the inhibition of EMP production which decreased pulmonary vascular wall damage in PAH.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (81370242 and 81101553), Guangdong Natural Science Foundation (S20130100-15005), Science and Technology Planning Project of Guangdong (2009B030801378 and 2012B031800224), Science and Technology Innovation Fund of Guangdong Medical College (STIF201128), Financial Foundation of Zhanjiang ([2011] 79 and [2010] 174), and American Heart Association (13POST17210033).

Disclosure of conflict of interest

None.

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