CASE REPORT

Analysis of cytokine/chemokine levels in bronchoalveolar lavage fluids from patients with acute respiratory distress syndrome


Abstract: Bronchoalveolar lavage (BAL) was performed in 5 patients with acute respiratory distress syndrome (ARDS; ARDS group hereafter) and 2 patients with respiratory failure (non-ARDS group hereafter) within 3 hours of intubation before ARDS treatment other than mechanical ventilation. ARDS was diagnosed based on the criteria defined by the American-European Consensus Conference. BAL fluid (BALF) and sera samples were obtained on the same occasion. The presence of 17 cytokines/chemokines in these specimens was simultaneously determined using a Bio-Plex® Suspension Array System. The levels of these cytokines/chemokines in BALF and sera samples were compared between the ARDS group and the non-ARDS group. The levels of chemokine granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1) (monocyte chemoattractant factor), and macrophage inflammatory protein-1β (MIP-1β) in the BALF samples were significantly higher in the ARDS group than in the non-ARDS group. The level of pro-inflammatory cytokine interleukin (IL)-6 in BALF was also higher in the ARDS group than in the non-ARDS group. In contrast, the levels of IL-1β and IL-8 in BALF were higher in the non-ARDS group than in the ARDS group. The levels of these cytokines in BALF were higher than the corresponding sera levels in both groups. These results indicate that severe initial alveolar injury significantly activates chemokines in patients with ARDS caused by direct lung injury (such as pneumonia), unlike the increased levels of the inflammatory cytokines IL-1β and IL-8 in BALF of patients with early ARDS.

Key words: ① acute respiratory distress syndrome (ARDS), ② cytokines/chemokines, ③ bronchoalveolar lavage (BAL)

Introduction

Acute respiratory distress syndrome (ARDS), which has various underlying etiologies, is a rapidly progressive disorder. The acute phase is characterized by the rapid onset of respiratory failure and arterial hypoxemia refractory to supplemental oxygen therapy. A new definition for ARDS was recommended by the American-European Consensus Conference Committee in 1994, enabling standardized clinical research and trials to become possible 1). The clinical disorders thought to be responsible for ARDS can be categorized into two groups: direct pulmonary injury, such as pneumonia (pulmonary etiology); and indirect lung injury, such as sepsis (extra-pulmonary etiology) 1). In the present study, we compared the cytokine levels of ARDS patients with pneumonia (no initial extra-pulmonary injury) and pneumonia patients without ARDS to clarify the factors responsible for the onset of ARDS.

The pathological findings of ARDS include diffuse alveolar damage and extravasation of the intravascular fluid. Inflammation was also identified as a key feature in the 1994 Consensus Conference on ARDS 1). The formation of inflammatory mediators is now widely regarded to play an important role in the pathophysiology of inflammation in ARDS 2). These mediators include tumor necrosis factor (TNF)-α and interleukin (IL)-1, IL-4, IL-6, IL-8, IL-10, and IL-13 3). C5a, the expression
of which is considered to be a major contributor to the inflammatory pathway in sepsis \(^4,5\) but is not involved in the final common pathway to acute lung injury (ALI) / ARDS, is complemented by the expression of chemokines, cytokines, and lipid signaling molecules \(^2,3\). On the other hand, the acute phase of ARDS is characterized by the influx of neutrophils as a consequence of the disruption of alveolar-capillary barrier \(^6\). Activated alveolar macrophages secrete cytokines, which activate the inflammatory cascade and neutrophils; this in turn stimulates the infiltration of neutrophils \(^7\). Although the levels of these cytokines/chemokines have been individually determined using enzyme-linked immunosorbent assay (ELISA) systems, the levels of inflammatory cytokines and chemokines must be simultaneously determined using a small sample to fully understand the involvements of inflammatory cytokines and chemokines in ARDS. Recently, the Bio-Plex\textsuperscript{®} Suspension Array System (Bio-Rad Laboratories, Inc., USA) has been established for the simultaneous determination of cytokines/chemokines in small samples of 15–50 \(\mu\)l, enabling a comprehensive analysis \(^8\).

In the present study, bronchoalveolar lavage (BAL) was performed in patients with ARDS or acute respiratory failure who were receiving mechanical ventilation. The levels of 17 cytokines/chemokines in BAL fluid (BALF) were compared between patients with ARDS and those with moderate respiratory failure arising from other causes to clarify the roles of cytokines and chemokines as inflammatory mediators in patients with ARDS.

### Materials and methods

**Patients:** This study was approved by the Ethics Committee of the International Medical Center of Japan (No. 449-H19). Written informed consent was obtained from all the patients.

Patients were diagnosed as having ARDS or ALI according to the definitions of the American-European Consensus Conference Committee \(^1\).

**Mechanical ventilation:** Mechanical ventilation was initiated in all the patients while they were in our hospital’s ICU; all the patients had severe arterial hypoxemia that was refractory to supplemental oxygen treatment. The mechanical ventilation parameters in the ARDS patients were decided based on the Lung Protective Strategy of the American-European Consensus Conference Committee \(^9\).

**BALF and sera samples:** BAL was performed within 3 hours of the start of mechanical ventilation and before the start of specific treatment for ARDS other than mechanical ventilation. The BAL procedure was performed using a fiber-optic bronchoscope with 3 aliquots of 50 m\(l\) of warm sterile saline, infusing 50 m\(l\) of warm sterile saline into a segment of the lung and aspirating the fluid back into each aliquot (more than 50% recovery). The first aliquot was discarded, and the third aliquot was saved and used for the assays. Sera samples were also obtained from the patients on the same occasion.

**Laboratory processing and assay:** The BALF and sera samples intended for the cytokine/chemokine assay were immediately processed and then stored at \(-80^\circ\)C.

The Bio-Plex\textsuperscript{®} Suspension Array System was used for the cytokine/chemokine assay, enabling the levels of 17 cytokines/chemokines; IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte macrophage colony stimulating factor (GM-CSF), interferon-\(\gamma\) (IFN-\(\gamma\)), TNF-\(\alpha\), IL-1\(\beta\), IL-5, IL-7, IL-12 (P70), IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1) (monocyte chemoattractant factor, MCAF), and macrophage inflammatory protein-1\(\beta\) (MIP-1\(\beta\)), to be measured simultaneously. The cytokines/chemokines were measured using the fluorescent microbeads method.

### Results

**Clinical data:** Seven patients were enrolled in this study. Their demographic data are shown in Table 1; 5 patients were diagnosed as having ARDS (ARDS group; P/F ratio was grade 4 in 4 patients and grade 3 in 1 patient according to the Murray Lung Injury Score \(^10\)), and the remaining 2 patients were diagnosed as having acute respiratory failure (non-ARDS group; 1 patient was within the definition for ALI [200 < P/F ratio < 300]). Of the 7 patients, 6 survived and were discharged from the ICU and 1 died while in the ICU. In the ARDS patients, mechanical ventilation was initiated using the controlled mechanical ventilation (CMV) (continuous positive pressure ventilation (CPPV) mode), and the initial ventilator settings were as follows: tidal volume, 6 m\(l\) \(\cdot\) kg\(^{-1}\);
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respiratory rate, 25–30 \( \text{min}^{-1} \); PEEP (positive end-expiratory pressure), 10–12 cmH2O; peak inspiratory pressure, below 30 cmH2O; FIO2, 1.0 (Table 2).

Cytokine and chemokine levels in BALF —ARDS and non-ARDS groups (Fig. 1): The levels of G-CSF (8,504 vs. 106 pg \( \cdot \text{ml}^{-1} \), mean values), MCP-1 (MCAF) (3,024 vs. 59 pg \( \cdot \text{ml}^{-1} \)) and MIP-1 \( \beta \) (1,181 vs. 126 pg \( \cdot \text{ml}^{-1} \)) in the BALF were higher in the ARDS group than in the non-ARDS group. The level of IL-6 was also elevated in the ARDS group, compared with that in the non-ARDS group (919 vs. 34 pg \( \cdot \text{ml}^{-1} \)). On the other hand, the levels of IL-1\( \beta \) (51 vs. 501 pg \( \cdot \text{ml}^{-1} \)) and IL-8 (761 vs. 2,247 pg \( \cdot \text{ml}^{-1} \)) were higher in the non-ARDS group than in the ARDS group.

Cytokine and chemokine levels in sera —ARDS and non-ARDS groups (Fig. 2): The serum levels of IL-7 (32 vs. 0 pg \( \cdot \text{ml}^{-1} \), mean values), IL-13 (21 vs. 4 pg \( \cdot \text{ml}^{-1} \)), GM-CSF (128 vs. 52 pg \( \cdot \text{ml}^{-1} \)) and G-CSF (364 vs. 86 pg \( \cdot \text{ml}^{-1} \)) were higher in the ARDS group than in the non-ARDS group. However, the serum levels of TNF-\( \alpha \) (15 vs. 94 pg \( \cdot \text{ml}^{-1} \)), IFN-\( \gamma \) (168 vs. 338 pg \( \cdot \text{ml}^{-1} \)), IL-6 (317 vs. 402 pg \( \cdot \text{ml}^{-1} \)) and MIP-1\( \beta \) (40 vs. 311 pg \( \cdot \text{ml}^{-1} \)) were higher in the non-ARDS group than in the ARDS group.

Discussion

ARDS is characterized by the acute onset of diffuse and severe inflammation \( ^7 \). The underlying causes have been classified into those causing direct lung injury and those causing indirect lung injury. Although some recent strategies and trials have been successful and have contributed to a decline in the mortality rate, the mortality rate of ARDS remains high \( ^{11} \)\( ^{13} \). The outcome of ARDS is considered to depend on the degree of alveolar epithelial injury. Clinical and experimental studies have demonstrated the evidence of neutrophil-mediated lung injury in ARDS and ALI \( ^{14} \). Neutrophils are recognized as major contributors in the pathogenesis of ARDS \( ^2 \), and a complex network of inflammatory cytokines plays an important role in the development of ARDS \( ^2 \)\( ^3 \).

One of the characteristic findings of this study was the remarkably elevated BALF levels of G-CSF, MCP-1 (MCAF) and MIP-1\( \beta \), which are all chemokines, in the ARDS group, compared with those in the non-ARDS group. These findings differ from those of previous reports, in which TNF-\( \alpha \) and IL-1\( \beta \) were described as being elevated in BALF and sera of patients with ARDS \( ^{15} \)\( ^{17} \).

TNF-\( \alpha \) and IL-1\( \beta \) are thought to be produced by activated macrophages during the acute phase of ARDS, leading to the induction of IL-6 and IL-8 and the subsequent activation of a network of cytokines \( ^{14} \)\( ^{16} \)\( ^{17} \). Once the inflammatory cascade has been activated by this cytokine network, neutrophils migrate into the alveolar space \( ^{17} \). Thus, in cases of ARDS with direct lung injury, the excessive activation of chemokines is thought to occur. The production of excess amounts of chemokines

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### Table 2  Characteristics of lung injuries and outcomes

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>Onset day</td>
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<td>3</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>2</td>
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ARDS, acute respiratory distress syndrome; CHF, congestive heart failure; CPPV, continuous positive pressure ventilation; SIMV, synchronized intermittent mandatory ventilation.
in the alveolar region is, in turn, thought to enhance the infiltration of neutrophils, resulting in the induction of direct damage to the lung.

Two possibilities might explain the extraordinarily high levels of chemokines in the BALF samples from the ARDS group in the present study. One is the different time-points at which the BALF specimens were obtained, and the other is the difference in the types of disorders that were associated with ARDS and acute respiratory failure in the study groups. In the present study, ARDS was associated with direct lung injury in all 5 cases. We performed BAL within 3 hours of the initiation of
mechanical ventilation in each patient; among the studies reported to date, this is the earliest reported time-point at which the BALF specimens were obtained \(^{14-16}\). In most previous studies, the BALF specimens were obtained within 24 hours. Thus, the BALF findings in the present study may not have been affected by ventilation-induced lung injury or ongoing inflammation. The levels of cytokines in BALF and sera might change drastically over the first 24 hours after intubation.

Rosseau et al. \(^{18}\) suggested that a significant correlation existed between the BALF MCP-1 concentration (reflecting a predominance of monocyte-like alveolar macrophages) and the severity of respiratory failure. In ARDS caused by direct lung injury, activated macrophages, monocytes and neutrophils cause inflammation of the lung, resulting in an increased production of G-CSF, MCP-1 and MIP-1β. Lung injury in cases with ARDS is more severe than that in cases without ARDS and is associated with high levels of G-CSF, MCP-1 and MIP-1β.

Another reason for the high levels of chemokines seen in our study may be the difference in the spectra of diseases associated with ARDS, since all the ARDS patients in the present study had direct lung injury. Elevations in cytokine/chemokine levels are thought to depend on whether the injury to the lung is direct or indirect. In ARDS associated with direct lung injury, the inflammation is thought to be more severe and to be associated with higher levels of cytokines/chemokines than that in ARDS associated with indirect lung injury.

Similar results to ours have been demonstrated in one previous study. Nakano et al. \(^{19}\) measured the levels of cytokines in 4 cases with ARDS and 8 normal controls and reported significantly higher levels of 16 cytokines/chemokines in the BALF specimens from the ARDS patients. Although the report did not describe the specific time-point at which the BALF specimens were obtained, the authors suggested that cytokine and chemokine influx from the plasma into the alveoli may be responsible for the excessive elevations of the cytokine/chemokine levels in BALF. According to our study, however, the cytokine/chemokine levels in BALF were higher than the cytokine levels in sera during the early phase of ARDS and were not the result of cytokine/chemokine influx from the plasma.

Among chemokines, IL-8 has one of the strongest effects on neutrophils. Although a previous report concluded that IL-8 was a reliable predictor of the development of ARDS \(^{20}\), our results suggest that this is not necessarily true. In our study, the levels of IL-1β and IL-8 in BALF were higher in the non-ARDS group than in the ARDS group. Because IL-8 is a chemotactic factor for neutrophils, the induction of IL-8 has already occurred once bacterial infection is observed in the lung (as in pneumonia). Thus, the levels of these cytokines are elevated not only in cases with ARDS, but also in cases with a high risk of ARDS, and elevated IL-8 levels might reflect the overall clinical severity, rather than pulmonary injury in ARDS \(^{21}\). The systemic levels of TNF-α and IL-1β have also been reported to reflect the severity of lung injury and not to have any diagnostic value \(^{15}\).

Sivelestat is known as a reversible, competitive inhibitor of neutrophil elastase. Although its efficacy remains controversial, it has been shown to be effective in animal models and has been demonstrated to have beneficial effects on pulmonary function \(^{22}\). However, the Sivelestat Trial in ALI Patients Requiring Mechanical Ventilation (STRIVE) study \(^{23}\) has demonstrated that the intravenous administration of a neutrophil elastase inhibitor had no effect on the 28-day all-cause mortality rate or the number of ventilator-free days in a heterogeneous group of patients with acute lung injury. Considering the pathogenesis of ARDS, neutrophil elastase inhibitors might be effective if they were used in cases of ARDS caused by direct lung injury.

Two possible limitations of this study should be mentioned: the number of cases was quite small, and ARDS was caused by direct lung injury in all of the reported cases. Further investigations using a larger number and a wider spectrum of ARDS patients, including cases caused by direct and indirect lung injury, are warranted.

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References


