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The expression of HSPs, anti-oxidants, and cytokines in plasma and bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome

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Accepted 21 January 2012
Available online 31 January 2012

Abstract

Objectives: We studied several acute inflammatory materials (AIM) such as various inflammatory cytokines, oxidative stress, and heat shock proteins in ARDS patients by simultaneously measuring from bronchoalveolar lavage fluid (BALF) and plasma.

Design and methods: AIM were measured by using plasma and BALF sampling obtained from ARDS group (n=12) and non-ARDS group (n=12).

Results: In the BALF, only HSP 47 was significantly related to HSP90, HSP 60 and GPx-3 in plasma but not in BALF. Ninety-day mortality was significantly related to HSP90, HSP 60 and GPx-3 in plasma but not in BALF.

Conclusion: Alteration of AIM levels in both BALF or plasma of ARDS group was not remarkable compared with the non-ARDS group. Our result suggests the need to reconsider ARDS pathophysiology and therapeutic strategy.

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Introduction

Acute respiratory distress syndrome (ARDS) is characterized by an overwhelming lung inflammation and increased microvascular permeability causing rapid onset of respiratory failure [1]. Although the current American-European Consensus Conference (AECC) definition of ARDS is widely accepted in the clinical setting, this allows an inclusion of highly heterogeneous group of critically ill patients as various types of lung injury [1]. Moreover, as diffuse alveolar damage is generally known as the histologic hallmark of ARDS, some questioned whether the concept of ARDS as a syndrome is appropriate [2].

We therefore studied the aspect of multiple AIMs as a whole in ARDS patients by measuring from bronchoalveolar lavage fluid (BALF) and plasma.

Methods

Study subjects

We prospectively enrolled 24 patients admitted to our Intensive Care Unit (ICU) from January to December 2009. Patients with ARDS (n=12) met the following diagnostic criteria for AECC ARDS definition [1,7]: acute onset, bilateral chest radiographic infiltrates, pulmonary artery occlusion pressure of ≤18 mm Hg, or no evidence of left atrial hypertension, and impaired oxygenation regardless of the PEEP level, with a PaO2/FIO2 <200. As a control group, we enrolled patients under mechanical ventilation with no obvious evidence of ARDS (non-ARDS group, n=12). This study was approved by the Korea University Anam Hospital Ethics Committee, and all patients gave written informed consent after given full explanation of this study.
Blood and bronchoalveolar lavage fluid (BALF) sampling

Blood and BALF samples were obtained as soon as the diagnosis of ARDS was established (i.e., within 48 h). The blood was collected in an ethylenediaminetetraacetic acid (EDTA)-2Na vacutainer tube and immediately kept at a temperature of 4 °C. After centrifugation at 1500 g for 10 min at 4 °C, the plasma was kept at −80 °C until measurement. At the same time, BALF was obtained. Study subjects were pretreated with atropine (0.5 mg) immediately before the BAL procedure. Bronchoscope (Olympus B2-10; Olympus Optical Co., Tokyo, Japan) was wedged into the bronchus of right middle lobe or lingular division, or the area of pneumonia. Instillation of 30 mL of warmed normal saline into bronchial trees for four times and gentle

Table 1
Baseline characteristics of patients with non-ARDS and ARDS.

| Variables            | Non-ARDS | ARDS  |
|----------------------|----------|-------|
| Patients             | 12       | 12    |
| Age, yr              | 61.4 ± 17.0 | 67.7 ± 8.0 |
| M/F gender           | 9/3      | 11/1  |
| Reasons for MV       |          |       |
| Pneumonia            | 0        | 12    |
| Volume overload      | 6        | 0     |
| Multiple trauma      | 2        | 0     |
| Neurosurgery         | 2        | 0     |
| Neuromuscular disorder | 1     | 0     |
| GI sepsis + atelectasis | 1   | 0     |

Data are presented as No. or mean ± S.D.
M = male, F = female, MV = mechanical ventilator, GI = gastrointestinal.

Fig. 1. Western blot analysis and relative intensities to α-tubulin of the expression of HSPs (A), anti-oxidants (B), and cytokines (C) in BALF. Only HSP 47 was significantly increased in the ARDS BALF than non-ARDS BALF among several HSPs, anti-oxidants, and cytokines. The data represent the means ± SD.
suction with a negative pressure below 150 mm Hg were done under bronchoscopic observation. A supernatant was separated from cell pellets by centrifugation at 500 g for 10 min. Supernatants were frozen and stored at −80 °C until study. No complication was experienced during all the BAL procedures.

Acute inflammatory materials (AIM) measurements

As HSPs, we measured glucose regulated protein (GRP)94, HSP 90, HSP70, HSP 60, HSP47, HSP40, and HSP27. Interleukin (IL)-1β, IL-6, IL-8, IL-12, and tumor necrosis factor (TNF)-α were measured as cytokines. On the other hand, glutathione-S-transferase (GST), glutathione peroxidase (GPx)-3, and superoxide dismutase (SOD) were measured as anti-oxidants.

Western blot analysis

BALF and plasma protein concentration of the total lysate was determined using a Bradford protein assay (Bio-Rad Laboratory, Richmond, CA, USA). An equal amount of protein per lane was then separated on 7.5–12% SDS polyacrylamide gels and subsequently transferred to polyvinylidene difluoride membranes (Millipore Corporation, MA, USA) at 400 mA for 2 h 40 min. The membranes were then blocked with blocking buffer (PBS-T (0.05% Tween 20 in PBS) containing 5% skim milk) for 1 h at room temperature, after which they were incubated with primary antibodies overnight at 4 °C; GRP94 (H-212; Santa cruz, 1:500 dilution), HSP90 (BD, 1:1000 dilution), HSP70 (K-20; S Santa cruz, 1:500 dilution), HSP60 (Abcam, 1:1000 dilution), HSP47 (E-1, Santa cruz, 1:500 dilution), HSP40 (N-19, Santa cruz, 1:500 dilution), HSP27 (cell signaling, 1:1000 dilution), IL-1β (H-153, Santa cruz, 1:500 dilution), IL-6 (Santa cruz, 1:500 dilution), IL-12 (Abcam, 1:500 dilution), GST (Z-5, Santa cruz, 1:500 dilution), GPx-3 (H-50, Santa cruz, 1:500 dilution), SOD-1 (FL-154; Santa cruz, 1:500 dilution). After washing with TBS-T, the membrane was incubated with secondary antibody (goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) that was diluted 1:2000. Bands were visualized by chemiluminescence (Amersham ECL kit) and analyzed by scanning (Epson Expression 10000× L, Epson), and the image files were transformed into TIFF format with linear gray scale values. Densitometric values were normalized using α-tubulin (Santa cruz, 1:1000 dilution) as an internal control. Scanning of the Western blots showed the curve to be linear in the range used for each antibody.

Statistical analysis

Statistic analyses were performed using SPSS version 14.0 (Chicago, IL, USA). Continuous variables were compared by Student’s t-test or Mann–Whitney test, as appropriate. Data are presented as means± standard deviation or as median (range). Statistical significance was defined as p<0.05.

Results

Baseline characteristics of the patients are shown in Table 1. The mean age was 61.4±17 in non-ARDS group and 67.7±8.0 in ARDS group. Both groups were male-dominant. All patients in ARDS group were diagnosed as pulmonary ARDS. In the non-ARDS group, main reasons for mechanical ventilation were volume overload, multiple trauma, and neurosurgery.

In terms of HSPs, only HSP47 was significantly increased in the ARDS BALF as compared with non-ARDS BALF (Fig. 1A). In plasma, on the other hand, GRP94, HSP90, HSP60 and HSP47 were increased with statistical significance in ARDS group than those in non-ARDS group (Fig. 2A). Among anti-oxidants, only GPx-3 in plasma was increased significantly in ARDS group (Figs. 1B and 2B). The intensity of IL-8 in plasma was exclusively increased in ARDS group among several cytokines (Fig. 2C). Meanwhile, there was no cytokine significantly increased in BALF from ARDS group compared with the non-ARDS group (Fig. 1C). In order to validate whether any AIM will function as a useful predictor of clinical outcome, a statistical analysis was performed; HSP90, HSP 60 and GPx-3 in plasma were associated with increased 90-day mortality. However, there was no AIM which was associated with increased mortality in BALF (Table 2).
Discussion

In the present study, only HSP 47 was significantly increased in ARDS BALF compared with non-ARDS BALF among several HSPs, anti-oxidants, and cytokines. In plasma, GRP94, HSP90, HSP60, HSP47, GPx-3, and IL-8 were increased significantly in ARDS group compared with non-ARDS group. Therefore, in contrary to our expectation, most of the HSPs, cytokines, and anti-oxidants in BALF or plasma were not significantly different in the ARDS group when compared with non-ARDS group. As a predictor of clinical outcome, only HSP90, HSP60 and GPx-3 in plasma but not in BALF among a number of AIM were associated with increased 90-day mortality. There have been several studies regarding cytokines, HSPs, or oxidative stress in human or animal ARDS. In our study, HSP47 was significantly increased both in BALF and plasma in ARDS group comparing to non-ARDS group. Similar to our results, Hagiwara et al. showed that HSP47 was up-regulated in an LPS-induced ARDS lung model in rat, especially in the process of lung fibrosis [12,13]. They suggested that HSP47 may play a major role in the processing and secretion of procollagen, which represents fibrosis. Meanwhile, in one human ARDS study, although basal HSP70 in peripheral blood monocytes were not different from those in the non-ARDS group, initial levels of inducible HSP70 were significantly decreased initially compared with non-ARDS. This HSP70 inducibility phenomenon gradually recovered over time [10]. In the present study, we utilized BALF instead of blood for measuring the levels of HSP70, and there was no significant difference between the two study groups. Singleton et al. showed that a deletion of HSP70 gene led to increased mortality and increased occurrence of lung injury in a sepsis induced by cecal ligation-and-puncture mice model [14]. It is not sure, however, whether the results from the above extrapulmonary ARDS mice model can be extrapolated to pulmonary ARDS. There has been controversy among researchers about the cytokine IL-8 in ARDS. Similar to our study, Bouros et al. observed that plasma level of IL-8 in ARDS was higher as compared to non-ARDS, but not in BALF [7]. On the contrary, another investigator showed that the IL-

![Western blot analysis and relative intensities to α-tubulin of the expression of HSPs (A), anti-oxidants (B), and cytokines (C) in plasma. GRP 94, HSP 90, HSP 60, HSP 47, GPx-3, and IL-8 in plasma were increased significantly in ARDS group compared with non-ARDS group. The data represent the means±SD.](image-url)
8 in BALF from patients with ARDS was higher than that from patients with cardiogenic pulmonary edema or from healthy volunteers without applying mechanical ventilation [12,15]. Meanwhile, these studies stressed that the level of IL-8 was more useful for prediction of outcomes such as survival rather than development of ARDS [7,15].

In our study, we measured several cytokines, anti-oxidants, and HSPs simultaneously in BALF and plasma. In theory, cytokines or HSPs responses will be naturally confined to the lungs, and therefore studying with blood specimens will merely provide an incomplete reflection of inflammatory events in lung. Accordingly, measurements of these AIMs in the lungs are likely to be more reasonable than utilizing plasma or serum as a specimen. On the other hand, measuring cytokines in the lungs may accompany some limitations, because cytokines exist not only in alveolar space but also in the tissue space (i.e., interstitium) [9]. Moreover, proteins or cytokines in alveolar fluids obtained by BAL are diluted for about 50–100 times during specimen handing [7,16]. Thus measuring AIMs in BALF may provide insufficient information about the concentration and function of a specific cytokine. Nevertheless, we believe that it is better way to estimate the extent of pulmonary ARDS than measuring AIMs in blood.

There are some limitations in our study. First, the sample size is small. We might consider the possibility that the fairly small sample size could give this study only a moderate statistical power. Second, when we initially designed this study, we did not consider what kind of nutritional support with nutrients is important nor has a certain influence on outcome of ARDS. Therefore, enrolled patients were

| Table 2 | Median (range) bronchoalveolar lavage fluid (BALF) and plasma intensities of the measured acute inflammatory materials (AIM) whether or not they survived at 90 days. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Survivors (n = 11) | Non-survivors (n = 10) | Survivors (n = 13) | Non-survivors (n = 11) |
| GRP94           | 1.68 (0.40–3.17)  | 1.54 (0.03–4.26)  | 0.98 (0.56–2.00)  | 1.60 (0.38–2.84)  |
| HSP90           | 0.85 (0.56–1.31)  | 1.02 (0.46–1.59)  | 1.12 (0.45–2.18)  | 3.48 (0.76–5.77)  |
| HSP70           | 0.78 (0.17–3.50)  | 0.27 (0.07–4.42)  |                 |                 |
| HSP60           | 1.01 (0.72–1.36)  | 0.90 (0.44–1.28)  |                 |                 |
| HSP47           | 1.19 (0.68–1.83)  | 1.59 (1.02–2.53)  | 1.03 (0.62–2.45)  | 1.91 (1.02–3.40)  |
| HSP40           | 1.03 (0.25–2.78)  | 1.21 (0.46–2.28)  | 0.80 (0.47–1.68)  | 1.08 (0.35–2.17)  |
| HSP27           | 1.04 (0.29–1.56)  | 0.70 (0.03–1.57)  | 0.81 (0.39–1.68)  | 0.88 (0.35–2.43)  |
| Catalase        | 0.97 (0.71–1.63)  | 1.35 (0.39–1.64)  | 0.76 (0.46–1.69)  | 0.88 (0.47–1.40)  |
| GST             | 1.03 (0.59–1.41)  | 0.97 (0.08–1.29)  | 0.86 (0.37–1.71)  | 1.13 (0.59–2.10)  |
| GPx-3           | 0.97 (0.68–1.24)  |                 | 0.97 (0.68–1.24)  | 1.37 (0.96–2.43)  |
| SOD-1           | 0.89 (0.13–1.32)  | 0.39 (0.04–1.99)  | 1.17 (0.69–1.60)  | 1.40 (0.34–2.56)  |
| IL-1β           | 1.09 (0.20–2.79)  | 1.21 (0.50–2.06)  | 2.07 (0.37–5.58)  | 2.16 (0.45–3.86)  |
| IL-6            | 0.96 (0.49–1.51)  | 1.08 (0.64–2.26)  | 1.31 (0.77–2.73)  | 1.43 (1.06–2.11)  |
| IL-8            | 1.08 (0.23–1.55)  | 0.94 (0.08–1.35)  | 1.76 (0.78–5.63)  | 2.31 (1.24–5.46)  |
| IL-12           | 1.21 (0.50–1.38)  | 1.04 (0.72–1.29)  | 3.01 (0.43–5.08)  | 3.84 (2.23–8.72)  |
| TNF-α           | 0.72 (0.23–1.82)  | 0.66 (0.10–1.27)  | 1.19 (0.68–3.23)  | 1.02 (0.69–2.04)  |

Comparisons were done using the Mann–Whitney test. GRP = glucose regulated protein, HSP = Heat shock protein, GST = glutathione-S-transferase, GPx = glutathione peroxidase, SOD = superoxide dismutase, IL = Interleukin, TNF = tumor necrosis factor

* P<0.05, versus survivors group.
not supplied with additional nutrients such as omega-3 fats or antioxidant vitamins. Pontes-Arruda et al. suggested that nutritional support with nutrients like omega-3 fats and antioxidant vitamins associated with reduction in the risk of 28-day mortality as well as relevant improvement in oxygenation and clinical outcomes including ventilator-free days, ICU-free days and risk of development of new organ dysfunction [17]. However, a recent study by Rice et al. suggests that twice-daily supplementation of omega-3 fats, γ-linolenic acid, and antioxidant may not be helpful for ARDS patients [18]. Based on these two conflicting results, nutrition may play a critical role in determining inflammation levels, morbidity and mortality in ARDS patients.

The current definition of ARDS is described clinical syndromes not by a specific pathophysiologic process which causes organ system dysfunction. Although the simplicity of current definition of ARDS has a strong point in easy application in the clinical setting, there are also several disadvantages and each manifestation of ARDS may encounter skepticism for the following several reasons [1,5]. First, several factors such as an underlying cause or existence of other organ failure, which are known to influence the outcome, do not need to be assessed [19,20]. In our study, even though we enrolled only pneumonia ARDS patients to the ARDS group to avoid potential heterogeneity in ARDS, we did not practically specify each pneumonic patient according to pathogens such as virus, Gram negative or positive bacteria. Second, the presence of bilateral pulmonary infiltrates on chest radiography can also manifest in various disease [5,21]. For instance, bilateral infiltrates can also be noted in patients with Goodpasture’s syndrome, Wegener’s granulomatosis, acute lupus pneumonia, and so on. The interpretation of bilateral infiltrates can be confused whether there is a cardiogenic pulmonary edema. Third, the severity of underlying disease is not properly described in the ARDS, and therefore a wide variety of disease severity can be included in the ARDS group. Fourth, although ARDS criteria contains the concept of time (i.e., acute onset), it lacks exact length of elapsed time as an outcome of an ARDS, and are inevitably arbitrary in the “real” world [5]. In contrast, most of the animal or cell-line investigations are fundamentally performed under the notion of punctuality when observing a consequence after an insult; this is practically impossible in the clinical domain.

Despite tremendous efforts, the pathogenic mechanisms that ARDS characterize are complex and still not fully understood. The diagnosis of ARDS contains a heterogeneous group of patients with various causes and pathophysiological mechanisms. Therefore it seems to be an unrealistic idea that a specific therapeutic agent that can successfully alter a single biological target in an animal model of ARDS will also reduce mortality of human subjects having an ARDS as well [14]. Therefore, we suggest that the current criteria for diagnosing ARDS justify a substantial modification, and the complex pathophysiology of this heterogeneous group disease needs to be more clearly defined.

Acknowledgments

This study was supported by a Korea University Grant

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