Inhibition of activin receptor-like kinase 5 induces matrix metalloproteinase 9 expression and aggravates lipopolysaccharide-induced pulmonary injury in mice


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MATERIALS AND METHODS: Anesthetized and endo-tracheally intubated C57BL/6 mice were randomized to three groups: the control group with intra-tracheal instillation of 1.5 mg/kg normal saline (NS); LPS stimulation group with intra-tracheal instillation of 3 mg/kg LPS (lipopolysaccharide); and LPS+SB431542 group with intra-peritoneal (i.p.) injection of 4.2 mg/kg SB431542 1 h before intra-tracheal instillation of 3 mg/kg LPS. The lung tissue was obtained 6 h after injury, and the degree of pulmonary injury was evaluated by pathologic scoring. The lung wet/dry weight ratio was measured. TNF-α, IL-1β, and MMP-9 (matrix metalloproteinase-9) mRNA expression levels were assayed by real time PCR (polymerase chain reaction). The content of MMP-9 total protein was measured by Western blotting. The content of active MMP-9 was detected by gelatin zymography. Location of MMP-9 in mouse lung tissue was monitored by immunohistochemistry.

RESULTS: The results showed that (1) pathologic changes including interstitial pulmonary edema, neutrophil infiltration, alveolar edema and hemorrhage were observed 6 h after LPS instillation. The lung wet/dry weight rate and pathologic scores confirmed that SB431542 administration aggravated LPS injury to the mouse lung; (2) the amount of TNF-α and IL-1β mRNA expression in LPS groups was significantly higher than that in the control group, and the highest in LPS+SB431542 group; (3) the amount of MMP-9 mRNA and MMP-9 protein expression and active MMP-9 in the lung tissue of LPS groups was significantly higher than that in the control group 6 h after injury, and the highest in LPS+SB431542 group; and (4) MMP-9 expression was mainly observed in the airway epithelial cells, vascular smooth muscle cells and cytoplasm of inflammatory cells as shown by immunohistochemistry, and brownish yellow uniform stained areas were also seen in the exudate from part of the alveoli.

CONCLUSIONS: These results indicate that blocking the activity of TGF-β/Smad pathway by specific inhibitor SB431542 of ALK5 promoted the releaser of large amounts of TNF-α, IL-1β and other pro-inflammatory cytokines from the lung tissue of mice sustaining acute lung injury (ALI). At the same time, the amount and activity of MMP-9 expression in the lung were increased, and MMP-9 expression was mainly located in the airway epithelial cells, vascular smooth muscle cells and inflammatory cells, causing increased permeability of the pulmonary blood vessels, degradation of the extracellular matrix and destruction of the normal lung tissue structures, which directly or indirectly promotes the progression of pulmonary inflammatory responses and aggravates ALI.

Key Words: Acute lung injury, SB431542, Activin receptor-like kinase (ALK), Matrix metalloproteinase 9 (MMP-9).

Introduction

Sepsis is a leading cause of death in critically ill patients and results in multi-organ injury. Of the major organs, the lung is most susceptible to...
attacks of various pathogenic factors, causing acute lung injury (ALI)\textsuperscript{2}. During the development and progression of lung injury, many inflammatory cells and inflammatory mediators constitute a highly complex “cellular network” of inflammatory responses through different signal transduction pathways\textsuperscript{3}.

Activin receptor-like kinase 5 (ALK-5) is the only TGF (Transforming Growth Factor) receptor-related kinase that mediates TGF biological signal transduction through the Smad signal transduction pathway\textsuperscript{4}. Bonniaud et al\textsuperscript{5} reported that TGF-\(\beta\) mediated cell proliferation and differentiation, embryonic development, wound repair, inflammatory response and other biological activities through members of the ALK family and TGF-\(\beta\)/Smad3 signaling pathway. Some researchers\textsuperscript{6} believed that inhibition of ALK-5 could attenuate tissue fibrosis including pulmonary fibrosis, and, therefore, selective interference with the activation of the ALK-mediated Smad-dependent signaling pathway may prove to be an effective strategy for the prevention of pulmonary fibrosis.

However, TGF-\(\beta\) has been implicated to play both an anti-inflammation role and a pro-inflammation role, and, therefore, whether single inhibition of ALK-mediated Smad activation and Smad-dependent signaling could effectively attenuate inflammation-induced pulmonary fibrosis is questionable. It was found in our preliminary experiment that prophylactic use of ALK-5 specific inhibitor SB431542 aggravated the severity of ALI. SB431542 is a small molecular weight inhibitor and is able to selectively inhibit the activity of endogenous ALKs and block the TGF-\(\beta\) signaling pathway without affecting the BMP (bone morphogenetic protein), ERK (extracellular signal-regulated kinases), JNK (c-Jun N-terminal kinases) and p38 MAP (mitogen-activated protein) signaling pathways\textsuperscript{7}.

It is generally believed that pro-inflammatory cytokines (such as TNF-\(\alpha\) and IL-1\(\beta\)) and inflammatory mediators (such as MMP-9 matrix metallopeptidase-9) would cause damage to lung epithelial cell, vascular endothelial cells and extracellular matrix, which further aggravates the severity of lung injury. Although the phenomenon of TGF-\(\beta\) preventing LPS (lipopolysaccharide)-stimulated mononuclear macrophage from releasing TNF-\(\alpha\) has been observed\textsuperscript{8}, it is still doubtful whether inhibiting TGF-\(\beta\) Smads signaling pathways could promote transcriptions of TNF-\(\alpha\), IL-1\(\beta\) and MMP-9. This study was designed to make a comprehensive evaluation on the role of ALK-5 in the development and progression of ALI by prophylactic use of SB431542 to selectively block biosignal transduction of the ALK-5 mediated TGF-\(\beta\)/Smad pathway, and subsequent detection of inflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\) expression and change in MMP-9 activity.

**Materials and Methods**

**Animal Grouping and Treatment**

Fifty-four healthy male C57BL/6 mice aged 8-10 weeks and weighing 18-25 g were provided by the Experimental Animal Center of the Second Military Medical University (Shanghai, China). All animal experiments were approved by the ethics Committee of the Second Military Medical University following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

After the animals were accustomed for 7 days with free access to standardized food and water in a 12:12 h diurnal circle before the experiments, they were equally randomized into three groups: control group, LPS stimulation group, and LPS+SB431542 group. One hour before the experiment, mice in LPS+SB431542 group received intraperitoneal (i.p.) injection of 4.2 mg/kg SB431542, and the other two groups received the same amount of the designated solvents i.p.

The animals were anesthetized with pentobarbital sodium (40 mg/kg) i.p.\textsuperscript{7}. A 1-cm midline cervical incision was made to expose the trachea. Using a bent 27-gauge tuberculin syringe, LPS (3 mg/kg) was administered intratracheally in both LPS group and LPS+SB431542 group, and NS (normal saline) (1.5 ml/kg) in the control group. The cervical incision was closed with 5.0 silk suture and the mice were returned to their cages. The mice from each group were sacrificed by inferior vena cava bleeding 6 h after LPS injection. Specimens from six mice in each group were fixed in 10% neutral formalin for pathological examination, specimens from additional six mice were sent for weight/dry ratio examination, and specimens from the remaining six mice were kept in liquid nitrogen for further analysis.

**Pathologic Evaluation of the Lung**

The lung tissue specimens were fixed in 10% neutral formalin for 48 h, dehydrated with ethanol, paraffin embedded, sliced into 6 \(\mu\)m sec-
Table I. Primer sequences, Tm, and length of amplified fragments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequences</th>
<th>Tm (°C)</th>
<th>Length (bp)</th>
</tr>
</thead>
</table>
| β-Actin  | F 5`CCTCTATGCCAACACAGTGC3` R 5`GTA
          | 58  | 211          |
| TNF-α    | F 5`GAGTCCGGGCAGTGCTACTTT3` R 5`
          | 58  | 235          |
| IL-1β    | F 5`GGGCTGACGTGTTTCTAAATGC3` R 5`
          | 57  | 134          |
| MMP-9    | F 5`CCTACTCGGCGCTCTTCAGA3` R 5`
          | 57  | 167          |

Western Blotting Analysis for Pulmonary MMP-9 Protein Expression, Gelatin Zymography Assay and Immunohistochemical Staining for Pulmonary MMP-9 Activity

Detection of Pulmonary MMP-9 Protein Expression by Western Blotting

Pulmonary MMP-9 protein expression was detected by Western blotting. The total protein content in the lysate was measured by bicinchoninic acid assay, and 30 µl protein per lane was added to the loading buffer. The lysate samples were loaded on 8% polyacrylamide gels under denaturing and reducing conditions, with addition of Laemmli buffer, blotted on a polyvinylidene difluoride membrane, and probed with anti-MMP-9 monoclonal antibodies at 1/1,000 final dilution. Bands were visualized by enhanced chemiluminescence. Immunoreactive bands were detected using an ECL (enhanced chemiluminescence) Western blotting detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Densitometric analysis of the bands was performed using a gel documentation system (Fluor-S MultiImager and Quantity One 4.4.0; Bio-Rad, Hercules, CA, USA). β-Actin served as an internal standard for protein loading and transfer.

Detection Pulmonary MMP-9 Activity by Gelatin Zymography

Pulmonary active MMP-9 level was evaluated by gelatin zymography as described[11,12]. The lysate samples were loaded on 8% polyacyr-
lamide gels containing 0.1% gelatin under non-denaturing and nonreducing conditions. After electrophoresis, gels were washed and incubated for 2 h at 80°C in a collagenase buffer. Densitometric analysis of the bands considered to reflect total enzymatic activity of MMP-9 was performed after generating TIFF (Tagged Image File Format) image files by a film scanner, using the software Quantity One (4.4.0 version, Bio-Rad, Shanghai, China). Pulmonary deposition activity was presented in volume that equals the product of the intensities of the pixels within the volume boundary multiplying pixel area.

**Detection of Pulmonary MMP-9 Activity by Immunohistochemical Staining**

For immunohistochemical staining, we adopted the method as follows: the tissue sections (5 µm) were dewaxed and rehydrated through graded ethanol washes. The slides were then incubated with 3% hydrogen peroxide for 20 min, placed in 10 mmol/L citrate buffer (pH 6.0), heated twice in a microwave oven for 5 min each, and finally blocked for 15 min with 10% normal goat serum. The sections were stained with an anti-MMP-9 monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA, USA). Subsequently, EnVision™ polymer reagent (Dako Corporate, Glostrup, Denmark) was added and visualized with peroxidase substrate (3,3'-diaminobenzidine tetrahydrochloride). The slides were counter-stained with hematoxylin, dehydrated in ethanol, and finally cleared with dimethylbenzene.

**Statistical Analysis**

All data were expressed as mean±SEM. A one-way ANOVA with SPSS for Windows software, version 14.0 (SPSS Inc., Chicago, IL, USA) was used to explore the differences of measurement data between groups. Comparison between the mean values of two samples was conducted by Student’s t-test using SPSS software, version 14.0. Difference was considered significant at \( p < 0.05 \).

**Results**

**Prophylactic Administration of SB431542 Aggravates LPS-Induced ALI**

After a 6-h period of LPS inhalation, severe lung injury was observed in LPS group, as indicated by extensive pulmonary edema, neutrophil infiltration, alveolar exudation and hemorrhage. These changes were significantly aggravated in mice administered with prophyllactic SB431542 (Figure 1). No evidence of marked lung injury was found in the control group. According to the results of pulmonary pathological score (Figure 2, Table II), LPS stimulation increased the score for ALI significantly compared with the control group (4.50±1.45 vs. 2.17±1.11, \( p < 0.05 \)). Prophylactic inhibition of ALK with SB431542 resulted in a further increase in the pulmonary pathological score (5.83±1.59 vs. 4.50±1.45, \( p < 0.01 \)). LPS inhalation also promoted pulmonary edema at 6 h after injury. The W/D weight ratio in LPS group increased significantly compared with the control group (4.21±0.28 vs. 3.85±0.22, \( p < 0.05 \)). Prophylactic administration of SB431542 aggravated the situation (4.98±0.23 vs. 4.21±0.28, \( p < 0.01 \)) (Figure 3, Table II).

**Prophylactic Administration of SB431542 Results in a Further Increase in LPS-induced up-regulation of Pulmonary TNF-α and IL-1β mRNA Expressions**

At 6 h after administration of 3 mg/kg LPS, TNF-α and IL-1β mRNA expression in LPS group was significantly higher than that in the control group (TNF-α mRNA, 14.05±0.64 vs. 11.10±0.99, \( p < 0.05 \); IL-1β mRNA 5.89±0.08 vs. 4.24±0.41, \( p < 0.05 \)). In vivo administration of SB431542 markedly increased the expression of TNF-α and IL-1β mRNA (TNF-α mRNA, 16.50±0.28 vs. 14.05±0.64, \( p < 0.01 \); IL-1β mRNA 9.79±0.30 vs. 5.89±0.08, \( p < 0.01 \)) (Figure 4, Table II).

**Prophylactic Administration of SB431542 Aggravates MMP-9 mRNA Expressions, with an Elevated Protein Content and Activity of MMP-9 and Mostly Located in the "key" Positions to Enhance LPS-Induced ALI**

Changes in pulmonary MMP-9 mRNA expression, MMP-9 content, and active MMP-9 level are shown in Table III. At 6 h after administration of 3mg/kg LPS, MMP-9 mRNA expression in LPS group was significant higher than that in the control group (16.50×10^2 ± 0.28×10^2 in LPS group vs. 10.85×10^2 ± 0.07×10^2 in the control group, \( p < 0.01 \)). Early inhibition of ALK with SB431542 aggravated MMP-9 mRNA expression (19.40×10^2 ± 0.14×10^2 in LPS+SB431542 group vs. 16.50×10^2 ± 0.28×10^2 in LPS group, \( p < 0.01 \)). Figure 5 shows the pulmonary MMP-9...
The MMP-9 content in the LPS group increased significantly compared to the control group (0.76±0.02 in the LPS group vs. 0.61±0.02 in the control group, \( p < 0.01 \)). Prophylactic administration of SB431542 increased the pulmonary MMP-9 content in the LPS+SB431542 group compared to the LPS group (0.84±0.07 in the LPS+SB431542 group vs. 0.76±0.02 in the LPS group, \( p < 0.05 \)). Figure 6 shows pulmonary active MMP-9 levels in different groups. MMP-9 activity in the LPS group was significantly higher than that in the control group (4.62±1.14 in the LPS group vs. 1.51±0.37 in the control group, \( p < 0.01 \)). Compared with the LPS group, i.p. administration of SB431542 markedly increased MMP-9 activity after LPS inhalation (7.79±2.36 in the LPS+SB431542 group vs. 4.62±1.14 in the LPS group, \( p < 0.05 \)).

The pulmonary MMP-9 content as detected by immunohistochemical staining in different groups is shown in Figure 7. The results showed that pulmonary MMP-9 in the LPS group increased significantly after LPS stimulation as compared with the control group. Meanwhile, prophylactic inhibition of ALK with SB431542 further increased MMP-9 in the LPS group, especially in the cytoplasm of pulmonary epithelial cells, vascular smooth muscle cells, and macrophages.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pathological score</th>
<th>W/D weight ratio</th>
<th>TNF-( \alpha \times 10^{-2} )</th>
<th>IL-1( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.17 ± 1.11</td>
<td>3.85 ± 0.22</td>
<td>11.10 ± 0.99</td>
<td>4.24 ± 0.41</td>
</tr>
<tr>
<td>LPS</td>
<td>4.50 ± 1.45*</td>
<td>4.21 ± 0.28*</td>
<td>14.05 ± 0.64*</td>
<td>5.89 ± 0.08*</td>
</tr>
<tr>
<td>LPS+SB431542</td>
<td>5.83 ± 1.59***</td>
<td>4.98 ± 0.23***</td>
<td>16.50 ± 0.28***</td>
<td>9.79 ± 0.30***</td>
</tr>
</tbody>
</table>

\(* p < 0.05\) vs control group; \( ** p < 0.01\) vs control group; \( ^{\#} p < 0.05\) vs LPS group; \( \#\# p < 0.01\) vs LPS group.
Discussion

In most cell types, ALK5 is the only TGF receptor-related kinase that mediates TGF biological signal transduction through the Smad signal transduction pathway. It is generally believed that TGF-β/Smad signaling pathway plays an anti-inflammatory role in inflammatory responses by inhibiting the pathophysiologic process. However, most recent studies on TGF-β/Smad signaling pathway were limited to the cell level, and few studies focused on organs of large animals. In this study, we established an ALI model in C57BL/6 mice by intratracheal administration of 3 mg/kg LPS. In addition, we prophylactically used SB431542 (a specific ALK5 activation inhibitor) to block kinase activity to further explore the role of TGF-β/Smad signaling pathway in ALI.

The lung has an abundant capillary network, and is the only organ that receives systemic blood reflux. It is an organ most susceptible to injury during the pathogenic process of sepsis and MODS (Multiple Organ Dysfunction Syndrome). It is generally accepted that the essence of ALI is an excessive and uncontrolled inflammatory response in the lung, and the core mechanism of the pathogenesis of ALI comprises two aspects: One is the uncontrolled systemic inflammatory response, as manifested by pulmonary inherent and chemotaxis wandering immunocytes that largely synthesize and secrete pro-inflammatory cytokines, such as TNF-α and IL-1β. The other is the increased number of destructive inflammatory mediators such as MMP, which promotes the injury of pulmonary epithelial cells and vascular endothelial cells, and finally causes degradation of extracellular matrix and destruction of lung tissues, thereby, promoting the duration of ALI.

It was found in this study that TNF-α and IL-1β mRNA expression increased significantly in the LPS group at 6 h after 3 mg/kg LPS administration as compared with the control group. In vivo administration of SB431542 markedly increased the expression of TNF-α and IL-1β. The pathological examination and the results of Caraway’s double-blind pathological score evaluation showed that LPS inhalation aggravated the histologic condition by promoting pulmonary edema, neutrophil infiltration, alveolar exudation and hemorrhage at 6 h after injury, and administration of SB431542 further worsened the situation. Although there are few data to explain the

### Table III. Changes of the MMP-9 mRNA, protein content and active MMP-9 expression in mouse lung.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MMP-9 mRNA (×10^-2)</th>
<th>MMP-9</th>
<th>Active MMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.85 ± 0.07</td>
<td>0.61 ± 0.02</td>
<td>1.51 ± 0.37</td>
</tr>
<tr>
<td>LPS</td>
<td>16.50 ± 0.28**</td>
<td>0.76 ± 0.02**</td>
<td>4.62 ± 1.14**</td>
</tr>
<tr>
<td>LPS+SB431542</td>
<td>19.40 ± 0.14**</td>
<td>0.84 ± 0.07**</td>
<td>7.79 ± 2.36**</td>
</tr>
</tbody>
</table>

*p < 0.05 vs control group; **p < 0.01 vs control group; *p < 0.05 vs LPS group; **p < 0.01 vs LPS group.
exact role of TGF-β/Smad signaling pathway in LPS-induced lung injury in vivo, the results from our study indicate that SB431542 treatment might change the course of Gram-negative pulmonary infection. Our findings indicate that prophylactic inhibition of TGF-β/Smad signaling pathway greatly intensified the LPS-induced over-expression of pulmonary TNF-α and IL-1β.

Figure 5. A, Expression of MMP-9 in the lung tissue in different groups which were tested by western blotting. B, Changes of MMP-9 levels in the lung tissue in different groups. **p < 0.01 when compared with the control group; *p < 0.05 when compared with the LPS group.

Figure 6. A, Results of active MMP-9 levels in the lung tissue in different groups which were tested by gelatin zymography. B, Changes of active MMP-9 levels in the lung tissue in different groups. **p < 0.01 when compared with the control group; *p < 0.01 when compared with the LPS group.
mRNA, which led to further disequilibrium between pro-inflammatory and anti-inflammatory systems, causing further deterioration of ALI.

MMP-9 is a protease first isolated from human neutrophils by Sopata et al.\(^1\), and can be synthesized and released by monocytes, neutrophils, fibroblasts and endothelial cells\(^2\). Active MMP-9 can degrade pulmonary endothelial cell basement membrane, thus causing injury to pulmonary epithelial cells and vascular endothelial cells, increasing the permeability of pulmonary vessels, and resulting in pulmonary microcirculation disorders\(^3\). At the same time, MMP-9 may also degrade extracellular matrix, causing further injury to the normal lung structures and thus promoting the progression of inflammatory lung injury\(^4\). The results of RT-PCR, Western blotting and gelatin zymography in our study showed that MMP-9 mRNA and protein expressions and MMP-9 activity in LPS group were significantly higher than those in the control group, while they were even higher in LPS+SB431542 group, indicating that intra-tracheal administration of LPS not only increased the synthesis and secretion of MMP-9 but increased MMP-9 activity. Inhibition of TGF-β/Smad signaling pathway by prophylactic use of SB431542 further enhanced the synthesis, secretion and activity of MMP-9. In addition, the results of immunohistochemistry also showed that the normal lung structures were damaged severely in LPS+SB431542 group, and large amounts of active MMP-9 were expressed in the tracheal epithelial cells, vascular smooth muscles cells and cytoplasm of inflammatory cells, which further confirmed that inhibition of TGF-β/Smad signaling pathway could increase the expression and activity of MMP-9, and the increased expression and activity of MMP-9 were mainly distributed in key cells related to lung injury, which directly or indirectly promoted deterioration of ALI.

**Conclusions**

In summary, inhibition of TGF-β/Smad signaling pathway by using ALK5 specific inhibitor SB431542 promoted release of large amounts of TNF-α and IL-1β in the lungs of ALI mice and increased the expression and activity of MMP-9. The increased expression and activity of MMP-9 were mainly distributed in key cells related to lung injury, which increased the permeability of pulmonary blood vessels, degraded the extracellular matrix and damaged the normal lung structures, which directly or indirectly promoted the progression of pulmonary inflammatory responses and aggravated ALI.

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