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Short communications

Effect of Ono-EI-600 elastase inhibitor on high-tidal-volume-induced lung injury in rats

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Abstract
We tested the effect of Ono-EI-600, an elastase inhibitor that suppresses cytokine release, on ventilator-induced lung injury in a rat model. After Wistar rats (aged 8–11 weeks) were anesthetized and tracheostomized, they were randomly assigned to four groups: high tidal volume (V_T) group (H group: $n = 10$) receiving peak inspiratory pressure (PIP) 30 cmH_2O for 240 min; high V_T with drug group (HD group: $n = 10$) receiving the same ventilation settings as H group and also intravenous infusion 10 $mg \cdot kg^{-1} \cdot h^{-1}$ of Ono-EI-600 during the protocol; the lower V_T group (L group: $n = 5$) receiving PIP 10 cmH_2O for 240 min; and control group (C group: $n = 5$) receiving the same ventilation as L group for 30 min. The cytokine levels (IL-6 and CINC-1) in the bronchoalveolar lavage fluid (BALF) of the H group were significantly higher than those of the C and L groups ($P < 0.05$). However, for the H and HD groups, no differences were found in arterial blood gas data, cytokine levels in BALF, and histological injury scores. Our experiment provided no evidence that elastase inhibitor Ono-EI-600 protects against lung injury induced by high V_T ventilation.

Key words Elastase inhibitor · Ventilator-induced lung injury · Lung injury score · BAL · Cytokine

Clinical studies have shown that ventilation with low tidal volumes (V_T) lessens mortality [1,2]. In animal models, high V_T induces mechanical stress injury to the pulmonary parenchyma and microvasculature from overdistension and causes the release of proinflammatory mediators [3–6].

Neutrophil reduction correlates with reduced lung injury, and it is accepted that neutrophils play an important role in the pathogenesis of acute lung injury/acute respiratory distress syndrome (ALI/ARDS) in animal studies [7–9]. The upregulated proteolytic activity of neutrophil elastase leads to a variety of changes relevant to the pathophysiology of ALI [9]. Consequently, neutrophil elastase inhibition may protect the lungs from injury produced by neutrophil elastase [10–12].

Several animal studies have shown elastase inhibition to be effective [13–17]. However, elastase inhibitor has not been shown to suppress the release of cytokines associated with ventilator-induced lung injury (VILI), and no reliably conclusive clinical study has yet been conducted [18]. In the present study, we investigated if a neutrophil elastase inhibitor, Ono-EI-600, affected rats with lung injury induced by very high V_T.

The study was approved by the Laboratory Investigation Committee of Osaka University Medical School. Specific pathogen-free male Wistar rats (age, 8–11 weeks; body weight, 359 ± 56 g) were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium and tracheostomized with an infiltration of local anesthetics. Each rat was kept in a supine position throughout the experiment.

After cannulation to the carotid artery, normal saline solution including 5 $mg \cdot ml^{-1}$ pentobarbital sodium, 0.1 $mg \cdot ml^{-1}$ pancuronium bromide, and 0.1 $mg \cdot ml^{-1}$ sodium bicarbonate was infused at 2 $ml \cdot h^{-1}$. Normal saline with 2 $U \cdot ml^{-1}$ heparin was also infused continuously at 1 $ml \cdot h^{-1}$ to compensate for blood sampling. The animals were connected via a neonatal ventilator circuit (compliance, 0.9 $ml \cdot cmH_2O^{-1}$) to a Servo 300 ventilator (Siemens-Elema AB, Solna, Sweden). At the following settings, all the rats were ventilated for 30 min: volume control ventilation; positive end-expiratory pressure (PEEP) of 5 cmH_2O; respiratory rate of 68 $breaths \cdot min^{-1}$; V_T titrated to achieve the displayed peak inspiratory pressure (PIP) of 10 cmH_2O; inspiratory-to-expiratory (I:E) ratio of 1:3; and the fraction of inspired oxygen ($F_{I_{O_2}}$) of 1.0.

During 10 cmH_2O PIP ventilation, we measured baseline blood gas levels as control values with a cali-

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brated blood gas analyzer (ABL505; Radiometer A/S, Copenhagen, Denmark). Then the animals were randomly assigned to four groups: high V_T group (H group: $n = 10$) at 12 breaths·min^{-1} and inspiratory V_T titrated to achieve the displayed PIP of 30 cmH_2O for 240 min; high V_T with drug group (HD group: $n = 10$), with same ventilation as H group and also 10 mg·kg·h^{-1} Ono-EI-600 infusion from a time before the commencement of ventilation to the end of the protocol; lower V_T group (L group: $n = 5$), with the same ventilation settings as the control period continued for 240 min. Settings were the same in all groups for $F_{I_{O_2}}$ 1.0, PEEP 0 cmH_2O, and I : E 1 : 3. Arterial blood gases were analyzed at 60-min intervals to the end of the protocol. Only the control group (C group: $n = 5$) continued the ventilation setting of the control period for only 30 min to obtain baseline values. Animals whose systolic arterial pressure decreased to less than 50 mmHg were excluded.

After the protocol, a blood sample was taken from each animal, which was then killed with 100 mg·kg^{-1} pentobarbital sodium injection before harvesting the bronchus and lungs. The excised right lungs were lavaged by a series of three injections and withdrawals of a 5-ml aliquot of phosphate-buffered saline. The samples were centrifuged at 3000 rpm for 5 min, and cellfree supernatant fluid was frozen and stored at –30°C until analysis.

Interleukin (IL)-1β, IL-6, cytokine-induced neutrophil chemoattractant 1 (CINC-1), IL-10, and tumor necrosis factor-alpha (TNF-α) concentration in bronchoalveolar lavage fluid (BALF) and in the serum were measured by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Rat Biotrak ELISA System; Amersham Biosciences, Bucks, UK).

The left lungs were fixed by immersion in 10% buffered neutral formalin. Paraffin-embedded sections of the lung tissues in the upper, middle, and lower lobes were stained with hematoxylin and eosin and examined by a pathologist who was blinded to the protocol and group source of the samples. Lung injury was scored according to the following four items: (1) alveolar congestion, (2) hemorrhage, (3) infiltration or aggregation of neutrophils in the airspace or vessel wall, and (4) thickness of the alveolar wall/hyaline membrane formation [19]. Each item was graded according to a five-point scale. A total score of 0 indicated normal histology, and that of 16 indicated maximal damage.

Lung injury scores (LIS) are shown as the median, whereas other data are presented as mean ± SD. Parametric data were analyzed with one-way or two-way analysis of variance (ANOVA) followed by post hoc analysis with the Tukey honest significant difference test. LIS was analyzed with the Kruskall–Wallis ANOVA median test followed by post hoc analysis with the Mann–Whitney *U* test. Statistical significance was accepted as $P < 0.05$.

Throughout the protocol, displayed V_T in L, H, and HD groups was 12 ± 2 ml·kg^{-1}, 70 ± 10 ml·kg^{-1}, and 69 ± 14 ml·kg^{-1}, respectively. Table 1 shows the basic characteristics of each animal, LIS, and the absolute levels of cytokines in BALF. IL-6 and CINC-1 were significantly higher in the H and HD groups than in the L group ($P < 0.01$). No differences in the levels of any type of

Table 1. Basic characteristics of animals and levels of cytokine and lung injury score (LIS) at the end of the ventilation protocol

Group	C	L	H	HD
Number	5	5	10	10
Weight (g)	374 ± 69	357 ± 51	351 ± 53	355 ± 69
Levels of cytokine in BALF				
IL-1β (pg/ml)	39 ± 38	21 ± 19	43 ± 37	50 ± 28
IL-6 (pg/ml)	240 ± 78	282 ± 84	2529 ± 1110*	2758 ± 1272*
CINC-1 (pg/ml)	101 ± 16	176 ± 60	1293 ± 519*	1230 ± 562*
IL-10 (pg/ml)	18 ± 13	10 ± 8	25 ± 8	27 ± 17
TNF-α (pg/ml)	57 ± 25	31 ± 21	95 ± 55	102 ± 61
LIS				
Upper		2	4.5*	3.5
Middle		2	4.5	3.5
Lower		2	7.5*	4

C, control group; L, lower tidal volume (V_T) group; H, high tidal volume group; HD, high tidal volume with drug; BALF, bronchoalveolar lavage fluid; IL, interleukin; CINC, cytokine-induced neutrophil chemoattractant; TNF-α, tumor necrosis factor alpha

Except LIS (median), values are expressed as mean ± SD. LIS was scored according to four criteria: alveolar congestion; hemorrhage; infiltration or aggregation of neutrophils in airspaces or vessel walls; and thickness of the alveolar wall/hyaline membrane formation

* Versus L (and C) group: $P < 0.05$, Kruskal–Wallis ANOVA, median test followed by post hoc analysis with Mann–Whitney *U* test

Table 2. Blood gas analysis and arterial pressure in each group

Group	C	L	H	HD
Pa_{O_2} (mmHg)				
Baseline	466 ± 43	401 ± 28	424 ± 26	422 ± 47
240 min		437 ± 30	299 ± 105	295 ± 118
Pa_{CO_2} (mmHg)				
Baseline	54.5 ± 4.5	53.6 ± 2.8	53.6 ± 4.3	52.9 ± 4.5
240 min		57.2 ± 6.1	58.5 ± 11.2	53.9 ± 21.1
pH				
Baseline	7.35 ± 0.04	7.40 ± 0.03	7.39 ± 0.05	7.40 ± 0.06
240 min		7.38 ± 0.03	7.37 ± 0.07	7.38 ± 0.08
BE (mmol/L)				
Baseline	2.9 ± 0.9	5.5 ± 1.8	5.1 ± 1.6	5.9 ± 2.8
240 min		6.8 ± 1.1	5.5 ± 1.7	7.1 ± 2.6
SAP (mmHg)				
Baseline	129 ± 9	147 ± 12	126 ± 9	119 ± 27
240 min		120 ± 19	97 ± 24	102 ± 26
MAP (mmHg)				
Baseline	121 ± 6	135 ± 8	103 ± 34	108 ± 24
240 min		112 ± 18	84 ± 22	90 ± 22

BE, base excess; SAP, systolic arterial blood pressure; MAP, mean arterial blood pressure
Values are expressed as mean ± SD

cytokine were found when comparing H and HD. LIS was not significantly different between H and HD. No significant differences in the absolute levels of each cytokine in serum were apparent between the groups (data not shown). Table 2 shows blood gas analysis and hemodynamics. These parameters were similar in each group during the ventilation protocol.

Our results showed that neutrophil elastase inhibitor Ono-EI-600 had no evidence of protection against VILI in healthy rats. Pretreatment with the elastase inhibitor neither extended the time for Pa_{O_2} to decrease below 300 mmHg nor decreased cytokine levels in BALF samples.

Low V_T has been found to improve the outcome of patients with ALI/ARDS [1]. The basic reason for this improvement is believed to be the reduction, with low V_T ventilation, of the mechanical stretch caused by high inspiratory pressure [2,20,21]. In vitro, stretching of alveolar epithelial type II cells or alveolar macrophages triggers the release of proinflammatory mediators, and low V_T ventilation is thought to reduce the release of these chemical mediators that aggravate lung injury [22,23]. In isolated lungs, high-volume or high-pressure ventilation has been found to elicit local and systemic concentrations of proinflammatory mediators [4]. In animal models of ALI [5,6], and in human patients with ALI [24], protective ventilation strategies increase survival and reduce mediator release. Dreyfuss et al. reported that IL-8 was the only mediator that was constantly released during overinflation [6]. The levels of IL-6 and CINC-1 were increased in BALF in H and HD, and it supported that the model in the present study was relevant to neutrophil elastase [12]. However, the cytokine levels in serum did not differ significantly between the groups. The effect of Ono-EI-600 was not sufficient to protect the lung against VILI, although there was no difference in LIS between the HD and L groups.

Abundant evidence from animal studies has shown that neutrophils play an important role in the pathogenesis of ALI/ARDS [9–12] and, more specifically, it has been suggested that elastase inhibitors have a positive effect on ARDS [11,12]. In the present study, we pretreated animals with neutrophil elastase inhibitor and ventilated them with injurious V_T. By contrast with lung injuries in previous studies, which were induced by intraperitoneal endotoxin, ischemia-reperfusion, and thrombin [13–17], we induced lung injury by high V_T ventilation that continues to injure the lungs during ventilation compared to previous studies. This mode of injury may explain the discrepancy in the results we achieved with Ono-EI-600. The dispersion of time course in the development of individual lung injury in each group might also have compromised our results. Even though the aforementioned Japanese research showed elastase inhibition to be beneficial in the pulmonary function of ALI/ARDS patients [25], the STRIVE study was unable to show that sivelestat was efficacious in a broad spectrum of ALI/ARDS cases [18]. The population under study may encompass too broad a range of pathogenesis to detect the effectiveness of treatment for specific conditions. This difference in sampling precision may account for the discrepancy in the findings of the STRIVE study [26] and the Japanese report.

The type of elastase inhibitor that we used may also have affected the findings. The molecular weight of

Ono-EI-600, which is the chiral variant of sivelestat, is almost the same (448.5 vs 528.5) as sivelestat, but it displays stronger inhibition of elastase than sivelestat [50% inhibitory concentration (IC_{50}), 24 vs 48 μM, rat, total blood in vitro]. We administered Ono-EI-600 in a dosage similar to that used in previous studies using sivelestat [14,16], so although we did not confirm the concentration of Ono-EI-600 in the blood, we assume that the dose was sufficient.

In conclusion, our study found no evidence that elastase inhibitor Ono-EI-600 protected the lungs against injury induced by very high V_T ventilation.

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