Vascular Endothelial-Cadherin Expression After Reperfusion Correlates With Lung Injury in Rat Lung Transplantation

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Background. Vascular endothelial-cadherin (VEC), composing the adherens junction of endothelial cells, has been shown to regulate vascular permeability. The aim of this study was to investigate VEC expression during cold ischemia (CI) and reperfusion and whether a dibutyryl cyclic adenosine 3',5'-monophosphate (db-cAMP) and nitroglycerin (NTG) additive to preservation solution can maintain VEC.

Methods. Rat left lung transplantation after CI was performed, and reperfusion was done for 2 hours. The experimental groups were Control (n = 5), CI3 (n = 5), CI6 (n = 6), and CI6-AMP/NTG (n = 6) with minimum ischemic period, 3 hours and 6 hours of CI, respectively, and 6 hours of CI with db-cAMP and NTG additive to preservation solution, respectively. Lung mechanics, lung wet-to-dry weight ratio (W/D), and the histologic findings of the graft were evaluated. The expression of VEC was evaluated by Western blot analysis of the lung tissue lysates.

Results. The CI3 group showed a decrease in dynamic compliance with perivascular edema. Dynamic compliance, graft oxygenation, and W/D were substantially deteriorated, and intraalveolar edema with neutrophil infiltration was recognized in the CI6 group. They were improved in the CI6 + AMP/NTG group. VEC expression was maintained during CI. After reperfusion, it reduced substantially in the CI6 group and was maintained in the CI6 + AMP/NTG group.

Conclusions. VEC expression was maintained during CI; however, it reduced early after reperfusion after 6 hours of CI, correlating with severe intraalveolar edema. db-cAMP/NTG additive to the preservation solution contributed to the maintenance of VEC expression after reperfusion.

Lung transplantation has been established as a life-saving procedure for patients with end-stage respiratory failure. Perioperative managements, including graft preservation, have been developed, but primary graft dysfunction is still a problem to overcome [1–6]. Primary graft dysfunction, caused mainly by ischemia/reperfusion injury (IRI), is associated not only with early rates of morbidity and mortality [3, 4] but also with chronic lung allograft dysfunction [3, 7]. Endothelial damage during ischemia is considered an important contributing factor to the occurrence of IRI [2, 6].

Vascular endothelial-cadherin (VEC) is the main component of adherens junction of the pulmonary endothelial cells, which has a pivotal role in controlling vascular permeability [8–10]. VEC expression was shown to decrease and contribute to increasing permeability during 6 hours of cold preservation of endothelial cells in vitro [11]. However, the in vivo dynamics of VEC in IRI have not been reported.

Our group has reported that the addition of dibutyl cyclic adenosine 3',5'-monophosphate (db-cAMP) and nitroglycerin (NTG) to the preservation solution ameliorated IRI because they serve as an intracellular cAMP and cyclic guanosine 3',5'-monophosphate (cGMP) provider [2, 12–15]. The increase in intracellular cAMP and cGMP was considered to be protective for pulmonary endothelial cells, but most studies were based on ex vivo circuits, and the influence on the adherens junction molecules has not been investigated.

This study aimed to test the hypothesis that decreased expression of VEC during cold ischemia (CI) contributes to IRI and that db-cAMP and NTG added to preservation solution can maintain VEC and ameliorate lung edema after reperfusion in a rat lung transplantation model.

Material and Methods

Animals

Specific pathogen-free inbred male Lewis rats (weight, 300 to 350 g) were used (Japan SLC Inc, Hamamatsu, Japan).
Japan). All animals received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals, prepared by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). This study protocol was approved by the Graduate School of Medicine Ethical Committee at Kyoto University (MedKyo15508).

Rat Transplantation Model Evaluating IRI

Donor rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg), tracheotomized, intubated with a plastic catheter, and mechanically ventilated (SN-480-7; Shinano Seisakusyo, Tokyo, Japan). Fraction of inspired oxygen was kept at 1.0, positive end-expiratory pressure at 2 cm H₂O, tidal volume at 10 mL/kg, and respiratory rate at 70 breaths/min. Laparotomy and median sternotomy were performed. The inferior vena cava, right auricle, and left atrial appendage were cut 5 minutes after the intravenous injection of heparin (300 IU). The bilateral lungs were flushed at 20 cm H₂O through the pulmonary artery with the use of an atraumatic metallic catheter by 20 mL of cold preservation solution (ET-Kyoto [ET-K]; Otsuka, Tokyo, Japan) with or without db-cAMP (2 mmol/900 mg/L) (Daiichi Sankyo, Tokyo, Japan) and NTG (100 mg/L) (Eizai, Tokyo, Japan). The trachea was clamped in the deep inspiratory phase, and the heart and lungs were harvested. The excised lungs were wrapped with gauze and soaked in a 4°C preservation solution.

Each recipient rat was anesthetized in the same manner as each donor rat. Left thoracotomy was performed, and the left hilum was exposed. Left pulmonary artery, bronchus, and pulmonary vein (PV) were dissected, and the left hilum was clamped. During clamping, tidal volume was decreased to 8 mL/kg, and respiratory rate was increased to 90 breaths/min. Anastomosis was performed with the cuff technique as previously described in the literature [16]. The left bronchus and PV were declamped, the transplanted lung was inflated, and retrograde reperfusion was confirmed. The PA was declamped last. The chest was closed with some sutures, and reperfusion was continued for 2 hours.

Experimental Groups

The experimental groups were as follows (Fig 1). In the Control group (n = 5), transplantation was performed with minimum ischemic period related to the transplantation procedure. In the CI groups, the period of cold preservation and warm ischemic time during anastomosis were set to 3 hours (CI3; n = 5) or 6 hours (CI6; n = 6). In these groups, the flushing and preserving of lungs were conducted with ET-K solution only. Flushing and 6 hours of preservation with the use of ET-K solution with db-cAMP and NTG were designated as the CI6+AMP/NTG group (n = 6). In the Sham group (n = 5), the animals were just anesthetized, thoracotomized, and ventilated for 2 hours.

Blood Gas Analysis and Lung Mechanics Measurement

After 2 hours of reperfusion, a blood sample was obtained from the left PV for blood gas analysis to evaluate graft oxygenation. Median sternotomy was performed to occlude the right hilum, and the animals were connected to a rodent ventilator (flexiVent, Scireq, Montreal, QC, Canada). After the stabilizing period, maximum airway pressure and dynamic compliance of the graft were measured.

Lung Wet-to-Dry Weight Ratio

After the measurement of lung function, the heart and lungs were harvested, and the graft was separated into three parts. The upper segment was used to calculate lung wet-to-dry weight ratio (W/D) for evaluating lung edema. The wet weight was measured soon after the harvest, and the dry weight was measured after the specimen had been dried overnight at 100°C. The ratio was calculated as wet weight divided by dry weight.

Histologic Analysis and Neutrophils Count

The lower part of the right lung before reperfusion and the middle part of the left lung after reperfusion were fixed in 10% formalin and stained with hematoxylin and eosin. Naphthol AS-D chloroacetate esterase stain was used for counting neutrophils infiltrating into the lung as previously described [17]. Three separate investigators...
(ST, KO, and TK) evaluated the samples in a blinded manner. The number of neutrophils was expressed by the average number of extravascular neutrophils in four randomly chosen high-power fields (HPFs) per section at ×400 magnification.

**Western Blot Analysis**
For the evaluation of the expression of VEC, Western blot analysis with the use of lung tissue lysates was performed. The upper part of the right lung before reperfusion and the lower part of the left lung after reperfusion, which had been stored at −80°C, were homogenized in RIPA buffer that contained a cocktail of protease inhibitors (Nacalai tesque, Kyoto, Japan). Tissue lysate was obtained, and protein concentration was determined by Bradford protein assay. The protein samples (15 μg) were loaded on sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and transferred to a polyvinylidene fluoride membrane. The membranes were incubated with anti-VEC antibody (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibody with the use of the standard procedure, and the blots were then detected with chemiluminescence agent. The same membrane was used for reprobing with anti–β-actin antibody (A5441; Sigma-Aldrich, St. Louis, MO). The procedures were done in quadruplicate, and the image was analyzed by densitometry (CS Analyzer 3; ATTO, Tokyo, Japan) to calculate the relative intensity of the band.

**Statistical Analysis**
Data were presented as the mean ± standard deviation. Statistical analysis was performed by JMP Pro 11.2.0 (SAS Institute Inc., Cary, NC). The Kruskal-Wallis test was used for comparing the three groups (Control, CI3, and CI6), and the Mann-Whitney test was used to compare the two groups (CI6 and CI6+AMP/NTG). Data of the Sham group were presented as a reference. The differences were considered to be statistically significant at \( p \) less than 0.05.

**Results**

**Graft Function**
The CI6 group showed higher maximum airway pressure than the Control and CI3 groups \( (p = 0.020 \text{ and } 0.012, \text{ respectively}) \) (Fig 2A). The CI6 group also showed lower dynamic compliance than the Control and CI3 groups \( (p = 0.020 \text{ and } 0.012, \text{ respectively}) \) (Fig 2B). These were improved in the CI6+AMP/NTG group \( (p = 0.023 \text{ and } 0.037, \text{ respectively}) \). Oxygenation of the graft was also significantly deteriorated in the CI6 group, compared with the Control and CI3 groups \( (p = 0.012 \text{ and } 0.012, \text{ respectively}) \), but was improved in the CI6+AMP/NTG group \( (p = 0.037) \) (Fig 2C). In a comparison between the Control and CI3 groups, maximum airway pressure and dynamic compliance were significantly deteriorated \( (p = 0.020 \text{ and } 0.020, \text{ respectively}) \).

**Perivascular Edema and Intraalveolar Edema of the Graft**
Edema of the graft evaluated by W/D was deteriorated in the CI6 group, compared with the Control and CI3 groups \( (p = 0.008 \text{ and } 0.008, \text{ respectively}) \), and was ameliorated in the CI6+AMP/NTG group \( (p = 0.013) \) (Fig 2D).

The histopathologic findings showed that only perivascular edema was recognized in the CI3 group, which did not affect W/D (Fig 3A–C). In the CI6 group, severe
perivascular and intraalveolar edema were recognized. Intraalveolar edema was ameliorated in the CI6 \(+\) AMP/NTG group (Fig 3D, E).

**Neutrophil Infiltration into the Graft**

Extravasation of neutrophils has been shown to play a pivotal role in IRI and the subsequent graft rejection [18], and so the number of neutrophils invaded in the graft was counted. The nuclei of the neutrophils in the graft were stained clear blue by naphthol AS-D chloroacetate esterase stain (Fig 4A–E). The mean number of neutrophils per HPF was 17.9/2.75 (Sham group), 16.6/2.70 (Control group), 21.8/5.70 (CI3 group), 42.9/5.70 (CI6 group), and 23.6/7.16 (CI6 \(+\) AMP/NTG group). In the CI6 group, there were more neutrophils invading in the graft, compared with the Control and CI3 groups (\(p = 0.030\) and 0.030, respectively). The number of neutrophils per HPF were significantly reduced in the CI6 \(+\) AMP/NTG group (\(p = 0.030\)) (Fig 4F).

**Expression of VEC during Cold Preservation and after Reperfusion**

The expression of VEC was evaluated by Western blot analysis (Fig 5). Relative intensity of VEC was calculated as VEC density divided by \(\beta\)-actin density. During the 6 hours of CI (Fig 5A), VEC expression was not changed. After reperfusion (Fig 5B), however, it was reduced in the CI3 group without significant statistical differences (\(p = 0.11\)) and was significantly decreased in the CI6 group compared with the Control group (\(p = 0.030\)). In the CI6 \(+\) AMP/NTG group, it was maintained after reperfusion (\(p = 0.030\)).

**Comment**

Past reports emphasized that endothelial damage during ischemia caused severe lung edema after reperfusion, focusing on the viability and the morphologic changes in endothelial cells during cold preservation [19, 20]. The molecular mechanism on the vascular endothelial junction that regulates vascular permeability has been clarified [8–10, 21]. VEC, composing the vascular endothelial adherens junction, was considered to play a pivotal role in regulating permeability [8–10]. The molecular mechanism of the stabilization, internalization, and degradation of VEC has come to be revealed [9, 22, 23].

In the field of transplantation, Trocha and colleagues [11] demonstrated that VEC expression evaluated by Western blot analysis was decreased with increased vascular permeability during 6 hours of cold preservation in an endothelial cell culture model. However, there have been no reports about VEC status in an in vivo IRI model. To our knowledge, this is the first report to investigate the VEC expression in an in vivo IRI model and its association with a detailed graft function and the severity of lung injury.
Fig 4. Naphthol AS-D chloroacetate esterase stain of the graft after reperfusion (original magnification, ×400). (A) Sham group. (B) Control group showed little neutrophil infiltration. (C) CI3 group also showed little neutrophil infiltration. (D) CI6 group showed the neutrophil infiltration increased (arrows). (E) In the CI6+AMP/NTG group, neutrophil infiltration decreased compared with the CI6 group. (F) The average number of neutrophils per HPF showed a significant increase in the CI6 group and a decrease in the CI6+AMP/NTG group. The box plot represents median, 25th and 75th percentiles, minimum, and maximum values. *p < 0.05. (CI3 = 3 hours of cold ischemia; CI6 = 6 hours of cold ischemia; db-cAMP = dibutyryl cyclic adenosine 3',5'-monophosphate; HPF = high-power field; NTG = nitroglycerin.)

Fig 5. Western blot analysis evaluating the expression of VEC. (A) During cold preservation (right donor lung), the expression of VEC was maintained. (B) After reperfusion (left donor lung), it reduced in the CI3 group without significant statistical differences and was significantly decreased in the CI6 group compared with the Control group. In the CI6+AMP/NTG group, it was maintained after reperfusion. The box plot represents median, 25th and 75th percentiles, minimum, and maximum values. *p < 0.05. (CI3 = 3 hours of cold ischemia; CI6 = 6 hours of cold ischemia; db-cAMP = dibutyryl cyclic adenosine 3',5'-monophosphate; NTG = nitroglycerin; VEC = vascular-endothelial cadherin.)
We hypothesized that the expression of VEC during CI was also reduced in vivo. In opposition to this hypothesis, VEC expression evaluated by Western blot analysis was not decreased during cold preservation up to 6 hours. After reperfusion, VEC expression seemed to be decreased in the CI3 group compared with the Control group, but it did not reach statistical significance. Only perivascular edema was recognized on histology in the CI3 group, and, interestingly, only the variables of ventilatory dynamics were substantially deteriorated in the CI3 group. Perivascular edema was reported to be sufficient to decrease compliance by the experiments that used the ex vivo circuit [24, 25]. In the CI6 group, the substantial decrease of VEC expression after reperfusion was correlated to developing intraalveolar edema with neutrophil infiltration into the graft. Adding db-cAMP and NTG to preservation solution as a provider of cAMP and cGMP contributed to maintaining VEC expression after reperfusion and the attenuation of intraalveolar edema. These results suggest that they might contribute to stabilizing VEC during cold preservation, which is supported by the reports that cAMP contributed to stabilizing VEC in vitro [26, 27]. A certain change of VEC such as destabilization could occur during cold preservation; however, internalization and degradation did not occur. It was possibly because a metabolic rate and biochemical reactions were reduced in cold preservation [2, 6]. This was not detected by the amount of VEC expression such as the Western blot analysis. Therefore, VEC expression during cold preservation might not be used as a predictor of lung injury after transplantation.

Recently, Bβ15-42, which is a VEC antagonist to preserve endothelial barrier function, has been tested by Tian and colleagues [28] to ascertain whether it could attenuate IRI in a rat lung transplantation model. Adequate effect was obtained only when it was given to both donor and recipient rats. VEC status in IRI was not investigated in that experiment. Combined with our results, therapeutic intervention for VEC should be done during cold preservation or ex vivo lung perfusion before restoring blood flow, which has recently evolved as a novel preservation method and a possible administration route for therapeutics [29, 30]. It would be worth evaluating the VEC status during normothermic ex vivo lung perfusion with maintaining active cellular metabolic functions.

Limitations were as follows. First, this was an in vivo experiment so the exact molecular mechanism regulating VEC dynamics during CI could not be evaluated. The detailed status of VEC was not measured solely by the amount of expression during cold preservation. VEC has been reported to stabilize intricately interacting with the intracellular molecules such as β-catenin [26]. It was not concluded that the decreased VEC expression and lung injury were a causal relation or coexisting phenomena in this experiment. Second, this model was subject to evaluating the effect of CI on lung injury. Clinically, the sequelae of brain death or agonal phase, which are known to establish proinflammatory state, cannot be ignored [1]. The status of VEC in the injured donor lungs might change during cold preservation; however, this model did not investigate such a situation. Third, both db-cAMP and NTG were simultaneously added to the preservation solution in this experiment. Therefore, it was not concluded that each agent had an individual or synergistic effect.

In conclusion, the expression of VEC was maintained during cold preservation; however, it was reduced early after reperfusion after 6 hours of CI in a rat lung transplantation model. Reduced VEC expression after reperfusion correlated with the severity of lung edema. Adding db-cAMP and NTG to the preservation solution alleviated the decrease in VEC after reperfusion but not during cold preservation.

References


