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Clinical grade allogeneic human mesenchymal stem cells restore alveolar fluid clearance in human lungs rejected for transplantation

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Submitted 10 December 2013; accepted in final form 11 February 2014

LUNG TRANSPLANTATION HAS BECOME the standard of care for selected patients with advanced lung diseases. However, patients are likely to die while awaiting lung transplantation, with waiting list mortality as high as 40% (12), in large part because of the limited availability of donor lungs. There is a worldwide gap between the supply and demand for transplantable organs, and this gap is enlarging (19). However, another major issue is that the use of marginal donor lungs is associated with an increased incidence of primary graft dysfunction, with poor early and long-term outcomes in recipients of such suboptimal organs (3). Hence, transplant teams tend to be conservative in the selection of donor lungs (29).

Primary graft dysfunction is the end-result of injury occurring from the time of brain death to the time of lung reperfusion after transplantation, including during cold ischemic storage of the lung allograft. The current clinical practice of organ preservation is cold static preservation. Hypothermia reduces metabolic activity to the point that cell viability can be maintained in the face of ischemia (20). Although hypothermia is essential for organ storage, it can be associated with a series of events (including cell injury and death) (13) that may lead to primary graft dysfunction, which represents a spectrum of injury ranging from mild pulmonary edema to a clinical picture similar to the acute respiratory distress syndrome (ARDS) (6). Longer ischemic times predispose to more injury, and more severe primary graft dysfunction (32).

There is recent interest in reconditioning lungs rejected for transplant using ex vivo lung perfusion (EVLP) (11). With EVLP, lungs are perfused and ventilated ex vivo at body temperature to mimic physiological conditions. EVLP can also facilitate delivery of a pharmacological, gene- or cell-based therapy to repair damaged lungs before transplant (23, 40). We previously reported that intravenous clinical-grade, cryopreserved allogeneic human mesenchymal stem (stromal) cells (hMSC) were effective in restoring alveolar fluid clearance (AFC) in a human EVLP model of acute lung injury (ALI) induced by endotoxin or Escherichia coli bacteria (22, 23). Thus, hMSCs may be a novel strategy to recondition lungs declined for transplantation. Mesenchymal stem cells (MSCs) have the ability to secrete multiple paracrine factors, including growth factors (10) that can potentially treat the major abnormalities that underlie lung injury and pulmonary edema during brain death, including impaired AFC. Specifically, keratinocyte growth factor (KGF) is known to be important in mediating some of the beneficial effects of hMSCs on repair in the...
ventilator-injured lung (10) and in restoring normal AFC in the human EVLVP model of ALI (22).

We hypothesized that hMSCs would be effective in restoring alveolar fluid transport in lungs rejected for transplantation. When the results demonstrated that hMSCs were efficacious for upregulating AFC, we then tested the potential role of KGF as a paracrine mechanism responsible for the therapeutic effect of hMSCs.

MATERIALS AND METHODS

Selection criteria for ex vivo perfused human lung preparation. Our laboratory receives one to two human lungs from brain-dead donors from the Northern California Transplant Donor Network weekly. For inclusion in this study, lungs must also have been rejected for transplantation by the Northern California Transplant Donor Network and approved for research use by the family or appropriate surrogate. We previously found that in northern and central California donor lung utilization rate was 14% (102/738) over a two-year period. Lungs from 312 donors therefore did not meet regional lung selection criteria and were rejected. The primary reasons for rejection of these lungs for transplantation were hypoxemia (PaO2/FIO2 < 300), smoking history > 20 pack-years, blunt injury, evidence of infection or aspiration, or chest radiographic infiltrates (38). Inclusion of lungs in the study was random and based solely on availability of a qualified surgical team to procure the lungs for the study. After informed consent from the brain-dead subject’s family to use lungs for research if they could not be used for transplantation, the lungs were resected on bloc without preservative flush, and inflated and transported at 4°C.

A previous study (38) found that most human lungs rejected for transplantation maintain alveolar epithelial fluid transport capacity. Furthermore, in a separate study, lungs rejected for transplant with measurable basal and stimulated fluid clearance had mean basal AFC rates of 19 ± 10%/h and mean stimulated (terbutaline) AFC of 43 ± 13%/h (17). Lungs were included in this study if the lung demonstrated impaired AFC, with lungs included if baseline AFC was < 10%/h and cold ischemia time < 48 h. Based on this criterion, from July 2011 to June 2012, ~50% of rejected lungs were eligible.

MSCs from the University of Minnesota National Heart Lung and Blood Institute Production Assistance for Cellular Therapy program. We established a formal collaboration with the University of Minnesota’s Molecular & Cellular Therapeutics Facility, the current Good Manufacturing Process facility of the University of Minnesota, through the National Heart Lung and Blood Institute Production Assistance for Cellular Therapy mechanism. Clinical-grade allogeneic bone marrow-derived hMSC, washed as if in a clinical trial and resuspended in PlasmaLyteA, were used in these studies.

Allogeneic bone marrow-derived human MCSs were manufactured at Molecular and Cellular Therapeutics of the University of Minnesota (FACT-accredited, CAP no. 18060–01, CLIA no. 24D0688128). Marrow was collected through Tissue Acquisition of Lonza Walkersville (Walkersville, MD). Enrichment of the mononuclear cell fraction of the marrow was accomplished using a manual separation method involving ficoll hypaque density gradient medium, specific gravity 1.077 g/dl (Isolymph; Gallard-Schlesinger Industries, Carle Place, NY). Analysis by flow cytometry was used to characterize the cell types within the final product. Positive markers include CD73, CD90, and CD105; negative markers include CD14, CD19, CD34, CD45, and HLA-DR. MSCs are also tested for their ability to differentiate into bone, fat, and cartilage as per International Society for Cell Therapy guidelines, for viability, using 7-aminoactinomycin D DNA staining and flow cytometry, for karyotyping, and lipopolysaccharide (LPS) and mycoplasma contamination.

Ex vivo perfused human lung and measurement of AFC. The lung to be used, either right or left, was chosen based primarily on a practical issue, whether or not the pulmonary artery could be cannulated with a catheter. The lung was first separated, and the pulmonary artery was cannulated by passing a Foley catheter 2–3 cm in the surgical stump, securing it in place with a purse-string suture. The Foley catheter was then connected to a peristaltic pump via polyvinyl chloride tubing (Nalgene, Rochester, NY). To measure pulmonary arterial pressure, a pulmonary artery catheter (Cook, Bloomington, IN) was passed through a side port in the tubing and advanced to the end of the Foley catheter. The right or left mainstem bronchus was then intubated with the tip of a size 7.0 endotracheal tube. The lung preparation was weighed and suspended within a sealed acrylic container from a mass transducer (Harvard Apparatus, Holliston, MA). The container was surrounded by a heated (38°C) water bath in which the inner container served as a reservoir for the perfusate solution (DME H-21 with 5% bovine serum albumin, 900 ml) (Fig. 1A). The lung preparation was slowly warmed over 1 h with the peristaltic pump until a flow rate of 0.3–0.4 l/min was achieved, giving an average mean pulmonary arterial pressure of 10–12 mmHg. Venous drainage or left atrial pressure was passive (0 mmHg) (22). When the temperature of the venous drainage reached 36°C, the lung was slowly inflated with continuous positive airway pressure (CPAP) at 10 cmH2O with 95% O2 and 5% CO2. Pulmonary arterial pressure and lung weight were then continuously monitored using a computer-integrated data acquisition system (Biopac, Goleta, CA). Perfusion pH, PO2, and PCO2 tensions were measured hourly with a blood gas machine (Bayer RapidLab 248, Erlangen, Germany). One hour following perfusion and oxygenation (CPAP), a lobe of the lung was...
cannulated with a PE catheter (240 tubing; BD, Franklin Lakes, NJ) and advanced until gentle resistance was encountered. Warmed normal saline with 5% albumin (125 ml, alveolar fluid solution) was instilled in the right or left upper lobe. AFC was measured by the change in protein concentration of a 5% albumin instillate in the lung lobe over 30 min and expressed as mean AFC ± SD (%/h) for each condition using the equation, as in our previous experiments (2, 3):

\[
AFC \times 100 = \frac{(C_i - C_f)}{C_i} \times 100
\]

where \(C_i\) is protein concentration at time 0 and \(C_f\) is protein concentration after 30 min. If the AFC of the lung lobe tested was <10%/h (final inclusion criteria), lungs received either intravenously hMSC (5 × 10^6 cells) added to the perfusate or the perfusion only as a control group. After 4 h, AFC in a separate lobe of the control and MSC-treated lungs was measured with the instillation of 125 ml of alveolar fluid solution (Fig. 1B).

Measurement of the receptor for advanced glycation end products in the bronchoalveolar lavage fluid. The alveolar fluid collected during the measurement of AFC in both the control and the MSC-treated lung lobes was used to measure the levels of the receptor for advanced glycation end products (RAGE), a marker of type 1 alveolar epithelial cell injury/activation, using an ELISA-based assay (Antigenix; R&D Systems).

KGF blocking antibody. To test the role of KGF in mediating the effect of MSCs, we used a KGF blocking antibody. In this set of experiments, we used human lungs that had been declined for transplantation with AFC <10%/h and >0%/h in our EVLP model. In the KGF neutralizing antibody experiments, the lungs received MSC (5 × 10^6 cells) added to the perfusate 1 h after administration of 2 μg/ml KGF neutralizing antibody (R&D Systems), or a control IgG antibody, intrabronchially.

Statistical analyses. Results were expressed as means ± SD if the data were normally distributed. Comparisons between two groups were made using the unpaired t-test. Comparisons with a sample over time were made by repeated-measures of ANOVA using the Bonferroni correction for multiple-comparison testing using Statview (SAS Institute).

RESULTS

Baseline data from donor lungs. Baseline demographic data and ischemia time for the 14 donor lungs are listed in Table 1. The right lung was used 35% of the time and the left lung 65% for the experiments. Donor age, evidence of radiographic abnormalities, PaO_2-to-FIO_2 ratio, and lung compliance (28) did not differ among the groups. The mean time from harvest to EVLP for the perfusion only group was 31 ± 6 h vs. 33 ± 31 h for the hMSC-treated group (\(P = 0.9\)).

Pulmonary arterial pressures. Pulmonary arterial pressures remained stable throughout the 4 h of EVLP and were not significantly different among the groups (Fig. 2). Pulmonary artery pressure remained stable throughout the protocol and were not significantly different between the groups (\(P = 0.4\) for pulmonary artery pressures, by repeated-measures ANOVA).

Table 1. Donor clinical and demographic data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Perfusion Only (n = 4)</th>
<th>MSC (n = 4)</th>
<th>MSC with KGF Antibody (n = 3)</th>
<th>MSC with IgG Antibody Control (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, yr</td>
<td>51 ± 5</td>
<td>51 ± 13</td>
<td>57 ± 5</td>
<td>38 ± 17</td>
</tr>
<tr>
<td>Female, %</td>
<td>25</td>
<td>50</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td>Donor P/F ratio</td>
<td>197.9 ± 81.1</td>
<td>190.9 ± 88.4</td>
<td>271.3 ± 114.3</td>
<td>375.7 ± 158.4</td>
</tr>
<tr>
<td>Donor Lung Compliance, ml/cmH_2O</td>
<td>40 ± 17</td>
<td>44 ± 6</td>
<td>37 ± 3</td>
<td>37 ± 11</td>
</tr>
<tr>
<td>PEEP</td>
<td>10 ± 2</td>
<td>7 ± 2</td>
<td>5 ± 0</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>CXR infiltrates</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Acute lung injury score</td>
<td>2.6 ± 0.7</td>
<td>2.1 ± 0.8</td>
<td>1.5 ± 0.8</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>Ischemic time, h</td>
<td>31 ± 6</td>
<td>33 ± 31</td>
<td>24 ± 14</td>
<td>29 ± 14</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD; n, no. of lungs. MSC, mesenchymal stem cell; KGF, keratinocyte growth factor; PEEP, positive end-expiratory pressure; CXR, chest X-ray. Acute lung injury score based on maximum of 4 points for the PaO_2-to-FIO_2 ratio (P/F), level of positive end-expiratory airway pressure, quasistatic respiratory compliance, and the extent of chest radiographic infiltrates.
plantation and had been subject to prolonged cold storage, 
~50% of the lungs demonstrated impaired AFC, a decreased 
capacity to reabsorb alveolar edema fluid, as defined as 
<10%/h. Treatment with allogeneic MSCs restored more nor-
mal AFC. This effect was mediated in part by KGF secretion. 
These results may have clinical implications because restoring 
more normal alveolar fluid transport in lungs before transplan-
tation may reduce their susceptibility to developing reperfusion 
pulmonary edema or primary graft dysfunction and may make 
marginal lungs suitable to be used for lung transplantation.

Primary graft dysfunction after lung transplantation mani-
fests itself initially as noncardiogenic pulmonary edema (6), 
and is a significant cause of morbidity and mortality (7). 
Experiments in a canine model of lung transplantation (31) and 
clinical observations in humans (35) suggest that impaired 
AFC contributes to the development of pulmonary edema in 
the posttransplant period. An intact alveolar epithelial barrier 
with preserved AFC has been associated with better clinical 
outcomes in patients with ARDS (37) and in patients with 
posttransplant reperfusion pulmonary edema (35).

Pulmonary edema is also a major factor contributing to poor 
donor oxygen indexes, resulting in refusal of procurement (14). 
In lungs from 29 donors that were rejected for transplantation, 
lung wet-to-dry weight ratio, a measure of pulmonary edema, 
was normal in only seven (24%) (38). In a recent trial of 
nebulized albuterol in brain-dead organ donors, mean lung 
weights were elevated by 250 grams compared with normal 
lung weight, suggestive of pulmonary edema, and chest radi-
ographs confirmed these findings, indicating that pulmonary 
edema is common in organ donors (34). Strategies to enhance 
the clearance of pulmonary edema could lead to higher rates of 
donor lung utilization and a reduced incidence of primary graft 
dysfunction in recipients. Several stimuli can upregulate AFC 
(which is driven in part by active vectorial transport of sodium 
across alveolar epithelial sodium channels), including cat-
echolamines and glucocorticoids (25). The compelling preclin-
ical data highlighting the importance of intact AFC to the 
resolution of pulmonary edema has led to clinical trials of 
β-agonists in ARDS (18, 24) and in brain-dead organ donors 
(36). The failure to demonstrate benefit for systemic or aero-
solized β-agonists in these populations suggests the need for a 
more innovative and fundamental therapeutic strategy. The 
present study highlights MSCs as a potential therapeutic agent 
to enhance pulmonary edema clearance in the marginal or 
injured donor lung before transplant.

MSCs have advantages over a single pharmacological ther-
apy, such as the ability to secrete a variety of immune-
modulating and tissue reparative factors, the ability to transfer 
mitochondria to injured cells, and the ability to home to sites of 
injury (27). Recent insights into stem cell biology have gener-
ated interest in the potential of MSCs to regenerate or repair 
damaged organs, including the lung (10). MSCs may directly 
attenuate bacterial pneumonia in donor lungs, a common cause 
of early morbidity and mortality after lung transplantation, via 
a number of mechanisms, including enhancement of phagocy-

Fig. 3. Effect of human MSCs on AFC in human lungs rejected for transplant 
with impaired AFC at baseline. Clinical-grade, cryopreserved human alloge-
nic MSCs injected in lung perfusate restored AFC after 4 h in lungs rejected 
for transplant and with impaired AFC at baseline (P < 0.001 for MSC vs. 
baseline and perfusion only by ANOVA).

Fig. 4. Effects of MSCs on the concentration of receptor for advanced 
glycation end products (RAGE) in bronchoalveolar lavage (BAL) fluid 
from baseline. MSCs did not significantly reduce the concentration of 
RAGE in BAL after 4 h of perfusion. (t-test, P value for change in RAGE 
concentrations = 0.17).

Fig. 5. Effects of human MSCs and the addition of an antibody (Ab) to 
keraatinocyte growth factor (KGF) on AFC in human lungs rejected for 
transplant with impaired AFC at baseline. The inhibition of KGF by a 
neutralizing antibody abrogated the effect of MSCs on AFC. For anti-KGF 
antibody, *P < 0.01 vs. MSCs treated with goat control IgG; n = 3–4 for each 
condition.
tosis and increased bacterial clearance (23), and antimicrobial peptide secretion (21). Both the distal lung epithelium and the pulmonary endothelium are selectively accessible to stem cell therapies during EVLP, via the intrabronchial route or the pulmonary vasculature.

Bone marrow-derived MSCs are known to produce several epithelial-specific growth factors, including angiopoietin-1, hepatocyte growth factor, and KGF. In the ex vivo perfused human lung, we previously reported that the intrabronchial instillation of hMSCs 1 h following endotoxin-induced lung injury restored AFC, an effect that was mediated in part by the secretion of KGF (22). More recently, we also reported that clinical-grade hMSCs, given intrabronchially or via the perfusate, were effective in restoring normal AFC following E. coli pneumonia-induced ALI (23). This effect was associated with increased bacterial phagocytosis and killing by monocytes and macrophages, was more effective than antibiotic therapy alone during prolonged pneumonia, and was abolished by the administration of anti-KGF antibody. In the current studies, the beneficial effects of MSCs were mediated in part by KGF in restoring AFC in donor lungs with impaired fluid clearance. The beneficial effect of exogenous KGF has been reported in several ALI models (39), and is currently being tested as a potential therapy for ALI (8). KGF has a wide variety of effects on lung epithelial cells that may mediate its protective effect in lung injury. In particular, KGF stimulates AFC (33). Although MSCs have been shown to be an important source of KGF, and KGF has been shown to mediate enhanced AFC in other models of ARDS (10, 22, 23), we cannot exclude the possibility that the KGF antibody used in these experiments acted on endogenous KGF produced in the donor lung to reduce AFC. In this scenario, the role of MSCs would be to enhance endogenous KGF production. This is unlikely, given that previous experiments have shown that endogenous KGF production occurs late after lung injury (9), and MSCs have been shown to produce larger quantities of KGF than other stromal cells (10, 22). We previously demonstrated that, in an E. coli endotoxin-induced lung injury in the ex vivo perfused lung, KGF small-interfering RNA pretreatment of MSCs eliminated the therapeutic effect of MSC-conditioned medium in restoring the AFC rate (22). In addition, in the endotoxin-injured lobe without treatment, there was no restoration of the AFC rate, although there was endogenous KGF expressed in the injured alveolus, suggesting that MSC KGF secretion was important for restoring AFC.

In addition to AFC, we also measured alveolar fluid concentrations of RAGE as a marker of type I alveolar epithelial cell injury/activation. Elevated plasma levels of RAGE are a marker of poor outcome after lung transplantation (5). Elevated airspace levels of RAGE were significantly correlated with impaired AFC in lungs declined for transplantation (4). Our data indicate a trend toward a reduction in the airspace levels of RAGE in lungs treated with MSCs.

There are some limitations to these experiments that will have to be addressed before clinical translation. First, the experimental protocol was designed to assess AFC only, and not alterations in gas exchange or overall lung fluid balance. The procedure to determine AFC requires the instillation of 125 ml of warmed normal saline with 5% albumin in the right or left upper lobe at baseline and after 4 h, which precludes accurate assessment of gas exchange; moreover, only CPAP was used in place of positive pressure mechanical ventilation. Furthermore, the perfusate used, DME H-21 with 5% albumin, is not optimized to maintain vascular integrity over time, which results in increased edema formation (32).

The method used to assess AFC may have some limitations. We have made the assumption that AFC measured in one lobe reflects what is happening in other lobes of the lung, which it might not. However, in earlier experiments with the ex vivo human lung where the AFC rate was intact in the control lobe, we consistently found that both the upper and lower lobes had AFC >10%/h (22). Furthermore, the use of a change of protein concentration in a test solution instilled in the injured lung as a measurement of AFC can be confounded in some situations. Because the concentration of protein in the instilled solution in the lung is equal to the protein concentration present in the circulation, it is unlikely that an increase in protein concentration in edema fluid can be due to an increased movement of protein from the interstitial space into the airspaces. However, movement of liquid from the interstitial space to the alveoli could potentially dilute the protein in the instilled solution. In this circumstance, it can be difficult to determine whether the decrease in clearance is due to a decreased reabsorption of liquid or an enhanced movement of liquid from the interstitial space. This is why a decrease in alveolar protein concentration cannot be used to calculate net fluid accumulation in the air spaces. A rise in alveolar protein concentration, however, as noted in this study, especially above the level of plasma protein concentration, means there must have been net AFC. This method has been validated first in normal lung (2) and has also been validated in multiple models of lung injury (26).

Despite these limitations, we believe that AFC is an important functional measure in the assessment of lungs for transplant. Current selection criteria are poor discriminators of pulmonary injury and infection and lead to the exclusion of potentially usable donor lungs (38). We (38) and others (16) have reported that physiological parameters in the donor, combined with AFC, could increase the rate of successful organ usage.

Although we administered MSCs directly in the perfusate, the primary site of action demonstrated in the study (improved AFC) was the epithelium. We have administered MSCs both intrabronchially in isolated human lungs injured by LPS (22) and intrabronchially and systemically (as in this current study in the perfusate) in isolated human lungs injured by E. coli bacteria (23). We observed similar efficacy with both routes of administration (23). This may be related to the paracrine mode of action of MSCs, as well as to the ability of MSCs to traffic to inflammatory sites. Systemically administered MSCs are also known to immediately accumulate in pulmonary capillary beds (15). Because intravenous administration had equal efficacy to intrabronchial delivery in our recent preclinical studies, and would be a more straightforward method of administration in patients, we delivered MSCs directly in the perfusate for these experiments. We believe, however, that the intrabronchial approach still has research and potential clinical value.

Finally, donor gender differences could have contributed to differences among the groups in regard to AFC. Specifically, the MSC with KGF antibody group was 66% female vs. 0% female in the MSC with IgG antibody control group. However, an increase in female donors in this group is more
likely to have resulted in lungs with enhanced, rather than reduced, AFC (1).

In conclusion, this study provides proof of principle that MSCs can enhance AFC in donor lungs with impaired AFC at baseline. Furthermore, it appears that one major factor responsible for this is secretion of KGF by MSCs. Organ availability continues to be a major impediment to more widespread use of lung transplantation. Only 15–25% of lungs from donors are transplanted, the lowest graft acceptance rate of any solid organ (30). EVLP has been suggested as a novel method to deliver therapeutic agents to marginal lungs. The current study provides support for the hypothesis that functionally impaired donor lungs could be improved during EVLP before transplantation with human bone marrow-derived allogeneic MSCs. Additional studies will be required to assess the effects of MSCs on oxygenation and lung compliance in clinical-grade EVLP systems using appropriate perfusion solutions and ventilation strategies before this therapy can proceed to clinical trials.

GRANTS

This work was supported by the following institutions and grants: National Heart, Lung, and Blood Institute (NHLBI) Grants HL-51854 and HL-51856 (M. A. Matthay) and HL-093026 and HL-113022 (J. W. Lee), the Nina Ireland Lung Disease Program (University of California, San Francisco), and the Northern California Transplant Donor Network (Oakland, CA). Clinical grade, cryopreserved human allogeneic MSCs were obtained from an National Institutes of Health-supported Production Assistance for Cellular Therapy grant. Additional studies were supported by a University of Toronto Clinician Scientist Transition Award and an International Anesthesiology Research Society Mentored Research Award.

DISCLOSURES

The authors of this manuscript have no competing interests to disclose.

AUTHOR CONTRIBUTIONS


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