A NEUTROPHIL ELASTASE INHIBITOR IMPROVES LUNG FUNCTION DURING EX VIVO LUNG PERFUSION

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Abstract

Objective:

Ex vivo lung perfusion (EVLP) has been used not only for graft evaluation but also for graft reconditioning prior to lung transplantation. Inflammatory cells such as neutrophils may cause additional graft injury during EVLP. Neutrophil elastase inhibitors protect lungs against neutrophil-induced lung injury, such as acute respiratory distress syndrome. This study aimed to investigate the effect of a neutrophil elastase inhibitor during EVLP.

Methods: EVLP was performed for 4 hours in bilateral pig lungs that had previously experienced warm ischemia for 2 hours with or without a neutrophil elastase inhibitor (treated and control groups, respectively; n = 6). Following EVLP, the left lung was transplanted into a recipient pig, and this was followed by observation for 4 hours. Pulmonary functions were observed both during EVLP and during the early post-transplant stage.

Results: During EVLP, decreases in neutrophil elastase levels ($P < 0.001$), the wet-dry weight ratio ($P < 0.05$), and pulmonary vascular resistance ($P < 0.01$) and increases in the PaO_2/FiO_2 ratio ($P < 0.01$) and pulmonary compliance ($P < 0.05$) were observed in the treated group. After transplantation, decreased pulmonary vascular resistance ($P < 0.05$) was observed in the treated group.

Conclusions:

A neutrophil elastase inhibitor attenuated the inflammatory response during EVLP and may decrease the incidence of lung reperfusion injury after transplantation.

Introduction:

Donor shortage continues to be a significant limiting factor for lung transplantation [1]. Lungs that satisfy the extended donor criteria, including those that were donated after cardiac death (DCD), have been used successfully and have contributed to an increase in the number of available donor organs. Ex vivo lung perfusion (EVLP) has been used to evaluate and recondition lungs from extended criteria donors. However, EVLP may injure the lung graft [2-4].

We have previously reported increased proinflammatory cytokine levels in the perfusate during EVLP [2]. Neutrophils can exist in the flushed lung graft and may play a role in proinflammatory cytokine release [5]. Neutrophil elastase inhibitors can protect lungs against neutrophilic lung injury such as acute respiratory distress syndrome [6-8]. Therefore, we speculate that neutrophil elastase inhibitors may protect lung grafts by decreasing neutrophil activity during EVLP. The aim of this study was to evaluate the efficacy of neutrophil elastase inhibitors during EVLP.

Methods:

The animals used in this study received humane care in compliance with *The Principles of Laboratory Animal Care* (formulated by the National Society for Medical Research) and *Guide for the Care and Use of Laboratory Animals* (prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health; NIH publication No. 86 –23,

revised 1996). The study protocol was approved by the Animal Care and Use Committee, Okayama University.

Experimental Design

Landrace pigs with a mean weight of 29.0 kg (27.0-32.0 kg) were randomly divided into 2 groups (treated and control groups, n = 6). In each groups, donor heart-lung blocks were harvested after 2 hours of warm ischemia and 4 hours of EVLP. In the treated groups, 0.5 g of neutrophil elastase inhibitor (sivelestat, Ono Pharmaceutical, Tokyo, Japan) was added to the perfusate both before and 2 hours after perfusion.

During EVLP, a gas analysis was performed, and the wet-dry weight ratios; fluid elastase levels; fluid inflammatory cytokine levels, including perfusate tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) levels; airway compliance; and pulmonary vascular resistance (PVR) were examined. After EVLP, the left donor lung was transplanted into the recipient pig, followed by a 4-hour observation period. Graft oxygenation and PVR were evaluated while clamping the right pulmonary artery for few minutes. The wet-dry weight ratio of transplanted lungs was assessed at the end of the observation period.

Donor lung retrieval

The donor pigs were pre-medicated with an intramuscular ketamine hydrochloride (10 mg kg^{-1})

injection (Ketaral, Daiichi Sankyo, Tokyo, Japan) and atropine sulfate (0.025 mg kg^{-1}) (Fuso, Osaka, Japan). The animals were then anesthetized by halothane inhalation (Fluothane, Takeda, Osaka, Japan), and intravenous pancuronium bromide (0.2 mg kg^{-1}) (Mioblock, Schering Plough, Osaka, Japan) was administered before intubation. In each groups, heparin (5,000 U/body IV) (heparin sodium, Ajinomoto, Tokyo, Japan) was administered and lungs were inflated with 50% oxygen prior to electrical stimulation-induced cardiac arrest. After 2 hours of warm ischemia, the donor lungs were started ventilation with a tidal volume of 10 ml/kg, a respiratory rate of 15 breaths/ min, a positive end-expiratory pressure of 5 cm H_2O, a fraction of inspired oxygen (FiO_2) of 0.5 and flushed with 1500 ml of a cold low-potassium dextran glucose solution (LPD), and the heart-lung block was then explanted and stored at 4°C for about 1 hour.

Heart-Lung Block Preparation

The main pulmonary artery was cannulated with a 24-F cannula (femoral cannula, Toyobo, Osaka, Japan). The heart was excised, leaving a generous left atrial cuff. A funnel-shaped plastic cannula (Xvivo perfusion) was then sewn to the atrial cuff. An additional 500 ml retrograde flush of a cold low-potassium dextran glucose solution was then performed.

Isolated Perfusion Circuit

The perfusion circuit consisted of a venous reservoir, a centrifugal pump (Bio-Console, Bio-Medicus, Eden Prairie, MN), a cardiopulmonary bypass membrane oxygenator (HPO-23RHF-C, MERA, Tokyo, Japan) as a deoxygenator, and a leukocyte filter (LG 6, Pall Corp, Port Washington, NY). Pulmonary artery flow was monitored using a flow meter (Bio-Prob, Bio-Medicus). The circuit was primed with 1.5 L of Steen solution (Xvivo perfusion, Gothenberg, Sweden), 1 g of Cefazolin (Cefamezin, Astellas, Tokyo, Japan), and 10,000 IU of heparin. Oxygen, carbon dioxide, and nitrogen were supplied to the oxygenator to achieve a partial pressure of 35 to 45 mmHg carbon dioxide in the perfusate that was taken from the inflow cannula.

Ex Vivo Lung Perfusion

The time between cold LPD flush and initiation of EVLP was about 3 hours. A low-flow perfusion (100 ml/min) at 25°C was initiated through the lungs, which were gradually warmed by increasing the perfusate temperature. The lungs were ventilated with a tidal volume of 8 ml/kg, a respiratory rate of 15 breaths/ min, a positive end-expiratory pressure of 5 cm H_2O, and a FiO_2 of 21% when the graft temperature reached 32°C. The pump flow was gradually increased and then maintained at 40% of the estimated donor cardiac output, but this value

remained below 20 mmHg, which is the peak arterial pressure (PAP).

Graft Variables

During EVLP, oxygenation was evaluated as the difference between the outflow and inflow oxygen partial pressures per fraction of inspired oxygen.

Elastase assay

Details of the elastase assay were described elsewhere [9]. Briefly, plasma neutrophil elastase was incubated with 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 M NaCl and 1 mM substrate at 37°C for 24 hours, and the amount of liberated *p*-nitroanilide was measured spectrophotometrically.

Cytokine assay

Small amounts of sodium citrate were added to the blood and perfusate samples, and the samples were then immediately centrifuged at 4,000 rpm for 5 minutes. The supernatant was frozen in liquid nitrogen and stored at -80°C. TNF-α, IL-6 and IL-8 levels were measured using enzyme-linked immunoassay kits (R&D systems, Minneapolis, MN). All of the samples were assayed in duplicate.

Wet-to-dry weight ratio

Specimens were obtained from the lower right lobe after 4 hours of EVLP and from the lower left lobe after transplantation. The specimens were dried for 3 days in an oven at 60°C and re-weighed to determine the wet-to-dry weight ratio.

Statistics

All of the results are expressed as the mean ± standard error of the mean. The data were analyzed using Statcel version 2 (OMS Publishing Inc, Japan). The differences were considered statistically significant if the p value was less than 0.05. For parametric data, the Mann-Whitney test was used for comparisons between groups. A repeated-measures analysis of variance (ANOVA) was used for the analysis of serial measurements.

Results

Baseline characteristics

The baseline characteristics of the donors and recipients, including weight, cardiac output, arterial PaO_2, and pulmonary artery pressure, were similar between the two groups (Table 1).

Perfusate elastase levels during EVLP

Perfusate elastase levels in the treated group were significantly lower than those in the control group ($P < 0.001$) throughout the perfusion period (Figure 1).

Perfusate cytokine levels during EVLP

Perfusate TNF-α, IL-6, and IL-8 levels increased gradually during EVLP (Figure 2). The IL-6 and IL-8 levels in the treated group were significantly lower than those in the control group ($P = 0.024$ and $P = 0.001$, respectively) (Figures 2B and 2C).

Lung functions during EVLP

The degree of oxygenation in the treated group was significantly greater than that in the control group ($P < 0.001$) (Figure 3A). The PVR in the treated group was significantly higher than that in the control group ($P < 0.001$) (Figure 3B). Additionally, the degree of pulmonary compliance in the treated group was significantly lower in the control group ($P = 0.002$) (Figure 3C).

Wet-to-dry weight ratio after perfusion

The wet-to-dry weight ratio of lungs from pigs in the treated group was significantly lower than that of lungs from pigs in the control group (4.65±1.98 versus 8.87±3.49, $P = 0.03$).

Graft functions after transplantation

The PVR in the treated group was significantly lower than that in the control group ($P = 0.02$) (Figure 4A). There were no differences in oxygenation or pulmonary compliance between the two groups ($P = 0.785$ and $P = 0.33$, respectively) (Figure 4B).

Wet-to-dry weight ratio after transplantation

There was no difference in the wet-to-dry weight ratio between the two groups ($P = 0.8$).

Discussion

EVLP is a novel technique that can be used to evaluate and recondition graft lung functions before transplantation [10-13]. However, previous studies demonstrated that proinflammatory cytokine levels increased gradually during EVLP and that graft lung function deteriorated after transplantation [2-4]. Therefore, longer EVLP has a potential risk for EVLP–induced lung graft injury [14]. In this study, perfusate neutrophil elastase activity increased during EVLP and was suppressed by a neutrophil elastase inhibitor. In addition, perfusate proinflammatory cytokines were suppressed and graft functions were ameliorated by neutrophil elastase inhibitor treatment during EVLP.

Historically, most experimental extracorporeal systems used to perfuse the lung have resulted in edema and graft degeneration, suggesting that ex vivo reperfusion negatively affects grafts [14]. The system, including the acellular perfusate, the perfusion techniques and the equipment, was improved by the Lund and Toronto groups [15]. However, our research group found that cytokine levels increased over time during EVLP [2], suggesting that neutrophils were present in the graft-EVLP circuit, where these cells should not exist. Barletta et al. reported that leukocytes, including NK cells, neutrophils, mononuclear myeloid cells, and T cells, were found in the vascular marginated compartment after the native mouse lungs were flushed [5]. These findings indicate that neutrophils in the vascular marginated compartment may release proinflammatory cytokines and cause graft injury during EVLP, despite the use of the acellular perfusate.

Acute lung IR injury in animals occurs in a biphasic pattern, with the early phase being mediated by macrophages and the late phase being mediated by neutrophils [16-18]. Early injury occurs when stimulated macrophages release superoxide and cytokines as a result of increased pulmonary vascular permeability. In the late phase, neutrophil elastase and other neutrophil granule protease products affect the lung. Neutrophil elastase is an important and pivotal mediator in acute lung injury. Neutrophil elastase induces the release of

proinflammatory cytokines, such as IL-6, IL-8, granulocyte-macrophage colony-stimulating factor and mucin, from epithelial cells [19, 20]. Therefore, neutrophil elastase inhibitors may suppress proinflammatory cytokine release.

In this experiment, the neutrophil elastase inhibitor was added to the perfusate twice- at the beginning of the experiment and at 2 hours after EVLP-, as the half-life of neutrophil elastase in plasma is approximately 130 minutes.

In this study, treatment with a neutrophil elastase inhibitor improved post-transplantation graft PVR, but values of post-transplantation graft oxygenation and pulmonary edema formation were not changed. Because post-transplantation lung function can be influenced by multiple factors other than proinflammatory cytokines. However, Aoki et al. reported that continuous neutrophil elastase inhibitor infusion after transplantation ameliorated lung function. Treatment with a neutrophil elastase inhibitor during combined EVLP and transplantation might ameliorate post-transplant graft functions. Further studies will be needed to clarify the effect of neutrophil elastase inhibitors on post-transplant graft functions.

Conclusion

Inflammatory cells such as neutrophils might cause additional injury to grafted lungs during

EVLP. Neutrophil elastase inhibitor attenuate the inflammatory response during EVLP and may ameliorate the functions of grafted lungs.

Conflict of interest statement

Masaaki Harada and other co-authors have no conflict of interest.

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