Pulmonary arterial reactivity during induced infection of single lung allografts

Hae Kyoong Kim1, Vinay P. Rao, Young-Sik Park2, Virginia M. Miller*, Henry D. Tazelaar, Christopher G.A. McGregor

Mayo Clinic College of Medicine, Rochester, MN 55905, United States

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Abstract

Objective: Infection is a major cause of mortality in the first year following single lung transplantation and is a distinct risk factor for the development of obliterative bronchiolitis. However, little is known about changes in pulmonary vascular activity in the setting of infection, which might affect and limit function of the graft. Therefore, the aim of this study was to determine how acute infection altered pulmonary arterial reactivity in single lung allografts. Such information could help to develop better diagnostic and therapeutic targets to improve outcome when grafts become infected. Methods: Following single lung transplantation, dogs were immunosuppressed with methylprednisolone acetate, cyclosporine and azathioprine. On postoperative day 5, infection was induced in one group of dogs by endobronchial inoculation of antibiotic resistant Eschericia coli (infection group, n = 5); in the second group, the same amount of culture media without bacteria was flushed into the bronchus (control group, n = 4). All animals were medicated under the same drug protocol. On postoperative day 8, lungs were removed, reviewed for histological assessment, pulmonary arteries were isolated, cut into rings and suspended for pharmacological characterization in organ chambers. Results: With acute infections, contractions to phenylephrine and angiotensin-1, but not endothelin-1, were reduced in pulmonary arteries with but not without endothelium. Inhibition of nitric oxide synthase with Nω-monomethyl-L-arginine, monoacetate salt (L-NMMA) restored these contractions. Endothelium-dependent relaxations to adenosine diphosphate and calcium ionophore, which stimulate release of endothelium-derived nitric oxide by a receptor and non-receptor mediated process, respectively, were not different between groups. Relaxations to nitric oxide also were not different between groups. Conclusion: These results suggest that acute infection selectively reduces contractions of pulmonary arteries in part through receptor-mediated release of nitric oxide from the endothelium. Inhibiting nitric oxide during episodes of acute infection may help to improve graft perfusion during episodes of acute infection.

Keywords: Endothelium-dependent relaxation; Histamine; Nitric oxide; Rejection

1. Introduction

Lung transplantation is a recognized therapeutic option for end-stage lung disease. Although a marginal improvement in 1-year survival has been noted over the years, infection and chronic rejection limit 5-year survival to approximately 45% [1]. Infections and primary graft failure are the major causes of mortality in the first year following transplantation [2], while obliterative bronchiolitis remains the major cause of morbidity and mortality in long-term survivors [2,3]. Episodes of infection increase mortality [4] and adversely affect the risk of this pathology following transplantation. Although precise mechanisms are unknown, infection could adversely affect the allograft through several mechanisms. IL-1 and TNF-α produced by macrophages infiltrating the alveoli through the activation of NF-κB reduce cell survival in infected lung allografts [5]. Circulating IL-1 and TNF-α upregulate expression of vascular adhesion molecules ICAM-1 and E-selectin, thereby promoting recruitment of neutrophils eventually leading to alveolar damage [6]. In addition, endotoxemia, resulting from gram-negative infections, causes a state of altered endothelial and vascular smooth muscle reactivity to circulating vasoactive substances that could decrease end-organ perfusion and cause irreversible graft damage [7]. Limited availability of donor organs implies a very low rate of retransplantation and, therefore, almost certain death for
those with failing grafts. Studies have shown preferential perfusion of the transplanted lung in single lung transplants reflecting an imbalance in vascular resistance associated with transplantation [8,9]. However, perfusion scintigraphy of the transplanted lung has revealed an association between relative hypoperfusion of the graft and episodes of both acute and chronic rejection [8,10]. These studies emphasize the importance of understanding mechanisms influencing graft perfusion during adverse events which affect viability of the perfused organ. This information would enable the design of effective therapeutic strategies to ensure long-term survival of patients with lung allografts. This study was designed to examine effects of infection on vascular reactivity in a large animal model of single lung transplantation. It was hypothesized that acute infection would reduce vascular reactivity in part through endothelium-dependent mechanisms.

2. Materials and methods

Male mongrel dogs of similar size and weight (20–25 kg) were used as donors and recipients. The dogs underwent single lung allotransplantation as previously described [11]. All dogs received intravenous heparin (1000 u) every 6 h for the first 24 h. Butrophanol tartrate (TorbogegicÂ®) 10 mg every 4–6 h was administered for analgesia during the first 24 h. Immunosuppression consisting of cyclosporine (SandimmuneÂ®; 10 mg/kg/day) and azathioprine (ImmuranÂ®; 2.5 mg/kg/day) was given every 12 h for 24 h, with the first dose given at the time of allograft reperfusion. Antibiotic therapy consisting of gentamycin sulfate 40 mg i.v. q12h, clindamycin phosphate (CleocinÂ®) 300 mg i.v. q12h, cefazolin sodium (CefazolinÂ®) 250 mg i.v. q12h and was administered to all animals for 5 days.

This protocol was approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and Foundation as being in compliance with the Animal Welfare Act of the United States.

2.1. Bronchial lavage and induction of allograft infection

Preparation of bacterial cultures and endobronchial inoculation of infection have been described previously [12]. Briefly, on postoperative day 5, animals were intubated and ventilated with 1.5–2.5% Halothane in oxygen. Chest radiography was performed to exclude pulmonary opacification. Using sterile technique, a tracheostomy was performed in randomly selected rings, the endothelium was removed by gentle abrasion using a pair of forceps [13]. Rings were suspended by two stainless steel wires inserted into the lumen of the ring between a fixed point and a force transducer (Hewlett Packard 7418A recorder) for the measurement of isometric force. Rings were equilibrated at a passive tension of less than 0.5 g for 30 min. Each ring was stretched progressively to the optimal point of its length-tension curve as determined by the tension developed to potassium chloride (20 mM/l) at each level of stretch. Organ chambers were then washed with control solution and rings were equilibrated until tension had returned to basal levels. Maximal contraction to potassium chloride (60 mmoles/l) was measured. This was followed by another equilibration period of 30 min in control solution. Cumulative concentration–response curves were obtained to phenylephrine (10−8 to 10−4 moles/l), an adrenergic agonist, angiotensin I (10−10 to 10−8 moles/l) to reflect activity of angiotensin converting enzyme, and endothelin-1, an endothelium-derivative contractile factor (10−11 to 10−7 moles/l). To study relaxations, cumulative concentration–response curves were obtained to adenosine diphosphate (ADP, 10−8 to 10−4 moles/l) and histamine (10−8 to 10−4 moles/l), substances released by platelets and leukocytes, respectively, in rings contracted with phenylephrine (10−8 moles/l). Responses to calcium ionophore A23187
(10^{-9} \text{ to } 10^{-6} \text{ moles/l}) \) and nitric oxide (10^{-8.5} \text{ to } 10^{-5} \text{ moles/l}) were obtained in rings with and without endothelium, respectively. Responses to all drugs were obtained in the absence and presence of \text{N}^\text{G}-\text{monomethyl}-\text{L-arginine}, monoacetate salt (L-\text{NMMA}, 10^{-4} \text{ moles/l}). Experiments in the absence and presence of inhibitors were conducted in parallel.

### 2.3. Histology

After the pulmonary arteries were removed from the lower lobes, the remainder of the heart—lung block was perfused with 10% buffered formalin via the bronchus and stored in 10% formalin. Tissues were fixed in formalin at least 24 h before sections were taken. Nine representative slices were obtained from transplanted and native lungs for histological evaluation. Hematoxylin and eosin staining was used to assess the severity of acute rejection from Grade 0 (no rejection) to Grade IV (severe rejection) according to the International Society for Heart and Lung Transplantation Working Formulation.

### 2.4. Infection grade

Infection was graded as described previously [12]. Briefly, a scale was developed based on the severity of neutrophil infiltration (<5 neutrophils/alveolus = 1, <5–10 neutrophils/alveolus = 2, <11–20 neutrophils/alveolus = 3, >20 neutrophils/alveolus = 4) and extent of the involved alveoli (<30 alveoli/high power field = 1, 30–50 alveoli/high power field = 2, >50 alveoli/high power field = 3). Total scores were derived by multiplying the severity \times number of involved alveoli. All scoring was performed by a pathologist blinded to the origin of the tissue.

### 2.5. Drugs and chemicals

The following drugs were used: Phenylephrine (Sigma Chemical Co., St. Louis, MO), angiotensin I (Sigma), endothelin-1 (Peptide Institute Inc., Osaka, Japan), adenosine triphosphate (Sigma), histamine (Sigma), A23187 (Sigma), \text{N}^\text{G}-\text{Monomethyl}-\text{L-arginine}, Monoacetate Salt (Calbiochem-Novabiochem Co., La Jolla, CA). Unless specified, drugs were prepared daily in distilled water. A23187 was dissolved in dimethyl sulfoxide (Sigma; final bath concentration, 8.2 \times 10^{-3} \text{ moles/l}). The dimethyl sulfoxide in the concentration used did not affect responses of tissues. Nitric oxide from a cylinder (Metheson gas products, Texas) was used to fill a glass bulb fitted with a silicone injection septum. A volume of gas was removed with a glass syringe and injected into another glass bulb that had been filled with 100 ml of distilled water (bubbled with helium for approximately 2 h) to give a stock solutions of nitric oxide (4 \times 10^{-5} \text{ moles/l}, 4 \times 10^{-4} \text{ moles/l}, 4 \times 10^{-3} \text{ moles/l}) [13]. All concentrations were expressed as the final molar (moles/l) concentration in the organ bath or incubation solution.

### 2.6. Statistical analysis

Results are expressed as means \pm standard error of mean. In all experiments, \( n \) equals the number of rings, each taken from different animals. Since rings with and without endothelium of the same blood vessel were studied in parallel in the presence and absence of inhibitors, Student’s \( t \)-test (two-tailed) for paired observations was used to compare areas under the concentration—response curves of rings within the same treatment group (control or infected) in the presence or absence of inhibitors. Student’s \( t \)-test for unpaired observations was used to compare areas under response curves or where appropriate, the effective concentration of causing 50% of the maximal response (EC_{50}) was calculated for individual concentration—response curves and means of these values reported as the negative logarithm of the molar concentration were compared. Values were considered to be statistically different when \( p < 0.05 \).

### 3. Results

#### 3.1. Histology

Rejection grade ranged from 0 to 1.5 in both groups. No infection was observed in transplanted lungs of animals in the control group and an infection grade of 12 was observed in all animals of the infected group.

On day 5, cell counts in the bronchial lavage were comparable between native lungs of both groups. However, leukocyte count was higher in the fluid from the transplanted lungs of the control group and an infection grade of 12 was observed in all animals of the infected group.

On day 8, BAL fluid monocytes/macrophages predominated in lavage from transplanted lungs of the control group, while neutrophils predominated in lavage of the transplanted lungs of the infected group.

#### 3.2. Organ chamber studies

##### 3.2.1. Contractions

Potassium chloride caused comparable contractions in pulmonary arteries of both groups. Contractions averaged 4.3 \pm 1.2 and 3.4 \pm 0.7 g in rings with and 4.0 \pm 0.2 and 4.3 \pm 1.2 g in rings without endothelium in transplanted lungs of the control and infected groups, respectively.

Phenylephrine (Fig. 2) and angiotensin-1 (Fig. 3) caused concentration-dependent contractions of rings with and without endothelium from transplanted lungs in each group. With infection, contractions of rings with but not without endothelium were reduced in infected compared with control animals. When rings with endothelium were

### Table 1

<table>
<thead>
<tr>
<th>Day 5</th>
<th>Day 8</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>Infected</td>
</tr>
<tr>
<td>Native lung</td>
<td>500 ± 161</td>
</tr>
<tr>
<td>Transplanted lung</td>
<td>367 ± 67</td>
</tr>
<tr>
<td>Native lung</td>
<td>391 ± 231</td>
</tr>
<tr>
<td>Transplanted lung</td>
<td>900 ± 333*</td>
</tr>
</tbody>
</table>

(* Denotes statistically significant difference from control groups at day 8; (+) Denotes statistically significant difference from day 5
incubated with L-NMMA, contractions of rings with endothelium to both drugs from infected dogs were comparable with those of control animals.

Endothelin-1 caused concentration-dependent comparable contractions in rings with and without endothelium from transplanted lungs of both groups. There were no statistically significant differences ($p = 0.07$) between groups in contractions in rings with or without endothelium or in rings with endothelium in the presence of L-NMMA (10$^{-4}$ moles/l) (Fig. 4).

### 3.3. Relaxations

#### 3.3.1. Endothelium-dependent relaxations

During contractions to phenylephrine, ADP caused greater relaxation in rings with compared with without endothelium in all arteries (not shown). These relaxations were not different between arteries from control and infected animals (Fig. 5; left panel). In rings contracted with phenylephrine, the calcium ionophore, A23187 also caused concentration-dependent decreases in tension which also were not different between transplanted lungs from control or infected groups (Fig. 5; middle panel).

#### 3.3.2. Endothelium-independent relaxations

Relaxations caused by exogenously applied nitric oxide were comparable in rings without endothelium from both groups of animals (Fig. 5; right panel). Histamine caused comparable relaxations of rings with and without endothelium in each group. These relaxations tended to be greater in arteries of transplanted lungs from

![Fig. 2. Concentration-dependent contractions to phenylephrine in pulmonary arterial rings from transplanted lungs of infected (n = 8) and control (n = 4) animals. Data are expressed as grams increase in tension and are shown as means ± standard error of the mean. Responses were determined in arterial rings with endothelium (left panel), without endothelium (middle panel) and in rings with endothelium in the presence of the L-NMMA (10$^{-4}$ moles/l; right panel). Maximal tensions were significantly reduced ($p < 0.05$) in rings with endothelium from infected animals; this difference was absent in the presence of L-NMMA and in rings devoid of endothelium.](image)

![Fig. 3. Concentration-dependent contractions to angiotensin-1 in pulmonary arterial rings from transplanted infected (n = 5) and control (n = 4) animals. Data are expressed as grams increase in tension and are shown as means ± standard error of the mean. Responses were determined in arterial rings with endothelium (left panel), without endothelium (middle panel) and in rings with endothelium in the presence of the L-NMMA (10$^{-4}$ moles/l; right panel). Maximal tensions were reduced significantly ($p < 0.05$) in rings with endothelium from infected animals; this difference was absent in the presence of L-NMMA and in rings devoid of endothelium.](image)
dogs with infection compared with those from the control group. L-NMMA did not significantly affect relaxations to histamine in rings without endothelium but reduced the variability in responses of arteries from control animals such that differences between groups reached statistical significance (Fig. 6).

4. Discussion

Results of this study indicate that infection reduces contraction of pulmonary arteries in transplanted lungs and that this attenuation is mediated by endothelium-derived nitric oxide and increased sensitivity of the smooth muscle to histamine. It is unclear at this time, if induction of nitric oxide in response to infection is due to increased receptor activation of the constituitive form of nitric oxide synthase (eNOS or NOS 2) or the inducible form of NOS (iNOS or NOS I). Neither is it clear if the endothelium is activated directly by the bacteria or indirectly from cytokines released from infection-activated leukocytes [14]. Most likely, both processes are involved, as both endothelium and macrophages express receptors of the toll family which are stimulated, in part, by bacterial-derived lipopolysaccharide [15—17]. Macrophage and infection-induced associated cytokines IL-1β, IL-6 and TNF-α induce NO and stimulate ET-1 from the vascular endothelium [18].

Infection seemed to differentially affect receptor-operated contractile responses. This conclusion is supported by the observation that contractions to phenylephrine and angiotensin-1 are reduced but contractions to KCl, which directly depolarizes vascular smooth muscle, were not altered by infection. It is unlikely that reduction in contractions to phenylephrine were due to reduction in
the number of α1-receptors as occurs in sepsis [19] as contractions were restored by inhibition of nitric oxide. Likewise it is unlikely that reduced contractions to angiotensin-1 are due to decreases in angiotensin converting enzyme (ACE) as in sepsis [20] as these contractions also were restored in the presence of L-NMMA.

Vascular endothelial responses are altered in non-infected denervated lungs as described in earlier work on single lung transplanted dogs [11]. Chronic denervation increases sensitivity to contractile agents most likely due to a decrease in receptor-mediated release of endothelium-derived relaxing factors and an increase in endothelium-derived contractile factors. An enhanced contractile response to norepinephrine in rings with endothelium and denervation alone may contribute to increases in perfusion pressure in the transplanted lung compared with the native lung in clinical studies [8,9]. Maximal contractions to endothelin-1, unlike contractions to norepinephrine, were reduced by denervation [11]. As responses to the alpha-adrenergic agonist (phenylephrine) in the present study were unlike those observed with denervation, this suggests that infection and not transplantation contributed to changes in reactivity. Also, as contractions to KCl did not differ between groups it suggests that responses of the smooth muscle to direct depolarization were not affected by infection.

Sensitivity of the smooth muscle to histamine was augmented in arteries from transplanted lungs of infected dogs. Histamine can stimulate a family of receptors which regulate cyclic adenosine monophosphate (cAMP) and cAMP-derived kinases [21,22]. Release of histamine by macrophages in the presence of infection could have caused an up-regulation of receptors leading to an augmented response of arterial rings in vitro. cAMP mechanisms were affected more than cGMP-associated mechanisms as relaxations to A23187, which releases NO from the endothelium or exogenous NO were not altered. Decreases in variability in rings without endothelium from control animals to histamines in the presence of L-NMMA may represent inhibition of inducible NOS by low-grade rejection in this group.

5. Conclusion

This study began to differentiate mechanisms by which infection, a known risk factor for lung graft failure, might affect graft vasoreactivity apart from acute rejection. Results show that infection selectively alters reactivity of pulmonary arteries to contractions induced by drugs of adrenergic nerve terminals (phenylephrine), water balance (angiotensin I), and leukocytes (histamine) (Table 2).

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Mechanism</th>
<th>Endothelium</th>
<th>Infection</th>
<th>L-NMMA</th>
</tr>
</thead>
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<td>Phenylephrine</td>
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<td>−</td>
<td>Not tested</td>
</tr>
<tr>
<td>Angiotensin-1</td>
<td>Receptor-mediated</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>Receptor-mediated</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ADP</td>
<td>Receptor-mediated</td>
<td>+</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
<td>A23187</td>
<td>Non-receptor-mediated</td>
<td>+</td>
<td>−</td>
<td>Not tested</td>
</tr>
<tr>
<td>NO</td>
<td>Non-receptor-mediated</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Histamine</td>
<td>Receptor-mediated</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

(∗) Differences in relaxations did not reach statistical significance from control animals; Symbols: +, with endothelium; −, without endothelium; NA, no affect; |, decreased response compared with that observed in arteries from control animals; ∗, increased response compared with that observed in the absence of L-NMMA.
Responses to acute infection were distinct from those described for sepsis and seem to affect ADP and receptor activated release of endothelium-derived nitric oxide and relaxations associated with cAMP compared with those activating cGMP [23]. Denervation following single lung transplantation is known to affect pulmonary vasoreactivity. However, changes in responses of arteries with infection were distinct from those of allografted denervated lungs. Direct comparison with responses with both infection and rejection need to be made to develop targeted diagnostic and therapeutic interventions to differentiate the two phenomena. As leukocyte counts were also elevated in native, non-transplanted lungs of the infected animals, arterial reactivity of the native lung may also be involved. Altered pulmonary vascular reactivity in response to repeated infection episodes could potentially affect post-transplantation vascular remodeling and lead to graft failure in the long-term. Therapeutic maneuvers to inhibit NO and increase pulmonary arterial tone may help to improve graft survival in the presence of acute infection.

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References