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ORIGINAL ARTICLE

Blockage of the macrophage migration inhibitory factor expression by short interference RNA inhibited the rejection of an allogeneic tracheal graft

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Summary

We investigated the inhibitory effect of blocking the macrophage migration inhibitory factor (MIF) on the fibrous obstruction of a transplanted allograft in a murine model of obstructive bronchiolitis (OB). Tracheal grafts from C57BL/6 mice were transplanted into a subcutaneous pouch of BALB/c. Three days after transplantation, liposome including short interference (si) RNA for MIF was injected into the lumen of the grafts. The allografts were then harvested 7, 14 or 28 days after transplantation for an evaluation of the morphological changes. The MIF expression, which was ubiquitously recognized in the epithelium of allografts, decreased after the *in vivo* transfection of MIF siRNA. OB formation was therefore inhibited significantly more by the treatment with MIF siRNA than the allografts injected with empty liposome on the 14th day, however, no difference was observed between them on the 28th day. Treatment with MIF siRNA inhibits the destruction of tracheal allografts and OB formation in the early phase, and MIF was thus found to be one of the major cytokines involved in the rejection of the allogeneic trachea.

Introduction

More than 1 year after lung transplantation many patients develop obliterative bronchiolitis (OB) [1,2], which is characterized by progressive dyspnoea and lung dysfunction, and finally which can lead to death. This is the major factor currently limiting the long-term survival of patients after lung transplantation and it is responsible for the 5-year survival rates that are approximately 30% lower than for other solid organ transplantations [3]. The therapies for OB, including immunosuppressive regimens or antibody therapies, are generally ineffective and the treatment of last resort is re-transplantation.

The heterotopic murine tracheal allograft model is a well-established animal model for the investigation of OB [4]. In this model, nonimmunosuppressed allografts develop a fibroproliferative lesion closely resembling the histological changes of OB in humans. Although OB is manifested as a progressive fibroproliferation of the small airways which are thought to be a consequence of the pathological repair process after lung injury, little is still known about the underlying mechanism of this disorder [5]. Both macrophage and T-cell activation have been postulated to play a critical role in the initiation of the delayed-type hypersensitivity reaction leading to the production of several cytokines or growth factors, which cause epithelial damage, smooth muscle cell proliferation and a gradual occlusion of the airway lumen [6].

Macrophage migration inhibitory factor (MIF) is a 12.5 kDa protein [7] that is encoded in the 1 Kb gene on chromosomes 22q11 and 21q22.3 [8], which are also known as a cytokine that is produced by activated T lymphocytes [9] and several human organs such as the anterior pituitary gland during inflammation [10], the adrenal gland [11], the bronchial epithelium [12], the brain [13], the islet beta cells [14], and so on. Although

the molecular functions of MIF have been yet to be completely elucidated, MIF is considered to inhibit the random migration of macrophages, while activating the T-cell functions [15], and also exhibiting enzymatic activities [16] and counter-regulating the immunosuppressive activities of glucocorticoids [17]. In addition, MIF has been shown to play a role in the pathogenesis of such types of inflammation as septic shock [18], inflammatory arthritis [19], glomerulonephritis [20], and allogeneic skin graft rejection [21]. Taking into account the fact that MIF is ubiquitously expressed in airway epithelial cells, we hypothesized that MIF might thus play an important role in the pathogenesis of untreatable OB in the transplanted lung.

Short interference RNA (siRNA) consists of short 20- or 21-mer double-stranded RNA, and it can be used to silence the expression of homologous messenger RNA [22]. This mechanism is highly sequence-specific and thus enables the expression of the target protein to be turned off. Many studies have demonstrated a high efficiency of gene silence by siRNA both *in vitro* and *in vivo* [23–25].

This study is the first report of the *in vivo* administration of siRNA for a tracheal transplantation model, and we herein attempted to down-regulate the MIF protein expression on the bronchial epithelium and the inhibitory effect on the formation of fibrotic occlusion in transplanted tracheal allografts in a murine model by siRNA in order to better understand the association between MIF with chronic allograft rejection in lung transplantation.

Materials and Methods

Mice

Both 15 BALB/c (H2-d) and C57BL/6 (H2-b) mice, respectively, (Charles River Japan, Kanagawa, Japan) were obtained from pathogen-free colonies and then were housed and used in accordance with the rules of the Institutional Animal Care Committee.

Tracheal transplantation

Donor mice (C57BL/6) were anaesthetized with ether, the trachea was dissected from the larynx to the carina and stored in saline. Recipient mice (C57BL/6, BALB/c) were anaesthetized with ether and then the tracheal graft was immediately transplanted into a subcutaneous pouch in the dorsal area of the mice. Three days after transplantation, the tracheal graft was then exposed and ian Opti-MEM 1 culture medium (Invitrogen Co., Carlsbad, CA, USA) either containing MIF siRNA or not was injected into the lumen of tracheal graft under directly vision. The mice were thereafter sacrificed at 7, 14 and 28 days after transplantation at which time the tracheal grafts were assessed.

Immunohistochemistry

The paraffin embedded sections were deparaffinized and stained using the streptoavidin–biotin–peroxidase complex method. Briefly, normal rabbit serum was applied for 20 min to block any nonspecific binding of the antibody. Next, each section was microwaved at 99 °C for 20 min in 0.01 M of Na-citrate buffer (pH 6.0) for antigen retrieval. These sections were incubated with 10% rabbit normal serum for 20 min. The first antibody used was goat polyclonal IgG against a murine MIF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dilution ratio of 1:300 at room temperature for 30 min. Hematoxylin was used for counter staining, while counterstaing was performed with hematoxylene.

Preparation for siRNA

TransMessenger Transfection Reagent was purchased from Qiagen (Valencia, CA, USA). The siRNA for murine MIF was designed according to the manufacturer's protocol. Briefly, we searched for the 23-nt sequence motif AA-(19 nucleotides)-TT and confirmed that the G/C-content of the arrangement was 50%. Using the BLAST-search of the NCBI database, the sequence was identified to be specific to the MIF gene. The siRNA sequences targeting murine MIF (Acc.No.BC024895) corresponded to the coding region 279–299 after the start codon. For annealing siRNA, 50 μM single strands RNAs were mixed with 5× annealing buffer (500 mM of potassium acetate, 150 mM of HEPES-KOH pH 7.4, 10 mM of magnesium acetate), and then were incubated for 1 min at 90 °C and for 60 min at 37 °C. Twenty μM of synthesized siRNA was preserved at −20 °C before use. An Opti-MEM 1 medium containing siRNA for transfection into the tracheal allograft was made according to manufacturer's TransMessenger protocol. Briefly, 1.2 μl of enhancer R and 26.8 μl of buffer EC-R were mixed with 2.0 μl of siRNA and then were incubated for 5 min at room temperature. Next, after incubation for 10 min with 2.4 μl TransMessenger Transfection Reagent, a total of 30 μl of transfection complex was added to 270 μl of Opti-MEM 1. This mixture was always made immediately before use.

Histological assessment

The harvested tracheal grafts were fixed in 10% formaldehyde, embedded in paraffin and then cross sections were made. These specimens were stained with hematoxylin and eosin and then were evaluated by light microscopy. Assessments of the tracheal grafts were done in a blinded fashion by two independent observers. The transplanted tracheal grafts were subjectively evaluated based on the

patency of the tracheal lumen, the viability of the tracheal epithelium, described elsewhere [26]. The patency of the tracheal grafts was assessed as follows: the patency of the tracheal lumen (%) = total area of the patent lumen × 100/total area of the tracheal lumen inside the basement membrane.

Results

Introduction of siRNA by lipofection

To optimize the concentration of the siRNA, excised tracheae were incubated in Opti-MEM 1 containing liposome (see Materials and methods) mixed with various concentrations (1, 2 and 4 μM) of rhodamine-labeled nonspecific siRNA (Fig. 1a–c), and then were observed by fluorescent microscopy. At a concentration of 2 μM (Fig. 1b), the rhodamine was clearly recognized as a red signal in the inner layer while at a concentration of 1 μM (Fig. 1a), no such signal was observed. In addition, the signal increased in the outer layer at a concentration of 4 μM (Fig. 1c). When 2 μM of rhodamine-labeled nonspecific siRNA prepared with liposome was injected into the transplanted trachea on the 3rd day *in vivo*, red signal was clearly observed in both the inner and outer layers of the tracheal graft on the next day (Fig. 1d).

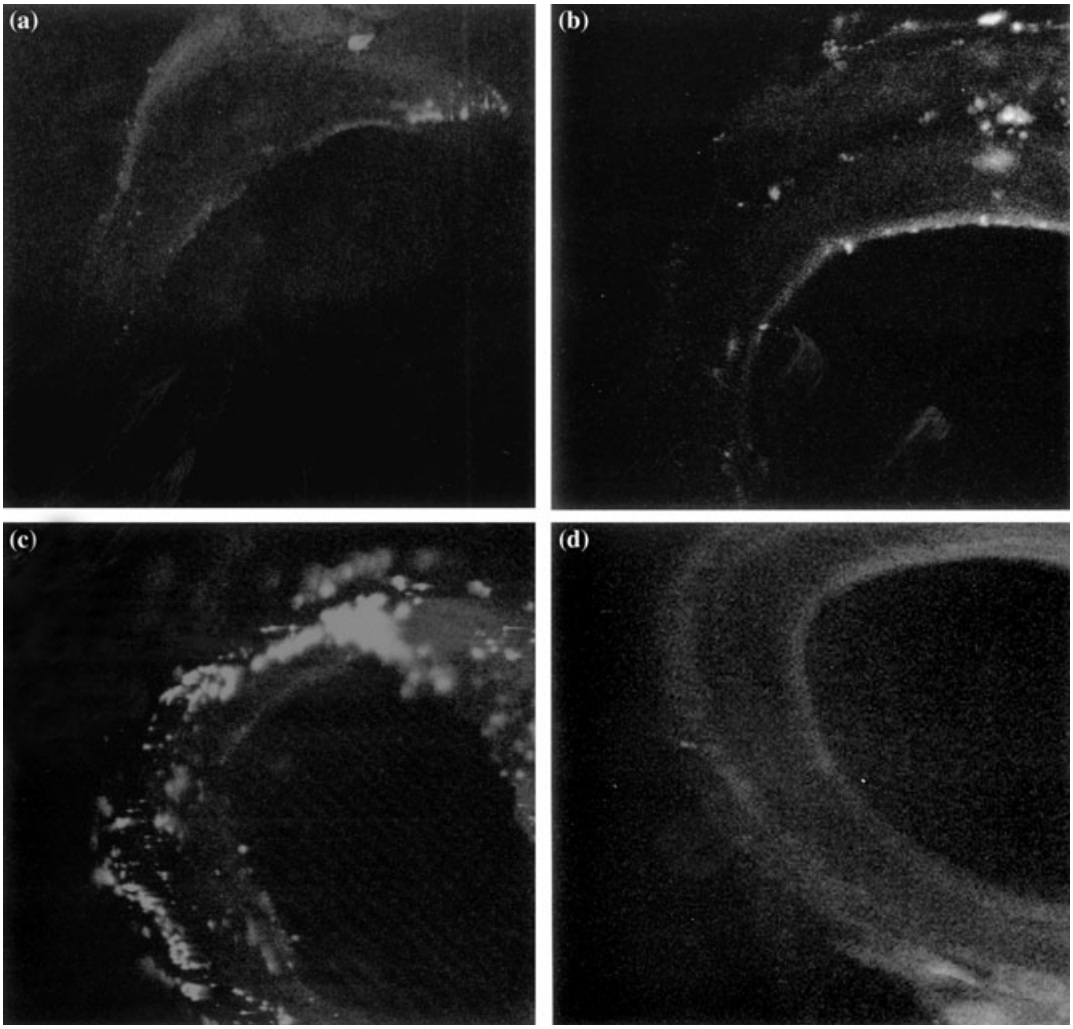

Figure 1 The administration of siRNA to induce MIF in the tracheal grafts. (a–c) Various concentrations (1, 2 and 4 μm) of rhodamine-labeled nonspecific siRNA were mixed with liposome, and then were transfected into the tracheal grafts *ex vivo*, and a red signal was observed by fluorescent microscopy (×100). (d) As 2 μm of siRNA was considered to be appropriate, it was injected into the transplanted tracheal grafts *in vivo*, and then was harvested on the next day. The red signal was recognized as a red signal not only in the inner layer but also in the outer layer of the tracheal graft (×100).

The effect of siRNA for MIF expression

To evaluate the silencing effect of MIF siRNA on the MIF expression in the transplanted tracheal grafts, we injected liposome either with or without MIF siRNA into the tracheal grafts. The initial ischaemic phase was seen during the first to the 7th day with a peak on the 2nd day after tracheal transplantation. MIF siRNA was injected on the 3rd day after transplantation as the transplanted grafts gradually became vascularized after the 3rd day and lymphatic infiltration occurred during the 3rd to the 14th day with a peak on the 7th day after transplantation [6]. The expression of MIF was recognized in the cytoplasm of epithelial cells as well as fibroblasts in both the syngeneic grafts (Fig. 2a) whereas the MIF was recognized in the fibroblasts but not in the epithelial cells in allogeneic tracheal grafts treated with control liposome on the 7th day (Fig. 2b). In the allogeneic grafts treated with liposome containing siRNA for MIF, the MIF was not recognized in either epithelial cells or fibroblasts (Fig. 2c).

The effect of MIF siRNA for the rejection of the allogeneic tracheal grafts

The transplanted allogeneic and syngeneic tracheal grafts were harvested and examined to determine the patency of the luminal area of the transplanted tracheal grafts, and the viability of epithelial cells on days 7, 14 and 28 after transplantation. No morphological changes were recognized in either the MIF-siRNA or the control grafts on the 7th day. On the 14th day, in contrast to the luminal fibrous airway obliteration of the allografts without MIF

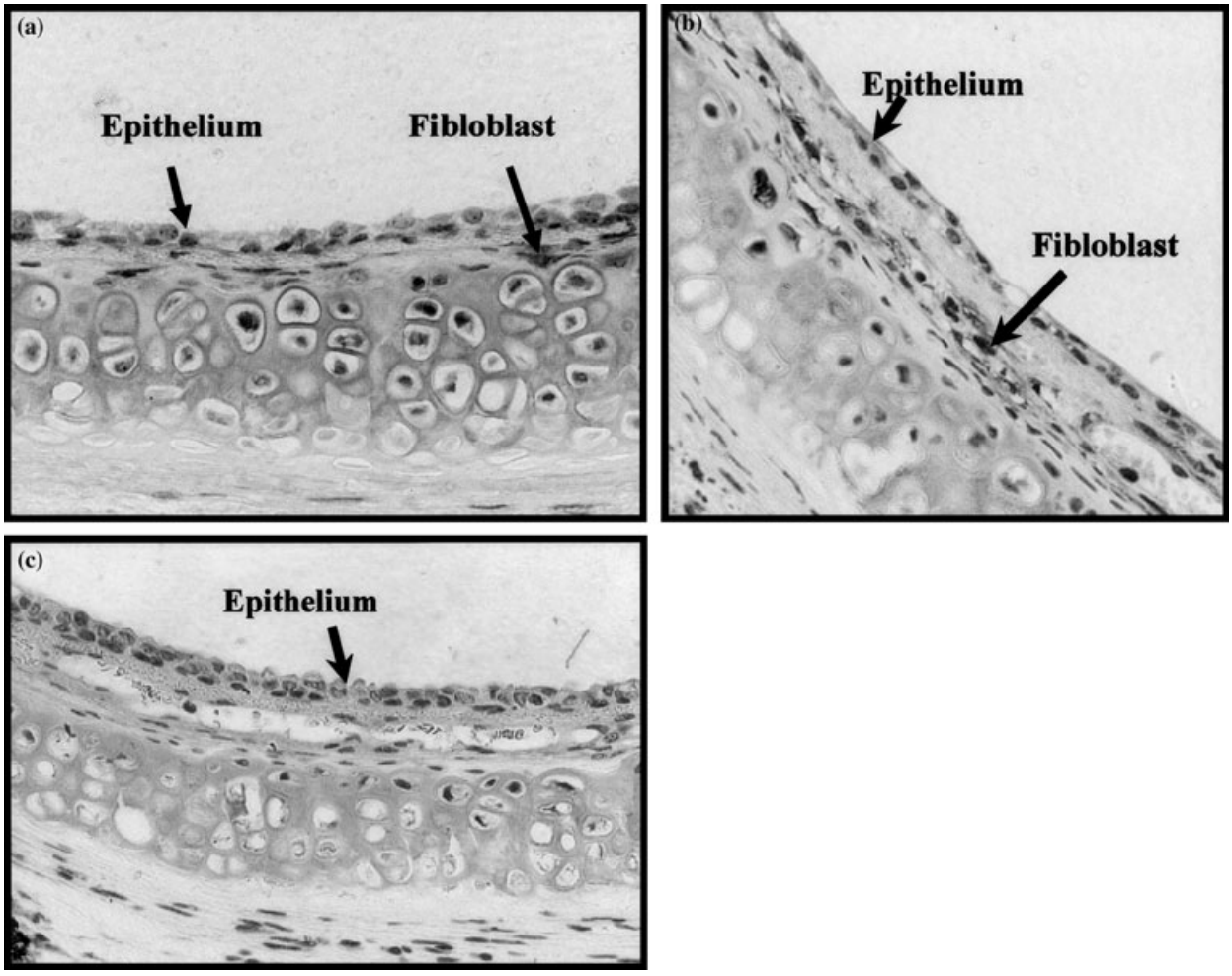

Figure 2 Immunohistochemical staining of MIF in the transplanted allogeneic and syngeneic tracheal grafts on day 7 after transplantation. (a) The MIF expression in syngenic tracheal graft was observed in both the epithelial cells and fibroblasts in the submucosal tissue (×200). (b) In allogeneic tracheal grafts, MIF was observed in fibroblasts, but not in the epithelial cells (×200). (c) The MIF expression of an allogeneic tracheal graft treated with siRNA for MIF was not observed in either the epithelial cells or the fibroblasts (×200).

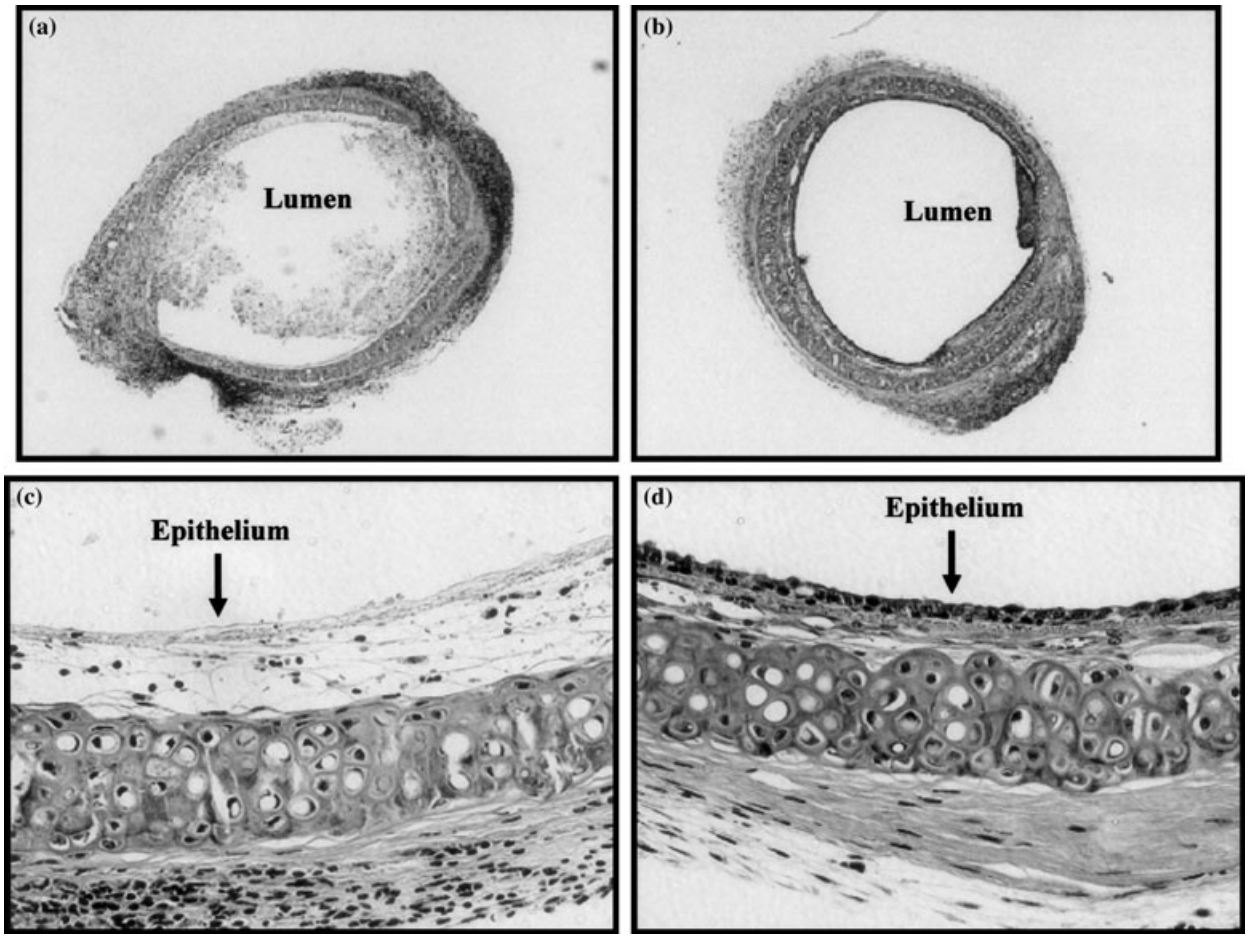

Figure 3 The effect of siRNA on MIF regarding the fibrous obliteration of the tracheal grafts harvested on the 14th day after transplantation and evaluated by Hematoxylene and Eosin staining. (a) (×100), (c) (×200) Allografts treated with empty liposome. Fibrotic luminal obstruction was noted with a marked destruction of the epithelial cells. (b) (×100), (d) (×200) Allografts treated with liposome containing MIFsiRNA showed a complete luminal opening with a well preserved epithelium.

Table 1. Morphological features among tracheal grafts treated and nontreated with siRNA for MIF.

Grafts	Patency (%)	Viability of epithelial cells (%)
Isograft day 7 ($n = 3$)	100	91.3 ± 4.2
Allograft day 7 ($n = 3$)	100	71.7 ± 8.1
Allo + siRNA day 7 ($n = 3$)	100	79.6 ± 5.7
Isograft day 14 ($n = 5$)	100	100**
Allograft day 14 ($n = 5$)	62.0 ± 16.7*	11.0 ± 24.6**,***
Allo + siRNA day 14 ($n = 5$)	100*	78.8 ± 44.1***
Allograft day 28 ($n = 5$)	0	0
Allo + siRNA day 28 ($n = 5$)	0	0

*$P = 0.007$, **$P = 0.0013$, ***$P = 0.0269$.

siRNA treatment, which exhibited a patency of 62.0 ± 16.7% (Fig. 3a), the allografts treated with MIF siRNA showed no luminal occlusion (Fig. 3b), which was the same as for the tracheal isografts (Table 1, $P = 0.007$). The epithelial cells were preserved to a much higher degree in the allografts treated with MIF siRNA (78.8 ± 44.1%) than in the tracheal allografts on the 14th day (11.0 ± 24.6%) ($P = 0.0269$, Fig. 3c and d). On the 28th day, the almost complete occlusion and destruction of the epithelial cells were recognized even in the MIF siRNA-treated grafts (Table 1).

Discussions

Obstructive bronchiolitis is the most significant problem in clinical lung transplantation, and it often leads to airway obliteration due to the proliferative response of fibroblasts, and the destruction of epithelium and the occurrence of inflammatory infiltrate. However, its detailed mechanism remains to be elucidated. To clarify

the pathogenesis and optimal therapy for OB, the heterotopic murine trachea transplant model was established and many experiments have been performed using this system. As Davreux *et al.* [27] first reported being able to prevent the generation of OB using cyclosporine and methylprednisolone, many studies on such immunosuppressive treatments have been reported [28–30]. Other studies have successfully induced tolerance by blocking the costimulatory pathway or by establishing chimerism in an animal model [31–35]. In this study, we demonstrated that the inhibition of MIF expressed in the airway of transplanted allogeneic tracheal grafts resulted in a decreased OB formation in a murine model on the 14th day, while also describing the *in vivo* introduction of siRNA in transplantation experiments for airway organs. In this study, the onset of OB occurred on day 14 after transplantation, and was earlier than several previous reports. It may be due to the tissue damage of the trachea by the *ex vivo* manipulation or liposomal transfection.

Migration inhibitory factor is one of the cytokines, first reported several decades ago, which are ubiquitously expressed in both immune and nonimmune cells included in various types of peripheral tissues. MIFs have interesting functions and they are known to play a role in inflammation, while also counter-regulating the immunosuppressive activities of glucocorticoids. Lan *et al.* [36] reported a marked up-regulation of MIF mRNA and protein expression in renal allograft rejection while other study groups have reported MIF to be an active participant in skin graft destruction [21]. We therefore hypothesized that MIF might thus be related to transplant rejection and thus decided to investigate MIF.

We introduced MIF siRNA into the tracheal graft to block the expression of MIF protein, and found that MIF was not as strongly recognized immunohistochemically in the treated allogeneic grafts as in the nontreated grafts on the 7th day. This result suggested that the introduction of MIF siRNA and the down-regulation of the expression of MIF protein is possible. Furthermore, allografts treated with liposome containing MIF siRNA showed no luminal occlusion and a better preservation of the epithelium on the 14th day after transplantation even though the nontreated allografts already demonstrated the onset of epithelial loss and luminal occlusion. MIF may therefore contribute to OB formation in the transplant trachea. However, the inhibitory effect of a single injection of the siRNA was only found to last for 14 days as the treated tracheal grafts showed OB on the 28th day as well as in the nontreated grafts (Table 1). Therefore, the introduction of siRNA in the transplant trachea with vector may be necessary for further examinations [37]. Recently, cholesterol-modified siRNA have been developed, and it has been shown to effectively silence the target gene expression of vascular endothelial cells after systemic administration in a rodent model [38]. If the pathogenesis of BO can be further clarified, then the blocking of several genes, including MIF, using such types of siRNA may thus be applicable in the clinical field. In addition, the blockage of MIF expression may also lead to a decrease in the quantity of methylprednisolon required to induce immunosupression in the future due to the ability of MIF to act as a counter-regulator of the glucocorticoid action.

In conclusion, the single treatment of MIF siRNA into the tracheal allografts was found to successfully inhibit fibrotic airway obliteration in a murine tracheal transplant model during the study period. Although further additional experiments will be needed, MIF may therefore be a useful new molecular target which can be used to prevent the development of OB in lung transplantation.

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