A novel cell culture model for studying ischemia-reperfusion injury in lung transplantation

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Cardella, Jonathan A., Shaf Keshavjee, Eric Mourgéon, Stephen D. Cassivi, Stefan Fischer, Noritaka Isowa, Arthur Slutsky, and Mingyao Liu. A novel cell culture model for studying ischemia-reperfusion injury in lung transplantation. J Appl Physiol 89: 1553–1560, 2000.—Many cell culture models have been developed to study ischemia-reperfusion injury; however, none is specific to the conditions of lung preservation and transplantation. The objective of this study was to design a cell culture model that mimics clinical lung transplantation, in which preservation is aerobic and hypothermic. A549 cells, a human pulmonary epithelial cell line, were preserved in 100% O2 at 4°C for varying periods in low-potassium dextran glucose solution, simulating ischemia, followed by the introduction of warm (37°C) DMEM plus 10% fetal bovine serum to simulate reperfusion. Cultures were assayed for cell attachment and viability. Sequential extension of ischemic times to 24 h showed a time-dependent loss of cells. There was a further decrease in cell number after simulated reperfusion. Cell detachment was due mainly to cell death, as determined by cell viability. The effects of chemical components such as dextran 40 and calcium in the preservation solution and various preservation gas mixtures were examined by use of this model system. With its design and validation, this model could be used to study mechanisms related to ischemia-reperfusion injury at the cellular and molecular level.

LUNG TRANSPLANTATION IS AN EFFECTIVE THERAPEUTIC MODALITY FOR PATIENTS WITH END-STAGE LUNG DISEASES (28, 33). However, this process is severely limited by the shortage of suitable donor organs (25). Only 10–25% of multiple-organ donors have lungs potentially suitable for transplantation; thus patients continue to die on transplant waiting lists (5, 32). Therefore, current organ preservation strategies must be improved to increase the potential donor pool by permitting the use of donor lungs that might otherwise be considered marginal.

The lung is a very sensitive organ with respect to ischemia-reperfusion (I/R) injury (24). I/R injury represents a major obstacle in lung transplantation, with mild to severe injury occurring in 20–30% of recipients. This is manifest as early posttransplant lung dysfunction (10). Clinically, patients who experience I/R injury typically present with pulmonary edema and decreased arterial Po2 levels. Furthermore, in animal and human studies of I/R injury, diffuse epithelial and endothelial cell damage and death are apparent (6, 26). Although many physiological phenomena have been associated with I/R injury, the cellular and molecular mechanisms resulting in poor lung function and cell death have been poorly characterized.

Lungs for transplantation are preserved by flushing the organ, through the pulmonary artery, with preservation solution. The lungs are then stored hypothermically, inflated with O2. During this period, the cells of the lung are able to undergo aerobic respiration. This is in contrast to other organs for transplantation, which are preserved under anaerobic hypothermic conditions. In our lung transplant program and others, lung preservation for transplantation involves the use of low-potassium dextran glucose (LPDG) preservation solution under hypothermic (4°C) conditions while the lungs remain inflated with 100% O2. It is during this period that the lung cells are able to undergo aerobic respiration (3). Lungs preserved with 100% O2 have been shown to be superior, postreperfusion, than those preserved with nitrogen or room air (35). There are studies indicating that LPDG is superior to other lung preservation solutions, which is why it was employed in this study (1, 15, 36).

Cell culture models have been used to study I/R injury in many organ systems. However, most of these models are not specific to lung transplantation in that they are hypoxia-reoxygenation models and use cells that are not pulmonary cells (9, 13, 17, 20, 27, 34). The models that do employ lung-specific cells use only cold preservation without simulated reperfusion (14, 18, 30,
31) or do not use 100% O$_2$ during simulated ischemia (7, 8).

The objective of this study was to develop an I/R injury model that mimics the stress and injury specific to lung transplantation. Specifically, we developed a model using a human pulmonary epithelial cell line (A549 cell line) with cold (4°C), aerobic (100% O$_2$) ischemia in LPDG solution followed by warm reperfusion using serum containing culture medium.

**MATERIALS AND METHODS**

**Cell culture.** A549 is a human pulmonary epithelial cell line established from lung carcinoma cells (American Type Culture Collection, Rockville, MD). Cells were grown and maintained in DMEM (GIBCO Life Technologies, Grand Island, NY) containing gentamicin and supplemented with 10% fetal bovine serum, referred to as D10. Cells were maintained in T75 flasks (Nunc, Naperville, IL) in a humidified atmosphere at 37°C and 5% CO$_2$. Cells were subcultured by using enzymatic digestion with 0.25% trypsin (GIBCO) and 1 mM EDTA (Sigma, St. Louis, MO) when cells were ~80% confluent.

A simulated I/R model system. A549 cells (5 × 10$^5$ cells/well) were plated in 24-well plates (Corning Costar, Cambridge, MA) and maintained in 1 ml of D10 in a humidified atmosphere at 5% CO$_2$ and 37°C for 18 h to allow cell attachment to the culture plate. Simulated cellular ischemia (referred to hereafter as ischemia) was achieved by the removal of D10 and introduction of 1 ml of cold (4°C) preservation solution. The cells were then placed into a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) filled with 100% O$_2$, or other gases as specified in the particular study group, and sealed. This high concentration of oxygen was used to ensure that the cells could maintain aerobic metabolism, mimicking the clinical situation of lung transplantation. Atmospheric pressure was maintained. The chamber was then stored and sealed at 4°C for ischemic times of 6, 12, 18, and 24 h. Simulated reperfusion (referred to hereafter as reperfusion) was performed by the removal of the preservation solution and the reintroduction of D10 at 37°C. The cells were then constantly bathed with 100% O$_2$ at 37°C for a period of 2 h (See Fig. 1). Cells were examined either 1) before hypothermic preservation as time = 0 controls, 2) after the simulated ischemia period, 3) after the 2-h simulated reperfusion period, or 4) after a period of time equal to the ischemic time plus the reperfusion time while cells were maintained at 5% CO$_2$ and 37°C.

**Evaluation of cell attachment.** As an indirect assessment of cell viability, cell attachment was quantified by using a Coulter counter (Coulter Electronics, Hialeah, FL). At each evaluation point, the solution covering the cells was removed, and the cells in each well were gently washed once with 1 ml of PBS. The PBS was then removed, and 1 ml PBS with 0.25% trypsin and 1 mM EDTA were added to each well. The cells were then incubated for 5 min at 37°C to detach the cells. An aliquot of cell suspension was added to a Dilu-Vial (VWR Scientific, Mississauga, ON) containing 10 ml of PBS. The cell number was then quantified using a Coulter counter. This value was converted to a total cell number per well. Each well was sampled twice, and each vial was quantified in duplicate. Four wells were used for each treatment, and every experiment was repeated at least three times.

**Fluorescein diacetate-propidium iodide staining.** To determine the viability of attached and detached cells, fluorescein diacetate (FDA)-propidium iodide (PI; FDA-PI) cell viability staining was used. After each evaluation point, the solutions covering the cells from four wells under identical treatment were pooled in a 15-ml tube and centrifuged at 2,000 rpm for 5 min. The supernatant was aspirated and discarded, and the pellet was resuspended in 100 µl of Dulbecco's PBS (GIBCO). Stock solutions of FDA (5 mg/ml in acetone) and PI (0.02 mg/ml in Dulbecco's PBS) were stored at 4°C in the dark. Staining was achieved by the addition of a final solution containing 2 µg of FDA and 0.6 µg of PI to the cell suspension. The solution was then gently mixed and allowed to stand for 3 min. The stained suspension was then mounted on a hemocytometer, and four fields were quantified under ×100 magnification for a percentage of living cells. The viability of cells was examined with a fluorescent microscope using 520 nm and 590 nm filters. Viable cells fluoresced green, whereas nonviable cells were red. Attached cells were attached.

**Fig. 1.** A cell culture model that mimics ischemia-reperfusion process in lung transplantation. A: human lung epithelial cells (A549) were seeded and allowed to acclimate to standard culture conditions for 18 h at 37°C, 5% CO$_2$ in DMEM containing 10% fetal bovine serum (D10). B: D10 was removed and replaced with cold (4°C) low-potassium dextran glucose (LPDG) lung preservation solution. C: cells were then placed into a modified incubator filled with 100% O$_2$ and kept at 4°C for various periods of time to simulate ischemia. D: LPGD was replaced with D10, and the culture was placed in a 37°C incubator with 100% O$_2$ constant flow for 2 h to simulate reperfusion.
assessed in a similar way, except the quantification was performed in situ with the cells still attached to the culture plate. Four microscope fields were assessed, and a percentage of living cells was ascertained.

**Preservation solutions.** In the initial studies, LPDG (Biophausia, Uppsala, Sweden) was used as the preservation solution to be tested. In addition, standard cell culture media, DMEM and D10, were also used for cells cultured under the standard (37°C, 5% CO2-95% room air) or simulated I/R conditions to determine the degree of stress that this non-physiological process exerted on lung cells. In subsequent experiments, to determine the effects of specific components, namely glucose, dextran, and Ca2+ in these solutions, several preparations were made with varying chemical compositions. The following modifications were made to the preservation solutions that were tested: D10 supplemented with 20 g/l of dextran 40 (Sigma), to examine the effect of dextran 40; low-potassium dextran (LPD) solution (i.e., no glucose); low-potassium glucose (LPG) solution (i.e., no dextran); and LPDG solution with 5 mM of Ca2+, to examine the contribution of Ca2+. The clinical laboratories at the Toronto General Hospital confirmed the actual concentrations of the solutions when possible; these are listed in Table 1. All preservation solutions were exposed to simulated ischemia for varying periods of ischemia at 4°C and 100% O2 preservation gas followed by 2 h of reperfusion.

**Preservation gases.** The concentrations of the preservation gas were changed from 100% O2 to 95% O2 with 5% CO2 as well as 21% O2 or 21% O2 with 5% CO2. The gas composition of the modified incubator was checked before and after simulated ischemia to ensure that there was no gas leaking. All preservation gases were tested by a preservation period of 24 h at 4°C in LPDG followed by 2 h of reperfusion. Standard culture media D10 and DMEM were also used to preserve cells but only at 4°C with 100% O2, or with 95% O2 plus 5% CO2, followed by reperfusion.

**Measurement of pH.** Measurement of pH was performed on preservation solutions after ischemia by using a CIBA-Corning 278 blood-gas system (Chiron, Markham, ON).

**Statistical analysis.** Statistical analysis was performed with SigmaStat 3.0 (Jandel Scientific, San Rafael, CA). One-way and two-way ANOVA were used, followed by Student-Newman-Keuls post hoc testing where appropriate. A P value <0.05 was considered significant. All data are shown as means ± SE.

### Table 1. Compositions of solutions

<table>
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<tr>
<th></th>
<th>LPDG</th>
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<th>LPG</th>
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<td>50</td>
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<td>271</td>
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</table>

All values were measured from prepared solutions (mM). LPDG, low-potassium dextran glucose solution; LPD, low-potassium dextran solution; LPG, low-potassium glucose solution. Selected chemical components are listed for comparison.

### RESULTS

**Aerobic hypothermic ischemia and reperfusion induce cell detachment.** A549 cells exposed to 100% O2 and preserved at 4°C in LPDG preservation solution (ischemia) showed a decrease in the number of cells that remained attached to the cell culture plate after 12 h of ischemia. After this, the amount of cell attachment leveled off (Fig. 2A). Reperfusion further reduced cell attachment as there was significantly (P < 0.05) less cell attachment after 24 h of ischemia in LPDG and 2 h of reperfusion than after ischemia only. Also there was significantly (P < 0.05) more cell attachment after 6 h of ischemia followed by reperfusion compared with 24 h of ischemia and reperfusion using LPDG as the preservation solution (Fig. 2B). This suggests that cell attachment after ischemia and reperfusion decreases in a manner dependent on ischemic time.
To determine the severity of cell injury that the simulated lung preservation and reperfusion process exerted on lung cells, cells were cultured with standard culture media, DMEM and D10. Significant cell loss was observed after 18 h of ischemia with or without reperfusion when DMEM was used (Fig. 2, A and B). When cells were cultured in D10, significant cell detachment was seen as early as at 12 h of ischemia with or without reperfusion (Fig. 2, A and B). In fact, cells exposed to ischemia alone in LPDG had significantly ($P < 0.05$) more cell attachment at 18 h and 24 h compared with those cultured in D10 and DMEM at the same time points (Fig. 2A). This difference persisted after reperfusion (Fig. 2B).

In contrast, when cell attachment was assessed on cells that were maintained under standard culture conditions (5% CO$_2$-95% room air and 37°C) for periods of time equaling their ischemic time plus 2 h to account for reperfusion time (i.e., for 24 h of ischemia, 24 h + 2 h = 26 h), cell attachment remained unchanged in all groups except during the evaluation of the 26 h control in which there was significantly ($P < 0.05$) greater cell attachment in the D10 group vs. the LPDG group (Fig. 2C).

Cell attachment provides an indirect quantification of cell viability. Cells were stained with FDA-PI staining to evaluate cell viability. Cells stained green by FDA-PI staining in situ are alive (Fig. 3A), whereas cells stained red by propidium iodide are dead (Fig. 3B). Cultures were assayed for viability both on cells that were attached to the culture plate and on those that were detached. The viability of attached cells preserved for 24 h in 100% O$_2$ and 4°C followed by reperfusion was 99% for cells preserved in LPDG, whereas viability decreased to 32% and 18%, respectively, when preservation was in DMEM or D10. For the detached cells receiving the same treatments, the viability was 18%, 5%, and <1% for LPDG, DMEM, and D10, respectively (Fig. 4). These data confirm that cell attachment is an indirect indicator of cell viability. In other words, the cell detachment due to ischemia and reperfusion was mainly due to cell death.

Effects of chemical components on cell viability. We sought to explore the reason behind the dramatic increase in cell death observed in the cells preserved in D10 and DMEM, compared with LPDG, after prolonged cellular preservation. This may provide useful information about factors that affect cell viability during preservation and reperfusion. The major chemical differences between DMEM and LPDG are that DMEM contains Ca$^{2+}$ and LPDG contains dextran 40. Preparations of D10 containing 20 g/l of dextran 40 were therefore studied to determine the effect of dextran on cell viability. This addition, however, did not provide significantly better cellular protection, in terms of cell attachment, than did D10 alone after 24 h of ischemia or after ischemia and reperfusion (Fig. 5).

Also, LPDG was prepared with 5 mM of Ca$^{2+}$ and compared with LPDG alone (Fig. 5). There was no difference in cell attachment after 24 h of simulated ischemia. However, after reperfusion of cells preserved in LPDG with 5 mM Ca$^{2+}$, cell attachment was significantly better ($P < 0.05$) vs. LPDG alone (Fig. 5).

Preparations of LPD and LPG solutions were tested against LPDG to determine the contributions of glucose and dextran, respectively, in cell preservation by LPDG (Table 1). After 24 h at 100% O$_2$ and 4°C (simulated ischemia), there was no significant change in the percentage of cells attached to the culture plate (Fig. 6). There was also no significant difference in cell attachment after ischemia and 2 h of simulated reperfusion. These data were positively correlated with cell viability staining (data not shown), in which the majority of cells attached to the culture plate were alive and the majority of detached cells were dead.

Preservation gas mixture has a marked effect on cell viability. To further examine the inadequate cellular preservation provided by D10 and DMEM, as well as the effect of various concentrations of O$_2$ and CO$_2$ in gas mixtures during preservation, special gas preparations were made. Cells exposed to ischemic conditions at 4°C for 24 h in D10 or DMEM with 95% O$_2$-5% CO$_2$ preservation gas had significantly ($P < 0.05$) better cell attachment than did cells preserved in the same solu-
tions using 100% O$_2$ preservation gas (Fig. 7). A similar, significant ($P < 0.05$) increase in cell attachment was seen after simulated reperfusion in the D10 and DMEM groups. Furthermore, the presence of 5% CO$_2$ in the preservation gas, when DMEM or D10 were used, provided equivalent cellular preservation to LPDG using 100% O$_2$ preservation gas, as determined by cell attachment (Fig. 7). These results were correlated with FDA-PI staining for cell viability (data not shown). Cells preserved in LPDG were similarly studied after 24 h of ischemia with a variety of inflation gas concentrations. No significant differences between groups were observed, with respect to cell attachment, after either ischemia or reperfusion (Fig. 8). However, there was a significant decrease ($P < 0.05$) in cell number, postreperfusion, for cells preserved in 100% O$_2$. This decrease was not seen in cells preserved for 24 h in LPDG and exposed to 95% O$_2$-5% CO$_2$, 21% O$_2$, or 21% O$_2$-5% CO$_2$.

**DISCUSSION**

We have developed a novel cell culture model that simulates the clinical process of lung transplantation. Most cellular models for I/R injury are not suitable for lung transplantation-related studies, because of the differences in ischemic conditions between transplantation and other clinical situations as well as between lungs and other organs preserved for transplantation. For example, in I/R injury triggered by stroke or myocardial infarction, ischemia is hypoxic and occurs at body temperature, whereas the reperfusion phase is characterized by the reintroduction of oxygenated blood. In transplantation, all organs are maintained by 10.220.33.1 on November 10, 2017 http://jap.physiology.org/ Downloaded from
without blood flow under hypothermic conditions. However, the lungs are inflated with O₂ and are able to maintain aerobic metabolism during ischemia, whereas other donor organs must undergo anaerobic metabolism (4). To our knowledge, the model described in the present study is the first cell culture model to combine aerobic, hypothermic ischemia with oxygenated warm reperfusion, a protocol mimicking the current clinical practice of lung preservation and transplantation.

In this model, with a short period of ischemia (6 h) at 4°C and 100% O₂ preservation gas followed by reperfusion, it is important to note that there was equivalent and minimal cell detachment in D10, DMEM, or LPDG preservation groups (Fig. 2, A and B). However, as preservation times were prolonged, the cell viability decreased in all groups, especially in the D10 and DMEM groups after ischemia or reperfusion. Furthermore, viability was further reduced after reperfusion (P < 0.05) in the LPDG group preserved for 24 h at 4°C in 100% O₂. This is similar to what has been noted in animal studies, in which reperfusion itself exacerbates the cell death after ischemia (29). Also, these results simulate the current lung transplantation practice in which short preservation times, in contrast to prolonged preservation times, yield adequate lung function after transplantation.

In this study we also used D10 and DMEM reagents, to determine the severity of the cell injury that the simulated lung preservation and reperfusion processes applied to cells. Cell detachment was extremely high after 18–24 h under these conditions. Interestingly, as the preservation time was extended to 18 and 24 h, LPDG afforded significantly better cell viability than did either DMEM or D10 after ischemia or reperfusion (Fig. 2, A and B). Although cell culture media are not designed for organ preservation, delin-
turn, an increase in reactive oxygen species formation occurs, which results in lipid peroxidative damage to mitochondrion. Overexpression of superoxide dismutase can counteract this effect in response to alka-loss. Alkalotic-induced, free radical-mediated damage could be a possible mechanism for the cell death seen after preservation in D10 and DMEM at 4°C and 100% O2. Currently, many lung transplant programs are still using organ preservation solutions with a bicarbonate buffering system (12). On the basis of the observations in this study, it is reasonable to suggest using a lung preservation solution with a phosphate buffering system, or, if a solution with a bicarbonate buffering system is used, CO2 should be considered in the preservation gas during aerobic lung preservation.

Reactive oxygen species during lung transplantation provide a source of cellular injury (2). This could be a result of the high O2 concentrations used to ensure aerobic conditions during lung preservation, as well as of the use of 100% O2 for mechanical ventilation during reperfusion. Therefore, we tested the effect of different gases on cellular preservation for 24 h using LPDG solution. We showed equivalent cellular preservation, defined by cell attachment to the culture plate, after 24 h of ischemia and after reperfusion, using preservation gases of 100% O2, 95% O2-5% CO2, 21% O2, or 21% O2-5% CO2 (Fig. 8). However, the significant reperfusion-induced decrease in cell attachment and viability seen using 100% O2 preservation gas was not significant in other groups. Thus decreasing the O2 concentration during lung preservation, although still supplying enough O2 to maintain aerobic conditions, could potentially decrease free radical production and should be considered clinically in the amelioration of I/R injury of lung transplants.

Lung transplant I/R injury has two primary sites of cell injury, the endothelial cells of the pulmonary capillaries and the pneumocytes (1, 23). Specifically, ischemia causes the loss of endothelial monolayer continuity, resulting in an increase in leaky junctions and subsequent pulmonary edema (11, 26). However, after 24 h of cold preservation and reperfusion in rat lungs, it has been shown that pneumocyte viability is decreased by three times compared with endothelial cell viability (29). Furthermore, in a rat model of I/R injury, diffuse epithelial cell damage was far more prominent than endothelial cell injury (26). These observations suggest that the maintenance of pneumocyte viability is imperative in ameliorating I/R injury. Therefore, we chose to study epithelial cells in the present study. The cell culture condition developed in this study is specifically designed for alveolar epithelial cells. However, endothelial cells or other cell types in the lung could be examined, using cell culture as a model, to elucidate their roles in lung transplant I/R injury. In these situations, the concept of aerobic hypothermic preservation followed by serum reperfusion at 37°C, developed in this study, should be considered.

In this model, to simulate reperfusion we used serum-containing media (D10) and changed temperatures from 4°C to 37°C. However, the reperfusion process during lung transplantation is more complicated than this. For example, after implantation the lung is mechanically ventilated. Airflow and cyclic stretch may have a significant impact on the function of lung parenchymal cells. Similarly, the reintroduction of blood flow-derived shear stress and stretch on the vascular wall may also affect the function of endothelial and smooth muscle cells. Furthermore, immune mediators and cell-to-cell interactions may be involved in the I/R injury. With the validation of the model, using epithelial cells, and establishing the significance of aerobic, hypothermic preservation in lung transplantation, contributions of these factors to lung transplant-related I/R injury could be examined by introducing mechanical stretch, shear stress, and other cell types, such as endothelial and smooth muscle cells. Therefore, this model, in future studies, may be a useful tool to study the cellular and molecular mechanisms associated with lung transplantation-related I/R injury. Furthermore, potential therapeutic interventions, such as antisense or gene therapy, can be tested with this model to evaluate their potential application in ameliorating I/R injury.

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