Antisense Intercellular Adhesion Molecule-1 (ICAM-1) Oligodeoxyribonucleotide Delivered During Organ Preservation Inhibits Posttransplant ICAM-1 Expression and Reduces Primary Lung Isograft Failure

Koichi Toda, Koichi Kayano, Ann Karimova, Yoshifumi Naka, Tomoyuki Fujita, Kanji Minamoto, Catherine Y. Wang, David J. Pinsky

Abstract—Transiently increased expression of leukocyte adhesion receptors after lung preservation contributes to early graft demise by recruiting leukocytes, activating complement, and causing microcirculatory stasis. We hypothesized that inhibiting intercellular adhesion molecule-1 (ICAM-1) expression even briefly may significantly improve lung graft function and that the preservation period might provide a unique window to deliver a therapeutic pulse of antisense oligonucleotide ICAM-1 to inhibit ICAM-1 expression after transplantation. Interleukin-1β–treated rat pulmonary endothelial cells given a 20-mer phosphorothioate oligonucleotide comprising an antisense span targeted to the 3′-untranslated region of rat ICAM-1 demonstrated an oligonucleotide dose–dependent reduction in ICAM-1 expression. Using a cationic liposomal carrier, this same antisense oligonucleotide (but not the sense control) instilled into the pulmonary vasculature at the time of preservation reduced subsequent graft ICAM-1 expression and graft leukostasis and markedly improved oxygenation, pulmonary blood flow, and graft survival. These experiments demonstrate that the preservation period presents a window during which to target an anti–ICAM-1 expression strategy to inhibit early adhesion receptor expression and improve functional outcome after lung transplantation. (Circ Res. 2000;86:166-174.)

Key Words: intercellular adhesion molecule-1 ▪ lung transplantation ▪ isograft ▪ leukocyte adhesion receptor

Clinical lung transplantation at its best is a harrowing experience, because lung grafts can fail catastrophically shortly after reperfusion for reasons that are often not understood. Clinical lung preservation strategies are directed toward maintaining proper electrolyte and osmotic homeostasis, but surprisingly little is done to protect the vast pulmonary vascular network on which the lung depends for both integrity and function. Because the early expression of the leukocyte adhesion receptor P-selectin can result in rapid sequestration of neutrophils (polymorphonuclear leukocytes [PMNs]) after lung transplantation, promoting microcirculatory stasis and local tissue destruction, we hypothesized that a strategy that protects the lungs from early leukocyte recruitment could confer clinical benefit. Because P-selectin places neutrophils into a favorable steric relationship for intercellular adhesion molecule-1 (ICAM-1) binding to β2-integrins, we hypothesized that early inhibition of inducible ICAM-1 expression might serve as a useful target for therapeutic intervention in lung transplantation to prevent acute graft injury. Toward this end, we have focused on the unique opportunity provided by the lung harvest procedure, during which it is possible to deliver agents (such as antisense oligodeoxynucleotides) ex vivo directly into the vasculature, which can block adhesion receptor expression during the first few critical hours after reperfusion. The current studies were designed to (1) elucidate the nature and functional relevance of endothelial ICAM-1 expression in the setting of isogeneic lung transplantation, which has not been established to date, and (2) to test the hypothesis that inhibiting ICAM-1 by pulsed delivery of an effective antisense ICAM-1 oligodeoxynucleotide during lung preservation can effectively block early ICAM-1 expression and improve early lung graft function and survival after transplantation.

Materials and Methods

Preservation Solution
For all transplant experiments, the basic preservation solution consisted of modified EuroCollins solution, modified by adding magnesium sulfate and glucose. This represents the standard formulation used in clinical lung transplantation.

Sense and Antisense Oligonucleotides
Sense and antisense phosphorothioate oligonucleotides were chemically synthesized and purified with high-performance liquid chro-
matography. The ICAM-1 antisense oligonucleotide was designed against the 3’-untranslated region of the rat ICAM-1 gene\(^5,6\) and comprised the following sequence: 5’-ACC GGA TAT CAC ACC TTC CT-3’. The complementary sense sequence was 5’-AGG AAG TGT TGA TAT CCG GT-3’.

Cationic Liposomal Carrier
To transfect the oligonucleotides into cells or pulmonary grafts, a cationic liposomal carrier was used that has demonstrated efficacy and lack of toxicity\(^7\) in pulmonary endothelial cells. The liposome formulation chosen for these experiments was the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) to enhance the oligonucleotide uptake by cells.

Endothelial Cell Experiments
Rat pulmonary microvascular endothelial cells were a generous gift of Dr Una Ryan (Avant Immunotherapeutics, Needham, Mass).\(^8\) Cells were grown to 60% to 70% confluency in 10-cm Petri dishes before use. The oligonucleotides were prepared for use as detailed in the accompanying electronic supplemental material. The oligonucleotide/lipofectin mixture was added to the cells for 3 hours of incubation, after which recombinant murine interleukin (IL)–1\(\beta\) was added to the culture medium to a final concentration of 2.5 ng/mL. Cultures were then incubated for an additional 16 hours, at which point cells were harvested for ICAM-1 protein and mRNA measurements.

Lung Transplant Experiments
Experiments were performed according to an institutionally approved protocol and in accordance with AAALAC guidelines.

Donor Lung Harvest
Inbred male Lewis rats were used. Donors were heparinized, after which the right pulmonary artery (PA) was ligated to restrict delivery of preservation solution to the donor lung used for grafting, and 7 mL of 4\(^\circ\)C preservation solution was administered into the main PA at a constant infusion pressure of 20 mm Hg. The left lung was then harvested and submerged for 4 hours in 4\(^\circ\)C preservation solution.

Transplantation
Orthotopic left lung transplantation was performed as described.\(^6\) For all experiments, the preservation duration was identical (4 hours). However, depending on the particular experiment, reperfusion durations ranged from 30 minutes to 24 hours (indicated in the figure legends). For those experiments in which oligonucleotides were studied, the base preservation solution was supplemented with lipofectin followed by the addition of either the sense or the antisense oligonucleotide construct and prepared fresh for each experiment. After lung transplantation, hemodynamic measurements were obtained as previously reported,\(^9\) with instrumentation beginning just before the 6-hour reperfusion time point. Thirty minutes after ligation of the native right PA (or at the time of recipient death, if it occurred before 30 minutes), transplanted lung tissue was excised for myeloperoxidase analysis or determining ICAM-1 expression.

Immunoblotting for ICAM-1 Protein
Protein was extracted by washing, scraping, and sonicating cells in the presence of protease inhibitors.\(^10\) Integral membrane proteins were extracted using a modification of a previously reported method,\(^11\) and protein concentrations determined according to the Bradford method.\(^12\) Afterward, samples were prepared for nonreduced SDS-PAGE; ICAM-1 was detected using a primary mouse monoclonal anti-ICAM-1 IgG (1A29 clone), with subsequent densitometric analysis of developed bands.

Northern Blotting for ICAM-1 mRNA
Northern blotting was performed using total cellular RNA, with RNA species of interest detected with either a rat ICAM-1 cDNA probe or a human \(\beta\)-actin cDNA probe, with subsequent densitometric analysis of developed bands.

Myeloperoxidase Assay
Myeloperoxidase assay was performed using a chromogenic assay, as previously described,\(^13\) and data are expressed as change in absorbance at 460 nm.

Immunohistochemistry
Lungs were prepared for immunohistochemistry by submerging the lung tissue in cold saline under pressure to evacuate air and fill alveoli with saline, embedded, frozen, and sectioned. A primary mouse monoclonal anti-rat ICAM-1 IgG was used, after which a secondary fluorescent antibody was applied.

Statistics
Significant differences between groups were tested for with the Mann-Whitney \(U\) test. Animal survival data were analyzed by contingency analysis using the \(\chi^2\) statistic. Values are expressed as mean±SEM, with differences considered statistically significant if \(P<0.05\).

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
Increased Early Expression of ICAM-1 in the Rat Lung Transplant Model
To determine whether ICAM-1 expression is increased in the setting of lung transplantation, an orthotopic rat lung transplant model\(^9\) was used and ICAM-1 expression examined. After transplantation, there was a significant increase in ICAM-1 mRNA levels as early as 30 minutes after reperfusion, apparent both on a representative Northern blot (Figure 1A) and on multiple blots (\(n=5\)) scanned for relative densitometric calculation (Figure 1B). ICAM-1 mRNA levels peaked at 3 hours of reperfusion (171±27% increase versus fresh lung, \(P<0.01\)) and subsequently tapering off to control levels by 12 hours. Although an insignificant increase in ICAM-1 protein expression was observed at 30 minutes, a significant increase in ICAM-1 protein expression was observed at 3 hours, peaking at 6 hours after reperfusion (179±25% increase versus fresh lung, \(P<0.01\)). These data are shown in a representative immunoblot (Figure 1C) as well as with a statistical analysis performed on the relative densitometric scans performed on immunoblots from 6 separate experiments (Figure 1D). Immunohistochemical examination for ICAM-1 using a primary monoclonal mouse anti-rat ICAM-1 IgG was performed to localize the sites of increased ICAM-1 expression. ICAM-1, which is constitutively expressed at low levels in unperturbed pulmonary endothelial cells and type I alveolar epithelial cells,\(^14,15\) was expressed primarily in the structures of the alveolar wall in the fresh lung (Figure 2A). However, in the transplanted lung, there was an increased intensity of alveolar wall staining and the appearance of strongly stained microvessels \(\approx\)100 \(\mu\)m in size (Figure 2B). When control sections were subjected to immunostaining procedures in the absence of the primary antibody, no staining was observed (data not shown).

In Vitro Efficacy of Antisense ICAM-1 Oligonucleotide on Rat Pulmonary Microvascular Endothelial Cells
To test the functional significance of ICAM-1 expression in lung transplantation, and to determine whether the preserva-
tion period presents an opportunity for therapeutic administration of an antisense ICAM-1 oligonucleotide, we first tested the in vitro efficacy of a 20-mer phosphorothioate oligonucleotide comprising an antisense span targeted to the 3'-untranslated region of rat ICAM-1. As ICAM-1 increases after pulmonary ischemia, and IL-1β is a known potent stimulus for endothelial ICAM-1 induction in vitro, IL-1β was therefore used as an ICAM-1-inducing stimulus for these studies. After exposure to 2.5 ng/mL of IL-1β for 16 hours, rat pulmonary microvascular endothelial cells exhibited a strong induction of ICAM-1 mRNA and protein compared with untreated cells (Figure 3, leftmost 2 lanes of both panels A and B). Application of the antisense ICAM-1 oligonucleotide in a cationic liposomal carrier (lipofectin) inhibited ICAM-1 protein expression in an oligonucleotide dose-dependent fashion (Figure 3A); at the 20 μg/mL dose, antisense ICAM-1 oligonucleotide inhibited the IL-1β-induced increase in ICAM-1 expression by 380% (n=5, P<0.05). No significant reduction in ICAM-1 expression was observed in rat pulmonary microvascular endothelial cells treated with IL-1β and an ICAM-1 sense construct spanning the region identical to the one the antisense construct used (Figure 3A, rightmost lane).

Because antisense ICAM-1 oligonucleotides targeted to the 3'-untranslated region of the ICAM-1 transcript have been shown to decrease mRNA stability and thereby reduce ICAM-1 protein levels, we examined the effects on mRNA levels of the antisense ICAM-1 oligonucleotide used in the current experiments, which was also targeted to the 3'-untranslated region of rat ICAM-1. These experiments demonstrated that this antisense oligonucleotide reduced the IL-1β-induced increase in ICAM-1 mRNA by 250% (n=5, P<0.05) (Figure 3B). No similar reduction in ICAM-1 levels was observed when the sense construct was used.

**Effects of Antisense ICAM-1 Oligonucleotide on Lung Graft ICAM-1 Expression**

Using the same antisense ICAM-1 oligonucleotide that had shown in vitro efficacy in rat pulmonary endothelial cells, experiments were performed to determine whether it could...
Inhibit the increased expression of ICAM-1 observed after transplantation. When the control (sense) oligonucleotide in a cationic liposomal carrier was flushed into the pulmonary vasculature at the time of lung harvest and allowed to remain in place for the 4-hour preservation period, ICAM-1 protein expression measured 6 hours after transplantation/reperfusion was markedly increased (Figures 4A and 4B). The degree of increased ICAM-1 expression in the sense-treated lungs was similar to that seen in untreated lung transplants (Figure 1). In sharp contrast, however, when identical procedures were performed using the antisense compound, ICAM-1 protein expression was markedly reduced, down to the background levels seen in untreated/nontransplanted (fresh) lungs (Figures 4A and 4B). As the in vitro experiments showed that the antisense oligonucleotide reduced ICAM-1 mRNA levels, mRNA levels were also examined in in vivo experiments. These experiments demonstrated that antisense (but not sense) ICAM-1 oligonucleotide blocked the increase ICAM-1 mRNA levels seen after lung transplantation (Figures 4C and 4D).

**Effects of Preservation-Delivered Antisense ICAM-1 Oligonucleotide on Posttransplantation Neutrophil Accumulation and Graft Function**

To gauge the functional effects of ICAM-1 expression and its blockade by antisense ICAM-1 oligonucleotide on pulmonary graft leukosequestration, myeloperoxidase activity was determined in (1) freshly harvested and transplanted isografts, as well as isografts subjected to hypothermic preservation after treatment with either (2) the cationic liposomal carrier to which no oligonucleotides were added or to which either (3) sense or (4) antisense ICAM-1 oligodeoxyribonucleotides were added. These experiments demonstrated a significant reduction in graft neutrophil accumulation by the antisense-treated, but not the sense-treated, grafts, suggesting that the antisense compound inhibited the expression of functional ICAM-1 protein (Figure 5A). To establish that the inhibition of ICAM-1 expression by the antisense compound is pathophysiologically significant in terms of protecting the graft against primary failure, experiments were performed using the rat lung transplant model in which, after transplantation, the nontransplanted lung was removed from the pulmonary circuit by ligating the right PA (as previously described). This ensures that subsequent functional measurements (including recipient survival) are entirely dependent on the function of the transplanted lung. In these experiments, treatment of the lung graft at the time of harvest with antisense ICAM-1 oligonucleotide resulted in significant improvements in arterial oxygenation (Figure 5B), reductions in pulmonary vascular resistance (Figure 5C) with increased pulmonary blood flow (5.5 ± 2.8 versus 11.0 ± 2.9 mL/min for sense versus antisense, respectively, P < 0.05), and markedly improved recipient survival (Figure 5D). In sharp contrast, treating lungs with either the liposomal carrier alone or the carrier to which the sense compound was added did not inhibit ICAM-1 expression; grafts so treated were associated with poor functional and survival outcomes after lung transplantation.

**Discussion**

Primary lung graft failure, which occurs in up to 20% of all cases shortly after transplantation, is a catastrophic clinical event that occurs unpredictably and for which there is no effective treatment. Because as an organ, the lung is one of the most richly vascularized, with nearly 40% of its parenchymal cellular mass composed of endothelial cells, it is not surprising that endothelial activation during the preservation or early transplantation period may represent a significant cause of primary lung graft failure. Because the lungs are detached from their native blood supply during the mechanical lung harvest procedure, we hypothesized that this would present an opportune time to deliver a therapeutic agent directly to endothelial cells to prevent their activation during or shortly after preservation. As we have shown neutrophil adhesion to activated endothelium is a culprit in early graft demise, we hypothesized that ICAM-1 would be a suitable target for genetic manipulation of the graft vasculature, because its expression is inducible. However, in the setting of primary lung graft failure after lung transplantation, it...
had not been previously established that ICAM-1 expression is augmented or relevant. In the current experiments, we demonstrate that ICAM-1 expression increases within the first several hours after lung transplantation and that this increase is pathophysiologically important. Furthermore, by administering an anti–ICAM-1 antisense oligonucleotide directly into the pulmonary vasculature at the time of harvest, we can virtually obliterate the posttransplant increase in ICAM-1 expression and effect a remarkable improvement in graft function and recipient survival compared with sense-treated controls. These data indicate that there exists a unique opportunity for genetic manipulation of the explanted graft vasculature that can translate into real, functionally relevant effects.

In the experiments performed in this study, an antisense approach was taken to reduce lung graft ICAM-1 expression. In this approach, hybridization of an antisense oligonucleotide to the complementary sense strand by normal Watson-Crick base pairing can block protein synthesis by steric hindrance causing translational arrest, inhibit RNA processing, and/or alter the susceptibility of targeted mRNA to degradation. The choice of using the 3’-untranslated region for the antisense oligonucleotide in our experiments was based on several published reports, in which an antisense oligonucleotide targeted to this region of human ICAM-1 was particularly effective at specifically inhibiting ICAM-1 expression, as well as a report showing efficacy in a renal ischemic injury model in the rat. The phosphorothioate composition of the oligonucleotide was selected to increase the stability of the compound against serum and other nucleases. To optimize conditions for study in the rat lung transplant model, we first used in vitro conditions with stimulated rat pulmonary microvascular endothelial cells. In this development stage of our project, IL-1β was chosen as the ICAM-1–inducing stimulus because it is a known potent inducer of endothelial ICAM-1 and because it may be relevant in the setting of pulmonary ischemia and reperfusion injury.

The cationic liposomal carrier delivery method we chose to use was selected because of the theoretical concerns that viral delivery systems, even though effective in delivering cytokines to the lungs, could promote postischemic vascular inflammation, which we wished to suppress, and because of reports that relatively high levels of transfection efficiency might be possible with the DNA/liposomal method. In addition, DNA/cationic liposomal complexes have been shown to successfully deliver genes to the lungs after aerosol or intravenous delivery without adverse effects in terms of pulmonary histology, lung compliance, or alveolar-arterial oxygen gradient. In mice, intravenous delivery of a reporter gene as DNA/liposome complexes was far more efficient than use of plasmid DNA alone. The cationic liposomal carrier delivery method we chose to use was selected because of the theoretical concerns that viral delivery systems, even though effective in delivering cytokines to the lungs, could promote postischemic vascular inflammation, which we wished to suppress, and because of reports that relatively high levels of transfection efficiency might be possible with the DNA/liposomal method. In addition, DNA/cationic liposomal complexes have been shown to successfully deliver genes to the lungs after aerosol or intravenous delivery without adverse effects in terms of pulmonary histology, lung compliance, or alveolar-arterial oxygen gradient. In mice, intravenous delivery of a reporter gene as DNA/liposome complexes was far more efficient than use of plasmid DNA alone. In a recent study, in which the transfection efficiency of a transforming growth factor-β (TGF-β) cDNA was examined in a rat lung transplant model, the use of the cationic liposomal carrier was shown to increase transfection efficiency up to 4-fold compared with the DNA/liposomal method.

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Figure 4. Inhibitory effect of antisense ICAM-1 oligonucleotide on pulmonary graft ICAM-1 expression. A, Effect on graft ICAM-1 protein expression, measured by Western blotting using a primary murine anti-rat ICAM-1 IgG. Samples were taken from fresh (nonpreserved) lungs or from lungs that had been treated with 7 mL of 50 μg/mL of either sense (S) or antisense (AS) oligonucleotide (50 μg/mL of cationic liposomal carrier) administered directly into the left PA at the time of lung harvest. Pulmonary arterial and venous clamps were placed to prevent fluid extravasation during the 4-hour submersion in 4°C modified EuroCollins preservation solution. Lungs were then transplanted orthotopically into an isogeneic recipient, reperfused for 6 hours, and then excised. Immunoblotting for rat ICAM-1 antigen was performed as described above (a representative blot is shown). B, Quantitative analysis of densitometric scans of multiple immunoblots showing the relative increase in ICAM-1 protein expression (vs fresh) seen when lungs were preserved with sense, but inhibited ICAM-1 expression when lungs were preserved with antisense. n = 7 for fresh lungs, and n = 12 transplants each for sense and antisense; data are mean±SEM; *P<0.005. C, Effect of sense or antisense on graft ICAM-1 mRNA levels, measured by Northern blotting, with β-actin as a control. To obtain RNA, separate additional experiments were performed as described in panel A, except that reperfusion duration was 3 hours. Representative Northern blot is shown. D, Relative ICAM-1 mRNA levels were evaluated in multiple blots by densitometry and expressed as relative increase compared with fresh (nonpreserved) lung samples. n = 2 for fresh lungs and n = 4 transplants each for sense and antisense groups; data are mean±SEM; *P<0.05.
Figure 5. In vivo functional effects of antisense ICAM-1 oligonucleotide in lung transplantation. Lungs were harvested and transplanted immediately (fresh, n=6, comprising untreated/nonpreserved isografts) or subjected to hypothermic preservation after treatment with vehicle+cationic liposomal carrier alone (Control, n=6; prepared as described in Materials and Methods) or vehicle to which either sense (S, n=12) or antisense (AS, n=12) ICAM-1 oligonucleotides were added. At 6 hours after reperfusion, the native right PA was ligated to allow examination of the function of the preserved/transplanted left lung independently of the native right lung. After the ligation procedure, hemodynamics were recorded until the final time at which the recipient was alive, or at the 30-minute time point if the animal survived until then, at which point the animal was euthanized and lung tissue collected. A, Effects on pulmonary graft neutrophil sequestration, measured with an assay of myeloperoxidase activity (MPO; ΔAbs 460 nm/min). B, Effects on pulmonary graft gas exchange, measured as arterial oxygenation (P_{O2}, mm Hg); recipients were ventilated with 100% O_2 throughout the posttransplant period. C, Effects on pulmonary graft vascular resistance (mm Hg/mL/min), measured by recording pulmonary arterial blood flow, pulmonary arterial pressure, and left atrial pressure. D, Effects on recipient survival; data are mean±SEM; *P<0.05.

The antisense ICAM-1 oligonucleotide we used in the current experiments not only reduced ICAM-1 protein levels, but ICAM-1 mRNA levels as well. In a recent study in which the molecular mechanisms by which antisense oligonucleotides inhibit ICAM-1 expression were examined, it was shown that human ICAM-1 antisense oligonucleotides inhibited ICAM-1 expression through 2 distinct posttranscriptional mechanisms. In the first, antisense oligonucleotides corresponding to the AUG translation initiation codon mask the ribosome recognition site and prevent the formation of the translation complex. In the second mechanism, an antisense oligonucleotide corresponding to the 3′-untranslated region of ICAM-1 mRNA (targeting similar to that of the antisense construct used in the current experiments) caused a marked increased susceptibility of ICAM-1 RNA to hydrolysis by RNase H, a ubiquitous enzyme that degrades the RNA strand of RNA-DNA hybrid molecules. Although experiments using antisense oligonucleotides must be interpreted carefully because of potential non–sequence-specific effects, these are not likely to apply to the current experiments for several reasons. First, we observed a direct inhibitory effect of the antisense oligonucleotide on both mRNA and protein levels of the targeted sequence (ICAM-1), which was not seen with control (sense) oligonucleotide. Although non–sequence-specific effects have been reported on the basis of the binding of phosphorothioate oligonucleotides to growth factors or to elements of the extracellular matrix, the lack of 4 consecutive guanosine residues (G-quartet) in the sequence used in the current experiments reduces the possibility of an aptameric effect.

In addition to demonstrating in vivo efficacy of an antisense ICAM-1 strategy in a clinically relevant scenario (lung transplantation), the experiments in this study offer the first direct proof of a functionally deleterious role for ICAM-1 expression in the setting of primary lung graft failure. Although selectins are responsible for initially decelerating circulating PMNs during their transit through the pulmonary vasculature, bonds formed between selectin glycoproteins on endothelial cells and their oligosaccharide counterligands on PMNs form and break readily to permit the PMN to roll to a stop along the endothelial surface. Selectins therefore position the PMN and the endothelial cell into a correct steric relationship so that the more potent adhesion receptor, ICAM-1, can interact with its PMN counterligands, LFA-1 and Mac-1. In fact, static incubation of activated PMNs on artificial lipid bilayers enables comparison of ICAM-1 and P-selectin–dependent adhesive forces; PMN binding through
the integrins LFA and Mac-1 to ICAM-1 is 100-fold more shear resistant than binding via CD62 (P-selectin). For these reasons, although selectin blockade can also be effective to diminish pulmonary graft leukostasis, 
blockade of ICAM-1 expression appeared to be a particularly attractive target to improve lung preservation. In addition, the pulmonary reperfusion microenvironment exhibits transient and inhomogeneous alterations in shearing forces that can transiently upregulate ICAM-1 expression. A rat model was chosen for study, although although adhesion receptor null mice have proven extremely useful for studying the pathophysiological roles of various adhesion receptors, lung transplantation is not technically possible in the mouse.

Although the present studies do not permit a direct comparison of the effects of functional blockade of P-selectin–dependent adhesion reactions via an antibody approach and the effects of an anti–ICAM-1 expression strategy provided by the antisense ICAM-1 approach, some rough comparisons can be made. In our previous work, in which we found that an antibody to P-selectin was effective at reducing the consequences of pulmonary ischemia/reperfusion injury, we looked at a 30-minute time point in the rat model (as P-selectin is rapidly expressed). However, in the current work, data show that ICAM-1 expression does not peak until 3 to 6 hours of reperfusion have passed, and we therefore examined the functional effects of ICAM-1 inhibition at the delayed (6-hour) time point. In Figure 5D, our data show that inhibiting ICAM-1 expression does not make a functional difference at the early (30-minute) time point, as would be expected from the time course of expression that we demonstrate in Figure 1. With P-selectin blockade alone and an experimental n of 4 (using the same model, but with a longer observation period), the mean survival time is 6 hours; with antisense ICAM-1, 75% of animals survive to 6 hours and presumably beyond (experiments were terminated according to protocol at 6 hours), suggesting that the antisense ICAM-1 approach may be more effective than that of providing a functionally blocking anti–P-selectin antibody.

There are other compelling reasons why we chose to study ICAM-1 in the setting of lung transplantation. Clinical studies have shown increased levels of soluble ICAM-1 after cardiopulmonary bypass, which causes pulmonary circulatory stasis and thereby exposes the lung to ischemia, as well as increased expression of ICAM-1 in posttransplant lung biopsy specimens. In other transplantation scenarios, such as cardiac or liver, ICAM-1 expression has also been implicated as a pathophysiological mediator of acute graft injury. In an in situ model of lung ischemia and reperfusion, ICAM-1 was shown to be upregulated within the pulmonary microvascular circulation and to contribute significantly to lung reperfusion injury. In the kidney, ICAM-1 expression is thought to be a mediator of posts ischemic renal injury and failure. In fact, a recent study showed that systemic (intravenous) administration of an antisense ICAM-1 oligodeoxynucleotide attenuates renal reperfusion injury and renal failure. The current experiments, however, differ from the work of Haller et al in that not only were different models and modes of antisense oligonucleotide administration used (ex vivo under hypothermic preservation conditions in the current study), but an entirely different vascular bed was the subject for study. The lungs are far more vulnerable to ischemia-reperfusion injury than are the kidneys; for instance, renal preservation may be successful after 24 hours, but lung grafts may not be used after 4 to 6 hours of hypothermic preservation because of the high incidence of primary lung graft failure. In addition, there can be significant differences between the phenotype of endothelial cells between vascular beds. For instance, the stimulus-specificity and selectin-dependence of leukocyte/endothelial adhesion differs between the pulmonary and the systemic vascular beds; in a model of muscle ischemia/reperfusion injury, local injury to the muscle was shown to be selectin-independent, yet injury to the lungs was selectin-dependent. In the case of integrins, the CD11/CD18 complex mediates PMN adherence in the systemic circulation, but in the pulmonary circulation, depending on the inciting stimulus, neutrophil adherence may be CD18-independent. Although our group has shown using ICAM-1 null mice that cardiac ischemia-reperfusion injury is ICAM-1–dependent, others have shown in a lung injury model (using control and ICAM-1–deficient mice) that cobra-venom factor–induced lung injury is ICAM-1–independent. A recent review by Rosenberg and Aird also provides insights into how the coagulant phenotype of the vessel wall can differ between vascular beds.

There are several theoretical reasons why an antisense approach to inhibit ICAM-1 expression may be preferable to an antibody-mediated approach. Because functionally blocking anti–ICAM-1 antibodies compete with integrin epitopes for binding, it may be difficult to achieve high enough antibody levels in the local ischemic vascular milieu (because of vasoconstriction, thrombosis, and cellular obstruction of microvascular lumina) to fully inhibit endothelial-neutrophil interactions. In addition, binding affinity and kinetics differ between various antibodies, which may account for experimental variability between them in different studies. In an isograft lung transplant model in which functionally blocking anti–ICAM-1 and anti–β2-integrin antibodies were studied, individual antibodies directed solely against ICAM-1, CD11a, or CD18 were far less effective than a combination of antibodies against all 3. Other studies, although an anti–ICAM-1 antibody approach did seem to improve the ability of isolated-perfused rabbit lungs to oxygenate, pulmonary vascular resistance, airway resistance, and lung edema were not improved, suggesting only partial effectiveness of the anti–ICAM-1 approach tested. Similar incomplete improvement of lung graft function was seen in a canine single-lung transplant model in which an antibody to CD18 was studied. Other potential detractions of an antibody-mediated approach include the potential for Fc-mediated activation of complement (as has been shown in liver transplantation). It is also possible that nonlocalized inhibition of ICAM-1–mediated leukocyte-endothelial interactions with an antibody may complicate issues related to clinical infection and immunosuppression.

The experiments shown here are the first to demonstrate a role for antisense ICAM-1 delivered during the preservation period to mitigate acute lung graft injury in the absence of an allogeneic immune response. Whether or not this approach will be useful in
lung allograft transplantation to reduce either primary graft failure or rejection remains an open question at this time. Although this is an extrapolation, it is interesting to speculate that the early suppression of graft ICAM-1 expression may have a longer-term immunomodulatory role. Blocking ICAM-1 expression with an antisense oligodeoxynucleotide (given intravenously over 1 to 2 weeks) in the setting of cardiac allograft transplantation was shown to have more of an immunomodulatory effect (to reduce rejection) rather than to reduce immediate graft injury and primary graft failure.50–61 In other experiments, anti-interferon-γ antisense oligodeoxynucleotides indirectly reduced ICAM-1 expression as well as cell surface induction of major histocompatibility complex (MHC) class I and II molecules.62,63 However, this alternative mechanism of action (interferon-γ inhibition) is not likely to apply to the current model, in which isogenic grafts (seen as “self” by the recipient) were used, diminishing a potential intermediary role for MHC class I or II molecule inhibition in reducing acute graft injury. Although in these experiments, the activity and potency of the oligonucleotide to inhibit induction of MHC class I and II molecules was sequence-dependent, the oligodeoxynucleotide appeared to exert its effects by inhibiting the association of interferon-γ with the cell surface. In the current studies, identification of diminished target (ICAM-1) mRNA and protein expression mitigates the need to invoke such alternative mechanisms of action for the ICAM-1 antisense oligodeoxynucleotide.

In summary, the data presented in this paper define the time course of ICAM-1 expression after lung transplantation, which is related to allogeneic-independent factors, and show for the first time the functional relevance of ICAM-1 expression in primary lung graft failure. Furthermore, guided by in vitro pulmonary endothelial cell experiments, this work identifies the pulmonary preservation period as a unique window of opportunity during which to deliver an antisense oligonucleotide to inhibit pulmonary ICAM-1 expression and to improve functional outcome after lung transplantation.

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**Materials:**

**Preservation solution:** For all transplant experiments, the basic preservation solution consisted of modified Euro-Collins solution obtained from Baxter Healthcare (Deerfield, IL; Na⁺ 10 mEq/L, K⁺ 115 mEq/L, Cl⁻ 15 mEq/L, HPO₄²⁻ 85 mEq/L, H₂PO₄⁻ 15 mEq/L, HCO₃⁻ 10 mEq/L), modified by adding 10 mL of 10% magnesium sulfate and 50 mL of 50% glucose solution per liter. This represents the standard formulation used in clinical lung transplantation⁴.

**Sense and antisense oligonucleotides:** Sense and antisense phosphorothioate oligonucleotides were chemically synthesized and HPLC-purified (GIBCO BRL, Grand Island, NY). The ICAM-1 antisense oligonucleotide was designed against the 3’-untranslated region of the rat ICAM-1 gene⁵,⁶, and comprised of the following sequence: 5’-ACC GGA TAT CAC ACC TTC CT-3’.

The complementary sense sequence used was 5’-AGG AAG GTG TGA TAT CCG GT-3’.

**Cationic liposomal carrier:** To transfect the oligonucleotides into cells or pulmonary grafts, a cationic liposomal carrier was used which has demonstrated efficacy and lack of toxicity⁷ in pulmonary endothelial cells. The liposome formulation chosen for these experiments was the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), and dioleoyl phosphatidylethanolamine (DOPE) (GIBCO BRL) to enhance the oligonucleotide uptake by cells.

**Other Materials:** Unless otherwise specified, chemicals were purchased from either Sigma Chemical Co. (St. Louis, MO) or Boehringer Mannheim Co. (Indianapolis, IN)

**Endothelial cell experiments:** Rat pulmonary microvascular endothelial cells (RPECs) were a generous gift of Dr. Una Ryan⁸. Cells were grown in Medium 199 (GIBCO BRL) supplemented with 10% fetal bovine serum and 100 U/mL of penicillin/streptomycin and placed in a 37 ºC humidified incubator with 5.0% CO₂ balanced with room air. Cells were grown to 60-70% confluency in 10 cm petri dishes, and washed twice with serum- and antibiotic-free Medium 199
immediately prior to their use in experiments. The oligonucleotides were prepared for addition to the washed cell cultures as follows. The dose of lipofectin to be used (GIBCO BRL) was added to 0.8 mL of serum-free medium and incubated at room temperature for 45 minutes. This mixture was then combined with the oligonucleotide (also in 0.8 mL of serum-free medium); this 1.6 mL mixture was allowed to incubate at room temperature for an additional 30 minute period, at which point 6.4 mL of serum-free M199 was added for a total volume of 8 mL. The amounts of oligonucleotides and lipofectin added to the initial aliquots were adjusted so that the final concentrations after addition to the cells ranged from 0-20 μg/mL, and are indicated in the Figures/Legends. This mixture was then added to the cells, and following 3 hours of incubation in the cell culture incubator, the medium was reconstituted with fetal bovine serum (to 10%), and an hour thereafter, recombinant murine IL-1β (Genzyme Co., Cambridge, MA) was added to the culture medium to a final concentration of 2.5 ng/mL. Cultures were then incubated for an additional 16 hours, at which point cells were harvested for ICAM-1 protein and mRNA measurements.

**Lung transplant experiments:**  Donor Lung Harvest: Inbred male Lewis rats (250-300 gms) were used for experiments according to a protocol approved by the Institutional Animal Care and Use Committee at Columbia University, in accordance with AAALAC guidelines. Donor rats were given 500 units of heparin intravenously, and following ligation of the right pulmonary artery (PA) (to restrict delivery of preservation solution to the donor lung used for grafting), 7 mL of 4°C preservation solution was administered into the main PA at a constant infusion pressure of 20 mm Hg. The left lung was then harvested, a cuff was placed on each vascular stump and the left bronchus, and the lung was submerged for 4 hours in preservation solution maintained at 4 °C. Transplantation was performed using gender/strain/size matched recipient rats which were anesthetized, intubated, and ventilated with 100% O₂ using a rodent ventilator (Harvard Apparatus, South Natick, MA). Orthotopic left lung transplantation was performed as described through a
left thoracotomy using a rapid cuff technique for all anastomoses, with warm ischemic times maintained below 5 minutes. The hilar cross-clamp was released, re-establishing blood flow and ventilation to the transplanted lung.

For all experiments, the preservation duration was identical (4 hours). However, depending upon the particular experiment, reperfusion durations ranged from 30 min. to 24 hours (indicated in the Figures/legends). For those experiments in which oligonucleotides were studied, the base preservation solution was supplemented with lipofectin (350 μg/0.8 mL of EuroCollins), which was allowed to equilibrate for 45 minutes at room temperature, followed by the addition of either the sense or the antisense oligonucleotide construct (350 μg in an additional 0.8 mL of EuroCollins); this 1.6 mL mixture was allowed to incubate at room temperature for 30 minutes, after which 5.4 mL of EuroCollins solution was added, and the entire mixture chilled to 4 °C. This was prepared fresh for each experiment. The site of injection was the left pulmonary artery prior to lung harvest, and the volume of the injectate was 7 mL of preservation solution containing 350 μg of either the sense or the antisense oligodeoxynucleotide compound.

After lung transplantation, hemodynamic measurements were obtained as follows. Immediately after surgical anastomoses were completed to finish the implantation procedure, a loose snare was passed around the right PA, and the thoracotomy loosely closed to prevent desiccation. Just prior to the 6 hour reperfusion time point, Millar catheters (2F; Millar Instruments, Houston, TX) were introduced into the main PA and the left atrium (LA) and a Doppler flow probe (Transonics, Ithaca, NY) was placed around the main PA. Online hemodynamic monitoring was begun using MacLab and a Macintosh IIci computer, and the loose snare around the right PA was tightened at 6 hours after transplantation. Measured hemodynamic parameters included LA and PA pressures (mm Hg), and PA flow (mL/min). Serial measurements were taken every five minutes until the time of euthanasia at 30 minutes (or until recipient death). Arterial oxygen tension (pO₂, mm Hg) was measured using a model ABL-30 gas analyzer (Radiometer, Copenhagen, Denmark) from a sample of left atrial blood taken at the final time point.
at which the recipient was alive (up to 30 minutes). Pulmonary vascular resistance (PVR) was calculated as (mean PA pressure - LA pressure)/mean PA flow and expressed as mm Hg/mL/min. Thirty minutes following ligation of the native right PA (or at the time of recipient death, if it occurred prior to 30 minutes), the transplanted lung was cross-clamped to divide it into equal parts, with one portion being excised, rinsed briskly in physiologic saline, and snap frozen in liquid nitrogen until the time of myeloperoxidase assay. The remaining portion of the transplanted lung was perfused with cold saline via the PA and snap frozen in liquid nitrogen until the time of assay for ICAM-1 expression.

**Immunoblotting for ICAM-1 Protein:** For cell culture experiments, total cellular protein was extracted by washing, scraping, and sonicating cells in the presence of protease inhibitors, similar to procedures previously described\(^\text{10}\). Membrane proteins were extracted by gentle shaking with 1% octyl-β-glucoside (OBG) for 3 hours, and the suspension was then centrifuged at 15,000 g for 5 minutes at 4 °C, and the supernatant recovered. For the lung transplant experiments, integral membrane proteins were extracted using a modification of a previously reported method\(^\text{11}\). For both cell culture and lung transplant experiments, protein concentrations were determined according to the Bradford method\(^\text{12}\). Afterwards, samples were prepared for nonreduced SDS-PAGE, with equal amounts of protein (20 µg) loaded onto on 7.5% Tris-Glycine gel, subjected to electrophoresis, and electrophoretically transferred onto a nitrocellulose membrane. After overnight blocking with nonfat dry milk, the membrane was incubated for 3 hours at room temperature with a 1:1000 dilution (in nonfat dry milk) of mouse monoclonal anti-ICAM-1 IgG (1A29 clone, Seikagaku, Ijamsville, MD), washed thrice, followed by a 1 hour room temperature incubation with a 1:1000 dilution of a horseradish peroxidase-conjugated goat anti-mouse IgG. Sites of primary antibody binding were visualized by the enhanced chemiluminescence method (for the cell culture experiments) (ECL kit: Amersham Corp., Arlington Heights, IL) or by a colorimetric method (for the transplant experiments) in which blots were developed with Diaminobenzidine in
Tris buffer (50 mM). Blots were scanned into a Macintosh computer for subsequent densitometric analysis.

**Northern blotting for ICAM-1 mRNA:** For analysis of ICAM-1 mRNA, total cellular RNA was extracted, equal amounts of RNA (20 µg) were loaded onto a 0.8% agarose gel containing 2.2 M formaldehyde for size fractionation, and then transferred overnight by capillary pressure to nylon membranes. An α32P-dCTP-labelled rat ICAM-1 cDNA probe (2.6 Kb, kind gift of Dr. Y. Kita) and subsequently a human β-actin cDNA probe (ATCC 77793, Rockville, MD) were used to probe blots. Blots were scanned into a Macintosh computer, and Molecular Analysis software used to calculate the density of each band.

**Myeloperoxidase assay:** Tissue was weighed and homogenized in 5 mL/g of phosphate buffered saline (50 mM, pH 5.5) containing hexadecyltrimethyl ammonium bromide (0.5%, Sigma). The assay was performed, as previously described, by thawing the sample, centrifuging at 40,000 g for 15 minutes, and decanting the supernatant, which was assayed for myeloperoxidase activity using a standard chromogenic spectrophotometric technique in which test sample (0.03 mL) was added to phosphate buffered saline (0.97 mL) containing O-dianisidine dihydrochloride and hydrogen peroxide (0.0005%). Change in absorbance at 460 nm was measured over 1 minute (increase in OD was linear over this time interval).

**Immunohistochemistry:** Lungs were prepared for immunohistochemistry by submerging the lung tissue in cold saline under pressure to evacuate air and fill alveoli with saline, embedded, frozen, and sectioned into 4-µm sections with a cryostat. Following acetone fixation/dessication, and rinsing, samples were incubated with mouse monoclonal anti-rat ICAM-1 IgG (1:100 dilution), washed, and incubated with FITC-conjugated goat anti-mouse IgG (1:120 dilution). Fluorescence microscopy was performed using an excitation wavelength of ≈ 475 nm and
an Olympus (BX60) fluorescence microscope (Nagano, Japan).

**Statistics:** Significant differences between groups were tested for using the Mann-Whitney U test performed with Statview™ software. Animal survival data was analyzed by contingency analysis using the Chi-square statistic. Values are expressed as means ± SEMs, with differences considered statistically significant if p<0.05.