Distinct time courses and pathogenic contributions of alveolar epithelial and endothelial injury in acute respiratory distress syndrome with COVID-19: evidence from circulating biomarkers

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Abstract

Background: In severe cases of coronavirus disease (COVID-19), acute respiratory distress syndrome (ARDS) with alveolar tissue injury occurs. However, the time course and specific contributions of alveolar epithelial and endothelial injury to the pathogenesis of COVID-19 ARDS remain unclear.

Methods: We evaluated the levels of a circulating alveolar epithelial injury marker (soluble receptor for advanced glycation end-products: sRAGE) and an endothelial injury marker (angiopoietin-2: ANG-2), along with an alveolar permeability indicator (surfactant protein D: SP-D) in 107 serum samples from nine patients with ARDS and eight without ARDS, all with COVID-19. We compared the initial levels of these markers between ARDS and non-ARDS patients, and analysed the temporal changes of these markers in ARDS patients.

Findings: All the initial levels of sRAGE, ANG-2, and SP-D were significantly higher in the ARDS patients than in the non-ARDS patients. The peak sRAGE level in the ARDS patients was observed at the very early phase of disease progression. However, the peaks of ANG-2 and SP-D were observed at a later phase. Moreover, the ANG-2 level was significantly correlated with the arterial oxygenation and the SPD level, but the sRAGE level was not.

Interpretation: Evaluation of circulating markers confirms that COVID-19 ARDS is characterised by severe alveolar tissue injury. Our data indicate that the endothelial injury, which continues for a longer period than the epithelial injury, seems to be the main
contributor to alveolar barrier disruption. Targeting the endothelial injury may, thus, be a promising approach to overcome ARDS with COVID-19.

Funding: None

Keywords

acute respiratory distress syndrome, alveolar epithelial injury, COVID-19, endothelial injury, SARS-CoV-2.
Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus is a novel strain of Coronavirus that has been spreading worldwide following its origin from Wuhan, China since late 2019, and its outbreak has provoked a public health emergency. Infection with SARS-CoV-2 causes Coronavirus disease (COVID-19), pneumonia, and in the most severe cases, the infection leads to acute respiratory distress syndrome (ARDS) that is characterised by severe pulmonary oedema with increased alveolar permeability[1]. The alveolar barrier is composed of alveolar epithelial and endothelial cells and separates the alveolar air space from pulmonary circulation[2]. Damage to the alveolar epithelial or endothelial cells is generally the main pathological signature of ARDS[3]. Several previous reports have demonstrated that the lungs from patients with ARDS with COVID-19 show diffused alveolar damage along with alveolar epithelial and endothelial injury[4,5] and the presence of viral RNA in epithelial and endothelial cells[6,7]. However, the time course and specific contribution of alveolar epithelial and of endothelial injury to the pathogenesis of COVID-19 ARDS remain unclear. Better clarity regarding these issues will help in improved understanding of the disease, and tell us what should be focused on for improved treatment possibilities.

There are several established circulating alveolar tissue injury markers[8–10]. The receptor for advanced glycation end-products (RAGE) is primarily expressed in alveolar type-1 cells, and the level of circulating soluble form of RAGE (sRAGE) correlates with type-1 alveolar epithelial injury[11,12]. Angiopoietin-2 (ANG-2) is a growth factor expressed
in endothelial cells and its elevation indicates endothelial injury in patients with ARDS[13,14]. Surfactant proteins, including surfactant protein D (SP-D), are secreted by type-2 alveolar epithelial cells into the alveolar space. In the case alveolar barrier is disrupted, SP-D leaks from the alveolar space into the blood stream, and increase in the concentration of circulating SP-D indicates the alveolar barrier permeability[15,16]. Evaluating the characteristics of these alveolar tissue injury markers can provide insights regarding the alveolar endothelial and epithelial injuries during COVID-19 ARDS progression[17].

Here, we investigated the levels of a circulating alveolar epithelial injury marker; soluble receptor for advanced glycation end-products (sRAGE), an endothelial injury marker; angiopoietin-2 (ANG-2), and an alveolar barrier permeability indicator; surfactant protein D (SP-D) in serum from COVID-19 patients with or without ARDS. Although both the alveolar epithelial and endothelial injury markers were markedly elevated in the COVID-19 ARDS patients, our data indicate that the endothelial injury, which continues for a longer period than the epithelial injury, seems to be the main contributor to alveolar barrier disruption.

Methods

Study Design

In this single-centre, retrospective observational study, we analysed the serum concentrations of sRAGE, ANG-2, and SP-D in serum from adult patients with COVID-19,
admitted to Yokohama City University Hospital from January to July 2020. The inclusion criteria were: 1) a diagnosis of COVID-19 by positive results of real-time polymerase chain reaction, 2) age 18 years old or over, 3) the residual serum samples were available. ARDS was diagnosed according to the Berlin Definition. The study protocol was reviewed and approved by the institutional review board of Yokohama City University Hospital (B200700100). Informed consent was waived in view of the retrospective observational nature of the study.

Clinical data collection

Clinical data were retrospectively collected from the medical charts of each patient. We collected data on patients’ basal characteristics, vital signs, laboratory tests, and blood gas analysis during the first 14 days after hospital admission.

Quantification of alveolar tissue injury markers in serum

Residual serum samples from the patients with COVID-19 were collected after daily laboratory tests and frozen. Serum sRAGE, ANG-2, and SP-D concentrations were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (human RAGE: DY1145, human ANG-2: DY623, human SP-D: DY1920, R&D systems, MN, USA) according to the manufacturer’s instructions.
We compared the initial concentrations of these markers on the first or second hospital day between ARDS and non-ARDS patients. Moreover, we analysed the temporal changes of these markers in ARDS patients.

**Statistical analyses**

The median ± interquartile range (IQR) values of the data were used for analyses. Comparisons of the serum levels of circulating alveolar tissue injury markers and various basal laboratory and physiological values between patients with ARDS and those without ARDS were performed using the Mann-Whitney U test. To analyse the correlation of serum levels of sRAGE or ANG-2 with SP-D levels or PaO\textsubscript{2}/fraction of inspired oxygen (P/F) ratios, the values of the serum markers were log-transformed to obey normal distribution, and the correlations were evaluated by linear mixed model analysis with random intercepts for individual patients. All statistical analyses were performed using R software (version 4.0.3). Statistical significance level was set at P < 0.05.

**Role of the funding source**

The authors have not declared a specific grant for this research from any funding agency in the public, commercial, or not-for-profit sectors.
Results

Clinical characteristics of patients

Nine ARDS and eight non-ARDS patients, all with COVID-19, were included in the study. Patient characteristics are shown in Table 1. The median ages of the ARDS and non-ARDS groups were 67 (IQR: 63–75) and 48·5 (30–73) years, respectively. All patients in the ARDS group were men, and six of eight patients in the non-ARDS group were men.

Three of the nine in the ARDS group (33·3%) died and all in the non-ARDS group survived. APACHE-2 scores at admission were significantly higher in those in the ARDS group than those in the non-ARDS group. Moreover, PaO₂/fraction of inspired oxygen (P/F) ratios at admission were lower in the ARDS group than in the non-ARDS group. However, creatinine and total bilirubin levels were not significantly different between the ARDS and non-ARDS groups. Laboratory data at admission demonstrate significantly lower lymphocyte count and higher C-reactive protein and D-dimer values in the ARDS group than those in the non-ARDS group, indicating that severe inflammatory and coagulation responses accompanied with acquired immunosuppression are hallmarks of ARDS with COVID-19.

Temporal changes of the organ function indicators of lung (P/F ratio), kidney (serum creatinine), and liver (serum total bilirubin) and Sequential Organ Failure Assessment (SOFA) score without Glasgow Coma Scale (GCS) in ARDS patients with COVID-19 for 14
days starting from admission are shown in Fig.1. Among patients with ARDS, only one patient developed acute kidney injury, and some patients showed only a small increase in total-bilirubin concentration. Thus, organ dysfunction in the patients was primarily limited to the lungs.

Circulating alveolar tissue injury markers

The initial serum levels of all markers; sRAGE, ANG-2, and SP-D, were significantly higher in the ARDS group than in the non-ARDS group (sRAGE: median: 2680 pg/mL, IQR:1522–5076 vs. median 701 pg/mL, IQR:344–1148.0, p=0.0152, ANG-2: median 699 pg/mL, IQR: 410-2501 vs. median 231 pg/mL, IQR: 64-584, p=0.0464, SP-D: median: 17542 pg/mL, IQR: 7423-22979 vs. 1771 pg/mL, IQR: 458-204, p=0.0274) (Fig.2).

The temporal changes in these markers in COVID-19 ARDS patients are shown in Fig.3A–C. The peak for serum sRAGE level was observed just after admission (median: day 1, IQR: day 1–3.5) (Fig.3D). In contrast, the peak timings of serum ANG-2 (median: day 4, IQR: day 2.5–6) and SP-D (median: day 5, IQR: day 3–7.5) and SP-D (median: day five after admission, IQR: day 3–7.5) levels were during a later phase of the disease (Fig.3D).

Linear mixed effect model analysis revealed that the serum ANG-2 levels had a significant negative correlation with P/F ratios (fixed effect: -29.9, 95%CI: -56.6–-3.3, p=0.030) (Fig. 4A) and a significant positive correlation with the SP-D levels (fixed effect:...
0.490, 95%CI: 0.184–0.797, p=0.002) (Fig. 4B) during first 14 hospital days; however, the sRAGE levels had no significant correlation with P/F ratios (fixed effect: -19.9, 95%CI: -51.2–11.3, p=0.215) (Fig. 4D), and an opposite correlation with SP-D levels (fixed effect: -0.557, 95%CI: -0.925–-0.189, p=0.004) (Fig. 4C).

**Discussion**

In ARDS with COVID-19, circulating alveolar epithelial and endothelial injury markers were markedly elevated, but not in the case of non-ARDS COVID-19, indicating that alveolar barrier tissue injury is a hallmark of COVID-19 ARDS pathogenesis. Interestingly, time courses of changes in alveolar epithelial and in endothelial injury markers were different from each other. The observed serum sRAGE level was highest on admission in most patients with ARDS. In contrast, ANG-2 level reached a peak at later time points. These data suggest that the alveolar epithelial injury in the COVID-19 ARDS already reached the maximum level before hospital admission. Meanwhile, the endothelial injury seems to continue to deteriorate for several days after admission. Therefore, it is expected that there is a potential therapeutic time window to alleviate the endothelial injury even after hospitalization. Moreover, the ANG-2 levels were significantly correlated with the P/F ratios and the SP-D
levels, suggesting endothelial injury, rather than alveolar epithelial injury, might be a main contributor that leads to deterioration of alveolar permeability during ARDS with COVID-19.

Several previous reports evaluating lung tissues obtained from patients who died from severe COVID-19 revealed diffused alveolar damage accompanied by epithelial and endothelial injuries [4,5] along with the presence of viral RNA in these cells [6,7]. Moreover, several studies demonstrated the blood levels of endothelial injury markers at admission were significantly increased [18–23]. However, the underlying mechanisms of alveolar epithelial and endothelial injury has not been fully elucidated. SARS-CoV-2 can infect alveolar type 1 and 2 epithelial cells and endothelial cells [24], and causes multiple cellular responses [25], which are potentially cytotoxic [26]. On the other hand, inflammatory responses caused by SARS-CoV-2, rather than the SARS-CoV-2 infection itself to the targeted cells, may be the main driver of the alveolar tissue injuries [27,28]. The present study clearly demonstrates the difference in the peak timing of epithelial and endothelial injury markers, indicating that there are distinct mechanisms for injury to each type of cell. Future studies to clarify each of the injury mechanisms, especially focusing the endothelial injury, can be a promising approach to find efficacious therapeutic targets for COVID-19 ARDS.

Only a small number of studies have evaluated the temporal kinetics of circulating alveolar epithelial and endothelial injury markers during ARDS caused by aetiologies other than COVID-19. Two reports have previously demonstrated that sRAGE levels in the blood
of ARDS patients with bacterial sepsis peak on the initial day after admission[29,30]. Thus, based on previous findings as well as our results, alveolar epithelial injury seems to occur during the initial phase of ARDS progression irrespective of the aetiology. On the other hand, the time course of circulating ANG-2 levels during ARDS has not been previously reported. Kumpers et al. have reported that circulating ANG-2 levels in patients with bacterial sepsis, 72 hours after intensive care unit admission, increased from the baseline level only in the non-survivors, not in the survivors[31]. In our study, the ANG-2 levels increased from baseline in most patients, including survivors, suggesting that SARS-CoV-2 induces more severe endothelial injury than other aetiologies for injury, such as bacterial infection. Several reports have demonstrated that lung vascular thrombosis is a pathological feature of ARDS caused by SARS-CoV-2[4,5,32]. Moreover, the occurrence of systemic thrombotic complications in patients with COVID-19 has been reported[33]. Endothelial injury caused by SARS-CoV-2 infection might be the basal mechanism of thrombosis formation[34].

Circulating markers of alveolar tissue injury can be easily measured in clinical settings. Our data suggest that initial levels of RAGE can be a prognostic marker of COVID-19. Moreover, patterns of these tissue injury markers might be useful for predicting or evaluating the responses to treatment for ARDS in the case of COVID-19. Recently, it has been shown that circulating biomarkers can distinguish sub-phenotypes among patients with ARDS[17,35]. It is possible that ARDS caused by COVID-19 also has several
sub-phenotypes, and different treatment strategies for each sub-phenotype might be necessary.

Further studies to evaluate the utility of these circulating markers are, thus, warranted.

There are some limitations to our study. First, the sample size was small because of the factor of availability of daily serum samples from patients with COVID-19 ARDS. This may have affected the statistical strength of our analyses. It is, hence, necessary to confirm our results using samples from large cohorts to obtain insights into the pathogenesis of alveolar tissue injury during COVID-19. Second, because of the observational nature of the study design, treatments for COVID-19 were not standardized. Therefore, it is possible that the treatment strategies might affect the kinetics of circulating alveolar tissue injury markers.

In conclusion, our evaluation of circulating alveolar tissue injury markers indicates that the alveolar epithelial and endothelial injury have distinct time courses and pathogenic contributions in ARDS with COVID-19. The injury to endothelial cells, which continues for a longer period than the epithelial injury, seems to be the main contributor to alveolar barrier disruption. Targeting the endothelial injury rather than the epithelial injury, may be a potential efficacious approach to overcome ARDS with COVID-19.

Acknowledgements

The authors wish to thank Department of Emergency Medicine (Prof. Ichiro Takeuchi), Department of Microbiology (Prof. Akihide Ryo), and Yokohama City University Center for
Novel and Exploratory Clinical Trials for collecting and providing the residual blood samples.

Footnotes

KT: conducted the study, performed ELISA, analysed data, and wrote the manuscript.

NY: performed ELISA and revised the manuscript

TM: analysed data, supervised statistical data analysis, and revised the manuscript

MA: collected patients’ clinical data and revised the manuscript

TG: supervised the study and revised the manuscript

Declaration of Competing Interest

The authors declare no conflict of interest.

Data sharing statement

De-identified patient data collected for the study will be rendered available by writing to KT (ktojo-cib@umin.net).

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Table 1

The clinical characteristics in ARDS and non-ARDS patients with COVID-19.
|                          | non-ARDS (n=8) | ARDS (n=9) | p-Value |
|--------------------------|----------------|------------|---------|
| Age (years)              | 49 (30-73)     | 67 (63-75) | 0.2634  |
| Males/Females (number)   | 6/2            | 9/0        | 0.2059  |
| APACHE2 score            | 7 (4.25-8.75)  | 11 (9.50-15.50) | *0.0290 |
| P/F ratio at admission,  | 389 (310-412)  | 270 (152-336) | *0.0073 |
| Mechanical ventilation use (number) | 8              | 0          | <0.0001 |
| Laboratory data on admission |               |            |         |
| WBC count (/μL)          | 4900 (2700-9500) | 6200 (5400-7350) | 0.7035  |
| Lymphocyte count (/μL)   | 1109 (965-1569) | 643 (321-760)  | *0.0003 |
| Platelet count (×10^3/μL)| 190 (120-306)  | 206 (170-256)  | >0.9999 |
| D-dimer (μg/mL)          | 0.33 (0.05-0.85) | 1.22 (0.65-7.135) | 0.0545  |
| CRP (mg/dL)              | 0.82 (0.15-1.26) | 14.62 (8.80-16.82) | *0.0002 |
| Creatinine (mg/dL)       | 0.72 (0.60-0.84) | 0.79 (0.67-0.91)  | 0.3212  |
| Total bilirubin (mg/dL)  | 0.55 (0.43-0.70) | 0.90 (0.50-1.00)  | 0.3788  |

**Figure Legends**

Figure 1

Temporal changes of (A) $\text{PaO}_2$/fraction of inspired oxygen (P/F) ratio, (B) serum creatinine, (C) serum total bilirubin, and (D) Sequential Organ Failure Assessment (SOFA) score without Glasgow Coma Scale (GCS) in ARDS patients with COVID-19 for 14 days starting from admission. Data were presented as median ± IQR.

Figure 2
Comparison of initial levels of circulating alveolar tissue injury markers in ARDS and non-ARDS patients, with COVID-19. A. sRAGE, B. ANG-2, and C. SP-D levels. Data were presented as median ± IQR. *p<0.05.

Figure 3

Temporal changes of (A) sRAGE, (B) ANG-2, and (C) SP-D levels in ARDS patients with COVID-19 for 14 days starting from admission. (D) The peak day of each of the alveolar tissue injury markers. Data were presented as median ± IQR.

Figure 4

The correlations between (A) ANG-2 and P/F ratios, (B) ANG-2 levels and SP-D levels, (C) sRAGE and P/F ratios, and (D) sRAGE levels and SP-D levels. Data were analyzed by linear mixed model analysis with random intercepts for individual patients. Each different colour represents the data from the individual patient.
