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Impact of Acute Pulmonary Embolism on Plasma and Tissue Hepatocyte Growth Factor: An Experimental Study

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Running title: Hepatocyte growth factor following PE

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Abstract

This study was designed to investigate the impact of acute pulmonary embolism (PE) on plasma and tissue hepatocyte growth factor (HGF). PE was established in 16 New Zealand white rabbits by intravenous injection of autologous blood clots. Another 16 sham-operated rabbits were used as control. Plasma HGF levels and tissue HGF expression was measured by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry, respectively. The plasma HGF levels in the PE group were elevated 1 h after PE ($P<0.01$). In the lung tissue samples, the positive HGF expression ratio was 91.7% and 20.8%, respectively, in the PE and the control group ($P<0.01$). The positive HGF expression ratio in the right ventricular tissue samples in the PE group was higher than in the control group (75.0% vs 20.9%, $P<0.01$). The positive HGF expression ratio in the liver samples in the PE and the control groups was 33.3% and 16.7%, respectively ($P<0.05$). In conclusion, acute PE was associated with a significant increase in plasma HGF. Acute PE was also associated with an enhanced HGF expression in the lungs, the right ventricle and the liver.

Key words: pulmonary embolism; hepatocyte growth factor; lungs; right ventricle; rabbit.
Introduction

Acute pulmonary embolism (PE) is potentially a fatal condition most commonly originating from deep venous thrombosis of the legs. The clinical manifestations of PE are variable, ranging from being asymptomatic to immediate death. The diagnosis of PE can sometimes be difficult, and several blood biomarkers have been investigated for their sensitivity and specificity in diagnosing PE.

Hepatocyte growth factor (HGF) is a large multidomain protein structurally similar to plasminogen. It was initially detected in the plasma of partially heptectomized rats as a potent mitogen for mature hepatocytes. HGF is also a pleiotropic factor that is produced by mesenchymal cells and acts on a wide variety of epithelial cells, including renal tubular cells, melanocytes and keratinocytes. More recently, HGF has been noted to function as a pulmotrophic factor for regeneration of an injured lung. A recent experimental study in rats has demonstrated that pulmonary ischemia, induced by ligation of the left pulmonary artery, leads to a rapid increase in plasma HGF, suggesting that HGF may be a potential biomarker for the diagnosis of pulmonary ischemia. However, the changes in plasma HGF following acute PE has not been fully investigated.

The primary aim of this study was to investigate the plasma levels and tissue expression of HGF following acute PE in a rabbit model.

Materials and Methods

Animal preparations

This study was approved by the institution review board of Liaocheng People’s Hospital. Thirty-two healthy New Zealand rabbits of both sexes (body weight, 3.2 ± 0.3 kg, age 4-6
months) were obtained from Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences, China. Animals were housed in groups at a constant temperature of 25°C (humidity 40%).

Animals were randomly divided into control (n=16) and PE (n=16) groups. In each group, the animals were divided into plasma HGF (n=8) and tissue HGF (n=8) subgroups.

Pulmonary embolism in the PE group was established by using a previously reported method. Under general anesthesia (sodium phenobarbital 150 mg/kg, ip), a 5 French catheter was inserted into the right femoral vein. The catheter was subsequently advanced to the right atrium for injection of emboli (1.0 ml/kg). The emboli were made of autologus blood clots of 1 x 3-4 mm in sizes as follows: Two milliliters of the venous blood was kept in a syringe for 45 min until coagulation was taking place. The sealed syringe was then left in a 70°C water bath for 10 min. The blood clots were then cut into small pieces (1 mm in diameter and 3-4 mm in length). The blood clots were washed with normal saline before injection.

The establishment of pulmonary embolism was verified by clinical signs, such as a reduction of blood oxygen saturation to 80-85%, and an increase in heart rate. It was confirmed in all PE animals by pulmonary tissue examination following the study (Fig 1). With the control group animal, normal saline was injected to the right atrium via the intravenous catheter.

After the establishment of PE, animals were allowed to recover under supervision. Respiration and electrocardiogram were monitored throughout the experiment.

Measurement of plasma HGF
Blood samples were collected from the ear vein before, and 1, 3, 6, 12, 24, 48 and 72h after the injection of blood clots or normal saline. The blood samples were kept in EDTA containing test tubes and were immediately centrifuged at 3000 rpm for 15 min. The products were stored at -80°C until HGF assay.

The plasma concentration of HGF was measured by enzyme-linked immunosorbent assay (ELISA), using commercially available testing kit (Jinan Medical Technology Co., Ltd., Shandong, China). The minimum detectable level of HGF with this ELISA kit was 0.2 ng/mL. Optical density was measured at 450/540 nm wavelengths by an automated ELISA reader. All samples were assayed at least twice and the average values of the two assays were used.

**Immunohistochemistry**

The animals from the control (n=8) and the PE (n=8) groups were euthanized 12 h after the catheterization. The lungs, right ventricle and the liver were harvested, after perfusion with saline through the right ventricle to remove the blood thoroughly from these organs. During the perfusion, the color of the liver tissue changed from dark red to light red as the blood was successfully removed from the systemic circulation, as well as from the pulmonary circulation. These tissues were fixed by 10% formaldehyde for at least 24 h. Each type of tissues was cut into three pieces and embedded with paraffin wax.

After deparaffinization, the tissue sections (thickness 4 μm) were incubated at 4°C overnight with a rabbit immunoglobulin G (IgG) against rat HGF (1:200) for the primary reaction. After three washes with phosphate-buffered saline (PBS), the sections were further
reacted with biotinylated goat anti-rabbit IgG at room temperature for 2 h. This was followed by further treatment with streptavidin–biotin complex (SABC, Wuhan Boshide Co. China) for 20 min. After washing the specimens with PBS for 4 times, freshly prepared diaminobenzidine (DAB) color liquid (Wuhan Boshide Co. China) was added to visualize HGF.

Positive HGF expression was presented as dark brown granules in the cytoplasm or the nucleus. The quantity of the positively stained cells was counted in each microscopic examination field by an investigator who was not aware of the identification of the tissue samples. The average number of the positive cells from five fields was used for that particular tissue sample. A HGF expression ratio was derived by dividing the number of HGF positive cells with the total number of the cells in the field. When a HGF expression ratio was less than 6%, it was defined as negative expression. A positive and a strong positive expression was defined as HGF expression ratio of 6-75% and more than 75%, respectively.

Statistical analysis
All data are presented as the mean±standard deviation (SD). Significance of differences was established by one-way analysis of variance (ANOVA) with multiple comparisons. Categorical data were analyzed by Chi-square test. \( P<0.05 \) was considered statistically significant.

Results
All animals survived the experiment with no mortalities within the first 72 h. Animals in the
PE group had dyspnea and cyanosis of lips and ears. There was also an increase in heart rate between 30-50 beats/min. The blood oxygen saturation rate was reduced by 10-15%. T wave inversion on electrocardiogram lead II was also noted. Blood clots were also found in pulmonary artery under microscope (Fig. 1). None of the above manifestations were found in the sham-operated animals. In the PE group, there was some reduction in cyanosis and increase in the blood oxygen saturation rate 24 h after the operation. At 72 h, the average blood oxygen saturation rate was 8±2% lower, whereas the average heart rate was 15±7 beats/min higher than the pre-operational values.

**Histological changes after acute PE**

The histological changes of lung tissues following acute PE are demonstrated in Fig 1. After 72 h, hyperemia of the alveolar septa and hypertrophy of the alveolar sacs were observed, with emboli in the small pulmonary arteries (Fig 1A). There was also significant infiltration of the leukocytes in the alveolar septa (Fig 1B). Pulmonary infarction or hemorrhagic necrosis was absent.

**Plasma HGF levels**

The plasma HGF levels were shown in Table 1. There was no significant difference in the plasma HGF between the two groups before the experiment (*P*>0.05). Between 1 and 72h following the PE procedure, the plasma HGF in the PE group was significantly higher than in the control group. The peak level of plasma HGF in the PE group occurred at 12 h following the PE procedure. The average plasma HGF at 72 h was lower than at the 48 h in the PE
group \( (P<0.01) \).

*Tissue expression of HGF*

Strong HGF expression was found in lung tissues 12 h the PE procedure (Fig 2). The types of cells showing positive HGF staining were the interstitial cells, fibroblasts, inflammatory cells (e.g. neutrophils) and alveolar epithelial cells. In the control group, some alveolar epithelial cells showed a weak HGF expression but other types of lung tissue cells showed no HGF expression.

In the PE group, strong HGF expression was also found in the interstitial cells of the right ventricle, but only a small number of the myocytes showed HGF expression. In the control group, there was little HGF expression in the right ventricular tissues. The HGF expression in the liver tissues of both groups was weak and was limited mainly to the cytoplasm of the hepatocytes.

Table 2 shows the number of tissue samples in which the level of HGF expression was identified in each group. In the lung tissues, positive HGF expression was found in 91.7% and 20.8% of the samples, respectively, in the PE and the control group \( (P<0.01) \). The positive expression ratio in the right ventricular tissue samples was higher in the PE group than in the control group \( (75.0\% \text{ vs } 20.9\%, \ P<0.01) \). There was no significant difference in the weak or strong HGF expression in the liver tissue samples \( (P>0.05) \). However, the total liver HGF expression in the PE group was higher than in the control group \( (33.3\% \text{ vs } 16.7\%, \ P<0.05) \).
Discussion

The major findings of this study are: 1) Acute PE was associated with an elevated plasma HGF level; 2) There was a significant increase in HGF expression in the lung and the right ventricular tissues following acute PE; 3) HGF expression in the liver was also elevated following acute PE.

Our study has demonstrated plasma HGF begins to increase within an hour of the blood clot injection and reaches peak in about 12 h. The plasma levels of HGF began to decline at 48 h, but were still elevated at 72 h. These results may have important clinical implications. The early onset and the rapid increase to peak plasma levels make plasma HGF a potential biomarker for PE screening or diagnosis, provided that the sensitivity and specificity for such diagnosis being confirmed in future studies.

The physiological or pathophysiological significance of HGF expression following PE is not entirely clear. HGF, originally discovered as a mitogen for hepatocyte regeneration,\textsuperscript{6, 7} is now recognized as a multifunctional mesenchymal factor for epithelial regeneration, including the regeneration of alveolar type II epithelial cells.\textsuperscript{8, 9} HGF markedly stimulated proliferation and DNA synthesis of rat tracheal epithelial cells \textit{in vitro} and \textit{in vivo}.\textsuperscript{8} The enhanced HGF production following lung injuries or insult are considered as a compensatory mechanism, because HGF would help the injured tissues or organs to regenerate.\textsuperscript{2,9-11} In patients with inflammatory lung disease, high level of HGF following anti-inflammatory treatment was associated with a poorer prognosis compared with those who had a rapid reduction in HGF after the same treatment.\textsuperscript{10} Recent studies indicate that HGF may be acting in an anti-inflammatory fashion in addition to pro-healing. HGF suppresses
inflammation by targeting multiple pathophysiological processes involved in inflammatory response, including activation of parenchymal cells, microvascular endothelial dysfunction, and leukocyte extravasation and chemotaxis. Administration of HGF effectively suppresses acute and chronic cardiac allograft rejection and autoimmune myocarditis, partially through suppression of T-cell-mediated immunity. The role of the elevated HGF in the plasma and the lung tissues in our study is not clear, but it may be important in the regeneration of the injured lungs after acute PE.

There has been little information about the origin of HGF following acute PE. HGF can be produced by the liver, lungs, spleen and kidney following nephrectomy, hepatic or myocardial ischemia. In a rat pulmonary ischemia model, the expression of HGF mRNA was elevated in the injured lung tissues, as well as in the liver, ahead of the serum HGF elevation. The HGF increase in the injured lung tissues was greater than in the healthy lung or liver tissues. Thus, the authors of this study concluded that HGF was mainly induced by paracrine mechanisms during pulmonary ischemia. In ischemia-reperfusion-induced lung injuries, there was an enhanced expression of HGF and DNA synthesis in the alveolar epithelial cells and in the macrophages.

In our study, we used immunohistochemistry to assess the tissue expression of HGF 12 h following acute PE. We found in PE animals, there were strong HGF expressions in the lung and right ventricular tissues, mostly in the interstitial cells of the lung and the heart, as well as in the abundant inflammatory cells in the lungs. Little HGF expression was found in the myocytes. Furthermore, the HGF expression in the liver was lower than in the lungs and the heart.
In conclusion, we have shown that plasma HGF was elevated following acute PE in rabbits. The plasma HGF elevation was accompanied by an enhanced expression of HGF in the ischemic lung tissues, as well as in the right ventricles and the liver. The early and rapid increase in the plasma concentration may also serve as a biomarker for the detection of pulmonary injuries. Further studies are required to clarify the sensitivity and specificity of plasma HGF in the diagnosis of PE.
References


16:388-97.


Table 1. Plasma HGF level (ng/ml) before and following acute pulmonary embolism (PE).

<table>
<thead>
<tr>
<th>Time</th>
<th>Control (n=8)</th>
<th>PE (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.34±0.26</td>
<td>0.49±0.33</td>
</tr>
<tr>
<td>Before</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>0.42±0.33</td>
<td>0.85±0.32*</td>
</tr>
<tr>
<td>3h</td>
<td>0.23±0.23</td>
<td>0.72±0.33*</td>
</tr>
<tr>
<td>6h</td>
<td>0.24±0.22</td>
<td>0.96±0.33**</td>
</tr>
<tr>
<td>12h</td>
<td>0.27±0.23</td>
<td>1.74±0.39***</td>
</tr>
<tr>
<td>24h</td>
<td>0.26±0.24</td>
<td>1.02±0.43**</td>
</tr>
<tr>
<td>48h</td>
<td>0.34±0.24</td>
<td>1.28±0.46**</td>
</tr>
<tr>
<td>72h</td>
<td>0.26±0.19</td>
<td>0.61±0.27*</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001.
Table 2. HGF expression in the lung, right ventricle and the liver tissue samples.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Control (n=24)</th>
<th>PE (n=24)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lung samples</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>19 (79.2%)</td>
<td>2 (8.3%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Weak positive</td>
<td>4 (16.7%)</td>
<td>5 (20.8%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Strong positive</td>
<td>1 (4.2%)</td>
<td>17 (70.8%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total positive</td>
<td>5 (20.8%)</td>
<td>21 (91.7%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>RV samples</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>19 (79.2%)</td>
<td>6 (25.0%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Weak positive</td>
<td>4 (16.7%)</td>
<td>9 (37.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Strong positive</td>
<td>1 (4.2%)</td>
<td>9 (37.5%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total positive</td>
<td>5 (20.8%)</td>
<td>18 (75.0%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Liver samples</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>20 (83.3%)</td>
<td>16 (66.7%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Weak positive</td>
<td>2 (8.3%)</td>
<td>5 (20.8%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Strong positive</td>
<td>2 (8.3%)</td>
<td>3 (12.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Total positive</td>
<td>4 (16.7%)</td>
<td>8 (33.3%)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

RV: right ventricle; PE: pulmonary embolism
Figure legends

Fig 1. Microscopic examination of lung tissues from an animal with acute pulmonary embolism (x200). A: An embolus is clearly seen in the pulmonary artery. B: Infiltration of inflammatory cells in the lung tissue.

Fig 2. Immunohistochemistry examination of tissue expression of HGF (x 400). A: the expression of HGF in the lung of the PE group; the horizontal arrows points to a fibroblast (interstitial cell) and the vertical arrow points to an inflammatory cell; B: chromatosis in the endochylema of the hepatic tissue of the PE group; C: expression of HGF in the interstitial cells of the right ventricle of the PE group; D: the expression of HGF in lung tissue of the control group; E and F: hepatic and right ventricular tissue of the control group.