Inhibition of nuclear factor κ B by I κ B superrepressor gene transfer ameliorates ischemia-reperfusion injury after experimental lung transplantation

Takaaki Ishiyama, MD,^a Sekhar Dharmarajan, MD,^a Makio Hayama, MD,^a Hisao Moriya, PhD,^b Kathleen Grapperhaus, BSc,^a and G. Alexander Patterson, MD, FRCS(C)^a

Objectives: Ischemia-reperfusion injury after lung transplantation is associated with significant morbidity and mortality. The activation of the transcription factor nuclear factor κ B is central to the 2 important pathways that characterize ischemia-reperfusion injury, namely the inflammatory response and apoptosis. The purpose of this study was to determine the effects of nuclear factor κ B inhibition on experimental lung transplant ischemia-reperfusion injury with gene transfer of the nuclear factor κ B inhibitor I κ B in a superrepressor form (I κ BSR).

Methods: An orthotopic left lung transplant model in isogeneic rats was used, with 18 hours of prolonged cold storage of donor lung grafts used to create severe ischemia-reperfusion injury. Donor rats underwent endobronchial gene transfection with saline alone or adenovirus encoding either β -galactosidase control or I κ BSR 48 hours before harvest. The function of transplanted lung grafts was assessed on the basis of isolated graft oxygenation, wet/dry lung weight ratio, and myeloperoxidase activity. Nuclear factor κ B activation was assessed by means of enzyme-linked immunosorbent assay. Apoptotic cell death was assessed by evaluating the levels of histone-associated DNA fragments and caspase-3 activity.

Results: Treatment of donor lung grafts with I κ BSR resulted in significantly improved oxygenation compared with that seen in control tissue 24 hours after transplantation. I κ BSR-treated lungs also demonstrated less pulmonary edema and reduced neutrophil infiltration 24 hours after reperfusion. Nuclear factor κ B activation and apoptotic cell death induction 2 hours after transplantation was significantly reduced in I κ BSR-treated lungs compared with in control lungs.

Conclusions: Inhibition of nuclear factor κ B activation by I κ BSR gene transfer improves transplanted lung graft oxygenation, decreases pulmonary edema and neutrophil sequestration, and reduces apoptotic cell death after experimental lung transplantation.

From the Division of Cardiothoracic Surgery, Department of Surgery,^a and the Department of Genetics,^b Washington University School of Medicine, St Louis, Mo.

Supported by National Institutes of Health grants RO1 HL41281 (GAP), NIH-NRSA 1 F32 HL074487-0 (SD).

Received for publication July 16, 2004; revisions received Feb 2, 2005; accepted for publication Feb 16, 2005.

Address for reprints: G. Alexander Patterson, MD, 3108 Queeny Tower, One Barnes-Jewish Hospital Plaza, St Louis, MO 63110-1013 (E-mail: pattersona@msnotes.wustl.edu).

J Thorac Cardiovasc Surg 2005;130:194-201
0022-5223/\$30.00

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doi:10.1016/j.jtcvs.2005.02.040

Lung transplantation has gained widespread acceptance for the treatment of many end-stage pulmonary diseases. Improvements in operative techniques, donor management, and immunosuppressive protocols have resulted in better short- and long-term survival after lung transplantation.¹ Despite of these improvements, however, ischemia-reperfusion (I/R) injury continues to cause significant morbidity and mortality in lung transplantation.

I/R injury after lung transplantation is a form of acute lung injury characterized by a dynamic and well-orchestrated combination of events involving a number of cell types and cytokine mediators. I/R injury after lung transplantation is thought to be a biphasic process. The initial phase of injury occurs soon after reperfusion (within 30 minutes) and is mediated primarily by activation of resident alveolar macrophages in the donor lung, which then produce several proinflammatory

cytokines and chemokines, including tumor necrosis factor (TNF) α , interleukin (IL) 1β , IL-8, and macrophage inflammatory protein 2α . These mediators result in the recruitment and activation of recipient neutrophils to the transplanted lung, eliciting a subsequent late phase of injury that generally occurs 4 to 6 hours after reperfusion. Activated neutrophils produce a number of proteolytic enzymes and reactive oxygen species that produce end-organ damage in the allograft.^{2,3}

Two important pathways that contribute significantly to the pathogenesis of I/R injury are the proinflammatory response and cell death in the form of apoptosis. In recent years, mechanisms leading to increased gene expression and biosynthesis of proinflammatory and apoptotic mediators have been well characterized. Nuclear factor κ B (NF κ B) is one such important rapid-response transcription factor that is critical to both the regulation of apoptosis and the maximal expression of many proinflammatory mediators.⁴ The activation of NF κ B has been shown to be central to the development of pulmonary inflammation and important in the pathogenesis of acute lung injury typical of I/R injury. In lung transplantation Ross and colleagues⁵ recently reported that NF κ B activation is an important event in initiating I/R injury in a porcine lung transplant model.

In unstimulated cells, NF κ B is bound to an inhibitor, I κ B, in the cytoplasm. I κ B prevents NF κ B from entering the nucleus, where it regulates gene transcription.⁶ When cells are stimulated, activation signals, such as reactive oxygen species, IL-1, and TNF- α , lead to signaling pathways involving the NF κ B/I κ B complex. By a specific kinase named IKK, I κ B is phosphorylated, then ubiquitinated, and finally rapidly degraded by proteasomes. After degradation of I κ B, NF κ B is released and enters the nucleus, where it binds to specific sequences in the promoter regions of target genes. The rapid transcription of proinflammatory cytokines (TNF- α , IL-1, and IL-6) and chemokines (IL-8 and macrophage inflammatory protein 1) begins, and these mediators stimulate neutrophil recruitment. Furthermore, NF κ B induces the production of cellular adhesion molecules (intercellular adhesion molecule 1 and E-selectin), which promote the binding and emigration of sequestered neutrophils. A positive feedback loop is created because IL-1 and TNF- α are important activators of I κ B, but they are also targets for NF κ B-mediated transcription. In addition, NF κ B regulates gene expression in apoptosis.^{3,6}

Programmed cell death, or apoptosis, can be initiated by a wide variety of stimuli, including I/R injury. Apoptosis has been reported to occur in animal models of I/R injury in the heart, kidney, retina, brain, liver, and adrenal gland.⁷ In both experimental and clinical lung transplantation, it has been shown that up to 30% of alveolar type II pneumocytes undergo apoptotic cell death after reperfusion.⁸ The pres-

ence of apoptosis and its effect on lung function after transplantation is still unclear, however.

In different cell types, NF κ B transcription has been reported to regulate the apoptosis pathway by either inducing or inhibiting it.⁴ Whether NF κ B promotes or inhibits apoptosis depends on the specific cell type and the inducing stimulus.⁴ A number of studies report that NF κ B activation can have a proapoptotic effect by means of downregulation of expression of antiapoptotic members of the bcl-2 gene family.^{9,10} Although some recent studies have reported on the separate contributions of both NF κ B activation and apoptosis to I/R injury in lung transplant models,¹¹ the effect of NF κ B activation on levels of apoptosis and lung function after lung transplantation has not been definitively studied.

We have recently demonstrated that several gene transfer strategies, such as overexpression of IL-10, heat shock protein 70, or soluble inhibitory receptors for TNF- α , protect lung grafts from I/R injury.¹²⁻¹⁷ Preliminary data from our laboratory indicate that these strategies act in part through a final common pathway of NF κ B inhibition. A superrepressor form of I κ B α (I κ BSR) containing serine-to-alanine mutations at amino acids 32 and 36 has been described, which inhibits signal-induced phosphorylation and subsequent degradation of I κ B α .¹⁸ In this study we investigated the effect of NF κ B inhibition by I κ BSR gene transfer on I/R injury after experimental lung transplantation. We also attempted to further elucidate the complex relationship between NF κ B activation, levels of apoptotic cell death, and graft function in lung transplantation.

Materials and Methods

Animals

Fischer 344 rats (Harlan Sprague Dawley, Inc, Indianapolis, Ind), weighing 250 to 280 g, were used in all experiments. All animal procedures were approved by the Animal Studies Committee at Washington University in St Louis. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Adenoviral Vectors

Adenovirus encoding I κ B superrepressor. Adenovirus encoding I κ B superrepressor (Ad5I κ BSR) is a recombinant, replication-deficient adenovirus encoding a dominant negative superrepressor form of I κ B. It is derived from E1-deleted adenovirus type 5 and driven by the constitutive cytomegalovirus promoter. The I κ B gene contains an extra 27-bp DNA nucleotide coding for a peptide derived from the hemagglutinin gene to discriminate between endogenous and exogenous I κ B. Ad5I κ BSR was purchased from the Gene Therapy Center Viral Vector Core Facility, University of North Carolina, with permission from Dr David A. Brenner.

Adenovirus encoding β -galactosidase. Ad5LacZ is an adenovirus control vector that produces a nonfunctional carbohydrate product. First-generation replication-deficient adenovirus serotype 5 carrying the *Escherichia coli* LacZ gene encoding for β -galactosidase (β -gal) and driven by the constitutive cytomegalovirus promoter was used in this study. Ad5LacZ was purchased from the Gene Therapy Center Viral Vector Core Facility at the University of North Carolina, Chapel Hill, North Carolina.

Purified viral aliquots were stored at -80°C in 10% glycerol buffered with 10 mmol/L tris (hydroxymethyl) aminomethane, 140 mmol/L sodium chloride, and 1 mmol/L magnesium chloride. These stocks were thawed and diluted in 0.1 mL of sterile isotonic sodium chloride solution immediately before use.

Donor Endobronchial Gene Transfection

Endobronchial gene transfection in rats is a technique that was developed in our laboratory and has been reported by us in detail previously.¹⁵ In brief, animals were anesthetized, intubated, and mechanically ventilated, and a right thoracotomy was performed. The carina was dissected, and a catheter was introduced through the endotracheal tube selectively into the left main bronchus. Adenoviral vector, 2×10^7 plaque-forming units, encoding either β -gal or I κ BSR or 0.1 mL of saline (vehicle alone) was then instilled through the catheter. The dose of 2×10^7 plaque-forming units was chosen on the basis of previous studies in our laboratory.¹⁵ After 10 minutes of bilateral ventilation after instillation, the left main bronchus was clamped for 60 minutes at the end-inspiratory phase of mechanical ventilation. After the left main bronchus was unclamped, the right thoracotomy was closed, and a temporary chest tube was placed. The chest tube was removed after recovery from anesthesia. Forty-eight hours after transfection, donor lungs were harvested for all experiments.

Orthotopic Left Lung Transplantation

We used the modified cuff technique for orthotopic left lung transplantation, which is well established in our laboratory and has been described in detail in previous reports.¹⁹ In brief, after general anesthesia with intraperitoneal pentobarbital (65 mg/kg), mechanical ventilation, systemic heparinization (300 units), and median laparosternotomy, donor rat lungs were flushed through the main pulmonary artery with 20 mL of cold (4°C) low-potassium dextran glucose solution at 20 cm H_2O pressure. The heart-lung block was then removed with the lungs inflated at end-tidal volume. The left lung graft was isolated, prepared, and stored in low-potassium dextran glucose at 4°C until transplantation.

For transplantation, recipient animals were anesthetized, intubated with a 14-gauge catheter, and underwent a left thoracotomy. The pulmonary vessels and bronchus were anastomosed with a modification of the previously described cuff technique.¹⁹

Functional Assessment of Transplanted Lung Grafts

Recipient rats were killed, and the function of left lung grafts was assessed 24 hours after transplantation. Recipient animals in each group were reanesthetized with pentobarbital. After tracheostomy, the animals were mechanically ventilated with 100% oxygen, and laparosternotomy was performed. The right main bronchus and pulmonary artery were clamped to isolate the left lung graft. The animals were ventilated for 5 minutes at a tidal volume of 1.5 mL,

a respiratory rate of 100 breaths/min, and 1.0 cm H_2O of positive end-expiratory pressure. Arterial blood gas analysis was performed with blood samples obtained from the ascending aorta. The lungs were flushed with 20 mL of cold (4°C) saline solution, and the lung graft was excised and divided into sections.

Myeloperoxidase Activity

Quantitative myeloperoxidase activity was determined as previously described.²⁰ Optical density was measured at 460 nm with a spectrophotometer (model PMQ II; Carl Zeiss, Oberkochen, Germany). Color development was linear from 5 minutes to 20 minutes. One unit of enzyme activity was defined as 1.0 optical density unit per minute per milligram of tissue protein at room temperature.

NF κ B p65 Transcription Factor Assay

NF κ B activation was measured in nuclear extracts of lung homogenates by a nuclear extraction kit (Active Motif North America, Carlsbad, Calif) per the manufacturer's protocol.

NF κ B binding to κ B sites was assessed by using the Trans-AM NF κ B p65 transcription factor assay kit (Active Motif North America), according to the manufacturer's instructions. In this assay an oligonucleotide containing the NF κ B consensus site is attached to a 96-well plate. The active form of NF κ B contained in cell extracts specifically binds to this oligonucleotide and can be revealed by incubation with antibodies by using enzyme-linked immunosorbent assay technology with absorbance reading.²¹

Determination of Histone-Associated DNA Fragments and Caspase-3 Activity

To evaluate induction of apoptosis, levels of histone-associated DNA fragments were determined in lung homogenates.²² DNA fragmentation was quantified by measuring histone-associated DNA fragments with an ELISA kit (Cell Death Detection ELISA^{plus}; Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Lung samples were homogenized with T-PER Extraction Reagent (Pierce Biotechnology, Rockford, Ill). To confirm results obtained with ELISA, active caspase-3 activity was determined in situ by means of immunohistochemistry with the Biotin Tyramide Signal Amplification kit (Perkin-Elmer Life Sciences, Boston, Mass).

Statistical Analysis

All values are described as means $\pm$ standard deviation. Statistical analysis was performed with 1-way analysis of variance for multiple group comparisons, with pairwise comparisons performed with the Fisher protected least significant difference (PLSD) test. Student *t* tests were used for 2-group comparisons. All analyses were performed on StatView software (SAS Institute, Inc, Cary NC).

Results

NF κ B Activation and Apoptotic Cell Death in Lungs After Cold Preservation and Transplantation

The activation of NF κ B was measured at various time points after reperfusion after 18 hours of cold preservation to evaluate the time point of maximal NF κ B activation in

Figure 1. Time course of NFκB activation in I/R injury after lung transplantation. After 18 hours of cold preservation, donor lung grafts were transplanted into recipients that were killed at 30 minutes and 1, 2, 4, 8, and 24 hours after reperfusion ($n = 3$ each). At the end of the reperfusion period, the lungs were flushed with 20 mL of cold (4°C) saline solution, and the transplanted lung graft was excised and frozen in liquid nitrogen for the Trans-AM NFκB p65 transcription factor assay. Nuclear extracts were analyzed by this assay to study activation levels of NFκB. NFκB activation occurs as soon as 30 minutes after reperfusion and is maximal between 30 minutes and 4 hours after reperfusion. * $P < .05$ versus the Normal, 8h, and 24h groups. OD, Optical density; 18hCP, 18 hours of cold preservation.

our model. NFκB was activated 30 minutes after reperfusion, and there was no significant difference in NFκB activation between lung grafts from the time points between 30 minutes after reperfusion and 4 hours after reperfusion (Figure 1). Maximal NFκB activation was detected from 30 minutes to 4 hours after reperfusion.

To examine the relationship between NFκB activation and apoptosis induction in I/R injury after experimental lung transplantation, we used quantitative analyses for both NFκB activation and apoptosis in lungs after cold preservation for various time periods and subsequent transplantation. All lungs were sampled after 2 hours of reperfusion because this has been shown to be the time point of maximal NFκB activation (Figure 1) and apoptotic cell death.¹¹ Figure 2 shows NFκB activation and apoptotic cell death induction in transplantation after varying amounts of cold preservation, ranging from 1 hour (immediate transplantation) to 18 hours. Additional groups titled Normal and 18hCP were used to study normal lungs and lungs after 18 hours of cold preservation alone without transplantation.

These experiments revealed an interesting difference between the activation of NFκB and apoptotic cell death in transplanted lungs. NFκB activation and apoptotic cell death are not observed during cold preservation for 18 hours without transplantation (compare the 18hCP group between Figure 2, A, and Figure 2, B). However, NFκB activation begins after transplantation alone with minimal cold pres-

Figure 2. NFκB activation and apoptosis induction after cold preservation and transplantation. Three lungs were flushed with 20 mL of cold (4°C) saline solution, were harvested, and underwent cold preservation alone at 4°C for 18 hours without transplantation. Groups of 3 rats were transplanted with lungs stored at 4°C for 1, 6, 12, and 18 hours by using the modified cuff technique for left lung transplantation. All lungs were sampled after 2 hours of reperfusion. A, The activity of NFκB in the lower third of the lung was analyzed as described in the "Materials and Methods" section. NFκB is activated by the process of transplantation or reperfusion alone, and there is no significant activation of NFκB during cold preservation. * $P < .05$ versus the Normal and 18hCP groups. B, Induction of apoptotic cell death in the middle third of the lung was measured as described in the "Materials and Methods" section. Apoptosis is minimal after cold preservation alone or reperfusion after minimal cold preservation (1 hour), but apoptotic cell death becomes significantly upregulated after reperfusion after 6 hours of cold preservation. * $P < .05$ versus the Normal, 18hCP, and 1h groups. OD, Optical density; 18hCP, 18 hours of cold preservation.

ervation (Figure 2, A, 1-hour group), whereas activation of apoptotic cell death is not observed at this time (Figure 2, B, 1-hour group). Significant NFκB activation and apoptotic cell death are observed in transplanted lungs with more than 6 hours of cold preservation (Figure 2, A, 6 hours to 18 hours, and Figure 2, B, 6 hours to 18 hours). These data indicate that events occurring because of transplantation itself (minimum cold preservation) can trigger the activation of NFκB, whereas additional events occurring during cold preservation are required to trigger apoptotic cell death. In

Figure 3. The influence of IκBSR gene transfection on NFκB activation in lung I/R injury. Male Fischer 344 rats were divided into 2 groups according to the transfecting vector. IκB-treated donors (n = 6) received Ad5IκBSR, and β-gal-treated donors (n = 6) received Ad5LacZ. Forty-eight hours after transfection, donor lungs were harvested and preserved for 18 hours at 4°C and then implanted into isogeneic recipients. Recipient rats were killed, and lung grafts were assessed 2 hours after transplantation. Two hours after reperfusion, the lungs were flushed with 20 mL of cold (4°C) saline solution, and the lung graft was excised and divided into sections. NFκB activity was measured as described in the "Materials and Methods" section. NFκB activation was significantly reduced in lungs transfected with IκB. **P* < .05 versus β-gal.

this study, the induction of apoptotic cell death occurred in lungs after 6 hours or more of cold preservation and not in lungs preserved only for 1 hour, although it is unknown whether any time points between 1 and 6 hours of cold preservation would be sufficient to induce apoptotic cell death.

NFκB Inhibition Ameliorates Lung Transplant I/R Injury

Functional assessment of recipient lung grafts was performed 24 hours after reperfusion to examine whether direct inhibition of NFκB activity by IκBSR gene transfer ameliorates lung transplant I/R injury. To evaluate the influence of IκBSR on NFκB activation in I/R injury after experimental lung transplantation, we analyzed the activation of NFκB. Reduced activation of NFκB in lungs transplanted after IκBSR gene transfection was confirmed (Figure 3). Next we confirmed the improvement of transplanted lung function after Ad5IκBSR gene transfection by isolated graft oxygenation (Figure 4, A), myeloperoxidase activity (Figure 4, B), and wet/dry lung weight ratio (Figure 4, C).

Isolated graft oxygenation for the IκBSR-treated group was significantly higher than that for the saline-treated and β-gal-treated groups (Figure 4, A). Myeloperoxidase activity is a reflection of tissue neutrophil sequestration. Myeloperoxidase activity levels were lower in the IκBSR-treated group than in the saline-treated and β-gal-treated groups, although this did not reach statistical significance (Figure 4,

Figure 4. The influence of IκBSR gene transfection on lung function in I/R injury. Male Fischer 344 rats were divided into 3 groups according to the transfecting vector. IκB-treated donors (n = 5) received Ad5IκBSR, β-gal-treated donors (n = 5) received Ad5LacZ, and saline-treated donors (n = 5) received vehicle alone (isotonic sodium chloride solution). Forty-eight hours after transfection, donor lungs were harvested and preserved for 18 hours at 4°C and then implanted into isogeneic recipients. Recipient rats were killed, and lung grafts were assessed 24 hours after transplantation, as described in the "Materials and Methods" section. Isolated graft oxygenation was significantly improved in IκB-treated lungs compared with in β-gal- or saline-treated lungs (A). The middle third of the lung graft was frozen in liquid nitrogen for myeloperoxidase activity assay (B). Myeloperoxidase activity was lower in IκB-treated lungs than in either of the control groups. The lower third of the graft was weighed, dried at 80°C for 48 hours, and then reweighed for calculation of the wet/dry weight ratio (C). Wet/dry weight ratios of IκB-treated lungs were lower than those of β-gal-treated or saline-treated lungs. A, **P* < .05 versus IκBSR. B, **P* = not significant versus IκBSR. C, **P* = not significant versus IκBSR. MPO, Myeloperoxidase; OD, optical density.

Figure 5. The influence of IκBSR gene transfection on apoptosis induction in lung I/R injury. **A**, Apoptotic cell death was measured as described in the “Materials and Methods” section by using the same lung samples as used in Figure 3. Apoptotic cell death was significantly reduced in IκB-treated lungs compared with in β-gal-treated control lungs. * $P < .05$ versus β-gal. **B**, Active caspase-3 activity, a marker for apoptotic cell death, was identified as described in the “Materials and Methods” section. Staining for caspase-3 was significantly less in IκB-treated lungs than in β-gal-treated control lungs, histologically confirming quantitative results obtained by means of enzyme-linked immunosorbent assay. OD, Optical density.

B). The wet/dry lung weight ratio is an indicator of tissue edema. Lungs in the IκBSR-treated group had lower wet/dry lung weight ratios than those of either the saline-treated or β-gal-treated groups, although this difference also did not reach statistical significance (Figure 4, *C*).

Apoptosis Induction Was Significantly Reduced After IκBSR Gene Transfection

To evaluate the influence of NFκB activation on apoptosis in I/R injury after experimental lung transplantation, we analyzed the induction of apoptotic cell death in the IκBSR-treated and β-gal-treated groups. Apoptotic cell death, as measured by means of histone-associated DNA fragments, was significantly reduced in the group treated with IκBSR (Figure 5, *A*). Immunohistochemical analysis for active caspase-3 activity, another indicator of apoptotic cell death, also showed a marked decrease in apoptotic cell death in the IκBSR-treated group (Figure 5, *B*). These data show that NFκB inhibition reduces apoptotic cell death in I/R injury after experimental lung transplantation.

Discussion

Inhibition of NFκB activation ameliorates lung I/R injury. We have demonstrated that several gene transfer strategies protect lung grafts from I/R injury.¹²⁻¹⁷ Preliminary data from our laboratory suggest that these strategies act in part through the final common mechanism of inhibition of NFκB activation (unpublished results). In this study, we were able to demonstrate significant improvements in isolated graft oxygenation in transplanted lungs after the inhibition of

NFκB activation by IκBSR gene transfer in experimental lung transplant I/R injury (Figure 3). Ross and colleagues⁵ showed that inhibiting NFκB with a nonspecific NFκB inhibitor, pyrrolidine dithiocarbamate, attenuates I/R injury in a porcine lung transplantation model. Together with our findings, these results demonstrate that NFκB is a major factor in the pathogenesis of lung transplant I/R injury and might provide a useful target for therapy to prevent I/R injury.

We were unable to demonstrate a significant reduction in myeloperoxidase activity and wet/dry weight ratio in treated lungs in this study, despite a significant reduction in NFκB activation and apoptotic cell death in these grafts. It is unclear whether these results imply differential effects on apoptosis and inflammation pathways mediated by IκBSR gene therapy. The most likely explanation for these differences is that these factors were measured in transplanted grafts after 24 hours of reperfusion. We and others have been able to show that many of the inflammatory changes in transplanted grafts begin to resolve by 24 hours after reperfusion. Therefore, it is likely that neutrophil activity and pulmonary edema in untreated control subjects is beginning to improve by this point and therefore is not significantly different from that of treated lung grafts. Another factor that might have contributed to these differences is adenoviral distribution after transfection. We have shown in prior publications that adenoviral transfection after endobronchial transfection is patchy rather than homogenous, and therefore depending on which portion of transfected grafts was

used for different assays, effects of treatment on measured parameters might vary.¹⁵

NF κ B Functions as a Factor Promoting Apoptosis in Lung Transplantation

We demonstrated a significant reduction in apoptotic cell death after inhibiting NF κ B (Figure 5), suggesting that NF κ B activation contributes to I/R injury by promoting apoptotic cell death. Other researchers have shown that NF κ B promotes apoptosis in a variety of neuronal cell injury models.²³ Meldrum and associates²⁴ showed that NF κ B inhibition by pyrrolidine dithiocarbamate prevents simulated ischemia-induced renal tubular cell apoptosis and that NF κ B activation is a proapoptotic signal in their model of simulated ischemia. Our results are similar to these conclusions in our in vivo lung transplant I/R injury model.

Two Independent Stimuli Activate Apoptosis in Lung Transplantation

Our results indicate that significant NF κ B activation occurs after transplantation alone (with minimal cold ischemia), whereas apoptotic cell death is activated only after transplantation after cold preservation of at least 6 hours in our experiment (Figure 2). We were also able to demonstrate that apoptotic cell death is significantly reduced by NF κ B inhibition (Figure 5). Taken together, these data indicate that there are at least 2 events necessary to activate apoptotic cell death in transplanted lungs. One event is triggered by transplantation by itself, which is most likely through an NF κ B-dependent pathway because NF κ B is activated by transplantation by itself. The other event is triggered during cold preservation. The time of cold preservation required to induce apoptotic cell death was found to be 6 hours or more, although time points between 1 and 6 hours of cold preservation were not evaluated in this study. In an experimental model of lung transplantation, Fischer and coworkers²⁵ demonstrated that with shorter periods of ischemia (6 or 12 hours), the mode of cell death was primarily apoptosis. Our results also demonstrate that apoptotic cell death is induced by 6 hours or more of cold preservation and reperfusion. There are two known pathways for apoptosis: the mitochondrial pathway and the death receptor pathway. The suggestion of these two events being necessary for the induction of apoptotic cell death might somehow be related to each of these two different pathways.

In conclusion, this study demonstrates that NF κ B inhibition with gene therapy prevents the induction of apoptosis and ameliorates I/R injury in an experimental rodent lung transplantation model. The present results suggest that NF κ B activation is a major factor in inciting I/R injury in lung transplantation by acting as a factor promoting apoptotic cell death and that at least two signals, one of which involves NF κ B activation, might be necessary for the induction of apoptosis in lung transplantation. The application

of gene therapy as a therapeutic strategy for lung transplantation will become more realistic as we solve the complicated mechanisms of the inflammatory cascade and apoptosis.

We thank David A. Brenner, MD (University of North Carolina, Chapel Hill, NC), for providing adenovirus encoding IkBSR. We also thank Richard B. Schuessler, PhD, for his guidance regarding statistical analysis; Camila Pflederer for her invaluable assistance with the assays used in this study; and Takashi Suda, MD, and Tsutomu Tagawa, MD, for their assistance with the technique of rat lung transplantation.

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