

Optimal Temperature of Graft Preservation after *ex Vivo* Gene Transfer in Lung Isografts

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The aim of this study was to determine the optimal temperature of graft preservation after *ex vivo* gene transfer to rat lung isografts. Left lungs were harvested and infused with cationic lipid/LacZ-DNA complex via the pulmonary artery, and the grafts were stored for 4h. The grafts (n=7) were allocated into groups I-IV according to the storage temperature: 4°C, 10°C, 16°C, and 23°C, respectively. Forty-eight h after orthotopic transplantation, the arterial blood gas was analyzed and the peak airway pressure (PAP) and the level of LacZ protein production in the grafts were measured by reverse transcription polymerase chain reaction. After reperfusion, the grafts were stained with hematoxylin and eosin. The grafts in groups III and IV showed more deterioration as evidenced by decreased arterial oxygen tension, increased PAP, and predominant infiltration of inflammatory cells compared with groups I and II. The level of LacZ production was significantly lower in group I than in groups II-IV. The optimal temperature of lung graft preservation after *ex vivo* gene transfer was determined to be 10°C, balancing considerations of lung injury and efficiency of transgene expression.

Key words: lung transplantation, gene transfection, optimal temperature, organ preservation

Lung transplantation has become an effective therapeutic option for selective patients with a variety of end-stage pulmonary diseases. Early graft dysfunction resulting from ischemia-reperfusion injury and chronic lung dysfunction, as manifested by bronchiolitis obliterans syndrome, accounts for the major part of mortality and significant morbidity after lung transplantation [1, 2]. Many experiments have been conducted with the goal of overcoming these obstacles [3-5], but few have been applicable to the clinical situation. A promising new approach in the field of organ transplantation is the use of gene therapy. The

application of gene therapy to organ transplantation has significant therapeutic possibilities for controlling adverse processes in lung transplantation. *Ex vivo* transfection of donor organs after harvest would enable targeted organ-specific transgene expression, and this could help to prevent graft damage. In addition, gene therapy has the potential to eliminate problems associated with immunosuppression by allowing the production of immunomodulatory proteins in the donor grafts resulting in local rather than systemic immunosuppression [6]. Several studies have already supported this possibility [7, 8].

One of the mechanisms involved in cationic lipid-mediated gene transfer is temperature [9], and a higher incubation temperature seems to be favorable for transgene expression [10-12]. In this regard, it

is desirable to keep the storage temperature high enough to elicit a good transfection; however, hypothermic preservation is the key to the maintenance of graft viability. Therefore, it is necessary to determine the optimal graft storage temperature in *ex vivo* gene transfection in order to apply this new modality in clinical settings. In the present study, we evaluated the influences among the storage temperature, the graft function, and the efficacy of transgene expression in a rat lung transplantation model.

Materials and Methods

Plasmid expression vector and cationic lipids. A eukaryotic expression vector plasmid containing the β -galactosidase gene, pCMV-SPORT- β -gal (Invitrogen Corp., Carlsbad, CA, USA), was used. The plasmid contains the reporter gene β -galactosidase from *Escherichia coli*. The cationic lipid reagent DMRIE-C (DMRIE: 1, 2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide/cholesterol) was purchased from Invitrogen Corp.

Preparation of cationic lipid/DNA complexes. The DNA /liposome complex for *ex vivo* administration was prepared as follows. A 5- μ l solution of cationic liposome containing 10 μ g of DMRIE-C reagent was added to 2 μ l (=2 μ g) plasmid DNA, which was then diluted with saline solution to a total volume of 300 μ l. We set the ratio of DMRIE-C reagent to plasmid DNA at 5 to 1 and the concentration of cationic lipid at 50 μ M, according to a previous report [13]. The resulting complex was mixed gently by drawing solution into a syringe and expelling it back into the total volume for 3 min. The mixed solution was incubated at room temperature for 6 min and gently injected into the left main pulmonary artery of the lung graft [14].

Animals. Male Crj: CD (SD) IGS rats weighing 240–290 g (Charles River Japan Inc. Osaka, Japan) were used in all experiments. All 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 Institute of Laboratory Animals Resources and published by the National Institutes of Health (NIH Publication No. 85–23, revised 1985).

Rat lung transplantation. Donor rats were

anesthetized, intubated, and heparinized and then underwent a median sternotomy-laparotomy. The abdominal aorta, inferior vena cava, and left atrial appendage were incised, and the lungs were flushed through the main pulmonary artery (PA) with 20 ml of cold (4°C) normal saline solution at 25 cmH₂O of pressure. After the heart-lung block was excised with the lungs inflated at end-tidal volume, the left lung was dissected away. The plasmid DNA/DMRIE-C complexes were administered via the left main PA, and the grafts were stored for 4 h in normal saline solution at various temperatures: 4°C (Group I, n=7), 10°C (Group II, n=7), 16°C (Group III, n=7), and 23°C (Group IV, n=7). Recipient rats were anesthetized, intubated, and subjected to a left thoracotomy and left pneumonectomy. Subsequently the grafts were implanted using the cuff technique [15]. Ventilation and perfusion to the grafts were restored, and a chest tube was inserted temporarily and then removed after the return of spontaneous respiration.

Assessment. Recipient animals underwent median sternotomy 48 h after transplantation. After the ligation of the right main bronchus and the right main PA, isografts only were ventilated with 100% oxygen for 5 min (tidal volume=1.5 ml, respiratory rate=100/min) while peak airway pressure (PAP) was measured by polygraph. An arterial blood sample was drawn from the ascending aorta and blood gas analysis was performed, and the left lung isografts were extracted and immediately flushed with 20 ml of cold PBS (–) from a height of 25 cm. The middle quarter of lung grafts was fixed with 20% formalin and stained with hematoxylin and eosin. The remaining lung grafts were frozen in liquid nitrogen and stored at –80°C for reverse transcription-polymerase chain reaction (RT-PCR) assessment.

Quantitative RT-PCR. We measured the expression of lacZ mRNA by quantitative RT-PCR assay. For the following reactions, a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Corp.) was used according to the manufacturer’s instructions. First-strand cDNA was generated by using a 20- μ l reaction volume containing 5 μ g of the extracted total RNA, 10 mM dNTP mix, 0.5 μ g per μ l Oligo (dT), 10 \times RT buffer, 25 mM MgCl₂, 0.1 molar dithiothreitol, 1 μ l RNASEOUT Recombinant RNase Inhibitor, and 1 μ l (50 units) of SuperScript II RT. Synthesis time was 50 min at

42°C. The cDNA was purified by incubation for 20min at 37°C with 1 μ l RNaseH. The purified cDNA was used for quantitative PCR. Quantitative PCR was performed in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Using this system, PCR products were quantified by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA for each cycle [16]. We used 50 μ l of reaction mixture containing 5 μ l of the cDNA acquired by RT described above, 25 μ l of the 2 \times SYBR Green Master Mix (Applied Biosystems), 3 μ l sense and anti-sense primers (5 μ M), and 14 μ l deionized water. The amplification program consisted of 2min at 50°C, 10min at 95°C, and 40 cycles involving denaturation at 95°C for 15sec and annealing/extension at 60°C for 1min. A semi-quantitative value for the initial target concentration in each reaction was determined on the basis of the kinetic approach using the GeneAmp 5700 software. GAPDH was used to normalize LacZ expression from the same cDNA sample.

cDNA primers for quantitative RT-PCR. LacZ primers for quantitative PCR were designed using the primer design software Primer Express (Applied Biosystems). LacZ primers were designed as follows: sense-primer, 5'-GGCAGTTATCTGGAAGATCAGGA-3'; antisense-primer 5'-CAACATGGAAATCGCTGATTTG-3'. Rodent GAPDH forward and reverse primers were purchased from Applied Biosystems.

Statistical analysis. Data are expressed as means \pm standard deviation. The groups were compared by an analysis of variance with the Tukey-Kramer test, and differences were considered significant when the probability was less than 0.05.

Results

The arterial oxygen pressure (PaO₂) levels in groups I and II were significantly higher than those in groups III and IV (mean PaO₂ \pm standard deviation [SD] in group I, II, III, and IV: 497.3 \pm 95.6 mmHg, 543.3 \pm 74.2 mmHg, 192.1 \pm 103.0 mmHg, and 129.1 \pm 42.6 mmHg, respectively; $p < 0.0001$, respectively). There were no significant differences between groups I and II ($p = 0.472$) (Fig. 1). There were no significant differences between the groups regarding the arterial carbon dioxide pressure

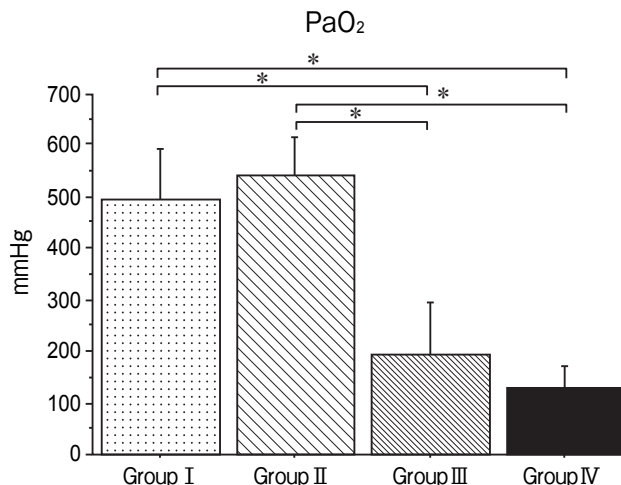


Fig. 1 Arterial blood gas analysis. Forty-eight h after lung transplantation the right main bronchus and the right main PA were ligated; 5min later, the PaO₂ of blood from those vessels was measured. Data are shown as means \pm standard deviation. (PaO₂ = arterial oxygen tension. *indicates $p < 0.01$)

(PaCO₂) levels (mean PaCO₂ \pm SD in groups I, II, III, and IV: 40.8 \pm 8.0 mmHg, 37.7 \pm 8.9 mmHg, 41.9 \pm 16.9 mmHg, and 24.2 \pm 19.8 mmHg, respectively). The PAPs in groups I and II were significantly lower than those of groups III and IV (mean PAP \pm SD in groups I, II, III, and IV: 24.2 \pm 4.1 mmHg, 24.8 \pm 3.9 mmHg, 36.4 \pm 8.6 mmHg, and 43.8 \pm 10.8 mmHg, respectively; $p = 0.0007$ and $p < 0.0001$: group I vs. group III and IV; $p = 0.0014$ and $p < 0.0001$: group II vs. group III and IV, respectively). There were no significant differences between groups I and II ($p = 0.999$) (Fig. 2). The gene expression in group I was significantly lower than in other groups and that in group II was significantly higher than in group IV (mean relative gene expression level \pm SD in groups I, II, III, and IV: 0.330 \pm 0.156 relative units, 0.964 \pm 0.246 relative units, 0.868 \pm 0.143 relative units, and 0.631 \pm 0.214 relative units, respectively; $p < 0.0001$, $p < 0.0001$, and $p = 0.037$: group I vs. group II, III, and IV; $p = 0.019$: group II vs. group IV) The difference between groups II and III was not statistically significant ($p = 0.812$) (Fig. 3). A histologic examination by a pathologist demonstrated that the graft lung tissue in groups III and IV showed higher degrees of severe injury to the lung parenchyma, suggesting the pathologic features of the lungs in acute respiratory distress syndrome (ARDS) (Fig. 4). The

alveolar ducts in group III appeared dilated as a result of the collapse and consolidation of adjacent alveoli. The graft in group IV showed a layering of the hyaline membranes on the alveolar septa. On the other hand, the grafts in groups I and II showed only a slight degree of hemorrhaging and alveolar edema.

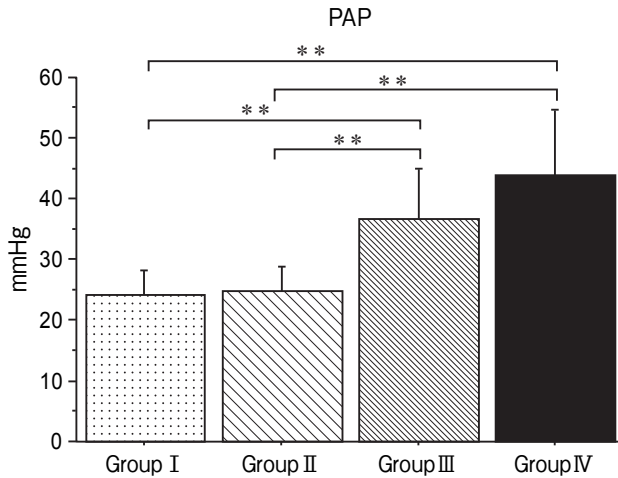


Fig. 2 Peak airway pressure. Forty-eight h after lung transplantation, PAP was measured by polygraph for 5 min after the ligation of the right main bronchus and the right main PA. Data are shown as means \pm standard deviation. (PAP, peak airway pressure; ** indicates $p < 0.05$.)

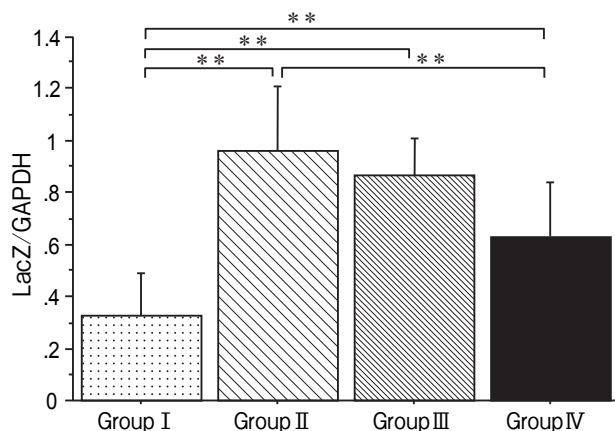


Fig. 3 Quantitative RT-PCR analysis of the expression of lacZ mRNA. Lungs were transfected *ex vivo*, stored for 4 hours at 4°C, 10°C, 16°C, and 23°C, and transplanted. The gene expression in the transplanted left lungs was assessed on postoperative day 2. Data are shown as means \pm standard deviation. (RT-PCR = reverse transcription-polymerase chain reaction; ** indicates $p < 0.05$)

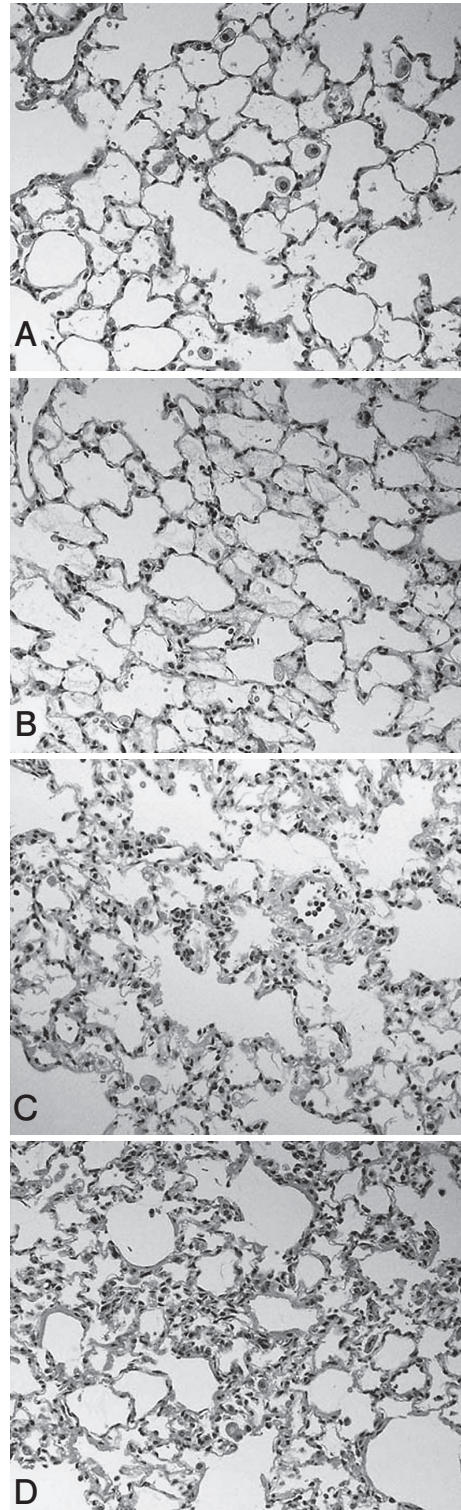


Fig. 4 Histologic findings of lung grafts tissue in (A) 4°C, (B) 10°C, (C) 16°C, (D) 23°C. Forty-eight h after lung transplantation, rat lung grafts were extracted and stained with hematoxylin and eosin. (Hematoxylin-eosin staining; original magnification $\times 200$.)

Discussion

As gene therapy principles and practices have improved, the application of gene therapy to transplantation has now become feasible in the laboratory and it is being used to study the mechanisms of transplantation tolerance, as well as to explore new strategies for tolerance induction. Although transient expression is regarded as one of the drawbacks of current gene delivery systems, this actually is desirable in the treatment of acute, self-limited conditions such as ischemia-reperfusion injury [17]. On the other hand, for chronic rejection, it is important that transgene expression exhibit long-term stability and that liposome-DNA complexes are repeatedly administered without inducing an inflammatory response. A second administration of viral vector DNA is not efficient owing to a significant inflammatory response, since the transduced cells express viral proteins and are promptly destroyed by the host immune system [18]. Canonico and associates [19] have demonstrated that weekly intravenous or aerosol delivery of cationic liposome DNA complexes in rabbits resulted in sustained transgene expression and did not affect lung histology, mechanism, or oxygenation. Therefore, cationic liposomes may be a more suitable vector for the treatment of chronic rejection than viral vector. This is the main reason that we chose cationic liposome as the vector for *ex vivo* transfection in this study.

In this study, 4 storage temperatures were chosen for the following reasons: 4°C is the temperature currently used for human lung preservation. Previous reports have insisted that the optimal preservation temperature is in the vicinity of 10°C [20, 21]. The temperature of 23°C is too warm to preserve the lung, but this group was included as previous studies have demonstrated that this temperature is good for transfection [10], and 16°C is equal to the mean of 10°C and 23°C. Hypothermia is better for organ preservation than normothermia [20]. Currently, 4°C is used as the storage temperature of lung grafts in clinical lung transplantation, as determined through extensive research efforts [22]. The higher the preservation temperature, the more the grafts deteriorate, as evidenced by a decreased PaO₂ level and increased PAP in the present study. A histologic examination in our study demonstrated that the grafts in groups III

and IV showed pathologic features of the lung similar to those observed in ARDS. There is no doubt that hypothermia is essential for ischemic organ preservation to slow the metabolic processes and maintain lung function; however, transgene expression is inhibited by decreased temperature, thus suggesting that the mechanism of nuclear import is energy dependent [9]. It is well known that as the phospholipid bilayers become heated, the membrane permeability to small molecules increases [23]. Therefore, we anticipated that a higher preservation temperature would lead to higher transfection efficiency. However, the transfection efficiency was the highest in group II, while the gene expression decreased in the groups at higher storage temperatures (groups III and IV), perhaps due to an impairment of the graft tissue caused by both the warm temperature during preservation and ischemia-reperfusion injury. The very low transfection rate of group I was probably due to a significant decrease of enzymatic activity in such hypothermic circumstances.

In summary, we judged the optimal temperature of graft preservation after *ex vivo* gene transfer to be 10°C, balancing the considerations of lung injury and the efficiency of transgene expression. Whether these levels of transgene expression are sufficient to produce beneficial effects in a graft still remains to be determined in future studies using functional genes, and new vectors must overcome the problems associated with the lack of specific cell targeting *in vivo*, low transfection frequencies, and the lack of a long-term stable expression.

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