Novel Small Interfering RNA–Containing Solution Protecting Donor Organs in Heart Transplantation

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Background—Ischemia/reperfusion injury is a major factor in graft quality and subsequent function in the transplantation setting. We hypothesize that the process of RNA interference may be used to “engineer” a graft to suppress expression of genes associated with inflammation, apoptosis, and complement, which are believed to cause ischemia/reperfusion injury. Such manipulation of pathological gene expression may be performed by treatment of the graft ex vivo with small interfering RNA (siRNA) as part of the preservation procedure.

Methods and Results—Heart grafts from BALB/c mice were preserved in UW solution (control) or UW solution containing siRNAs targeting tumor necrosis factor-α, C3, and Fas genes (siRNA solution) at 4°C for 48 hours and subsequently transplanted into syngeneic recipients. Tumor necrosis factor-α, C3, and Fas genes were elevated by ischemia/reperfusion injury after 48 hours of preservation in UW solution. Preservation in siRNA solution knocked down gene expression at the level of messenger RNA and protein in the grafts after transplantation. All grafts preserved in siRNA solution showed strong contraction, whereas grafts preserved in control solution demonstrated no detectable contraction by high-frequency ultrasound scanning. siRNA solution–treated organs exhibited improved histology and diminished neutrophil and lymphocyte infiltration compared with control solution–treated organs. Furthermore, the treated heart grafts retained strong beating up to the end of the observation period (>100 days), whereas all control grafts lost function within 8 days.

Conclusion—Incorporation of siRNA into organ storage solution is a feasible and effective method of attenuating ischemia/reperfusion injury, protecting cardiac function, and prolonging graft survival. (Circulation. 2009;120:1099-1107.)

Key Words: gene silencing [ischemia] [organ preservation] [reperfusion] [RNA, small interfering] [transplantation]

Organ transplantation is the only effective treatment for patients with end-stage organ failure. Although this procedure has been significantly improved over the past 70 years, there are still many challenges to overcome.1 One of them is ischemia/reperfusion (I/R) injury, which is an unavoidable reality of transplantation.1 Traditionally, refrigeration and instillation of specialized preservation solutions have been used to mitigate damage during the ex vivo preservation period. The principle underlying cold preservation is that through slowing the metabolism and reducing cell swelling with a specialized preservation solution, the viability and quality of organs will be preserved. Unfortunately, metabolic activity is not completely halted at cold temperature,2 and anaerobic cellular metabolism continues, albeit at a reduced level. This leads to an accumulation of toxic and harmful metabolites during preservation, which serve to activate immunological processes during reperfusion, causing inflammation and organ injury.3,4

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Many organ preservation solutions have been developed and applied in transplantation, but despite advances, cold ischemic storage of the heart is still limited to 4 to 6 hours because the preservation solutions are unable to significantly inhibit the ensuing I/R injury.5,6 Consequently, storage beyond the 4- to 6-hour window leads to delayed organ function and even organ failure. The lack of ability to preserve organs...
for longer periods contributes to the shortage of donor organs and increased number of patients on the waiting list for heart transplantation. Therefore, developing a novel organ preservation solution that can reduce I/R injury and extend organ preservation time is critical for clinical organ transplantation.

I/R injury leads to organ damage in various ways. First, increased permeability of the endothelial cells occurs, causing edema. Second, the endothelial cells acquire an activated state, as defined by upregulated expression of adhesion molecules, increased inflammatory cytokines production, and reduced nitric oxide synthesis. The activated endothelium initiates recruitment of monocytes and neutrophils that release reactive oxygen species and proteolytic enzymes that induce significant damage to the parenchymal tissue and vascular cells. Third, the adherent leukocytes mechanically obstruct the blood vessels, increasing the ischemic state. Despite the multiple effects of I/R injury on transplanted organs, the molecular mechanisms associated with this form of tissue injury are relatively well defined. In particular, apoptosis, complement activation, and inflammatory pathways are the best-defined and well-studied causative factors directly related to I/R. Accordingly, we sought to attenuate I/R injury through silencing tumor necrosis factor-α (TNF-α), complement 3 (C3), and Fas genes using small interfering RNA (siRNA).

Gene silencing with siRNA is one of the most efficient methods for selectively knocking down gene expression. Previously, we and others have demonstrated that systemic administration of siRNA can interrupt apoptosis and complement pathways and possesses therapeutic effects in preventing I/R in mouse liver and kidney models. Although these findings are promising in RNA interference–based therapy, systemically treating donors with siRNA is not practical in clinical transplantation. In this study, we developed a novel solution consisting of siRNA that targets TNF-α, C3, and Fas genes. We perfused donor organs with siRNA solution and silenced I/R-associated genes in the preservation period. The siRNA-based preservation solution was capable of prolonging the preservation of donor heart organs by reducing cold ischemia injury in mice.

Methods

Animals

Male BALB/c mice weighing 25 g (Jackson Laboratories, Bar Harbor, Me) were kept in filter-top cages at the Animal Facility of the University of Western Ontario according to National Canadian Council for Animal guidelines. Mice were fed food and water ad libitum and were allowed to settle for 2 weeks before the initiation of experimentation, which had ethics approval from the university board. The experimental protocol for this study was approved by the Animal Care Committee of the University of Western Ontario, and the study was conducted in accordance with the guidelines established by the Canadian Council on Animal Care.

siRNA Design

siRNA with 19-nt length, targeting TNF-α, C3, and Fas gene, respectively, was designed and synthesized by Dharmaco Inc (Lafayette, Colo). The sequences of sense were as follows: TNF-α, 5'-GACAACCAACUCUGUGUCC-3'; C3, 5'-GUGCAAGACUUCCUAAAGA-3'; and Fas, 5'-GUGCAAGUGCACAACCAGAC-3'.

Gene Transfection In Vitro

Macrophage cells or L929 cells were transfected with TNF-α–siRNA, C3-siRNA, or Fas-siRNA with Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, Calif). The vehicle alone and scrambled (nonsense) siRNA were used as negative controls. Briefly, cells were plated onto 12-well plates (3×10⁵ cells per well) and allowed to grow overnight to reach 90% confluency. Cells were transfected with 2 μg of the above siRNA or negative control siRNA in serum-reduced medium for 5 hours and then incubated in complete medium for 24 hours. All RNAs were prepared for subsequent analysis.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from cultured cells or tissues with TRIzol (Invitrogen Life Technologies) according to the manufacturer’s protocol. To generate the first-strand complementary DNA, the SuperScript Preamplification System (Invitrogen Life Technologies) was used. Briefly, 0.5 μg oligo(dT) (12 to 18 bp) and 100 U of SuperScript-II reverse transcriptase were incubated with 1 μg DNA-free total RNA for 50 minutes at 42°C in the presence of 0.5 mmol/L dNTP, 10 mmol/L DTT, and 1× first-strand buffer. Primers used in this study included the following: TNF-α: sense, 5'-GCCCTTCTTCTACATCG-3'; and antisense, 5'-CCGTTATCTCCTCCCTCCTCCCTCCT-3'; C3: sense, 5'-CCCTGCCTCTCCTCCCTCCCTCCTCCT-3'; and antisense, 5'-CGTACTGTGCCCCCTCTCTCTG-3'; Fas: sense, 5'-AAAGGAAAGGTACATGACAC-3'; and antisense, 5'-GTATGGTTTACGACTGGAGGTCTG-3'; and GAPDH: sense, 5'-TGTAGACATCAAAGGAATGTGGTGA-3'; and antisense, 5'-TCTTTGGAGCCATGACACTCAT-3'. Quantitative polymerase chain reaction (qPCR) was performed on a Stratagen MX 4000 PCR Instrument (Stratagen, Cedar Creek, Tex) in a 20-μL volume with 2× Universal SYBR Green PCR Master mix (Stratagen). This process was followed by 40 cycles consisting of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute. A relative quantitative assay was adopted. Mouse GADPH messenger RNA was used for normalization to ensure equal amounts of starting RNA. Each sample was tested in triplicate. Samples were obtained from at least 3 independent experiments to calculate the mean and SD.

Donor Organ Preservation and Cardiac Transplantation Procedure

Adult BALB/c mice weighing 25 to 30 g were used as donors and recipients. Atropine (0.04 mg/kg SC) is given to all animals before they are anesthetized. Ketoprofen 5 mg/kg is given at the time of sedation or induction. The general anesthesia was induced by a combination of xylazine 5 mg/kg IM and ketamine 100 mg/kg IM and then maintained by inhalant halothane and oxygen. The recipients were kept on a warming blanket during surgery.

Heterotypic cardiac transplantation was performed in a syngeneic recipient as previously described. Heart grafts were harvested in a mostly standard fashion, with a minor modification of placing a tiny tube (5×1 mm) into the inferior vena cava below the heart. The hearts were perfused with siRNA solution through the inferior vena cava and aorta until the vessel of the heart turned clear. The tube was kept there to support the lumen of the inferior vena cava tied with silk suture. The donor hearts were then excised and immersed in siRNA solution at 4°C for 48 hours. Before the anastomosis to the recipient was performed, the donor heart was flushed with fresh siRNA solution again through the tube to wash out the potentially harmful cellular metabolites that accumulate during the period of cold ischemia. Then, the tube was removed and the inferior vena cava was ligated permanently. The graft was revascularized with end-to-side anastomoses between the donor aorta and recipient abdominal aorta and between the donor pulmonary artery and recipient inferior vena cava. All anastomoses were performed with 11-0 nylon sutures. The abdomen was closed in 2 layers. Survival was judged by the quality of heart beat and ultrasound scanning.
Clinical Criteria for Graft Function After Transplantation
Heart graft viability was monitored daily by direct abdominal palpation. The degree of pulsation was scored as follows: A, beating strongly; B, noticeable decline in the intensity of pulsation; or C, complete cessation of pulsation.

In Vivo Imaging of Cardiac Grafts With Ultrasound Scanner
A high-frequency ultrasound scanner (Vevo 770, VisualSonics Inc, Toronto, Ontario, Canada) system was used to assess the overall function of a transplanted secondary heart. Both 30- and 40-MHz probes were used to obtain anatomic B-mode, ventricular wall functional M-mode, and blood velocity spectral Doppler information. Other technical specifications related to M-mode and spectral Doppler function modalities were followed according to the methods described by Zhou et al.14,15

An anesthetic induction with a 4% isoflurane and oxygen mixture was initially used on the mice to prepare for imaging. Mice were then transferred to a heated platform where they were secured in a supine position and anesthesia was maintained with 1.5% inhaled isoflurane; all 4 paws were attached to ECG electrodes embedded on the platform, which fed signals from both the native and transplanted hearts to the onboard ultrasound computer system. Physiological parameters were monitored throughout imaging. The ECG, representing the combined electric activity of the primary heart and the graft, was recorded16 and displayed with the Doppler spectrum. Mouse abdominal hair was cleanly removed with hair-removal cream. Prewarmed ultrasound gel was place on the abdomen for coupling the transducer to the tissue. The mouse body temperature was monitored by a rectal thermometer and maintained between 36°C and 38°C. Respiration also was monitored throughout ultrasound imaging.

The orientation of the cardiac graft was judged by palpation. The graft was usually found to be situated in the right-middle portion of the abdomen, with the cardiac base at midline and the apex pointing inferiorly and to the right. The transducer was first oriented to obtain a long-axis view of the graft, showing the flow channel from its anastomosis with the primary abdominal aorta, retrograde through the ascending aorta and aortic orifice, to the left ventricular outflow tract. The Doppler sample volume was placed at the middle portion of the ascending aorta to record a flow velocity spectrum. Primary heart function was compared with the transplanted heart. The sample volume was moved to the aortic orifice, slightly on the ventricular side, to assess the secondary heart left-side function and to record the aortic regurgitant jet. If left ventricular blood flow was visible or left ventricular wall motion was detected, M-mode measurements were made to assess left heart function. Right heart function also was investigated with the use of both Doppler and M-mode measurements at the tricuspid outflow tract and right ventricle, respectively.

Histology and Neutrophil Detection
On day 7 after transplantation, heart grafts were dissected from mice, and tissue slices were fixed in 10% formalin and processed for histology examination using standard techniques. Formalin tissue was embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin using standard techniques. These sections were examined in a blinded fashion by a pathologist. The percentage of histology changes in grafts was scored with a semiquantitative scale designed to evaluate the degree of infarction, ischemia, and cast formation on a 5-point scale based on injury area of involvement as follows: 0, <10%; 1, 10% to 25%; 2, 25% to 50%; 3, 50% to 75%; and 4, 75% to 100%. A pathologist quantitatively assessed neutrophil infiltration by counting the number of neutrophils per high-powered field (×400) over 5 fields and then averaging neutrophil numbers.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay
Cell apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using the terminal deoxynucleotidyl transferase–DNA FragEL detection kit (Calbiochem, San Diego, Calif). Paraffin sections of the hearts were in situ permeabilized and inactivated with endogenous peroxidase, followed by DNA fragment labeling, termination, and detection of labeled DNA according to the manufacturer’s instructions.

Statistical Analysis
Graft survival was compared between experimental groups with the log-rank test. Data for gene silencing and ultrasound scanning were analyzed with 1-way ANOVA, followed, if necessary, by the Newman-Keuls test. Differences at P<0.05 were considered significant.

Results
Validation of Gene Silencing by siRNA Specifically to TNF-α, C3, and Fas Genes
As a proof of principle, we validated gene silencing activity of siRNAs designed to target TNF-α, C3, and Fas transcripts. Fas and TNF-α siRNAs were transfected into L929 murine fibroblasts that express high levels of Fas and TNF-α genes. C3 siRNA was transfected in a macrophage cell line that highly expresses C3. Twenty-four hours after transfection of siRNAs, the expression of TNF-α, C3, and Fas was significantly knocked down as detected by qPCR compared with expression on the cells transfected with control scrambled siRNA (Figure 1). Therefore, the siRNAs used for the following in vivo experiments possessed potent gene knockdown capacity.

Effectiveness of siRNA Incorporation in Heart Tissues
To deliver siRNA into cardiac tissues effectively, we perfused hearts with UW solution containing 100 μg/mL siRNAs specific to TNF-α, C3, and Fas genes (siRNA solution). To determine siRNA uptake by myocardial cells of the donor heart, the heart was perfused with a Cy3-labeled siRNA solution after preservation in the same siRNA solution. Forty-eight hours after preservation, the hearts were harvested and sectioned. Bright red fluorescence from the Cy3-labeled siRNA was detected in the heart tissue under fluorescent microscopy (Figure 2A). No red fluorescence was detected in the heart tissue preserved in the UW solution alone (Figure 2B). These data suggest that siRNA could be delivered to cardiac tissue through perfusion and preservation in siRNA solution.

Gene Knockdown by siRNA Solution
Accumulating studies have shown that long-term cold preservation and subsequent reperfusion are associated with a specific gene-expression profile.17 Given the ability of the organ storage solution to allow intracellular entry of siRNA, we next assessed functionality of siRNA introduced via this method. Hearts were taken from BALB/c mice and preserved in siRNA solution containing siRNAs specific to TNF-α, C3, and Fas genes at 4°C for 48 hours, followed by syngeneic heart transplantation. After 48 hours, hearts were harvested and gene expression was examined at the messenger RNA
level with qPCR. As shown in Figure 3, the gene expression of TNF-α, C3 and Fas was dramatically upregulated in the control grafts preserved in UW solution alone or control siRNA solution compared with the hearts without I/R injury. In contrast, the relative quantities of TNF-α, C3, and Fas messenger RNAs were significantly decreased in the grafts preserved in the siRNA solution. Thus, it appears that siRNA solution can knock down the gene expression induced by I/R injury.

Protection of Cardiac Function by siRNA Solution

Next, we evaluated the effects of siRNA in protecting graft heart function using the same syngeneic murine heart transplantation model described above. Donor hearts from BALB/c mice were dissected and preserved in the above-described siRNA solution, control siRNA solution, or UW solution at 4°C for 48 hours, followed by a syngeneic heart transplantation. To assess graft function accurately and quantitatively, a high-frequency ultrasound scanning technique was applied in this study on day 7 after transplantation. Real-time B-mode imaging, M-mode imaging, and pulsed-wave Doppler were used to assess graft function. Real-time B-mode imaging, M-mode imaging, and pulsed-wave Doppler were used to assess graft function. Real-time B-mode and M-mode images qualitatively classified that most grafts treated with UW solution did not beat; only 1 grafted heart showed moderate but asynchronous cardiac contractility. In contrast, the grafts treated with siRNA solution had moderate to strong heart contractions on day 7 (Figure 4A). Pulsed-wave Doppler showed that the peak outflow velocity of the grafts preserved in siRNA solution (>20 cm/s) was significantly higher than the UW or control siRNA grafts (<2 cm/s; Figure 4B). The 20-cm/s outflow velocity produced by the siRNA-treated grafts is close to the outflow velocity typically produced by a heart graft without cold ischemia injury. Pulsed-wave Doppler peak velocities in the outflow and inflow tracts of the right ventricle were recorded, and the ratio of outflow to inflow was calculated (Figure 4C). The ratio of outflow to inflow for the grafts...
preserved in UW solution or control siRNA solution was 0, whereas outflow velocity was approximately equal to inflow for siRNA solution–treated hearts. Speed of contraction was determined from M-mode measurements of the right ventricle of the secondary heart (M-mode images obtained in the long-axis view). As shown in Figure 4D, the speed of contraction of grafts in siRNA solution was 13.99/11006.145 mm/s, whereas the speed of contraction of heart grafts preserved in control siRNA solution or UW solution was 0. For comparison, the speed of contraction of heart grafts without cold ischemia measured by M-mode was 13.71±3.88 mm/s. Similar outcomes were found when the ratio of the heart rates for the secondary and primary hearts was compared (Figure 4E). The results from ultrasound scanning demonstrated that siRNA solution greatly protects the heart graft function.

Histological Analysis of Protected Grafts
To study the cellular changes associated with protection from I/R injury mediated by the siRNA solution, grafts were harvested on day 7 after transplantation for histological analysis. Compared with grafts without cold I/R (Figure 5Aa), grafts preserved in UW solution (Figure 5Ab) or control siRNA solution (Figure 5Ac) exhibited hemorrhage, inflammatory cell (including neutrophils) infiltration, and severe infarction, accompanied by multifocal massive necrosis of myocytes in the area of the inflammatory cellular infiltration, perivasculitis, and endotheliitis (as summarized in the Table). In contrast, these pathological changes were significantly attenuated in the siRNA solution–preserved hearts (the Table). The structure of grafts preserved in siRNA solution showed no significant necrosis (Figure 5Ad).

We further detected apoptosis in the grafts using TUNEL assay. The hearts without cold ischemia showed minimum apoptosis (Figure 5Ba). The hearts preserved in the solution without siRNA or with control siRNA displayed massive apoptosis in the grafts (Figure 5Bb and 5Bc). In contrast, the hearts preserved in siRNA solution showed a significant decrease in apoptosis (Figure 5Bd).

Prolongation of Graft Survival by siRNA Solution
To further assess the protective ability of the siRNA solution, syngeneic heart transplantation was performed with the donor organs preserved with siRNA solution or control solution at 4°C for 48 hours. The survival of the cardiac grafts was compared between the 3 experimental groups over a 100-day period. As shown in Figure 6, for the heart grafts preserved in the siRNA solution, 87.5% of grafts retained their function after 100 days. In contrast, most heart grafts preserved in the control siRNA solution or UW solution lost their function within 8 days after transplantation (Figure 6).

Discussion
I/R injury represents a major clinical challenge. In the context of organ transplantation, it is documented that this form of injury not only decreases graft quality but also increases immunogenicity, thus leading to increased probability of immunologic rejection.18 “Danger signals” are released by the organ as a result of I/R injury, including heat shock protein 72, high-mobility group box 1, and hyaluronan fragments, which are known to activate Toll-like receptors and to increase propensity for adaptive immune recognition.19 I/R injury also is a fundamental component in exacerbating organ damage in other medical situations such as postinfarct revascularization in both the heart and brain.20

Given that I/R injury is fundamentally associated with a key set of genes being upregulated, we decided to target 3 main candidates that have been well defined as culprits of this inflammatory process—TNF-α, C3, and Fas—using siRNA. TNF-α is produced locally and systemically after cardiac I/R.

![Figure 3](http://circ.ahajournals.org/)

Figure 3. Gene silencing in cardiac grafts. Donor hearts were preserved in siRNA solution, control siRNA solution, or UW solution for 48 hours. The preserved hearts were implanted into syngeneic mice. Forty-eight hours after transplantation, grafts were harvested and total RNAs were extracted. Transcripts of TNF-α, C3, and Fas and GAPDH were determined by qPCR. TNF-α, C3, and Fas expression was compared between the siRNA group (n=6 per group) and control groups that were treated with UW solution or control siRNA solution. Relative quantity of TNF-α (A), C3 (B), and Fas (C) messenger RNA was expressed as mean±SEM. *P<0.05.
injury by cardiac mast cells, macrophages, and endothelial cells in response to activation of various innate immunity-associated receptors such as Toll-like receptor-4. Deleterious effects of TNF-α release are mediated directly by cytotoxicity to cardiomyocytes and by initiation and amplification of inflammatory processes such as endothelial cell activation and chemoattraction of immunocytes. The functional importance of TNF-α in I/R has previously been demonstrated in experiments with TNF-α knockout mice in which endothelial responsiveness was preserved in the knockout but not wild-type heart after 30 minutes of global ischemia. On the other hand, apoptosis is another main pathological change in I/R injury. Fas possesses proapoptotic properties during I/R injury. For example, it is reported that I/R injury results in upregulation of both Fas and FasL in a Stat-1–dependent manner in cardiomyocytes and that apoptosis can be inhibited by blocking this interaction at the cellular membrane. In addition to apoptosis/necrosis, the complement cascade has been found to be activated, causing release of toxic C3a, C5a, and membrane attack complex in the tissue during I/R and inducing cell infiltration. The fundamental importance of C3 in I/R damage was demonstrated by us and others. Thus, we initially sought to knock down these I/R–associated molecules in this study.

Other groups have demonstrated that inhibition of Fas with siRNA can significantly prevent I/R injury; however, this was performed with systemic administration, which is not clinically feasible. We theorized that the large amount of siRNA may be administered ex vivo in the organ storage solution. To test this possibility practically, we began by optimizing concentrations of the siRNA required. We tested concentrations of 25 to 200 μg/mL siRNA delivered in UW solution in a procedure that mimics the process of clinical organ harvesting and preservation. The optimal concentration for achieving maximal transfection with minimal siRNA was determined by assessment of the fluorescent signal in organs treated with labeled siRNA (Figure I of the online-only Data Supplement). We found that the 100-μg/mL concentration was optimal and that this concentration also was effective at substantially attenuating target gene expression as assessed by real-time PCR. Treatment was correlated with improved pathology score as assessed by a reduction in infiltration, infarction, and necrosis. The protective effects of the siRNA solution were demonstrated by attenuated I/R injury and prolonged graft survival (Figure 5). The histological results showed that the siRNA-containing solution decreased necrosis and apoptosis in heart tissues. Currently, cold ischemic storage of the heart is limited to 4 to 6 hours. This short time window contributes to the shortage of donor heart organs and subsequently an increased number of patients on the waiting list for heart transplantation. Therefore, a solution that can prevent organ injuries from occurring in the cold storage and reperfusion stages is in high clinical demand. This study is the first to report a novel siRNA-containing solution that can attenuate cold ischemia injury and prolong the preservation time of donor heart organ.
criteria have expanded to include older and marginal donors, improvement of preservation solutions is a necessity. Several strategies are being explored to reduce I/R injury and to enhance organ preservation. The first strategy applied is adding a range of chemical compounds (e.g., superoxide dismutase, lazaroids, and calcium channel blockers) to preservation solutions to prevent I/R injury.34,35 One group used chemical caspase inhibitors to prevent the increase in caspase-3, -2, -8, and -9 activity and apoptosis in the cold ischemic mouse kidney.36 However, these modifications achieved only marginal improvement in preventing ischemia damage. More important, the preservation time is still counted in hours rather than days.33 The second strategy is modulation of the organ during cold ischemia to improve responses to the graft after reperfusion. Perfusion of kidney with intercellular adhesion molecule-1 antisense oligonucleotides or antibody effectively blocked intercellular adhesion molecule-1–Mac-1 interaction and prevented I/R injuries in animal models.37–39 However, these strategies have been used only at the reperfusion stage and not during the cold preservation period.34 Genetic modification of the organ with the adenovirus delivery system is an alternative strategy to reduce I/R injury and graft immunogenicity. Prolongation of graft survival with adenovirus-mediated gene transfer of CTLA4-Ig, interleukin-10, Fas ligand, and intercellular adhesion molecule-1 into cold preserved organs has been achieved.40–43 Although promising and effective in small-animal models, there are several issues with this strategy in clinical use. For example, the protocol requires treatment of the donor at least 2 days before transplantation because the vectors used have limited transfection efficiency during cold storage and need time to translate to protein. Second, significant amounts of recombinant protein are required for protection. This is a problem because protein production is minimal by the time of reperfusion, even if gene transfection occurs during cold storage. Thus, this restricts the therapeutic effects.34

In our study, we combined both of the above strategies into a novel preservation solution. By implementing siRNA technology, we were able to downregulate the expression of certain genes involved in cold ischemic injury and leukocyte-mediated tissue injury. siRNA directly decreases gene expression in the cytoplasm of the cell. siRNA has the ability to inhibit even very low-level metabolism of target genes under hypoxic conditions. We targeted Fas, C3, and TNF-α for their involvement with the apoptosis, complement, and inflammatory response pathways. By comparing heart organs preserved in control solution and siRNA solution, we demonstrated the potential implications of siRNA technology to organ preservation. The ultrasound scans demonstrated that the siRNA-containing solution can partially prevent I/R injury and showed improved heart graft function. Furthermore, the prolongation of cardiac grafts after they were preserved in siRNA-containing solution supports the protective effect of the siRNA solution. The siRNA organ solution that we developed in this study consists of 3 different siRNAs targeting 3 different pathways that are crucial to I/R injury. The siRNA solution efficiently and specifically attenuates complement activation, apoptosis of cells, and inflammation of grafts.

Clinical translation of the present research would require substantial amounts of siRNA and attention to several considerations. For an example, in this study, we demonstrated that suppression of I/R injury was achieved by perfusing the heart with siRNA at a concentration of 100 μg/mL. Clinical trials would require a much higher volume, which would cause the total dose administered to be in the milligram range if converted on a per-weight basis. Large amounts of siRNA have previously been used for local delivery such as a clinical trial involving aerosol-delivered siRNA for respiratory syncytial virus in which dose escalation up to 150 mg per patient was achieved without reported treatment-associated adverse reactions.44 Although this is obviously a different system than
ex vivo cardiac perfusion, the feasibility of other trials to administer high concentrations of siRNA provides rationale for continued exploration of this approach in large-animal studies. Clinical translation of the present work has certain limitations that need to be addressed: lack of knowledge about species-specific siRNA transfection differences, need for development of scalable siRNA material under good manufacturing practices, unknown tissue distribution and washout of siRNA subsequent to reperfusion, unknown impact of gene silencing in the context of allogeneic transplantation, and elucidation of a “maximally tolerated dose” equivalent. Another practical consideration is the large-animal models chosen for preclinical studies; eg, porcine hearts are known to possess an inherent resistance to ischemic damage compared with human hearts. With these considerations and potential hurdles in mind, studies are currently being performed to elucidate the concentration of siRNA entering the organ and postimplantation pharmacokinetics. We also are examining methods of decreasing the amount of siRNA needed through the use of liposomal and frequency-dependent transfection methodologies.

Conclusion

This study demonstrates a novel siRNA solution that can decrease cardiac I/R injury, protect cardiac function, and prolong graft survival in heart transplantation. This can potentially have significance in clinical application.

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Disclosure

None.

References


Table. Pathological Change in Grafts on Day 7 After Transplantation

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<th>Treatment</th>
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<th>Hemorrhage</th>
<th>Neutrophil Infiltration</th>
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Values are mean±SEM. *P<0.05, †P<0.01, ‡P<0.001 versus groups of UW solution or control siRNA solution.


**CLINICAL PERSPECTIVE**

The present study provides a framework for development of a clinically applicable ex vivo method of modifying organ grafts to decrease immunogenicity. These findings support further investigation into the feasibility of large-organ manipulation through the perfusion method. Current organ storage solutions have the limitation of providing physical protection from hypothermia-associated changes but do nothing to inhibit ischemia/reperfusion injury or the ensuing rise in immunogenicity. By demonstrating that gene silencing can be induced in the context of this clinically applicable scenario, we can develop new targets and approaches to improve graft quality before transplantation, which would expand the use of marginal donors and increase organ transportation time.
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Supplementary Methods

Adult BALB/c mice weighing 25-30 g were anesthetized and heart organs were harvested as described in the Materials and Methods. The hearts were perfused with different concentration of Cy3 labeled siRNA solution through the inferior vena cava (IVC) and aorta until the vessel of the heart turned clear. The donor hearts were then excised and immersed in siRNA solution at 4 °C for 48 h. Heart tissues were sectioned and fluorescence of Cy3-siRNA was determined with an inverted microscope IX50 Olympus (Hitech Instruments Inc, Broomall, PA) with Image Pro (Media Cybernetics Inc., Bethesda, MD).

Supplementary Figure 1

Supplementary Figure 1. Determination of concentration of siRNA in preservation. Donor hearts were taken from the mice and preserved in a UW solution containing 25, 50, 100 and 200 μg of Cy3-labeled siRNA at 4 °C, as described in Materials and Methods. Forty-eight hours after preservation, hearts were harvested and sectioned. The distribution of siRNA was tracked by red fluorescence using fluorescence microscopy.