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# Fas ligand gene transfer combined with low dose cyclosporine A reduces acute lung allograft rejection

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**Abstract** The interaction between Fas and its ligand (FasL) induces apoptosis in the Fas-expressing cell. We hypothesized that liposome-mediated FasL gene transduction to the lung allograft, in addition to low-dose immunosuppression, might reduce acute rejection. Orthotopic left lung allotransplantation was performed in male rats (Brown Norway to Fischer F344). FasL gene transfer was performed by use of the plasmid pBCMGSNeo carrying the gene coding for murine FasL and the cationic liposome GL#67:DOPE. Six hundred and sixty micrograms of DNA in 250 µl $H_2O$ and 0.5 µmol GL#67 in 250 µl $H_2O$ were diluted to 5 ml with saline solution. This emulsion (20 °C) was instilled retrogradely through the left pulmonary vein after flushing with LPD solution (20 ml, at 4 °C). Subsequently, the graft was stored at 10 °C for 3 h. A single dose of cyclosporine A (CsA; 2.5 mg/kg i.m.) was given to all groups 48 h after the transplantation. In group 1 ($n = 6$), FasL/GL#67 was instilled as described. In group 2 ($n = 5$), GL#67 was given without DNA. Group 3 ($n = 5$) animals received CsA only. Five days after transplantation, gas exchange was assessed after exclusion of the contralateral native lung ($FiO_2 = 1.0$). Grafts were flushed with saline solution and fixed in formaldehyde for histological evaluation. No statistical difference in gas exchange ($PaO_2$) between the two control groups 2 (6.4 ± 0.4 kPa) and 3 (7.4 ± 0.4 kPa) could be detected 5 days postoperatively ($P = 0.9$). In contrast, grafts transduced with FasL (group 1) had significantly better gas exchange on postoperative day 5 ($PaO_2$: group 1 37.0 ± 10.6 kPa vs group 2 6.4 ± 0.41 kPa; $P = 0.002$). Two animals in group 1 revealed no or only minimal improvement in gas exchange. Histologically, all lung specimen of all groups showed signs of acute rejection (A2). Leukocyte infiltrates, rated by two independent observers, were less severe in all group 1 animals. Liposome-mediated FasL gene transfer at the time of harvest in combination with low-dose CsA reduces acute rejection in four out of six animals in this model of rat lung allotransplantation.

**Key words** Fas ligand · Gene transfer · Low-dose immunosuppression · Lung allograft

## Introduction

Gene transfer to solid organ allografts represents a new approach to modifing the biological response following transplantation. Hyperexpression of specific genes introduced at the time of harvest might protect the allograft from posttransplant ischemia/reperfusion injury and acute rejection [1]. Fas ligand (FasL), one of the receptors of the TNF receptor family, induces apoptosis in activated, Fas-expressing cytotoxic T-lymphocytes [2].

Previous studies demonstrate that genetically engineered FasL-expressing myocytes, which were cotransplanted with pancreatic islets into diabetic mice, protected the allogeneic islets from acute rejection rendering the mice normoglycemic for over 80 days [3]. A number of experiments followed with the aim of inducing local immunosuppression to allografts by transduction of the FasL gene. The results, however, were contradictory [4, 5]. In the present study we evaluated the modulation of acute lung allograft rejection by liposome-mediated FasL transduction at the time of organ harvest in combination with low-dose cyclosporine A (CsA) in a rat model of unilateral lung allotransplantation.

## Material and methods

### Surgical procedure

Orthotopic single left lung allotransplantation was performed. Donor lungs from male Brown Norway rats were transplanted into Fischer (F344) rats weighing 200–250 g. In this strain combination (major histocompatibility complex missmatch), complete rejection of the graft occurs within 5 days (120 h) [6]. For the vascular anastomoses, a cuff technique was employed, as described by Mizuta et al. [7]. All animals received humane care in compliance with the European Convention of Animal Care. The protocol was approved by the local Animal Study Committee.

### Donor

The animal was anesthetized by intraperitoneal administration of pentobarbital at a dose of 50 mg/kg body weight and heparinized (500 IU/kg). A tracheotomy was carried out and the animal was ventilated ($FiO_2$ 1.0, $f_R = 65$/min, tidal volume 10 ml/kg body weight) with a Harvard rodent ventilator (Harvard Apparatus, South Natick, Mass., USA). After cutting the inferior vena cava and left appendix of the heart, a small silicon hose was inserted into the main pulmonary artery. Both lungs were flushed with 20 ml LPD solution (Perfadex; XVIVO Transplantation Systems, Gothenburg, Sweden) at a pressure of 20 cm $H_2O$ and a temperature of 4 °C. The trachea was tied in end-inspiration, and the heart-lung block was removed. Cuffs made from 14 gauge cannulas were placed on the pulmonary artery and the pulmonary vein, and the vessels were inverted and tied onto the cuff.

### Recipient

The recipient was anesthetized by breathing Halothane in a glass chamber, intubated, and anesthesia was maintained with Halothane 2–2.8%. Ventilation parameters were the same as in donor animals. A left lateral thoracotomy was performed in the fourth intercostal space. The left hilum was dissected. After clamping the pulmonary artery and vein with removable microclips, the pulmonary vein was opened, flushed with heparinized saline solution, and the cuff inserted and fixed with 6–0 silk. In the same way, the pulmonary artery was anastomosed. The recipient's native left lung was removed and the bronchial anastomosis performed with a running over-and-over suture with 9–0 Monosof (generously provided by Autosuture, Kendall Medical, Wollerau, Switzerland). The lung was ventilated and then reperfused. A chest tube was inserted and the thoracotomy closed. The chest tube was removed after restoration of sufficient spontaneous breathing a few minutes after extubation.

### Study groups

Three groups were studied. In group 1, FasL gene transfer was performed as described below. In group 2 grafts were flushed with the liposome emulsion only, not containing AdvFasL. Group 3 underwent transplantation without administration of either the liposome or the adenovirus. All recipients received a single dose of CsA (2.5 mg/kg i.m.) 48 h after transplantation.

### Gene transfer

For FasL gene transfer, a cationic liposome (GL#67:DOPE, generously provided by Genzyme, Framingham, Mass., USA) was used which demonstrated high transduction rates in the lung [8]. The murine FasL cDNA was previously subcloned into the Xho I and Not I sites of the pBCMGSNeo expression plasmid [9]. Six hundred and sixty micrograms of plasmid DNA in 250 µl $H_2O$ and 0.5 µmol GL#67 in 250 µl $H_2O$ were diluted to 5 ml with saline solution at 20 °C. This emulsion was instilled retrogradely through the left pulmonary vein. After infusion of the lipid/DNA suspension, the graft was stored in LPD solution at 10 °C until implantation. Total ischemic time (exposure time of the plasmid to the graft) was 3 h.

### Assessment

The recipient animal was reanesthetized by intraperitoneal administration of pentobarbital (50 mg/kg) 120 h after implantation. A tracheotomy was performed and the animal was ventilated with $FiO_2 = 1.0$, a frequency of 100/min, a tidal volume of 8 ml/kg, and positive end-expiratory pressure of 0.5 cm $H_2O$. The right hilum was dissected and the right pulmonary artery and the right main bronchus were occluded with microvessel clips for functional assessment of the graft. Five minutes after occlusion, an arterial blood gas sample was collected from the thoracic aorta. After measurement of the blood gas and heparinization (500 IU/kg) the lungs were flushed via the pulmonary artery with 20 ml saline solution. The heart-lung block was excised and the lungs were fixed overnight at room temperature with 10% buffered formalin. Formalin was instilled through the trachea at a pressure of 20 cm $H_2O$ to ensure equal expansion of all grafts.

### Blood gas analysis

For the assessment of $PaO_2$, an automated blood gas analyzer (AVL 993, AVL List, Graz, Austria) was used.

### Histology

Histological slides, stained with hematoxylin & eosin, were evaluated in blind fashion by two independent pulmonary pathologists according to the guidelines of the International Society for Heart and Lung Transplantation [10]. In addition, terminal deoxynucle-

otidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) of apoptotic cells was performed in slides of each animal using a standard technique as described previously [11].

### Statistical analysis

All values are given as the mean ± SEM. One-way analysis of variance (ANOVA) between groups with planned comparison (contrast analysis) was applied (Statistica 4.5 software; Statsoft, Tulsa, Okla., USA). A $P$ value of less than 0.05 was considered significant.

## Results

### Experimental groups

The operation time for implantation was 50 ± 5 min and did not differ between groups. Warm ischemic time was 23 ± 0.5 min in the control group and 23 ± 0.4 min in the treatment group, with no statistical difference between groups.

### Arterial blood gas analysis

There was no statsitically significant difference in oxygenation at day 5 postoperatively between groups 2 and 3 (group 2: 6.4 ± 0.4 kPa vs group 3: 7.4 ± 0.4 kP; $P = 0.900$; Fig. 1 a). Grafts transduced with FasL (group 1) had a significantly better gas exchange on day 5 (PaO2: group 1: 37.0 ± 10.7 kPa vs group 2: 6.4 ± 0.4 mmHg; $P = 0.002$). This statistically significant result could be demonstrated in spite of the fact that two animals in group 1 revealed no or only minimal improvement in gas exchange (Fig. 1 b).

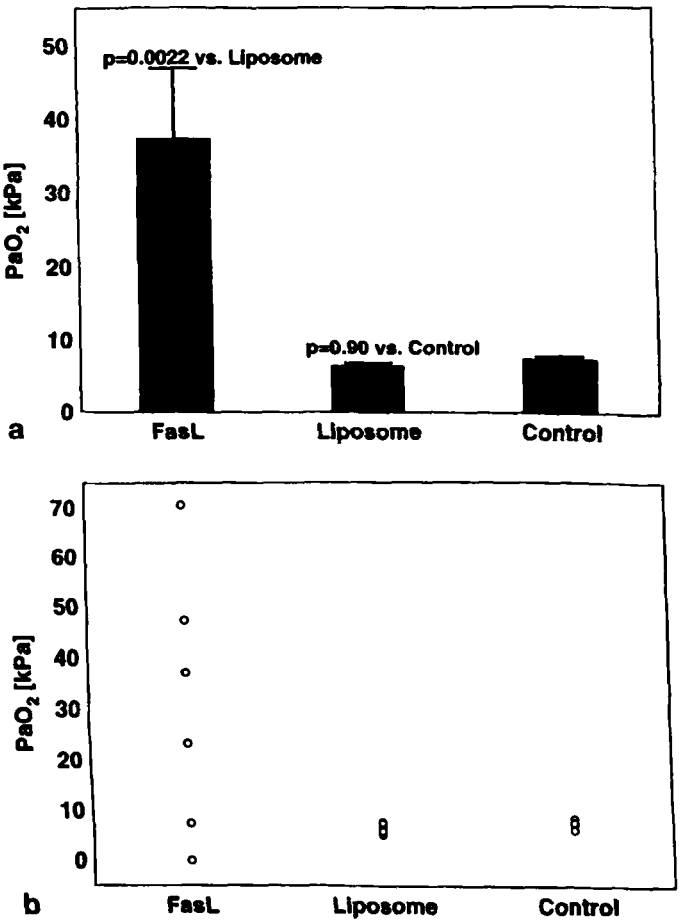


**Fig. 1 a, b** Gas exchange of the lung graft after exclusion of the right native lung 5 days after transplantation. **a** In Fas ligand (FasL)-transduced grafts (group 1) oxygenation ($PaO_2$) was significantly improved ($P = 0.002$; mean ± SEM). **b** The variability of oxygenation on the 5th day ($PaO_2$) in the FasL group is large, as demonstrated in the scatter plot

### Histological grading

Histologically, all lung specimen demonstrated acute rejection (A2). Leukocyte infiltrates were less extensive in all FasL-treated animals, as rated by two independent observers. Three of the FasL-treated animals exhibited acute bronchiolitis and bronchopneumonia.

### TUNEL

In group 2 animals a few apoptotic T-lymphocytes were seen. In group 3 animals, however, clusters of apoptotic cells were detected (Fig. 2).

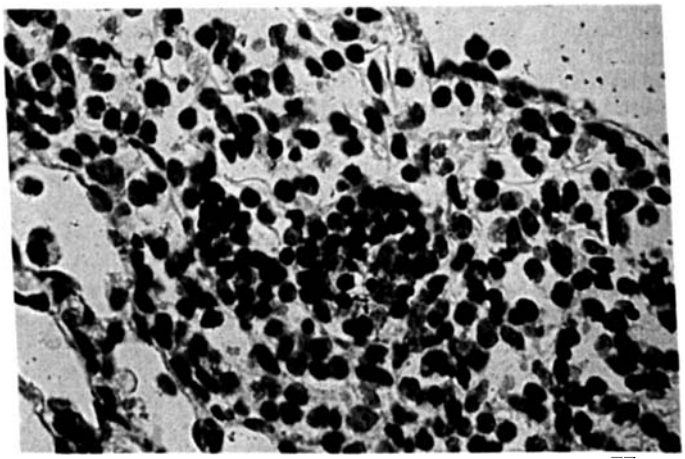

**Fig. 2** Nick-end labeling (TUNEL) showed clusters of apoptotic cells in FasL-tranduced grafts on the 5th postoperative day, which were not seen in control animals

## Discussion

We demonstrated that liposome-mediated FasL gene transfer at the time of harvest in combination with low-dose CsA reduces rejection in a small animal model of lung allotransplantation. Cytotoxic T-lymphoctes are the main effector cells of acute rejection following organ transplantation. FasL is a receptor of the tumor necrosis factor family and mediates apoptosis in a large number of cell types. T-cells use FasL to induce apoptosis in Fas-expressing cells [12]. Inversely, it is known that in immunoprivileged tissues, as in the testis and the cornea, FasL is expressed [13]. It is therefore thought that hyperexpression of FasL induces apoptosis in infiltrating lymphocytes in these tissues. The same mechanism may also protect transplanted allogenic organs from infiltrating lymphocytes and reduce acute rejection [14].

A number of investigators could demonstrate this effect in rodent models. Lau et al. reported that cotransplantation of genetically engineered syngenic myocytes expressing FasL constitutively with allogenic islets into diabetic mice inhibited rejection of the islets, rendering the diabetic mice normoglycemic for more than 80 days [3]. Similarly, Swenson et al. described, in a rat model, improved survival after transplantation when the allogenic kidney was transduced with FasL [15].

Because of vigorous discussions of the latter paper and the fact that other groups could not demonstrate the effect of FasL gene transduction, we decided to evaluate this mechanism in our system, which has the advantage that gas exchange,as a direct functional parameter of the graft,can be measured.

Our work proceeded from basic experiments of gene transfer in rat lung transplantation using adenoviral vectors at the time of organ harvest [16]. Transfer of the gene encoding for chloramphenicol acetyltransferase (CAT) resulted in expression up to 20 days following syngenic transplantation in inbred rats [17]. In subsequent experiments performed by Boasquevisque et al. it has been demonstrated that liposome-mediated gene transfer of TGF$\beta$ at the time of harvest reduces rejection in allogenic rat lung transplantation [18]. Liposomes have some advantages over adenoviral vectors, as infectious complications in the immunosupressed recipient might be a major limitation to the use of adenoviral vectors in the transplant setting.

In preliminary studies without low-dose immunosuppression, we could not demonstrate an effect of FasL transduction [4]. This is in contrast to the mentioned studies with renal grafts [15], but may be explained by the difference in donor and recipient strain combination or less FasL expression. In another series of preliminary experiments we noted a substantial variability in transgene expression in our system using GL#67 and CAT as reporter gene (data not shown). We speculate that gene transfer in the two 'non-responders' was insufficient. On the other hand we can not exclude that more FasL was expressed in these animals, which might have damaged the graft [5], as, again in contrast to the studies by Swenson et al., we could not demonstrate increased FasL expression with RT-PCR in the transduced grafts. Substantial FasL expression was also present in the untreated rejected lung allografts, most probably on infiltrating T-lymphocytes.

Gas exchange and the macroscopic appearance demonstrated an impressive reduction of rejection by FasL gene transfer in combination with low-dose CsA in the majority of the animals. Histologically the differences were less impressive, but a clear reduction of lymphocyte infiltrates in the FasL-transduced grafts was noted. This seems not surprising, as leukocyte migration to the graft is not reduced by FasL transduction. Interestingly, TUNEL in the FasL-transduced lungs showed clusters of apoptotic cells, which were not noted in the untreated grafts (Fig. 2). In conclusion, we could demonstrate that liposome-mediated FasL gene transfer in combination with low-dose immunosuppression reduces acute rejection in this model of lung allotransplantation. The mechanism of the additive effect of CsA and the reason for the large variety of the results in the FasL-treated group need further evaluation.

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