LUNG TRANSPLANT REPERFUSION INJURY INVOLVES PULMONARY MACROPHAGES AND CIRCULATING LEUKOCYTES IN A BIPHASIC RESPONSE

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Objective: Both donor pulmonary macrophages and recipient circulating leukocytes may be involved in reperfusion injury after lung transplantation. By using the macrophage inhibitor gadolinium chloride and leukocyte filters, we attempted to identify the roles of these two populations of cells in lung transplant reperfusion injury.

Methods: With our isolated, ventilated, blood-perfused rabbit lung model, all groups underwent lung harvest followed by 18-hour cold storage and 2-hour blood reperfusion. Measurements of pulmonary artery pressure, lung compliance, and arterial oxygenation were obtained. Group I (n = 8) served as a control. Group II (n = 8) received gadolinium chloride at 14 mg/kg 24 hours before lung harvest. Group III (n = 8) received leukocyte-depleted blood reperfusion by means of a leukocyte filter.

Results: The gadolinium chloride group had significantly improved arterial oxygenation and pulmonary artery pressure measurements compared with control subjects and an improved arterial oxygenation compared with the filter group after 30 minutes of reperfusion. After 120 minutes of reperfusion, however, the filter group had significantly improved arterial oxygenation and pulmonary artery pressure measurements compared with the control group and an improved arterial oxygenation compared with the gadolinium chloride group.

Conclusions: Lung transplant reperfusion injury occurs in two phases. The early phase is mediated by donor pulmonary macrophages and is followed by a late injury induced by recipient circulating leukocytes. (*J Thorac Cardiovasc Surg* 2001;121:1069-75)

Despite considerable advancements in organ preservation techniques and solutions, transplanted lungs remain vulnerable to reperfusion injury, with severe graft dysfunction occurring in 20% of lung transplant recipients.¹⁻⁵ Severe graft dysfunction after lung transplantation can be reversible; however, it is often associ-

ated with the need for prolonged intensive care and increased mortality.⁶⁻⁸ Although ischemia is clearly involved, it has become increasingly evident that reperfusion is responsible for the majority of injury after lung transplantation.⁹ In addition, studies have confirmed that the lung injury is a result of up-regulation of inflammatory mediators after reperfusion.^{10,11}

Neutrophils have long been recognized as critical components of the inflammatory cascade, but their role in the pathophysiology of lung reperfusion injury has been a source of controversy. Evidence that neutrophils play an important role in lung reperfusion injury has been shown in recent investigations using leukocyte depletion and in studies with monoclonal antibodies directed against adhesion molecules on leukocytes and endothelial cells.^{2,9-14} In contrast, some investigators have demonstrated that significant reperfusion injury can occur without neutrophil participation and that neutrophils may have no effect at all in some models of lung reperfusion injury.^{15,16}

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Fig 1. Diagram of the isolated, blood-perfused rabbit lung model. P, Pressure transducer; N₂, nitrogen gas; O₂, oxygen gas.

Recent work with cytokine antibodies suggests that pulmonary macrophages in donor lungs may have a role in reperfusion injury.¹¹ Macrophages could potentially initiate reperfusion injury with further escalation induced by circulating leukocytes. Gadolinium chloride (GdCl₃), a rare lanthanide earth salt, inactivates pulmonary macrophages by suppressing phagocytic, immune, and inflammatory responses.¹⁷ This compound has been used in recent studies to inhibit alveolar macrophages.^{18,19} The goal of this study was to discriminate between the roles of pulmonary macrophages and circulating leukocytes in a lung model of transplant reperfusion injury by using cell-specific inhibition with GdCl₃ and leukocyte filtration, respectively.

Materials and methods

Experimental protocol. Three experimental groups were compared in an isolated, blood-perfused, ventilated rabbit lung model to study lung transplant reperfusion injury. Group I lungs (control group, n = 8) were harvested and then stored for 18 hours at 4°C before reperfusion with whole blood. Group II rabbits (GdCl₃ group, n = 8) were given intravenous GdCl₃ (14 mg/kg; Sigma Corporation, St Louis, Mo) 24 hours before harvest, followed by lung storage for 18 hours at 4°C and reperfusion with whole blood. Group III lungs (filter group, n = 8) underwent 18-hour storage at 4°C followed by reperfusion with whole blood that was first passed through a leukocyte-depleting filter (Pall Purcell RCG, East Hills, NY).

Harvest procedure. Adult New Zealand White rabbits of both sexes weighing 3.0 to 3.5 kg were randomly assigned to the 3 experimental groups. Animals were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg). A tracheostomy was performed, and mechanical ventilation was instituted with a constant pressure ventilator (RSP1002; Kent Scientific Corporation, Litchfield, Conn) by using room air and a rate of 20 breaths/min. A median sternotomy and thymectomy were then performed. The 2 superior and 1 infe-

rior venae cavae were loosely encircled with ligatures, and the pericardium was opened. Both the pulmonary artery (PA) and aorta were dissected free and similarly encircled. A purse-string suture was placed in the free wall of the right ventricle (RV), and intravenous heparin was administered (500 units/kg). After injection of 30 µg of alprostadil (prostaglandin E₁; Upjohn Company, Kalamazoo, Mich) into the PA, the cavae were interrupted and onset of ischemia was noted. The PA was then cannulated through a right ventriculotomy placed in the center of the purse-string suture. Both the RV and PA ligatures were tied to secure the cannula. After venting of the left ventricle (LV) with a left ventriculotomy and ligation of the aorta, 50 mL/kg of Euro-Collins (Hamburg, Germany) preservation solution at 4°C was infused into the PA from a height of 30 cm. Topical cooling was achieved with cold saline solution slush. During the PA flush, the left atrium was cannulated through the left ventriculotomy with an outflow catheter and a catheter to directly transduce left atrial pressures. A purse-string suture was placed to secure these cannulas. After completion of the PA flush, the inflow and outflow cannulas were clamped. The heart-lung block was excised, and the tracheostomy tube was clamped at end-inspiration. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals," published by the National Institutes of Health (NIH publication 85-23, revised 1996).

Reperfusion procedure. After organ harvest and ischemic storage, the heart-lung block was suspended in a warm, humidified tissue chamber and ventilation was re-established with a 95% oxygen and 5% carbon dioxide gas mixture at a respiratory rate of 20 breaths/min by using the constant pressure ventilator (Fig 1). The inflow and outflow cannulas were then connected to a venous blood reperfusion circuit. New Zealand White rabbits weighing 3.5 to 5.0 kg served as fresh venous blood donors. The lungs were reperfused with venous blood from a main reservoir. A second nonrecirculated venous blood reservoir was used to challenge the lungs and determine the single pass oxygenation values during reperfusion. In the experimental group designated to undergo leuko-

Fig 2. The GdCl₃ group had a significantly improved Po₂ after 30 minutes of reperfusion compared with control and filter groups. However, after 120 minutes of reperfusion, the filter group had a significantly higher Po₂ compared with the GdCl₃ and control groups. **P* < .001 GdCl₃ vs all; ***P* < .001 Filter vs all.

Fig 3. The control group had a significantly higher PVR compared with the GdCl₃ and filter groups after 30 and 120 minutes of reperfusion. There was a trend toward a lower PVR in the GdCl₃ group compared with the filter group after 30 minutes of reperfusion (*P* = .17). **P* < .001 Control vs all; ***P* < .001 Control vs all.

cyte-depleted reperfusion, venous blood was passed through a leukocyte-depleting filter (Pall Purcell RCQ) before being added to both blood reservoirs of the circuit. The circuit (Kent Scientific Corporation) was designed to recirculate 150 mL of warmed blood by using a roller pump (7521-40; Cole Palmer Instrument Company, Chicago, Ill) at 60 mL/min.

Continuous recordings of pulmonary artery pressure (PAP), left atrial pressure, and air flow were performed with a dynamic data acquisition program (Workbench PC; Strawberry Tree, Inc, Sunnydale, Calif) on a personal computer (470A; Compaq Prolinea, Houston, Tex). This program automatically calculated and displayed tidal volume, pulmonary vascular resistance (PVR), and dynamic airway compliance. Pulmonary venous blood samples were collected for blood gas analysis (Corning 178pH/Blood Gas Analyzer; Corning Inc, Corning NY) after 10, 20, 30, 60, and 120 minutes of reperfusion. At each sampling interval, inflow from the main reservoir was temporarily interrupted and the circuit

was filled with nonrecirculated blood from the second inflow reservoir. A 30-mL sample of venous blood was passed through the pulmonary vasculature at each interval to obtain accurate measurements of pulmonary venous oxygen content. Oxygen contact with exposed blood surfaces was minimized by continuous passive infusion of 100% nitrogen.

Lung tissue myeloperoxidase. A myeloperoxidase assay (MPO) was performed to quantify neutrophil sequestration. Lung tissue was placed in 5 mL of 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mmol/L potassium phosphate solution (pH 7.4) and disrupted by homogenizing at 4°C. The solution was centrifuged at 15,000g for 15 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in 2 mL of 0.5% HTAB in 50 mmol/L potassium phosphate solution (pH 6.0) and homogenized. Tissue was disrupted further by sonication and 3 freeze-thaw cycles (liquid nitrogen bath/37°C water bath). The solution was again centrifuged at 15,000g for 15 minutes at 4°C. Aliquots

Fig 4. Light micrograph of lung tissue from the control group that shows severe leukocyte infiltration and edema formation in the alveolar spaces (hematoxylin-eosin stain; original magnification $\times 40$).

Fig 5. Light micrograph of lung tissue from the GdCl₃ group that shows decreased leukocyte infiltration and edema formation in the alveolar spaces compared with the control group (hematoxylin-eosin stain; original magnification $\times 40$).

(0.1 mL) of supernatant were added to the assay buffer of *O*-dianisidine dihydrochloride, H₂O₂, and 50 mmol/L potassium phosphate (pH 6.0). Absorbance at 460 nm was measured during a period of 2 minutes by spectrophotometry (LKB model 4050, Cambridge, United Kingdom). Protein concentration for each of the lung samples was measured by using the BCA protein assay kit from Pierce (Rockford, Ill). Protein concentrations were calculated by comparing the absorbance at 595 nm of the experimental samples with that of known bovine serum albumin standard concentrations in the same assay. Lung tissue MPO activity was expressed as change in absorbance per gram of protein per minute.

Lung wet/dry weight ratios. Lung wet/dry weight ratios were used as a measurement of pulmonary edema. Samples of lung tissue were weighed immediately after reperfusion. These samples then underwent passive desiccation at room temperature until a stable dry weight was achieved. The weight immediately after reperfusion and the stable dry weight were then used to calculate the lung wet/dry weight ratios.

Histopathology. Samples of lung tissue underwent fixation with a 10% buffered formalin phosphate solution followed by hematoxylin-eosin staining.

Statistical analysis. Statistical analysis was performed with analysis of variance (ANOVA) on SPSS software (SPSS Inc, Chicago, Ill). In addition, a repeated-measures ANOVA was performed for data with multiple time points. Significant differences were determined with the Bonferroni multiple comparison test. Data are expressed as the mean $\pm$ the standard deviation.

Results

Hematologic data. White blood cell (WBC) count of the blood reperfusate before leukocyte filtration averaged 3400/ μ L. Leukocyte filtration substantially reduced the WBC count of the blood reperfusate to 120/ μ L. The mean hematocrit value remained relatively constant, with an average of 39% before filtration and 35% after filtration. However, the average platelet count of the blood reperfusate dropped from 112,000/ μ L to less than 10,000/ μ L after leukocyte filtration. Donor rabbits in the GdCl₃ group were not noted to have any adverse events within the first 24 hours after drug treatment.

Physiologic parameters

Arterial oxygenation. Arterial Po₂ was significantly higher in the GdCl₃ group (357.13 ± 126.87 mm Hg, $P < .001$, Fig 2), which received the macrophage inhibitor GdCl₃, compared with the control (75.25 ± 21.34 mm Hg) and filter (leukocyte filter, 76.25 ± 20.32 mm Hg) groups after the initial 30-minute reperfusion period. Conversely, during the late phase (2 hours) of reperfusion, arterial Po₂ was significantly higher in the filter group (222.63 ± 43.87 mm Hg, $P < .001$) than in the control (96.38 ± 42.76 mm Hg) and GdCl₃ (100.88 ± 38.80 mm Hg) groups.

PVR. Mean PVR was significantly lower in the GdCl₃ ($32,262 \pm 12,022$ dynes $\cdot$ sec $\cdot$ cm⁻⁵, Fig 3) and

filter ($41,558 \pm 5847$ dynes $\cdot$ sec $\cdot$ cm⁻⁵) groups compared with control group ($50,185 \pm 1949$ dynes $\cdot$ sec $\cdot$ cm⁻⁵, $P = .001$) at 30 minutes of reperfusion. There was also a trend toward a lower PVR in the GdCl₃ group compared with the filter group after 30 minutes of reperfusion ($P = .17$). After 120 minutes of reperfusion, PAP was significantly lower in the GdCl₃ ($38,387 \pm 13,205$ dynes $\cdot$ sec $\cdot$ cm⁻⁵) and filter ($33,176 \pm 5892$ dynes $\cdot$ sec $\cdot$ cm⁻⁵) groups compared with the control group ($53,025 \pm 3532$ dynes $\cdot$ sec $\cdot$ cm⁻⁵, $P < .001$). Although the average PVR was lower in the filter group compared with the GdCl₃ group after 120 minutes of reperfusion, the difference did not reach statistical significance.

Dynamic airway compliance. There was a trend toward improved airway compliance in the filter group (1.20 ± 0.17 mL/mm Hg) compared with the control group (1.08 ± 0.08 mL/mm Hg, $P = .23$) after 120 minutes of reperfusion. There were no significant differences in airway compliance in the GdCl₃ group (1.13 ± 0.08 mL/mm Hg) compared with the filter and control groups.

Lung wet/dry weight ratio. The mean wet/dry weight ratio of the filter group (6.41 ± 0.91 , $P = .013$) was significantly lower than the wet/dry weight ratios of the control (7.84 ± 0.76) and GdCl₃ (7.50 ± 1.05) groups. There were no significant differences in wet/dry weight ratios between the GdCl₃ and control groups.

Tissue MPO activity. There was a trend toward lower MPO activity in the filter group (1078 ± 297 Δ absorbance/g protein/min) than in the control group (1512 ± 458 Δ absorbance/g protein/min, $P = .16$). MPO activity for the GdCl₃ group (1188 ± 501 Δ absorbance/g protein/min) was not significantly different from MPO activity in control and filter groups. In addition, in a preliminary experiment, control lungs displayed a significant rise in tissue MPO activity between 30 minutes and 120 minutes of whole blood reperfusion ($P = .05$).

Histopathology. Control lungs (Fig 4) showed more severe leukocyte infiltration and edema formation in alveolar spaces after 2 hours of reperfusion compared with the GdCl₃ (Fig 5) and filter (Fig 6) groups.

Comment

During the past few years many of the cellular and molecular events mediating the inflammatory response to reperfusion injury have been elucidated.^{2,20,21} One of the critical steps responsible for reperfusion injury is believed to be the interaction between WBCs and the vascular endothelium during reperfusion. Neutrophil participation in at least one

Fig 6. Light micrograph of lung tissue from the filter group that shows decreased leukocyte infiltration and edema formation in the alveolar spaces compared with the control group (hematoxylin-eosin stain; original magnification $\times 40$).

phase of reperfusion injury is established in the current study and in work by others using various models of lung reperfusion injury.¹⁰⁻¹⁴ Neutrophils may contribute to lung injury during reperfusion in several ways. One of the primary mechanisms by which neutrophils cause injury is through the release of toxic oxygen metabolites, such as superoxide anion, hydroxyl radical, and hydrogen peroxide, that can damage pulmonary endothelium directly or indirectly.² Elastase and other proteases that are products of neutrophil granules can also directly injure pulmonary endothelial and parenchymal cells. Finally, a third method by which neutrophils can cause or contribute to reperfusion injury is capillary plugging (no-flow phenomenon). Activated neutrophils become less deformable and may then be permanently trapped in alveolar capillaries, contributing to poor reflow during reperfusion.^{21,22}

Although many investigations in addition to the current study have confirmed the role of neutrophils in reperfusion injury, some have questioned neutrophil involvement. Deeb and colleagues¹⁶ demonstrated early on that neutrophils are not necessary to induce reperfusion injury in an ex vivo rat lung preparation with isolated blood cell components. Their study reported attenuation of injury with the addition of red

blood cells or catalase. They concluded that a non-neutrophil source of oxygen metabolites, such as lung macrophages, was responsible for the observed injury. Steimle and colleagues¹⁵ also demonstrated neutrophil-independent reperfusion injury by using neutrophil antibodies in an *in vivo* rat lung model at 90 minutes of reperfusion. Their study showed no accumulation of neutrophils in the damaged lungs of the non-neutrophil-depleted rats when compared with the injured lungs of the neutrophil-depleted rats on the basis of histologic and electron microscopic findings. Eppinger and colleagues¹⁰ performed a similar *in vivo* study and found that neutrophil depletion had no protective effect after 30 minutes of reperfusion but did attenuate injury after 4 hours.

A recent investigation by Eppinger and colleagues¹¹ strengthens the likelihood that lung macrophages are involved in the early phase of lung reperfusion injury. In their study the chemical mediators of reperfusion injury in the rat lung were characterized. Tumor necrosis factor- α (TNF- α), interferon- γ (INF- γ), and monocyte chemoattractant protein-1 (MCP-1) were shown to be required for early injury by using cytokine specific antibodies. One possible mechanism for the decreased injury with anti-TNF- α and anti-INF- γ is through suppression of macrophage function. Both TNF- α and INF- γ are known to be important factors in the respiratory burst activity and other inflammatory functions of macrophages. They also found that anti-MCP-1, which is an antibody against a highly specific macrophage activator and has no activity on neutrophils, dramatically decreased the early phase of injury. They concluded that early lung injury is in large part determined by products of activated macrophages, whereas delayed injury is mediated mostly by products of activated and recruited neutrophils.

The current study supports this bimodal concept of reperfusion injury, consisting of both early and late phases. At 30 minutes of reperfusion, macrophage inhibition with GdCl₃ significantly attenuates the poor oxygenation and elevated PAP measurements observed in control lungs. This effect is substantially decreased, however, after 120 minutes of reperfusion. For the filter group, leukocyte depletion showed no improvement in arterial oxygenation compared with the control group during the early phase (30 minutes) of reperfusion injury. Significant improvement in oxygenation, however, was observed in the late phase (2 hours) of reperfusion. Tissue MPO activity tended to be lower in the filter group than in the control group, a finding consistent with depletion of circulating WBCs. If this experiment had been performed for a longer time peri-

od, the control group likely would have had a further increase in neutrophil sequestration, resulting in a statistically significant difference in MPO activity between the control group and the filter group.

In a separate study combining GdCl₃ and leukocyte filtration, arterial oxygenation and PVR measurements were similar to the GdCl₃ group after 30 minutes of reperfusion (248.71 ± 46.16 mm Hg and $31,082 \pm 5434$ dynes $\cdot$ sec $\cdot$ cm⁻⁵, respectively). Similarly, the combined GdCl₃ and leukocyte filtration group had arterial oxygenation and PVR measurements that were similar to the filter group after 120 minutes of reperfusion (320.83 ± 43.70 mm Hg and $39,934 \pm 8609$ dynes $\cdot$ sec $\cdot$ cm⁻⁵, respectively). In addition, our studies on MPO in control lungs at 30 and 120 minutes showed significantly increased MPO activity after 2 hours of reperfusion compared with 30 minutes. This finding further suggests neutrophil involvement in reperfusion injury occurs during the late phase of reperfusion.

Most of the research in the area of lung reperfusion injury has focused on the recipient inflammatory system. However, activation of donor, resident alveolar macrophages could be the initiating factor in lung damage. This model would suggest that macrophages in donor lungs are activated early on by preservation and reperfusion. These cells subsequently release cytokines, chemoattractants, and proteolytic enzymes that induce an early reperfusion injury. This early damage is then followed by a cascade of events leading to activation of the recipient inflammatory system against the already damaged lung tissue.^{2,11} This model of lung reperfusion injury helps explain the early, neutrophil-independent injury reported by some groups.

In conclusion, pulmonary reperfusion injury is a complex process, likely involving many cell types, cytokines, and mechanisms. Both macrophages and neutrophils play major roles in lung injury after transplantation. The effect of each cell type can be reversed with subsequent improvement in lung function at specific time points. Macrophage inhibition with GdCl₃ significantly attenuates the early (30 minutes) phase of reperfusion injury. Leukocyte filtration with a leukocyte filter significantly attenuates the late (2 hours) phase of reperfusion. Clinical application of GdCl₃, however, is unlikely in the transplant setting because it must be given 24 hours in advance. However, administration of other macrophage inhibitors, such as intratracheal antimacrophage antibodies, may have a potential role in clinical lung transplantation.

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