Title: Sivelestat Prevents Cytoskeletal Rearrangements in Neutrophils Resulting from Lung Re-expansion following One-Lung Ventilation during Thoracic Surgery

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Abstract

Patients undergoing lobectomy are at risk of developing acute lung injury resulting from one-lung ventilation (OLV) during surgery. We investigated the morphological and functional behavior of neutrophils in patients who underwent lobectomy and assessed the ability of sivelestat to inhibit neutrophil activity. This was a blinded randomized study. Sixteen patients who underwent lobectomy were given intravenous sivelestat (n = 8) or intravenous saline (n = 8). We studied the cytoskeletal rearrangements of circulating neutrophils by determining the localization of filamentous actin (F-actin). Pulmonary oxygenation was evaluated by measuring the partial pressure of arterial oxygen. We found that the number of circulating, F-actin-rimmed neutrophils increased during OLV and after lung re-expansion. Our results suggest that, in addition to the surgical procedure and OLV, re-expansion of the remaining lung after lobectomy increases the neutrophil activation levels. Furthermore, administration of sivelestat limited neutrophil activation and improved pulmonary oxygenation in our patients.

Keywords: Acute lung injury; Cytoskeletal rearrangement; Lung re-expansion, One-lung ventilation; Sivelestat

Introduction

One-lung ventilation (OLV) is a major cause of acute lung injury (ALI) following thoracic surgery [1]. Neutrophils play a key role in the development of ALI [2], and previous studies have shown that OLV induces alveolar damage via neutrophil recruitment and cytokine production [3].

Cytoskeletal rearrangements in circulating neutrophils can be induced by various stimuli, including inflammatory cytokines and reactive oxygen species (ROS), which can, in turn, cause a decrease in the pliability of neutrophils. Neutrophils have to change their shape in order to pass through lung capillaries, and a decrease in their pliability results in neutrophilic build-up in the lung capillaries [4; 5]. Thus, the cytoskeletal rearrangements that occur in circulating neutrophils are thought to initiate the build-up of these cells in lung capillaries. In a preliminary study with rats, we found that (1) the number of F-actin-rimmed neutrophils increases during OLV and rise further after re-expansion, (2) the number of neutrophils in pulmonary capillaries increases after lung re-expansion, and (3) the plasma levels of cytokine-induced neutrophil chemo-attractant 1 (CINC-1), the equivalent of human interleukin 8 (IL-8), increase during OLV and then to further increase after lung re-expansion (personal observation). Furthermore, we previously reported that during OLV in patients undergoing thoracic surgery, IL-8 levels increase in the epithelial lining fluid of the collapsed lung and in plasma [6]. However, thus far, the association between the cytoskeletal rearrangement of neutrophils and their

recruitment in lung capillaries during and after OLV in patients undergoing thoracic surgery is not fully understood.

The release of neutrophil elastase (NE) is thought to be an important mechanism by which neutrophils cause lung interstitial and alveolar damage [7], and the selective NE inhibitor, sivelestat (sodium-[2-[4-(2,2-dimethylpropionyloxy) phenylsulfonylamino]benzonyl]amino-acetatetetatrahydrate) (Ono 5046; Ono Pharmaceutical, Osaka, Japan), has been indicated as a potential treatment for patients at risk for ALI [8; 9]. Studies with sivelestat have reported that this drug can prevent changes in neutrophil pliability, induced by both inflammatory mediators as well as dysfunctional pulmonary microcirculation [10; 11]. Results from these studies indicated that sivelestat may well be able to suppress neutrophil recruitment in lung capillaries and thus prevent the development of ALI. However, this has not yet been confirmed.

The data from previous investigations with sivelestat served as a basis for the 2 hypotheses that we tested in the current study. Firstly, we hypothesized that cytoskeletal changes in neutrophils are induced by OLV, and secondly, we hypothesized that treatment with sivelestat would improve the pliability of neutrophils. To test our hypotheses, we designed a prospective randomized clinical study of patients who underwent lobectomy during thoracic surgery. We also investigated the role of

neutrophil chemo-attractant and ROS as possible mediators for the damaging effects of OLV.

Patients and Methods

Study design

From October 2006 to February 2008, 16 patients with primary lung tumor or metastatic lung cancer, who were scheduled for lobectomy at Shinshu University Hospital in Japan were enrolled in this study. Ethical clearance for the study was obtained from the Ethics Committee of Shinshu University School of Medicine, and the study was conducted in accordance with the Declaration of Helsinki. All patients gave informed consent.

Patients were randomly assigned to a control or experimental group. Patients (n = 8) in the experimental group were given intravenous (IV) sivelestat sodium at a dose of 0.2 mg/(kg·h), whereas patients in the control group (n = 8) received IV saline. The sivelestat sodium and the saline control were continuously infused at a rate of 10 mL/h during the surgery. During mechanical ventilation, the O_2 tension was set at 100%, the respiratory rate ranged from 8 breaths per min to 12 breaths per min, and the positive end-expiratory pressure ranged from 0 to 5 cm H_2O. One-lung ventilation was initiated after the first skin incision, and mechanical ventilation was maintained with a tidal volume ranging from 6 to 8 mL/kg. After re-expansion, two-lung ventilation (TLV) was

maintained with a tidal volume of 10 mL/kg. Arterial blood samples were taken on 3 occasions: just before the start of OLV (point 1); just before lung re-expansion (point 2); and 30 min after lung re-expansion (point 3, Figure 1). For all patients, whole blood was obtained at each of these points.

Total and F-actin-rimmed neutrophil counts

Total neutrophil counts were calculated using an automated cell counter (Sysmex XE2100^{TM}; Sysmex, Kobe, Japan). Neutrophil cytoskeletal rearrangements were assessed by observing any changes in the localization of F-actin [12; 13]. Neutrophils were categorized according to the presence or absence of an F-actin rim, as previously described by Yoshida *et al.* [13]. F-actin rimmed neutrophil counts were obtained by multiplying the total neutrophil count by the percentage of neutrophils that contain an F-actin rim.

Partial pressure of arterial oxygen (PaO_2) and interleukin 8 (IL-8) and malondialdehyde (MDA) levels in plasma

PaO_2 was measured with a blood analysis system (TRU-Point ^{TM}; Irma, OH, USA). The data are expressed as the rate of change of PaO_2. IL-8 levels in plasma were measured with the use of a commercial ELISA kit (Human Interleukin-8 Immunoassay;

Quantikine, R&D Systems, Inc., NE). MDA levels in the plasma were measured with the use of a commercial thiobarbituric acid reactive substances assay kit (TBARS assay kit; ZeproMetrix, Co, NY).

Statistical analysis

All data are presented as the mean ± standard error. The repeated parameters of the F-actin-rimmed neutrophil counts, plasma IL-8 levels, and plasma MDA levels were evaluated using Friedman test with Scheffe's analysis. The comparison of the time courses of those parameters between the 2 groups was evaluated using a repeated measures two-way repeated analysis of variance (ANOVA) with Scheffe's analysis. The rate of change of the PaO_2 was evaluated using an unpaired *t*-test. Statistical significance was set at $P < 0.05$.

Results

Clinical characteristics of the patients undergoing thoracic surgery

Our study included 7 men (44%) and 9 women (56%) between 53 and 85 years of age (mean, 71.4 years). All of the patients had a normal immune status. Right-sided lobectomy was performed in 94% (n = 15) of patients. Seven patients (44%) were smokers or ex-smokers. Three patients (19%) had diabetes mellitus, and 7 (44%) had

hypertension. Fifteen patients (94%) had a diagnosis of lung cancer, while 1 patient (6%) had a metastatic lung tumor derived from a primary colon cancer. None of the patients had any major surgical complications such as ALI, pneumonia, or bronchopleural fistula during or after surgery. There were no significant differences between the 2 groups in terms of age, sex, side of lobectomy, smoking index, preoperative %FVC, preoperative FEV1.0%, preoperative PaO_2, OLV period, blood loss, or resected lung volume (Table 1).

Total neutrophil counts

The total neutrophil counts are shown in Table 2 and Figure 2a. When compared with baseline counts, in both groups, the total neutrophil count tended to increase at point 2, and there was a further increase at point 3. The total neutrophil counts at point 3 were significantly higher than the baseline both in the 2 groups ($p = 0.0009$ and 0.0009, respectively). There was no significant difference in the time courses of the 2 groups.

F-actin rimmed neutrophil counts

The F-actin-rimmed neutrophil counts are shown in Table 2 and Figure 2b. In the control group, the F-actin-rimmed neutrophil counts tended to increase at point 2, when compared with baseline and then to increase further at point 3. Patients in the sivelestat

group did not show an increase in the number of F-actin-rimmed neutrophils. There was significant difference in the time courses of the 2 groups ($p = 0.0061$).

The rate of changes of the PaO_2

The rate of change of the PaO_2 in the control and sivelestat groups was 0.81 ± 0.07 and 0.98 ± 0.05, respectively, and differed significantly between the 2 groups (Figure 3).

IL-8 levels in the plasma

Plasma levels of IL-8 did not increase significantly during or after OLV in patients in the control or experimental groups, and there was no difference in this respect between the 2 groups (Table 2).

MDA levels in the plasma

In the control group, the MDA level at point 3 was significantly higher than that at baseline. In the sivelestat group, the MDA levels did not increase during surgery. However, the time course of MDA expression did not differ between the 2 groups (Table 2).

Discussion

The results of our study show that the number of circulating F-actin-rimmed neutrophils increases not only during OLV but also after lung re-expansion following lobectomy. This finding suggests that, in addition to the surgical procedure and OLV, lung re-expansion following OLV also leads to an increase in neutrophil activation levels. Furthermore, inhibition of neutrophil elastase by sivelestat was able to limit the number of circulating F-actin-rimmed neutrophils and improve pulmonary oxygenation of patients.

Our results clearly show that the NE inhibitor sivelestat can suppress cytoskeletal rearrangements in circulating neutrophils. Previous investigations have also reported that sivelestat may have inhibitory effects on cytoskeletal rearrangement in neutrophils [10; 11]; however, it is not yet known whether NE directly changes the neutrophil cytoskeleton or whether this occurs via the activation of other mediators such as ROS or pro-inflammatory cytokines. With regard to neutrophil sequestration in lung capillaries, we speculate that this is a secondary effect of the decreased pliability of circulating neutrophils. However, previous investigators indicated that NE promotes neutrophil adhesion to lung capillaries by cleaving ICAM-1 from pulmonary endothelial cells [14] and by binding integrins on the neutrophil surfaces to regulate integrin activity [15]. These findings suggest that sivelestat may prevent neutrophil sequestration by inhibiting these functions of NE. However, we did not evaluate this possibility in our current

experiments, and further investigation should be performed to elucidate this mechanism.

We also found that sivelestat improved pulmonary oxygenation in patients undergoing OLV. Previous studies reported that neutrophil sequestration can have serious effects upon pulmonary microcirculation [16] and can increase pulmonary vascular resistance [17], while others reported that sivelestat improved blood flow in the pulmonary microcirculatory system as well as pulmonary oxygenation [18]. Taken together, these findings suggest that sivelestat improves pulmonary oxygenation via the inhibition of neutrophil sequestration.

For this study, we also investigated neutrophil chemo-attractant and ROS as possible mediators for the damaging effects of lung re-expansion. IL-8 is known to have a potent chemotactic ability that can rapidly induce neutrophil cytoskeletal rearrangement [19]. IL-8 also plays an important role in ischemia/reperfusion injury [20]. We previously reported that during OLV in patients undergoing thoracic surgery, IL-8 levels increase in the epithelial lining fluid of the collapsed lung and in plasma [6]. An animal study with rats also showed that plasma CINC-1 level increased during OLV (personal observation). However, in the present study, there was no evidence of an increase in plasma IL-8 levels during thoracic surgery. It remains unclear why there is a discrepancy between our previous studies and this current study. Misthos et al. measured MDA, a lipid peroxidation product, during lung re-expansion after OLV and

found evidence of severe oxidative stress as indicated by the levels of MDA [21]. We detected elevation of plasma MDA after re-expansion in patients in our control group, but not in patients given sivelestat. Thus far, it is unclear whether NE inhibition causes oxidative stress suppression, because we could not find a significant difference between the time course of MDA elevation in patients in the control and experimental groups.

Further studies are clearly necessary to elucidate the physiological mechanism underlying NE inhibition and neutrophil cytoskeletal rearrangement. In addition, we were unable to detect NE enzymatic activity in the peripheral arterial blood of patients undergoing lobectomy. Because of this, we speculate that OLV cannot elevate NE activity in the peripheral blood because endogenous protease inhibitors are so abundant in circulation that free NE is rapidly inactivated [22]. However, in lung capillaries, NE released from sequestered neutrophils might not get inactivated by endogenous protease inhibitors because these inhibitors are too large to enter the narrow spaces between neutrophils and endothelial cells. We propose that the molecular size of sivelestat allows for its entry between neutrophils and endothelial cells and thus protects against lung injury. Further studies investigating NE inhibition are necessary to elucidate this premise. Our study was constrained by the small number of enrolled patients, and future studies with a larger sample size will further elucidate the underlying causes of OLV. All the patients in the present study underwent lung lobectomy, and the surgical procedure

could have affected the behavior of neutrophils. Another study with a different design might thus be required to verify the exclusive impact of OLV on lung injury.

Conclusion

We found that lung re-expansion following OLV induces cytoskeletal rearrangements in circulating neutrophils and that sivelestat can prevent these changes in neutrophils and improve pulmonary oxygenation in patients undergoing thoracic surgery for lobectomy.

We propose that cytoskeletal changes in neutrophils initiate postoperative lung injury in patients undergoing OLV and that perioperative administration of sivelestat can prevent postoperative complications.

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Conflicts of interest

There are no conflicts of interest to declare.

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Table 1. Clinical characteristics of the thoracic patients analyzed in this study

	Control group	Sivelestat group	*P*-value
Number	8	8	
Age (year)	72.1 ± 3	72 ± 2.8	0.3046
Gender (M/F)	4/4	3/5	0.3210
Side of operation (R/L)	7/1	8/0	0.2225
Smoking index (pack-year)	23 ± 13	13 ± 8	0.5024
%FVC (%)	104 ± 4	114 ± 5	0.3666
FEV_1% (%)	78 ± 2	74 ± 1	0.0702
PaO_2 (mmHg)	84 ± 3	79 ± 3	0.3787
OLV period (min)	217 ± 17	189 ± 19	0.1040
Blood loss (ml)	166 ± 50	148 ± 26	0.4507
*Resected volume	7 ± 1	9 ± 1	0.1951

* The quantity of subsegments that were included in the resected lobe

Table 2. Time course data of total and F-actin-rimmed neutrophil counts and interleukin 8 (IL-8) and malondialdehyde (MDA) levels in the plasma

	Point 1	Point 2	Point 3	*P value
Total neutrophils (/μl)				
Control	2450 ± 216	6275 ± 659	6875 ± 811[#]	0.6485
Sivelestat	3061 ± 234	5211 ± 529	6429 ± 475[#]	
F-actin rimmed neutrophils (/μl)				
Control	376 ± 108	1975 ± 398	2701 ± 606[#]	0.0061
Sivelestat	268 ± 41	612 ± 91	961 ± 239[#]	
IL-8 levels in plasma (pg/ml)				
Control	10.1 ± 4.2	13.5 ± 4.5	11.3 ± 4.0	0.7868
Sivelestat	12.0±3.2	14.0 ± 4.0	14.1 ± 1.2	
MDA levels in plasma (pg/ml)				
Control	37.1±3.1	44.3±4.1	55.4 ± 6.6[#]	0.5135
Sivelestat	40.6±4.1	46.7±3.2	53.0 ± 11.7	

*P value in comparison of the time courses between the 2 groups

[#]Significant difference compared with the baseline

Figures

Figure 1. Time course of the protocol

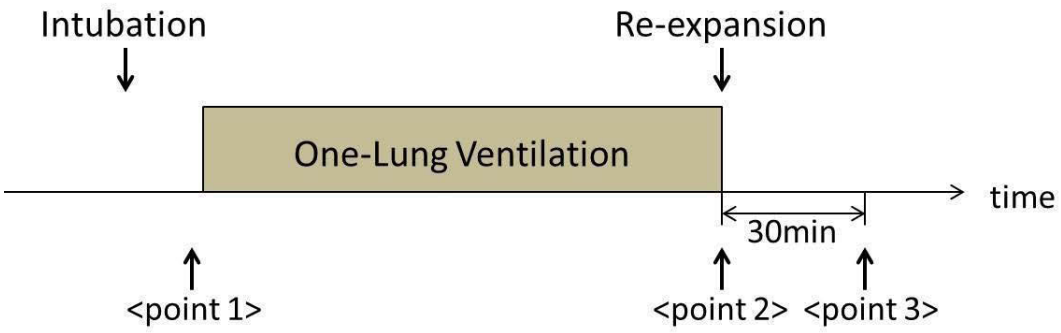
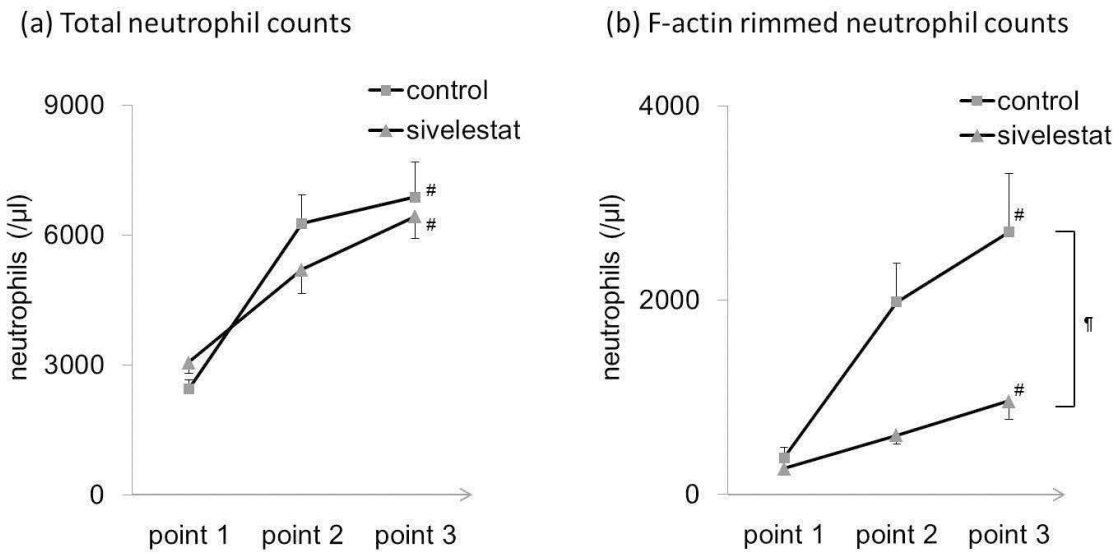


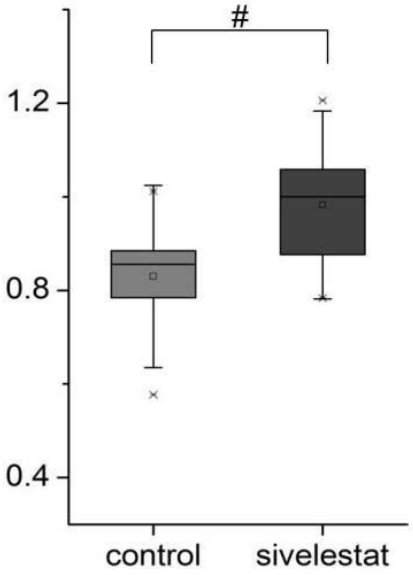
Figure 2. Total and F-actin rimmed neutrophil counts



#Significant difference compared with the baseline

¶Significant difference between the 2 time courses

Figure 3. The rate of change of the PaO_2



#Significant difference between the 2 groups