Interleukin 10 gene transfection of donor lungs ameliorates posttransplant cell death by a switch from cellular necrosis to apoptosis

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Background: We have previously shown that cell death is a pathophysiologic consequence of ischemia-reperfusion and that interleukin-10 gene therapy improves the function of transplanted lungs. Interleukin-10 downregulates the inflammatory response and can inhibit apoptosis. The objective was to determine whether donor lung transfection with the interleukin-10 gene ameliorates lung dysfunction by decreasing cell death after transplantation.

Methods: Single lung transplants were performed in 3 groups of rats ($n = 5$ each): AdhIL-10, transtracheal administration of Ad5E1RSVhIL-10 (5×10^9 pfu); EV, empty vector; and VD, vector diluent (3% sucrose). After in vivo transfection, donor lungs were excised, stored at 4°C for 24 hours, and then transplanted. After 2 hours of reperfusion, lungs were flushed with trypan blue and fixed. TUNEL staining was used for the detection of apoptosis. This combined staining technique allows one to determine the mode of cell death by distinguishing apoptotic dead cells from necrotic dead cells.

Results: Lung function was superior in the interleukin-10 group ($P = .0001$) vs the EV and VD group (Pao_2 : 240 ± 31 mm Hg vs 98 ± 17 mm Hg vs 129 ± 11 mm Hg, respectively). Although the total number of dead cells (as percent of total cells) was similar in all groups ($32.7\% \pm 3.2\%$, $30.2\% \pm 2.5\%$, and $30.3\% \pm 3.8\%$), interestingly, apoptosis was highest in interleukin-10 lungs (9.7 ± 1.9 vs 2 ± 1.9 and 1.8 ± 2 , $P = .0001$), and necrosis was lowest in the interleukin-10 group (20.6 ± 5.7 vs 28.3 ± 3.1 and 30.3 ± 4.2 , $P = .01$).

Conclusions: AdhIL-10 gene transfection improves function of transplanted lungs. Although the total number of cells dying as a result of the transplant process did not change, the *mode* of cell death appears to have been modified. It is possible that AdhIL-10, by decreasing proinflammatory cytokine production, ameliorates the overall injury and preserves the ability of damaged cells to undergo a more quiescent and less tissue-damaging mode of cell death—apoptosis, rather than necrosis.

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Lung transplantation is now accepted as a standard treatment modality for patients with a number of end-stage lung diseases.¹ The long-term outcome after clinical lung transplantation, however, is still not satisfactory, with a current 5-year survival of only approximately 55%.²

It is clear that an improved understanding of the underlying mechanisms of the injury that is inflicted on pulmonary grafts by ischemia, the transplant process itself, and reperfusion of the transplanted lung—referred to as ischemia-reperfusion (IR) injury—is required. In examining processes related to the induction of cell death in human lungs during the transplantation period, we have previously shown that approximately 30% of graft cells die within the first 2 hours after graft reperfusion by “programmed cell death” or apoptosis, whereas almost no signs of apoptosis occurred in same lungs neither during 1 to 6 hours of cold ischemia before the transplant nor during the implantation process.³ This degree of cell death was seen even in patients who did very well after lung transplantation.

Having discovered this remarkable loss of cells in transplanted lungs, which has also been described to various degrees in other solid organs after transplantation, such as the liver,⁴ kidney,⁵ and the heart,⁶ in a second study we attempted to answer the question of what determines the mode of cell death after IR injury in transplanted lungs and how various factors such as ischemic time might influence the amount of cell death that occurs. In a rat lung transplant model, we determined that apoptosis is the predominant mode of cell death in transplanted and reperfused lungs after short and clinically relevant ischemic times (6–12 hours), whereas in transplanted lungs after extended cold ischemic periods (18 and 24 hours) necrosis is the predominant mode of cell death with almost no apoptosis. The total amount of dead cells in grafts, however, was similar in all study lungs.⁷ Interestingly, the amount of necrotic cells negatively correlated with posttransplant graft function, but the degree of apoptosis did not.⁷

From this study we learned that severe graft injury, experimentally induced by a prolonged pretransplant graft ischemic period, appears to be associated with severe cell damage with no ability for the damaged cell to recover. The severely injured cells undergo an uncontrolled and undirected pathway to death, which is necrosis. The cell membranes disrupt, intracellular proteins are released in the surrounding tissue, and a massive inflammatory response is initiated that affects neighboring healthy cells, which may be secondarily injured and may also undergo cell death. In case of milder IR injury, such as in lungs after short ischemic times, however, the injured cells appear to have preserved their ability to either recover or, if unable to recover, to undergo the more quiescent mode of cell death—apopto-

sis—by initiating internal genetic suicide programs, which follow complex pathways.⁸

In another study, we transfected donor rat lungs transtracheally in vivo with adenoviral vectors carrying the gene that encodes for the human anti-inflammatory cytokine interleukin 10 (IL-10), also named *cytokine release inhibitor factor*. Indeed, we were able to demonstrate that IR injury in IL-10-transfected lungs was significantly improved compared with lungs that were transfected with the empty vehicle or the vehicle diluent only.⁹ This positive effect was seen in physiologic lung function, histologic examination, and the expression of the proinflammatory cytokines interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), which are early-onset mediators of IR injury.⁹ The fact that IL-10 gene therapy improves the function of the transplanted lung having been demonstrated, the goal of the current study was to determine whether this intervention had any effect on the degree or mode of cell death associated with IR injury in lung transplantation.

Methods

Animals

Experiments were performed in male inbred (250–350 g) Lewis rats (Charles River Inc, Montreal, Quebec, Canada). All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985, US Government Printing Office, Washington, DC 20402-9325), and the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council on Animal Care. The experimental protocol was approved by the Animal Care Committee of the Toronto General Hospital Research Institute.

In Vivo Transfection Procedure

For the in vivo gene transfection procedure, donor animals were anesthetized in a halothane chamber, orotracheally intubated with a 14-gauge intravenous cannula, and connected to a volume-controlled ventilator (Harvard Rodent Ventilator, model 683, South Natick, Mass). All animals were ventilated with an inspired oxygen fraction (FIO₂) of 1.0 and a tidal volume of 10 mL/kg at 80 breaths/min.

A 1-mL syringe containing 0.5 mL of the transfection solution was connected to the lateral outlet of a 3-way stopcock placed in the circuit at the endotracheal catheter. For intratracheal injection, the ventilator outlet of the 3-way stopcock was closed and the solution was injected. Ventilation was then continued until the animal resumed spontaneous breathing (approximately 60 to 90 seconds after intratracheal injection). All transfected animals were kept in microisolators for 24 hours until graft retrieval. Food and water was supplied ad libitum.

Lung Transplantation Procedure

Harvest and storage. We used a rat left single lung transplant model. Donor rats were anesthetized by an intraperitoneal injection of 1 mL of sodium pentobarbital (Somnotol, MTC Pharmaceuti-

cals, Cambridge, Ontario, Canada) and intubated through a tracheostomy with a 14-gauge intravenous catheter. The tracheostomy tube was then connected to a volume-controlled ventilator (Harvard Rodent Ventilator, model 683) and the animals were ventilated at a rate of 70 breaths/min, tidal volume of 10 mL/kg, FiO_2 of 1.0, and a positive end-expiratory pressure (PEEP) of 2 cm H_2O . After this, a median laparo-sternotomy was performed and 300 USP units of heparin (Hepalean, Organon Teknika, Toronto, Ontario, Canada) was injected into the inferior vena cava (IVC). After a period of 5 minutes, 0.5 mL of arterial blood was taken from the abdominal aorta for baseline blood gas analysis.

For the retrieval of the heart-lung block, the IVC was incised, the left atrial appendage was excised, and a 14-gauge intravenous catheter was placed into the main pulmonary artery (PA) through an anterior incision in the right ventricular outflow tract. The lungs were then flushed through this catheter with 20 mL of low-potassium dextran glucose (LPDG) preservation solution (Perfadex, Biophausia, Uppsala, Sweden) containing 500 $\mu\text{g/L}$ of prostaglandin E_1 (PGE-1, Prostin VR, Upjohn, Don Mills, Ontario, Canada) from a height of 30 cm. This is the same preservation protocol that is currently used in our clinical lung transplant program.⁹ Immediately after the lungs were flushed, the tracheostomy tube was clamped after inspiration to preserve the lungs in the inflated state. The heart-lung block was then removed and placed in iced LPDG at 4°C. The left lung was prepared for transplantation with the placement of three 14-gauge cuffs into the left PA, left pulmonary vein (PV), and the left main bronchus (MB), respectively. Left lungs were placed into 40 mL of LPDG at 4°C for 24 hours before transplantation.

Transplantation. Recipient animals were anesthetized and a tracheostomy was performed as described for the donor animals. The recipient animals were ventilated with a gas mixture of 75% oxygen and 25% room air at a rate of 70 breaths/min and tidal volume 10 mL/kg. A left thoracotomy was performed through the fifth intercostal space. The left lung was mobilized by dividing the pulmonary ligament. The hilar structures were then dissected free. The left PA, PV, and MB were identified and clamped with microsurgical aneurysm clamps. A ventral incision was made in each of these structures. The cuffs on the donor lung structures were placed into the corresponding recipient structures through each incision. The anastomoses were secured with 7-0 polypropylene ties. The implantation time, or warm ischemia time, was standardized at 20 minutes; this is the period when the deflated donor lung is in the chest at body temperature and the anastomotic procedure is underway. After the standardized 20-minute period of warm ischemia, the transplanted lung was reinflated after removal of the MB clamp and blood was reintroduced by unclamping first the PV, then the PA. The animal was ventilated with an FiO_2 of 1.0 at a rate of 70 breaths/min, tidal volume of 10 mL/kg, and PEEP of 2 cm H_2O during the 2 hours of the reperfusion period. One minute into the reperfusion period, each animal received 1 mL of 0.9% normal saline solution intraperitoneally for volume replacement. Heparin was not given to the recipient animals, because it is not routinely administered to lung transplant patients before graft reperfusion. Each animal was covered during the reperfusion period to prevent hypothermia. Two hours after graft reperfusion, 0.5 mL of blood was taken from the left PV of the transplanted lung distal to the cuff anastomosis for blood gas analysis.

Tissue Treatment

All study lungs were flushed for 5 minutes with 20 mL of a 500 $\mu\text{mol/L}$ trypan blue (Sigma Chemical Co, St. Louis, Mo) solution through the main PA, followed by 20 mL of 0.9% normal saline solution and 10 mL of 4% paraformaldehyde. Trypan blue was dissolved in Krebs-Henseleit buffer (pH 7.4; Sigma Chemical Co). The lungs were then fixed in 10% formalin. The middle third of the left lungs was used for histologic examination as that section is representative of peripheral and central parenchymal areas.

Histologic Evaluation and Viability Assessment: Triple Staining Technique

The triple staining technique for the quantification of apoptotic and necrotic cells as compared to all nucleated graft cells (dead and alive) in the same microscopic section has been previously described.⁷

In brief, formalin-fixed lung tissues were embedded in paraffin and cut into 4- μm tissue slices. These were mounted onto saline-treated glass slides for histologic assessment.

Apoptosis detection by in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) was undertaken using the ApopTag Kit (Oncor, Gaithersburg, Md) according to the manufacturer's instructions. Sections were deparaffinized and rehydrated. Protein digestion was carried out by application of proteinase K (20 $\mu\text{g/mL}$) to the slides for 15 minutes at room temperature followed by 4 washes in distilled water for 2 minutes each. Equilibration buffer was applied to the sections and these were incubated in a humidified chamber for 3 minutes. The sections were then incubated with TdT enzyme in a humidified chamber at 37°C for 1 hour. This method is based on the enzymatic ability of TdT to catalyze a template-independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA. Anti-digoxigenin-fluorescein was applied to the sections and these were then incubated in a humidified chamber for 30 minutes at room temperature. The sections were then washed with phosphate-buffered saline, and antifade containing propidium iodide (PI) (Oncor, Gaithersburg, Md) was applied for nuclear staining.

TUNEL-stained tissue sections were examined with fluorescent microscopy. First, the PI staining (red) was examined through a 520-nm filter at a magnification of 100 $\times$. PI stains (red) all nucleated cells (alive, necrotic, and apoptotic) in the same manner. The magnification was increased to 400 $\times$ and a color photomicrograph was taken. Then, the *same* area viewed for the PI staining was similarly examined for apoptotic staining (bright green) using a 590-nm filter at a magnification of 400 $\times$. After a color photomicrograph was taken through this filter system, the filters and the ultraviolet light were turned off and a third picture of the *same* area was taken to detect the trypan blue stain (dead cells) with standard light microscopy. Two randomly chosen areas from each slide were examined using this triple staining method.

The cell counts were performed by 2 researchers in a blinded fashion. The slides were projected onto a grid comprised of 12 fields so that the cells could be counted. Six randomly chosen fields were used. These 6 fields were used for all study slide counts. PI-stained cells were counted first, followed by TUNEL-positive cells, and finally the trypan blue-stained cells. Only cells that could clearly be identified as individual cells were counted as

cells. If they did not fulfill this criterion, they were considered to be background staining.

The PI-stained count represents the total number of nucleated graft cells (total cells: alive + necrotic + apoptotic). The TUNEL-positive count represents the number of apoptotic cells. The trypan blue count identifies the number of dead cells (necrotic + apoptotic). Therefore, the trypan blue count (necrotic + apoptotic) minus the TUNEL-positive count (apoptotic) equals the number of necrotic cells. The numbers of necrotic and apoptotic cells are given as percentages of the total number of cells (PI-stained count).

Optimization of hIL-10 Gene Transfection and Expression in Donor Lungs

IL-10 gene transfection of donor lungs and expression of the transfected protein were optimized in our previous studies on in vivo adenovirus-mediated gene transfection through the transtracheal route.⁹⁻¹²

Generation of Recombinant Adenovirus Expressing Human IL-10

Adenoviral vectors (serotype 5) containing the human IL-10 gene with an RSV promoter (Ad5RSVhIL-10) and “empty” vectors (Ad5BGL2) were constructed at the Gene Transfer Vector Core of the University of Iowa College of Medicine, Iowa City, Iowa. The hIL-10 adenoviral construct will be referred to as “AdhIL-10” and the Ad5BGL2 will be referred to as “empty vector.”

Human IL-10 (hIL-10) cDNA was obtained by polymerase chain reaction (PCR) with 5' and 3' flanking primers (5'-hIL-10BamHI:5'-CGCGGATCCCATGCACAGCT-CAGCACTG-3'; 3'-hIL-10BamHI:5'-CGCGGATCCGCCACCCTGATGTCTCAGT-3'), using the clone pSR α hIL-10 as template (kindly provided by E. Field, University of Iowa). The PCR product was cloned, using the BamHI restriction-site tails added to the oligonucleotide sequences, in a shuttle plasmid (pAdRSV4). This shuttle plasmid contains the Rous sarcoma virus promoter, the SV40-poly A signal, and the genomic adenoviral sequences from 0 to 1 and 9 to 16 map units of human adenovirus type 5. Recombinant adenovirus expressing IL-10 was generated by homologous recombination between pAdRSVhIL-10 and human adenovirus serotype 5 derivative d1309, using standard methods.¹³ AdBGL2 has the same viral backbone as AdRSVhIL-10.

In Vitro and in Vivo Evaluation of hIL-10 Bioactivity after Ad5RSVhIL-10 Gene Transfer

An in vitro functional assay for hIL-10 was performed by Gudmundsson and colleagues at the University of Iowa College of Medicine to test the activity of Ad5RSVhIL-10 on the basis of the ability of IL-10 to inhibit the synthesis of IFN- γ by lectin-stimulated spleen cells.¹³ The in vitro study showed that Ad5RSVhIL-10 is bioactive in vitro, and inhibits IFN- γ expression in lectin-stimulated murine spleen cells in a dose-dependent manner. To test the bioactivity of Ad5RSVhIL-10 in vivo, IL-10 knockout (KO) mice were injected with 5×10^8 pfu of Ad5RSVhIL-10 or AdRSVLacZ via a tail vein. Six days later hIL-10 levels in blood were detected by ELISA. In a separate experiment, IL-10 KO mice that received intravenous administration of Ad5RSVhIL-10 survived after intravenous lipopolysaccharide injection, whereas IL-10 KO mice that received AdRSVLacZ

Figure 1. In vivo transtracheal adenoviral mediated IL-10 gene transfection of donor lungs improves posttransplant lung function. Study groups: EV, empty vector; VD, vector diluent; IL-10, adenovirus-mediated interleukin-10. * $P = .0001$ versus other groups.

died of the endotoxin, which demonstrates the bioactivity of Ad5RSVhIL-10 in vivo.

Measurement of Lung Graft Function

Graft function was assessed by Pao₂ levels in blood taken from the graft pulmonary vein at the completion of the 2-hour reperfusion period under direct vision with a heparinized syringe.

Statistical Analysis

All data are expressed as mean $\pm$ SD. A 1-way analysis of variance (ANOVA) was used to determine statistical significance. When the test of equal variance or the normality test failed a Kruskal-Wallis 1-way ANOVA on ranks was performed. When statistical significance was reached, it was followed by a post hoc analysis using the Student-Newman-Keul method. The SigmaStat software package version 1.0 (Jandel Scientific, San Rafael, Calif) was used for all statistical analyses.

Results

Graft Function

Donor and recipient rats were size-matched in study groups. All animals in this study survived for the individual study periods. At the completion of the 2-hour reperfusion period, blood samples were taken from the pulmonary graft vein and analyzed for the partial pressure of oxygen (Pao₂). The ability of a transplanted lung to oxygenate blood remains a critical measurement for the evaluation of posttransplant pulmonary graft function.

The mean Pao₂ level in arterial blood from donors in all 3 study groups was 572 ± 41 mm Hg. The donor Pao₂ levels were not significantly different among the individual study groups. After the completion of the 2-hour reperfusion period, Pao₂ levels in IL-10 lungs were significantly higher ($P = .0001$) than in empty vector (EV) and vector diluent (VD) lungs (240 ± 31 vs 98 ± 17 mm Hg and 129 ± 11 mm Hg, respectively, Figure 1).

Figure 2. IL-10 in vivo donor lung transfection leads to a switch from necrosis to apoptosis in transplanted and reperfused lungs. Note that the total number of dead cells is similar in all groups, but that the AdIL-10 group has significantly less necrosis and more apoptosis (* $P = .0001$; # $P = .01$) compared with the other groups.

Cell Death

We have previously described the time course of apoptosis induction in transplanted lungs during ischemia and in the early phase after transplantation.³ We have further shown that the mode of cell death (apoptosis/necrosis) in lungs after transplantation is dependent on the length of the cold ischemic graft preservation time (CIT) before transplantation in that apoptosis appears after transplantation and reperfusion in lungs that have been stored for relatively short periods of CIT, whereas necrosis is the predominant mode of cell death in lungs after extended CIT.⁷ We also demonstrated that necrosis, but not apoptosis, correlates with posttransplant lung function.⁷ Cox has previously demonstrated that IL-10 enhances the resolution of pulmonary inflammation in vivo by promoting apoptosis of neutrophils.¹⁴ Furthermore, IL-10 has an inhibitory effect on TNF- α -induced programmed cell death, as shown by Rojas and associates.¹⁵

Apoptosis levels in the EV ($1.8\% \pm 2\%$) and VD ($2.0\% \pm 1.9\%$) group in this study were as low as seen in our previous study after similar CIT, when necrosis was the predominant mode of cell death.⁷ In the IL-10 group, however, apoptosis levels reached $9.7\% \pm 1.9\%$ of total graft cells, which was significantly different than the other 2 study groups ($P = .0001$). This in fact does not necessarily support a proapoptotic effect of IL-10. Rather, it suggests that IL-10 reduced the injury in lungs after prolonged preservation and transplantation and preserved the ability of damaged graft cells to undergo a less tissue destructive mode of cell death (apoptosis) as compared with necrosis, which causes further inflammation and tissue injury. Note that the total number of cell that died (through apoptosis or necrosis) was constant in all groups ($32.7\% \pm 3.2\%$ in EV,

$30.2\% \pm 2.5\%$ in VD and $30.3\% \pm 3.8\%$ in IL-10 lungs) as depicted in Figure 2. The number of necrotic cells was lowest in IL-10 lungs ($20.6\% \pm 5.7\%$) compared with VD lungs ($28.3\% \pm 3.1\%$) and EV lungs ($30.3\% \pm 4.2\%$), ($P = .01$).

Discussion

IR injury remains a significant problem and can result in pulmonary failure, multiorgan failure, and death after lung transplantation. Clinical and experimental studies have identified many of the mediators that are involved in the regulation of pulmonary IR injury.^{16,17}

We have previously described the role that apoptosis plays in lung transplantation.⁸ Ischemia has been shown to be a potential inducer of apoptosis.¹⁸ Because apoptotic cells in an organ system are usually phagocytosed by macrophages before their membranes break down and intracellular enzymes are released, this mode of cell death does not lead to significant tissue inflammation, which is characteristically prominent with necrosis.¹⁸ In human lungs, we have previously shown that apoptosis does not occur in grafts during cold or warm ischemia. In the early phase after graft reperfusion, however, the number of apoptotic graft cells increased to 34% of cells.³ As a consequence of this novel observation in lung transplantation, we have chosen to focus our efforts on the regulatory mechanisms that are involved in this dramatic loss of cells in lung grafts with the ultimate hope to develop strategies to prevent cell death in this setting.

However, we have demonstrated that, in addition to apoptosis, cellular necrosis also plays a very important, if not more important, role in posttransplant graft function and that the mode of cell death (apoptosis or necrosis) after transplantation is dependent on the length of ischemic graft preservation before lung transplantation.⁷ Interestingly, the amount of necrotic cells in transplanted lungs negatively correlated with posttransplant graft function, whereas the amount of apoptosis did not. This intriguing finding suggests that the 2 different modes of cell death differentially affect overall graft function. This is consistent with the general understanding that apoptosis is a relatively "quiescent" form of cell death as compared with necrosis, which induces significant inflammation and cytokine release. Obviously, organ transplantation at this stage is not possible without some injury to the graft or graft cells. Modern preservation strategies in clinical lung transplantation continue to focus on the reduction of graft preservation injury.¹⁹ However, our experimental and clinical observations suggest that graft cell injury still remains a significant problem. Ideally, we strive to minimize *all* cell death related to the injury of transplantation. However, if the process of transplantation inflicts a degree of injury such that an inevitable amount of cell death must occur, it would seem to be

protective to the organism overall if cells underwent apoptosis rather than necrosis.

IR injury is an insult to the transplanted lung that occurs at a predictable time point. This lends itself to the application of preventive strategies that can be applied before the insult occurs. We have previously described the protective effect of adenovirus-mediated transtracheal transfection of the human IL-10 gene to donor lungs in vivo on posttransplant lung function in the early phase after graft reperfusion.⁹ This strategy uses the graft's own cellular machinery to upregulate expression of a protective gene and to produce the desired protein like human IL-10 in the current study, such that it is available in appropriate protective quantities at the time of the initiation of the insult.

From experimental studies, it is evident that proinflammatory cytokines such as TNF- α and IFN- γ , produced by macrophages, epithelial cells, and monocytes, play important key roles in IR injury in lung transplantation.²⁰ We have shown that IL-10 gene transfection of donor lungs leads to a significantly reduced synthesis and release of proinflammatory cytokines in transplanted lungs after 24 hours of cold preservation⁹ and that, as a result of the severe insult during 24-hour preservation, the majority of dead graft cells after transplantation are necrotic.⁷

Because IL-10 is widely known to inhibit the release of these proinflammatory cytokines, we hypothesized that reduction of the inflammatory response by IL-10 gene therapy in this rat model of severe posttransplant lung injury might affect the amount of cell death and may improve lung function. What we have found is indeed intriguing and not what one might have expected. Although the total number of dead cells does not change in the IL-10-treated group compared with the control groups, in a third of all dead graft cells the mode of cell death was switched from necrosis to apoptosis in the IL-10-transfected lungs. This suggests that possibly by the reduction of the severity of the inflammatory response to IR injury, IL-10 gene upregulation significantly protected the graft such that a significant amount of cells, which were perhaps irreversibly damaged beyond recovery, retained their ability to undergo apoptosis, rather than necrosis.

This study once again underscores the importance of improving our understanding of the mechanisms of cell death in transplantation medicine. In future studies the underlying regulatory pathways of the observed switch from necrosis to apoptosis need to be clarified in greater depth to develop more specific approaches to the prevention of cell death in lung grafts and to prolong the maximally tolerable ischemic time for lungs, which eventually will help to increase the number of donor organs available and the success of clinical lung transplantation.

In summary, transtracheal transfection of the lung can be used to genetically modify donor lungs to express a desired

protein before transplantation and graft reperfusion. We have shown that the in vivo transtracheal delivery of the gene that encodes for IL-10 to donor rat lungs significantly improves graft function after lung transplantation. This improved function is associated with a switch from cellular necrosis to apoptosis in a significant number of graft cells.

The viruses used in this study (AdhIL-10 and AdBGL-2) were prepared by the Gene Vector Core (Director: B. Davidson, PhD) of the University of Iowa Faculty of Medicine, Iowa City, Iowa. We also acknowledge the professional assistance by Dr Beverly Davidson (Gene Vector Core of the University of Iowa Faculty of Medicine, Iowa City, Iowa) for her advice on planning the experiments and discussing the results and Dr Ioan Mates (DVM) for his support in conducting the animal procedures.

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Discussion

Dr Frank W. Sellke (*Boston, Mass*). With the total number of dead cells the same between groups, how do you explain the dramatic functional difference? Second, did you evaluate the expression of inflammatory cytokines such as TNF- α and IL-2 to see whether the IL-10 transfection had an effect in this area?

Dr Fischer. Regarding the second question, we have measured the expression of TNF- α , not in this study but in the first IL-10 study that I mentioned, and TNF- α and other proinflammatory cytokines were indeed significantly lessened regarding their expression.

The first question was regarding lung function, why we see such a difference in lung function even though we have the same amount of cell death. I think it is not only dependent on the number of cells that are there but also on the inflammatory response around the cells. A cell may be in an area that is very inflamed. These areas may not be well perfused with blood, and that might be a reason for this huge difference in lung function.

Dr Sellke. Did you evaluate the inflammatory response histologically? Was there a difference between the groups? Did you observe more inflammation and membrane thickening, which may impair lung function in the absence of cell death?

Dr Fischer. We saw much more hemorrhage on simple hematoxylin-eosin staining, and we saw much more edema. Also, wet/dry weight ratios indicated a significant difference in the groups.

Dr G. Alexander Patterson (*St. Louis, Mo*). I have a follow-up question to Dr. Sellke's. With 30% of cells dead, it is hard to understand that the lung is actually functioning so well. Which cells are dead? You put the animals to death 2 hours after the reperfusion. Do you have any data to indicate what the story would

be if you put the animals to death at 1 hour or 12 hours or 24 hours? It seems like a very brief snapshot at one time point.

Dr Fischer. Thank you very much. Your question regarding which cells undergo cell death is a very important one. First, we were surprised by the high number of dead cells that we discovered in transplanted human lungs. We looked at the dead cells by electron microscopy and found that the vast majority of dead cells or apoptotic cells were alveolar type II cells. We saw some interstitial cells and we also saw apoptotic neutrophils, which may not be native cells but immigrated cells.

Regarding the second question, we have looked at later outcome after 1 day of transplantation and reperfusion in the IL-10 study. We found, first, that gene expression continued after transplant, either cold preservation or transplantation, but we have not looked at cell death at this moment after a day. It is a very important point, and ongoing studies now are definitely focusing on the question; whole lung with 30% dead cells recovers from this dramatic injury, and I think that is a very important and complex problem by itself. We have actually several ongoing studies focusing on this recovery story.

Dr Minoru Ono (*Tokyo, Japan*). I am worrying about the suppression of the inflammatory cytokine protection induced by IL-10. It has other proinflammatory cytokine effects. Did you check the survival model using the IL-10 while in transfection? If you did not check the survival model, do you have any idea about the IL-10 effect on the infection of the transplanted lung?

Dr Fischer. Is your concern that the IL-10 expression does not reduce the release or expression of proinflammatory cytokines?

Dr Ono. No. My concern is that IL-10 induction may cause the suppression of the inflammatory cytokine expression, including IL-6 or TNF- α or IL-2. That may impair the resistance to infection in the survival model.

Dr Fischer. In the first IL-10 study that I mentioned we performed several survival studies in pilot studies, and we found that the suppression of the inflammatory response by IL-10 gene transfection continues until day 1 after transplantation. We have not performed survival studies beyond the first day of reperfusion because that is probably another point that has to be focused on, which was not the initial question of our study. That is the only information I can provide so far to your question.