ELEVATED HIGH MORBILITY GROUP BOX PROTEIN 1 ON PATHOLOGICAL FINDINGS IN SUBCLINICAL INTERSTITIAL PNEUMONIA DURING EARLY PHASE OF THORACIC SURGERY

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Abstract

[Background] Some cases of post-surgical acute respiratory distress syndrome (ARDS) may be an acute exacerbation of idiopathic pulmonary fibrosis (IPF) including subclinical IP, and pathological interstitial pneumonia (IP) is a predictor of the ARDS high-risk group. Although one-lung-ventilation (OLV) has become established procedure during thoracic surgery, there is little data on the re-expansion of pulmonary edema after surgery. In the present study, we evaluated the possibility that occult lung injury occurs in pathological IP patients during the early phase of thoracic surgery.

[Methods] Study was performed in 22 patients who underwent pulmonary resection at our institute. Plasma levels of interleukin (IL)-6, IL-8 and high mobility group box protein-1 (HMGB1) were measured during the pre-, peri- and postoperative periods.

[Results] Serum levels of IL-6 and IL-8 increased after surgery ; however, there were no significant differences between the IP and non-IP groups. Meanwhile, serum levels of HMGB1 were significantly higher at 30 min after OLV in IP group, and those in IP group were continuously higher during the peri- and postoperative periods.

[Conclusions] This study suggests that elevation of HMGB1, which might influence occult lung injury, occurred during OLV in IP group, and that it already existed before lung re-expansion.

Key words : lung injury, interstitial pneumonia, ARDS

Introduction

Despite advances in surgical technique, anesthetic management, and postoperative care, the mortality and morbidity associated with pulmonary complications, particularly acute respiratory distress syndrome (ARDS), remain significant after pulmonary resection[1-4]. The overall incidence of post-surgical ARDS shows a wide range (1.4-3.6%) with a mortality of 40-88%[1, 3-5], and ARDS is the cause of almost 72.5% of total mortality cases after pulmonary resection[2].

Acute interstitial pneumonia (AIP) is a type of post-surgical ARDS that generally presents with progressive respiratory insufficiency and is responsible for approximately 30% of hospital deaths following pulmonary re-

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section[6]. The feasibility of numerous factors as possible predictors of post-surgical ARDS has been studied ; acute exacerbation of idiopathic pulmonary fibrosis (IPF) may occur following surgery, chemotherapy, or radiotherapy[2]. However, for patients who develop ARDS following pulmonary resection, IPF is not always discovered prior to surgery. It has recently been reported that localized (not diffuse) usual interstitial pneumonia (UIP) can, in some cases, progress to IPF[7]. UIP is the most commonly seen of several recognized patterns of interstitial pneumonia (IP). Patients with localized UIP are not always recognized as having IPF because the lesions are localized and symptomless (subclinical IP). One of the factors in this progression of localized IP to exacerbation may be re-expansion pulmonary vascular endothelial cell injury on the residual lung through the pulmonary resection process[8,9] ; however, the mechanisms have not been fully investigated.

Although one-lung-ventilation (OLV) is a common surgical procedure used to obtain an optimal surgical field, there is little data on the re-expansion pulmonary edema after surgery. Experimental and clinical studies have shown that OLV induces a proinflammatory reaction in the lung[9-11]. In addition to mechanical stretching and shear stress, inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8[12] are produced in the lung. High mobility group box-1 (HMGB1) is a nuclear protein produced by nearly all cell types[13], and has been identified as a late mediator in sepsis-associated lung injury in mice and in patients with sepsis[14]. Receptors for HMGB1 are thought to include receptor for advanced glycation products (RAGE), Toll-like receptor (TLR) 2, and TLR4[15]. Binding of HMGB1 to its receptors activates endothelial cells to up-regulate adhesion molecules, and activated macrophages to release inflammatory cytokines. Therefore, HMGB1 has multiple functions in infection, tissue injury, inflammation, apoptosis and immune responses[16]. Elevation of serum HMGB1 levels in septic patients is a marker of poor prognosis[14], and plasma HMGB1 levels increase in patients with ALI[17]. Necrotic or injured cells can also passively release HMGB1[18].

It has been reported that pneumonectomy causes neutrophil and macrophage infiltration, capillary hyperpermeability and edematous changes in the residual lung, which is associated with a significant increase in plasma levels of HMGB1, indicating occult lung injury, which might be one of the factors in developing ALI[19]. Although several proinflammatory cytokines, including HMGB1, that may contribute to the development of ALI have been investigated, little is known about its role in IPF[20], or its influence on subclinical IP patients during thoracic surgery. In the present study, we evaluated the possibility that the presence of occult lung injury may develop to ALI in subclinical IP patients during the early phase of thoracic surgery.

Methods

Study population

This study was approved by our Institutional Review Boards and written informed consent was obtained from all patients. A prospective study was performed in 22 patients who underwent pulmonary resection at our institute. Data was evaluated by dividing patients into two groups based on the presence of histological IP [histological IP (n=10) and non-IP (n=12)]. We excluded cases that underwent pneumonectomy, previous lung resection, and preoperative administration of steroids or immunosuppression, those that showed clinical signs of systematic or pulmonary infection before surgery, and those that were diagnosed with IPF prior to surgery.

Surgery

All patients received standard premedication with diazepam (0.1 mg/kg orally 2 h before induction). Anesthesia was performed using propofol, remifentanil and sevoflurane. A thoracic epidural catheter was inserted (T4/5 to T6/7), and at the beginning of surgery, 0.2% ropivacaine was started, and was maintained for 2-4 days until the chest tubes had been removed. Patients were intubated using either a left- or right-sided double-lumen endotracheal tube. The affected lung was deflated as soon as the pleural space was opened and deflation was maintained for most of the operative time. The lung was reinflated at the end of resection in order to check for air leakage and closure. The fraction of inspired oxygen (FiO_2) during surgery ranged from 0.3 to 1.0 accord-

ing to intra-operative blood gas analysis evaluation. Surgery was most commonly performed via postero-lateral thoracotomy. In general, patients were extubated at the end of surgery and were transferred to the ward after a brief stay in the recovery area.

Collection of samples

Baseline blood samples were obtained immediately after induction of anesthesia. Additional blood was sampled at 30 min after OLV was started, at 5 min before and after re-expansion, and at 1, 6 and 24 h after surgery. All plasma samples were stored in a freezer at −80°C until analysis.

Bronchoscopic microsampling (BMS) of the pulmonary epithelial lining fluid (ELF) using a microsampling probe (Olympus, Tokyo, Japan), which consists of a 1.7-mm diameter polyethylene outer sheath and an inner fiber rod probe of 1.2 mm in diameter and 30 mm in length attached to a stainless steel guide wire 100 cm in length, obtained from the lung on the surgical side was obtained after induction of anesthesia and at 1 h after surgery. Briefly, the probe was inserted into the channel and gently advanced. While the outer sheath was held at the target in the subsegmental bronchus, the inner probe was advanced slowly into the peripheral airway until it was in contact with the mucosal surface and was maintained in that position for 5-7 s, thus allowing the fiber rod to absorb up to 20 μl of ELF. The inner probe was then withdrawn into the outer sheath, and both were removed together. The wet inner probe was cut, placed in a tube, and stored in a freezer at −80°C until analysis. The stored frozen probes were weighed before the ELF saline suspension was prepared. Diluted ELF solutions were prepared for biochemical measurements by placing frozen probes into a 15-ml polyethylene tube containing 3 ml of saline and vortexing for 1 min. The solution was centrifuged for 15 min at 1,400×g, and the supernatant was collected. The probe was dried and weighed in order to calculate the ELF volume recovered from the BMS probes. The dilution factor was calculated as follows : ELF volume (ml)/[3 ml+ELF volume (ml)] [21,22].

Enzyme-linked immunosorbent assay for HMGB1 and cytokines

Levels of HMGB1 in plasma and ELF were measured by enzyme-linked immunosorbent assay (ELISA) (Shino-test Corporation, Kanagawa, Japan) according to the manufacturer's instructions. The lower detection limit of HMGB1 was 1 ng/ml. IL-1β, 6 and 8, and TNF-α levels in serum and ELF were measured at the same time points with a Single-Analyte ELISArray kit (SABiosciences, Frederick, MD, USA). We performed all assays in duplicate, and the mean of the two data points was determined for individual samples.

Histological examination

Histopathological examination was performed using two randomly selected sections of resected lung (not occupied by cancer), including a suitable lesion if findings of IP were detected on preoperative CT. The lung samples were fixed with 10% formalin overnight and embedded in paraffin. Whole-field sections were observed and IP findings were examined (×400) by a pathologist and by the authors in order to divide patients into of the non-IP (Figure 1a) or IP groups (Figure 1b). Briefly, histopathological IP was diagnosed according to the following criteria that the pathological finding with fibrosis, thick of lung parenchyma and infiltration of lymphocytes into the interstitial space. Meanwhile, a blind coexperimenter counted the number of macrophages and neutrophils per square millimeter in 10 randomly selected views.

Immunohistochemistry for HMGB1

Immunohistochemical staining was performed with monoclonal mouse anti-human HMGB1 antibody (clone J2E1, Advanced Technology Research Center, Gyeonggi-do, South Korea) in order to localize the expression of HMGB1 in the air space. Briefly, 3-μm paraffin sections were adhered to a slide pretreated with poly-L-lysine. After deparaffinization, sections were incubated with an anti-HMGB1 antibody (1 : 200 dilution) for 6 min at room temperature. For control incubations, the specific antibodies were replaced by nonimmune serum. Sections were then incubated with peroxidase-conjugated anti-rabbit/mouse immunoglobulins (Dako ChemMate Envi-

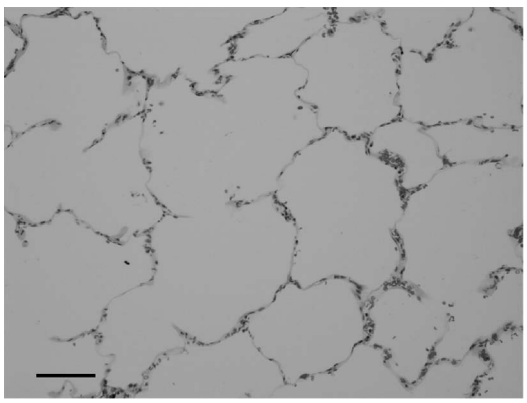

Fig. 1a

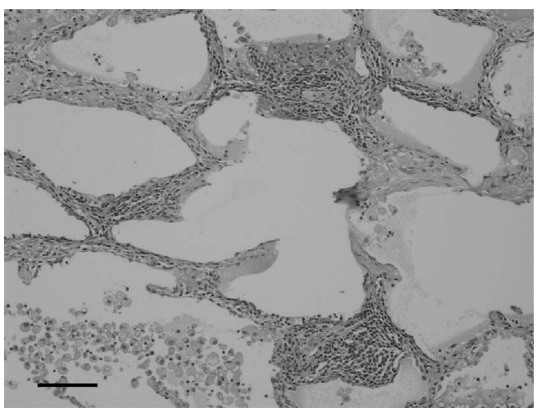

Fig. 1b

Fig. 1. Histopathological slide shows non-IP (a), and IP-positive findings (b) with fibrosis and infiltration of lymphocytes into the interstitial space (HE stain). Original magnification, ×100 ; bar = 100 μm.

sion detection kit ; DAKO Japan Corporation, Kyoto, Japan) for 30 min at room temperature, and were developed with 3,3´-diaminobenzidine tetrahydrochloride, followed by counterstaining with hematoxylin.

Statistics

We used JMP IN 8.0.1 (SAS Institute Inc., Cary, NC, USA) statistical software for statistical evaluation. Groups were compared by chi-squared test and one-way analysis of variance (ANOVA) in combination with Dunnett's method. Differences between the groups were considered to be significant at $p<0.05$. Values are expressed as means±SD.

Results

Patients characteristics

A total of 22 patients undergoing sublobular resection and lobectomy at our institute were enrolled in this study. Data were evaluated by dividing patients into the histological IP ($n=10$) and non-IP ($n=12$) groups. Clinical outcome was not differences in both groups. None of the patients showed any signs of preoperative infection, and there were no differences in patient clinical background or surgical data between the two groups, except for lower values of $FEV_{1.0\%}$ (62.3±11.5) and $\%FEV_{1.0}$ (90.0±23.9) in pathological IP group (Table 1). As we excluded cases that were already diagnosed with IPF prior to surgery, pathological IP in this study was seen because : 1) subclinical, asymptomatic and localized IP that was not detectable by chest X-ray, although some localized IP was detectable chest CT ; and/or 2) localized IP resulting from pathological IP would be masked preoperatively by emphysematous changes in patients with COPD.

Inflammatory mediators of serum and ELF

The IL-6, IL-8 and HMGB1 levels in plasma during thoracic surgery are shown in Figure 2. Serum levels of IL-6 and IL-8 increased after surgery ; however, there were no significant differences between the IP and non-IP groups (Figure 2a, 2b). Serum IL-1β and TNF-α levels were undetectable throughout the experimental period (data not shown). However, serum levels of HMGB-1 were significantly higher at 30 min after OLV started (particularly before re-expansion) in the IP group (IP/non-IP : 4.6/3.0 mg/ml), and serum HMGB-1 levels in the IP group were continuously higher than in the non-IP group during the peri- and postoperative periods (Figure 2c). On ELF, levels of IL-6, IL-8 and HMGB1 at 1 h after surgery are shown in Table 2. Data are expressed as mean values in the IP group/mean values in the non-IP group. There were no significant differences observed in IL-6, IL-8 between the IP and non-IP groups. IL-1β and TNF-α in ELF were not detectable at this time point in this study. However, ELF levels of HMGB-1 were significantly higher at 5 min before reexpansion in the IP group (IP/non-IP : 2.04) at 1 h after surgery (IP/non-IP :

Table 1. Clinical details of all patients who underwent pulmonary resection.

	non-IP	IP	p-value
n	12	10	
age (year)	67.4 ± 12.6	68.4 ± 10.7	0.921
gender (M/F)	10/2	9/1	0.658
case of malignancy (%)	66.7	90	0.204
case of sublobular resection (%)	50.0	30.0	0.353
operation time (min)	171.3 ± 78.3	205.6 ± 80.9	0.307
one lung ventilation time (min)	117.9 ± 61.9	140.5 ± 53.6	0.222
blood loss (ml)	82.0 ± 79.6	92.9 ± 73.7	0.644
VC (ml)	3288.3 ± 940.2	3458.0 ± 900.1	0.742
%VC (%)	104.1 ± 16.6	107.5 ± 18.5	0.644
$FEV_{1.0\%}$(G) (%)	82.3 ± 4.2	62.3 ± 11.5	0.001 *
$\%FEV_{1.0}$ (%)	118.8 ± 21.2	90.0 ± 23.9	0.015 *

Data are expressed as means±SD. * indicates significant differences between both IP and non-IP groups ; $p<0.05$. VC : vital capacity ; FEV : forced expiratory volume.

Table 2. Levels of cytokines and HMGB1 on ELF in pathological IP ($n=10$) and non-IP groups ($n=12$) before surgery, at 5 min before re-expansion and at 1 h after surgery.

ELF (IP/non-IP)	before surgery	5 min before reexpansion	1 hr after surgery
TNF-α	ND	ND	ND
IL-1β	ND	ND	ND
IL-6	ND	ND	1.10
IL-8	1.14	1.03	1.11
HMGB-1	0.86	2.04*	1.44*

Data are expressed as mean values of IP group/mean values of non-IP group. * indicates significant differences between IP and non-IP groups ; $p<0.05$. ND : cytokine levels were not detectable in samples at this time point.

1.14).

Histopathological examination

Histopathological examination was performed using two randomly selected sections of resected lung (cancer-free areas). Cell counts of macrophages and neutrophils per square millimeter in 10 randomly selected views are shown in Figure 3. Alveolar macrophages and intracapillary neutrophil counts on resected lung were significantly higher in the IP group. In order to identify the source of HMGB1 in the serum and lungs during surgery, we performed immunohistochemistry on resected lung tissue using anti-HMGB1 antibody. Alveolar macrophages showed a small amount of staining for HMGB1 in the non-IP group (Figure 4a), but alveolar macrophages and intracapillary neutrophils were prominently stained for HMGB1 in the IP group (Figure 4b). Positive staining in cytoplasm was also noted in some of these cells. Resected lung samples were obtained before lung re-expansion.

Discussion

A prospective study was performed in 22 patients to evaluate occult lung injury in pathological IP patients during the early phase of thoracic surgery. Our results

Fig. 2a

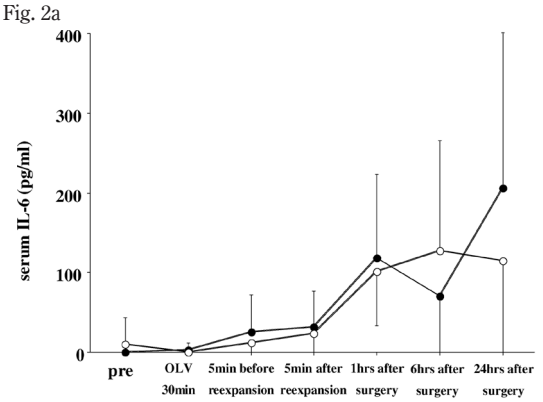

Fig. 2b

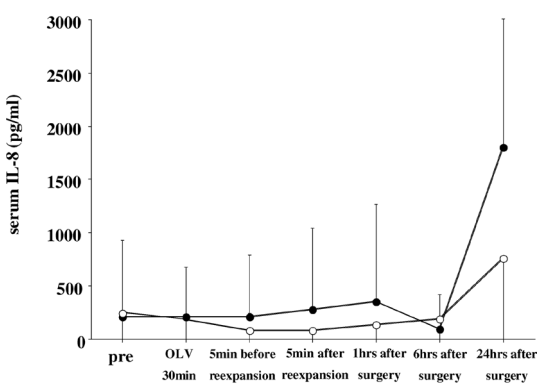

Fig. 2c

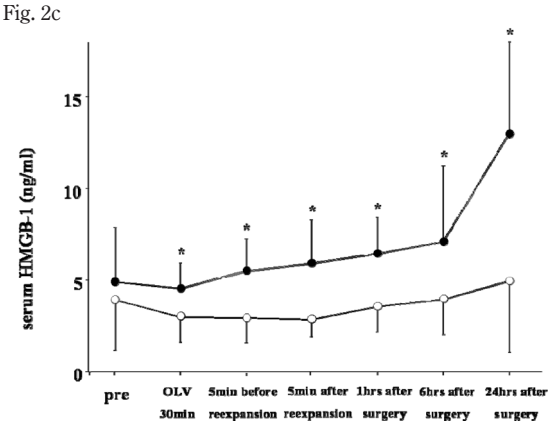

Fig. 2. Time course of IL-6 (a), IL-8 (b) and HMGB1 (c) levels of plasma in the pathological IP (closed circle, $n=10$) and non-IP groups (open circle, $n=12$) at baseline (pre), 30 min after OLV started (OLV 30 min), 5 min before lung re-expansion (5 min before re-expansion), 5 min after lung re-expansion (5 min after re-expansion), and 1, 6 and 24 h after surgery (1 h after surgery, 6 h after surgery, 24 h after surgery). Data was expressed as means±SD. * indicates significant differences between IP and non-IP groups ; $p<0.05$.

Fig. 3a

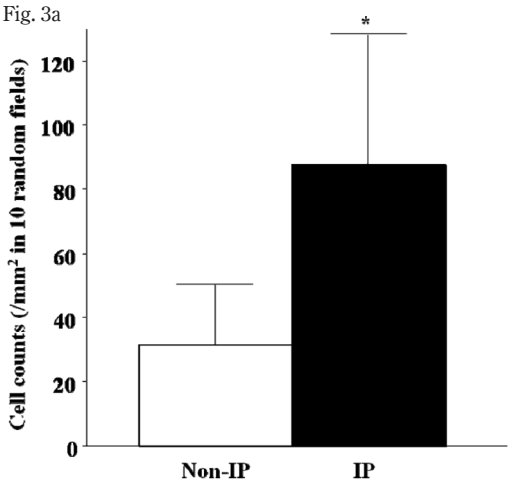

Fig. 3b

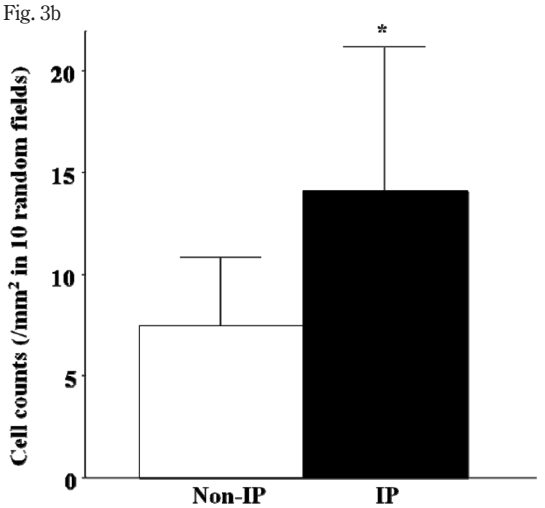

Fig. 3. Histopathological examination was performed using two randomly selected sections of resected lung (not occupied by cancer). A blind coexperimenter counted the macrophages (a) and neutrophils (b) per square millimeter in 10 randomly selected views in the pathological IP (closed bar, $n=10$) and non-IP groups (open bar, $n=12$). Data are expressed as means±SD. * indicates significant differences between IP and non-IP groups ; $p<0.05$.

showed that serum levels of IL-6 and IL-8 increased after surgery ; however, there were no significant differences between the IP and non-IP groups. Serum levels of HMGB1 were significantly higher at 30 min after OLV

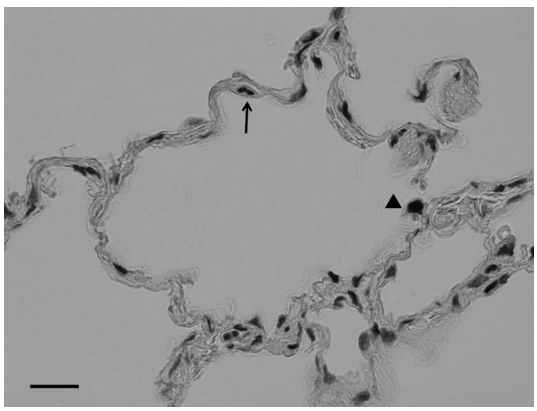

Fig. 4a

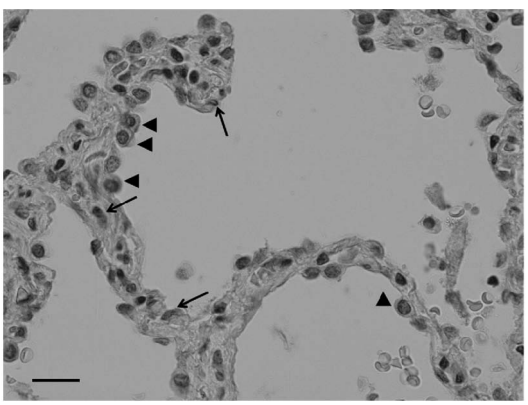

Fig. 4b

Fig. 4. Representative results of immunohistochemistry of HMGB1 staining (IP group, $n=10$; non-IP group, $n=12$) of lung tissues from patients in the (a) non-IP and (b) IP groups. Arrows indicate intracapillary neutrophils and arrow heads indicate alveolar macrophages. Some cell fractions showed strong staining in nuclei or cytoplasm, particularly in (b). Original magnification, ×400 ; bar=20 μm.

began in the IP group, and serum HMGB1 levels in the IP group were continuously higher than in the non-IP group during the peri- and postoperative periods. Alveolar macrophages and intracapillary neutrophil counts in resected lung were significantly higher in the IP group. This study suggested that elevation of HMGB1, which may influence occult lung injury, occurred during OLV in the IP group, and that it was present before lung re-expansion.

A number of factors have been investigated for the purpose of explaining and predicting the occurrence of post-surgical ARDS following pulmonary resection[1,2,7,23] ; however, pathogenesis as a predictor of post-operative ARDS has not been fully investigated. A previous study reported that the overall incidence of post-surgical ARDS was significantly higher in patients with IPF undergoing pulmonary resection for non-small-cell lung cancer than in those without IPF (21% vs 3.7%, respectively)[2]. In both post-surgical ARDS caused by acute exacerbation of IP and other forms of ARDS, histopathological findings showed the same diffuse alveolar damage in the final phase of severe cases. At this point, it is difficult to distinguish the two types. Patients with IP-positive findings were more susceptible to post-surgical ARDS because of surgical stress, as compared to patients without IP-positive findings. In the present study, serum levels of HMGB1 were significantly higher at 30 min after OLV started in the IP group. Elevated serum HMGB1 levels, which may influence occult lung injury, suggest that acute exacerbation of IPF may be a factor in post-surgical ARDS.

HMGB1 was predominantly found in the nuclei of infiltrating inflammatory cells, alveolar macrophages and some neutrophils, and positive staining in cytoplasm was also found in some of these cells. These results suggest that HMGB1 expression is up-regulated in affected areas and that secreted HMGB1 may play a role as a proinflammatory cytokine in interstitial lung diseases. Cytoplasmic staining for HMGB1 in lung tissues appears to be reflected by the high levels of HMGB1 in ELF and inflammatory cell accumulation in strongly stained areas, and may contribute to occult lung injury, which may develop into acute lung injury.

An alternative source of HMGB1 is neutrophils ; however, our immunohistochemistry experiments showed that a fraction of neutrophils were positive for HMGB1. At the site of inflammation, neutrophils can become necrotic instead of undergoing apoptosis[24]. During apoptosis, the surface of neutrophils remains intact, and they can be cleared by macrophages without leaking their potentially injurious contents. However, if macrophages fail to rapidly clear apoptotic neutrophils, the latter undergo secondary necrosis. Intracapillary and alveolar neutrophils that undergo necrosis cannot be stained in

immunohistochemical studies ; however, leakage of HMGB1 from necrotic cells contributes to increases in the serum and BALF in the early period, despite HMGB1 being known as a late-phase pro-inflammatory mediator. The present study showed that serum levels of HMGB1 were significantly higher in the early phase, particularly at 30 min after OLV. The mechanisms have not been clarified, although the above pathway is a possible explanation.

In clinical settings, OLV has become a common procedure in thoracic surgery, and in most cases, is performed without postoperative pulmonary complications, although there is little data on re-expansion pulmonary edema after surgery. One of possible mechanism is vascular endothelial damage after reperfusion injury in areas of previous hypoxic vasoconstriction, with the resulting reactive oxygen species altering the permeability of the vascular endothelium[9,10]. This study demonstrated that proinflammatory mediators, including HMGB1, are not elevated during OLV in the non-IP group ; thus, OLV is not an invasive procedure in normal subjects. Serum levels of HMGB1 were significantly higher in the IP group, and a previous report found that HMGB1 levels in BALF were significantly elevated in IPF patients when compared to control subjects[20]. It is possible that deflation of the lung stimulates localized and subclinical IP, which may exacerbate and lead to ARDS, resulting in elevated levels of proinflammatory mediators such as HMGB1. Moreover, this study showed that serum levels of HMGB1 were significantly higher before lung re-expansion of lung. In previous animal experiments, activated neutrophils were sequestered in the pulmonary capillary in collapsed lung in a rat atelectasis model[25], in which neutrophils are primed for respiratory bursting. During atelectasis, p47-phox, a nicotinamide adenine dinucleotide phosphate oxidase subunit, was translocated to the plasma membrane from the cytosol of trapped neutrophils[25]. In this study, histological examination of the resected lung showed that intracapillary neutrophils were significantly higher in the IP group. This suggests the possibility that primed and activated neutrophils in subclinical IP lung tissues during lung deflation elevate the HMGB1, and this elevation of HMGB1 as proinflammatory mediator may contribute to the exacerbation of localized IP and lead to ARDS. The above pathway is a possible explanation, however the mechanisms have not been clarified and the further investigation is needed.

The use of corticosteroids in the treatment of post-surgical ARDS after lung resection remains controversial[26,27]. We speculate that early and perioperative administration of steroids would contribute to a decrease in mortality only if it were possible to preoperatively identify patients with UIP progressing to ARDS. Further randomized controlled studies are needed to determine whether steroid administration is beneficial in the treatment of post-surgical ARDS.

We acknowledge that the case series reported here includes only 22 patients, and as such, suffers from the limitations inherent to all small case series. However, we demonstrated that serum levels of HMGB1 were significantly higher at 30 min after OLV began in the IP group, and serum HMGB1 levels in the IP group were continuously higher than in the non-IP group during the peri- and postoperative period. This study suggested that elevation of serum HMGB1, which may influence occult lung injury, occurred during OLV in the IP group, and that it was already present before lung re-expansion.

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