Relationship between the Eosinophilia of Bronchoalveolar Lavage Fluid (BALF) and the Severity of Pulmonary Fibrosis Induced by Bleomycin in Rats

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A large number of studies have demonstrated that the presence of eosinophils in the lungs of patients with pulmonary fibrosis correlates with poor prognosis or resistance to therapy. However, direct evidence of the relationship between the influx of eosinophil and pulmonary fibrosis has not yet been described experimentally. In this article, pulmonary fibrosis was induced by different doses of bleomycin (BLM) and using different aged rats. On selected days afterwards, the lungs were lavaged and harvested for analyzing fibrosis, eosinophil influx and cytokine expression. There was a significant relationship between eosinophilia and the pulmonary fibrosis (r=0.98, p<0.01). In spite of the fact that there was no significant increase in hydroxyproline of the lung, eosinophil influxes of bronchoalveolar lavage fluid (BALF) was maximal 7 d after BLM administration. Moreover, there were similar patterns among transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF) and eosinophil influx of BALF in that they were dependent on dose of BLM and age. These findings, together, have suggested the causal correlation of eosinophilia during the early stage with subsequent pulmonary fibrosis. The possible role of eosinophils in the pathogenesis of pulmonary fibrosis might contribute to not only TGF-β, but also HGF production.

Key words eosinophilia; pulmonary fibrosis; transforming growth factor beta (TGF-β); hepatocyte growth factor (HGF); bronchoalveolar lavage fluid (BALF); bleomycin

A series of recent studies have associated eosinophilia with fibrotic conditions of different etiology, including endomyocardial fibrosis, wound repair, tissue remodeling and pulmonary fibrosis. Although there have been clinical demonstrations that the presence of eosinophils in the lung of patients with pulmonary fibrosis correlates with a worse prognosis or resistance to therapy, direct evidence of the correlation of eosinophilia with pulmonary fibrosis has not yet been described experimentally.

Eosinophils are thought to be a key source for a number of fibrogenic cytokines with known inflammatory and fibrosis promoting activities, such as transforming growth factor-beta (TGF-β) and Monocyte Chemoattractant Protein (MCP)-1. In particular, TGF-β1 was a potent profibrotic cytokine which stimulated fibroblasts and promoted the synthesis and secretion of many proteins of the extracellular matrix. In addition, it was found that other cytotoxic proteins released from eosinophil granules, such as eosinophil-derived neurotoxin (EDN), major basic protein (MBP), and eosinophil cationic protein (ECP), may also promote tissue injury and exacerbate the alveolitis, which drive the fibrotic response, suggesting the role of eosinophil in the pathogenesis of pulmonary fibrosis.

On the other hand, recent studies have also demonstrated that some of the eosinophil mediators such as interleukin (IL)-2, interferon (IFN)-γ and collagenase have antifibrotic properties, and that activated eosinophils expressed not only TGF-β1 but also hepatocyte growth factor (HGF). HGF is known to act not only as a mitogen but also as a morphogen on many kinds of epithelial cells in a paracrine fashion. Simultaneous or delayed administration of HGF also suppressed pulmonary fibrosis induced by bleomycin (BLM). These findings, taken together, have suggested the complex role of eosinophils in the processes of pulmonary fibrosis.

The studies presented in this paper were designed to examine the correlation of eosinophilia with pulmonary fibrosis during the development of lung fibrosis.

MATERIALS AND METHODS

Animals Male Wistar rats were purchased from NSC, Japan. The rats were 5, 10 and 15 weeks old, and their body weights ranged from 95—110, 235—260 and 290—320 g, respectively, at the start of the study.

Animal Husbandry The animals were housed in pairs in cages (280 (w)×440 (D)×180 (H) mm) in an animal room with a temperature of 22±2 °C, husbandry 51±10 (%). Fluorescent light provided an 11 h light (07:00—18:00 h) and 13 h dark cycle, and air was recycled 15 times/h. The rats were fed a solid stock food (MF, Oriental Yeast Co., Ltd. Tokyo, Japan) and were allowed free access to water. All the experiments conformed to the Japanese law concerning animal care and use, following the Guidelines for animal experimentation recommendations, and were approved by the institution’s animal care and use committee.

Induction of Bleomycin-Induced Pulmonary Fibrosis Male Wistar rats, aged 15 weeks old (NSC, Japan), were randomly divided into four experimental groups: A) control: treated with sterile saline alone (n=9); B) treated with 0.30 U/100 g body weight (BW) of BLM (n=10); C) treated with 0.75 U/100 g BW of BLM (n=10); D) treated with 1.20 U/100 g BW of BLM in 0.3 ml sterile saline (n=10). In contrast, Male rats aged 5 and 10 weeks old, were divided into control (n=8) and 1.20 U/100 g BW of BLM treated groups (n=10) respectively.

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Pulmonary fibrosis was induced on day 0 by an intratracheal injection of BLM, at doses of 0.30, 0.75 and 1.20 U/100 g BW of Bleomycin hydrochloride (Nippon Kayaku Co., Tokyo, Japan) in 0.3 ml of sterile saline under Nembutal anesthesia using pentobarbital (50 mg/kg BW). Control animals received 0.3 ml of sterile saline only, in a manner similar to the BLM-treated rats.

Bronchoalveolar Lavage Fluid (BALF) Collection Animals were killed by abdominooartiac exsanguination, and blood was collected at 1, 3, 7, 14 and 28 days after saline or BLM administration under pentobarbital (50 mg/kg BW) anesthesia. Briefly, the lungs were exposed and lavaged 5 times. with endotoxin-free saline (Otsuka Pharm, Tokushima, Japan) of 10 ml/100 g BW. The solution with lavage material was immediately aspirated back after each aliquot. The lavage fluid recovery was about 90%. The BALF was centrifuged at 4°C for 10 min at 300 g. The supernatants were used to prepare duplicate cytocentrifuge preparations, which were stained with Wright–Giemsa for light microscopy. A cell differential count was obtained by randomly reading at least 200 cells per slide on a hemocytometer, and expressed according to the percentage of macrophages, polymorphonuclear leukocytes, lymphocytes and eosinophils.

Histopathologic Evaluation At designated time points (1, 3, 7, 14 and 28 days after BLM administration), rats were killed by abdominooartiac exsanguination under Nembutal anesthesia. After the BAL procedure, the left lung was tied at the main stem bronchus and removed. The left lung was then immediately wrapped in foil and placed in liquid nitrogen before storage at −80°C for later hydroxyproline measurement. The right lung was perfused with 2 ml of 10% neutral formalin through a tracheal cannula, the trachea was then clamped, and the right lung was removed from the chest cavity and placed in fresh fixative overnight before processing in paraffin. Sequential 4-μm sections of lung were stained with hematoxylin and eosin (H&E). The severity of fibrosis was assessed semi-quantitatively according to the method described by Ashcroft.10) The grade of pulmonary fibrosis was scored in a blinded fashion on a scale of 0 to 8 by the method described by Ashcroft.15) The grade of pulmonary fibrosis was assessed semi-quantitatively according to the stained with hematoxylin and eosin (H&E). The severity of...
mean ± S.E. from 10 samples. Data were evaluated by one-way analysis of variance and, where appropriate, further evaluated with the non-parametric Mann–Whitney rank sum test. \( p < 0.05 \) were considered significant.

RESULTS

Differential Cellular Analysis of BALF BALF obtained from control animals consisted of more than 99.5% macrophages. Neutrophils began to appear in significant amounts in the lavage fluid 1 d after BLM administration. The peak infiltration of PMN occurred within 3 d and returned to normal by 28 d after BLM administration.

Significant increases in the lymphocytes were observed 3, 7, and 14 d after BLM administration. A minor increase in the eosinophils of BALF was found 1 d after BLM administration (Fig. 1). In spite of aging of rats, the peak infiltration of eosinophils occurred 7 d, and returned to normal by 1 month after BLM administration (data not shown). The peak influxes of eosinophils were shown to be dependant on dose of BLM and aging (Table 1).

Effect of BLM-Doses and Aging of Rats on the EPO Activities The EPO activity, an indicator of eosinophil influx, was measured 7 d after BLM administration. The levels of EPO activities were enhanced dose-dependently (0.127, 0.421, 0.56 respectively) in response to BLM at 0.30, 0.75 and 1.20 U/100 g, suggesting a similar pattern to eosinophil counts of BALF (Fig. 2).

Similar EPO activities were observed between 10- and 15-week-old rats (21.58 and 23.63 times vs control, respectively), and the levels of EPO activities in both 10 and 15-week-1.20 U were significantly higher than in 5-week-old rats (Fig. 2).

Kinetics of Hydroxyproline of Lung in Response to BLM Pulmonary fibrosis was assessed by quantifying the hydroxyproline content of the left lungs, and was confirmed by Ashcroft score in the lung tissue section 28 d after BLM administration. In spite of the fact that there was a maximal increase in eosinophils of BALF, no significant increase in hydroxyproline of the lung was observed 7 d after BLM administration, suggesting the causal correlation of pulmonary fibrosis with the influx of eosinophils of BALF during early stage of pulmonary fibrosis. In this study, hydroxyproline content began to increase 14 d after BLM administration (Fig. 3).

Correlation of EPO Activity with Hydroxyproline during Development of Pulmonary Fibrosis In this study, the correlation of EPO activity with hydroxyproline was evaluated in response to three different doses of BLM and three different aged rats.

There was a significant relationship between EPO activity and hydroxyproline (Fig. 4).

Table 1. Effect of BLM Doses and Aging on the Differential Cells of BALF 7 d after BLM Administration

<table>
<thead>
<tr>
<th>Age (week)</th>
<th>BLM (U/100 g)</th>
<th>Total cell ( \times 10^6 )</th>
<th>Cell differential count (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAM</td>
<td>PMN</td>
<td>Ly</td>
</tr>
<tr>
<td>15</td>
<td>Control</td>
<td>2.16±0.10</td>
<td>99.5±0</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>3.18±0.36</td>
<td>64.51±1.46</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>4.33±0.25</td>
<td>59.62±2.23</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>4.21±0.30</td>
<td>60.49±1.67</td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>1.63±0.12</td>
<td>99.5±0.00</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>4.55±0.30</td>
<td>51.75±1.27</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>2.16±0.10</td>
<td>99.5±0.00</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>2.06±0.23</td>
<td>61.52±2.90</td>
</tr>
</tbody>
</table>

PAM: pulmonary alveolar macrophages; PMN: polymorphonuclear cells; Ly: lymphocytes; Eo: eosinophils. *\( p < 0.01 \) indicate significant differences from 5-week-old rats.
Changes in TGF-β₁ and HGF of BALF Showed a Similar Pattern with the Pulmonary Fibrosis The levels of both hydroxyproline and Ashcroft scores were shown to be dependent on dose of BLM and aging (Table 2).

There was a similar pattern among the level of TGF-β₁, HGF and pulmonary fibrosis, suggesting the causal correlation of pulmonary fibrosis with TGF-β₁ and HGF of BALF during the pulmonary fibrosis process (Table 2).

**DISCUSSION**

Accumulation of eosinophils in alveolar space and parenchyma has been observed in various pathological situations, such as allergic inflammation, parasitic infections, and neoplastic diseases. A series of recent studies has associated eosinophilia with fibrotic conditions of different etiopathology, including endomyocardial fibrosis, wound repair, tissue remodeling and pulmonary fibrosis. Four lines of evidence have suggested the importance of eosinophils in the pathogenesis of pulmonary fibrosis. First, eosinophil influxes were found in many fibrotic conditions, including those induced by BLM. Second, these cells were known to be a key source for a number of fibrogenic cytokines such as TGF-β₁, MCP-1. Third, there was evidence that other cytotoxic proteins secreted from eosinophil granules may promote tissue injury and exacerbate alveolitis, which drives the fibrotic response. Fourth, the presence of these cells correlates with either a worse prognosis in terms of progressive fibrosis or a resistance to current modalities of therapy. In this study, the control animals consisted of more than 99.5% macrophages. Differential analysis of the inflammatory cells showed that PMNs were the first cells to appear in response to injury. The peak infiltration of PMNs was found at 3 d, and then progressively returned to normal 28 d after BLM administration. Lymphocytes were the second cell group to appear in the BALF, and made up approximately 7% of the cells 7 d after BLM administration. Meanwhile, the peak infiltration of eosinophils was found 7 d after BLM administration.

### Table 2. Effect of Both BLM and Aging on the Markers of the Processes of Pulmonary Fibrosis Induced by BLM

<table>
<thead>
<tr>
<th>Age (week)</th>
<th>BLM (U/100 g)</th>
<th>Hydroxyproline (µg/lung)</th>
<th>Ashcroft score</th>
<th>TGF-β₁ (pg/ml)</th>
<th>HGF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Control</td>
<td>662.89±38.59</td>
<td>0.48±0.09</td>
<td>12.03±1.68</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>907.36±53.81</td>
<td>1.67±0.09</td>
<td>17.14±1.27</td>
<td>54.24±10.17</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>995.4±32.10</td>
<td>1.88±0.18</td>
<td>31.35±3.58</td>
<td>84.09±30.79</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>1140.15±68.88*</td>
<td>2.56±0.24*</td>
<td>0.72±7.50*</td>
<td>49.23±35.06*</td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>593.13±22.57</td>
<td>0.42±0.13</td>
<td>7.36±2.36</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>1010.88±65.25*</td>
<td>2.34±0.31*</td>
<td>8.82±10.75*</td>
<td>18.98±10.55*</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>538.23±19.75</td>
<td>0.37±0.08</td>
<td>5.13±0.55</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>656.70±24.10</td>
<td>1.44±0.11</td>
<td>7.50±0.60</td>
<td>36.09±13.13</td>
</tr>
</tbody>
</table>

The levels of both TGF-β₁ and HGF in BALF were measured, respectively, 3 and 7 d after BLM administration. The samples of BALF concentrated 10-fold using a YM 30 filter. The levels of HGF of BALF were below the detection limit in the control. *p<0.01 indicates significant differences from 5-week-old rats.
administration. Increasing scientific evidence defines the importance of differential cells of BALF in the pathogenesis of pulmonary fibrosis. The focus of this study was on the correlation of eosinophils with pulmonary fibrosis induced by BLM. The hydroxyproline contents began to increase 14 d after BLM administration, and in spite of the fact that there was no significant increase in hydroxyproline of the lung, eosinophil influxes of BALF were shown to be maximal 7 d after BLM administration. There was a significant correlation of eosinophilia with pulmonary fibrosis (r=0.98, p<0.05). These findings suggest that the influxes of eosinophils during the early stage of pulmonary fibrosis might be correlated with the subsequent pulmonary fibrosis induced by BLM. In contrast, previous studies have also demonstrated eosinophils to be another sources of cytokines, such as IL-2, IFN-γ, and collagenase, which have antifibrotic properties.10,11 Activated eosinophils expressed HGF which is known to act as a mitogen or a morphogen on many kinds of epithelial cells in a paracrine fashion.12,13,14 Clinical studies also demonstrated that there was an irreversible alveolar change in pulmonary architecture and relative good prognosis in contrast to idiopathic interstitial tissue pneumonia in eosinophil infiltration lung disease.25,26 In this study, there was a similar pattern among TGF-β, HGF and eosinophila of BALF during the development of pulmonary fibrosis, suggesting complex roles for eosinophils in the pulmonary fibrosis: eosinophils were likely to play a role in not only up-regulation but also down-regulation of pulmonary fibrosis, and TGF-β might work cooperatively with HGF against the fibrosis process by various mechanisms. On the other hand, it has been recently shown that the eosinophil may not be a critical cell type for the development of pulmonary fibrosis and asthma. Treatment with anti-IL-5 completely prevented BLM-induced pulmonary eosinophilia; however, anti-IL-5 failed to block BLM-induced pulmonary fibrosis.27 Clinical trial of an IL-5 blocking monoclonal antibody showed that a single dose of the antibody decreased blood and spumt eosinophils in patients with mild allergic asthma, but there was no significant improvement in asthma symptoms nor an effect on the late asthmatic response or airway hyperresponsiveness to histamine after antigen challenge.28 These data suggest that eosinophils are not essential for pulmonary fibrosis and asthma, and the actual role of eosinophils needs to be found.

Pulmonary fibrosis was different according to different ages, species and strains of animals.29 One of the characteristics of aging is immunosenescence, and the decline of the immune system is related to an increased susceptibility to infectious disease, cancer and autoimmunity.30,31 In this study, pulmonary fibrosis was different among three different ages of rat, suggesting the characteristics of aging.

In summary, eosinophil infiltration was found in various pathological situations. It is important to comprehensively evaluate the usefulness of the correlation between eosinophils and pulmonary fibrosis with other indicators, such as TGF-β and HGF of BALF.

REFERENCES