RESEARCH ARTICLE

Airway-directed gene transfer of interleukin-10 using recombinant Sendai virus effectively prevents post-transplant fibrous airway obliteration in mice

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Bronchiolitis obliterans (BO) after lung transplantation prevents a satisfactory prognosis, and recent studies suggested that interleukin-10 (IL-10) gene transfer to distant organs could inhibit BO in rodent models. Although delivery of the therapeutic gene to a local airway would be favored to minimize systemic effects, current limitations include lower gene transfer efficiency to airway epithelium. As recombinant Sendai virus (SeV) can produce dramatically efficient gene transfer to airway epithelium, we determined if SeV-mediated IL-10 gene transfer to the local airway would inhibit bronchial fibrous obliteration in murine tracheal allografts. Administration of cyclosporine A (CsA) significantly promoted not only recovery of the injured airway epithelium but also SeV-mediated IL-10 expression (CsA–versus CsA+= 228 ± 78

versus 3627 ± 1372 pg/graft with 5×10^7 pfu), thereby suggesting the requirement of epithelia for efficient gene transfer. Even at the highest expression, no significant leakage of IL-10 was evident in the systemic circulation, and the induction of interferon- γ was completely diminished on day 7 by IL-10 gene transfer. As a result, luminal loss was significantly prevented in allografts treated with SeV-IL-10 (luminal opening, all control groups: 0% respectively, and SeV-IL-10 5×10^7 pfu: $25.7 \pm 10.5\%$), an effect that was enhanced by short-term CsA treatment (SeV-IL-10 5×10^7 pfu with CsA: $63.7 \pm 12.7\%$). We propose that SeV is a useful vector that can target airway epithelium to prevent BO avoiding putative systemic effect.

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Introduction

Lung transplantation can be effective for patients with end-stage pulmonary diseases; however, post-transplantation bronchiolitis obliterans (BO), a form of chronic graft rejection, occurs in approximately 50% of recipients surviving over 3 months after receiving a graft. Although improvement in surgical techniques, careful immunosuppression and postoperative management have improved the outcome after lung transplantation,^{1,2} preventive treatment for BO has not been available.

The heterotopic tracheal transplantation model in rodents has been used in laboratory studies.^{3–5} A recent study showed that during development of airway obliteration, a strong Th1 response in allografts was observed whereas no significant changes in Th2 response was reported, and another study showed that an immunosuppressant could inhibit BO in this model, suggesting that the Th1 response against tracheal allografts is likely to be critical in the development of BO.^{6,7} Interleukin-10 (IL-10), an anti-inflammatory

cytokine, is a molecule that modulates cellular and hormonal immunity against an allograft by suppressing the expression of IL-2, IL-4, and interferon- γ (IFN- γ).^{8,9}

Several studies on experimental gene therapy using IL-10 to prevent post-transplant fibrous airway obliteration were also reported. A study demonstrated that intramuscular administration of an adenovirus encoding IL-10 (Ad-IL-10) prevented post-transplant fibrous airway obliteration in a rat model, as a result of increased circulating IL-10 protein levels.¹⁰ Although this encouraging study showed the proof of principle of IL-10 gene therapy to prevent post-transplant fibrous airway obliteration, it remained unclear whether increased concentrations of the immunosuppressant IL-10 in systemic circulation are relevant in a clinical setting. In our pilot study, mice with intramuscular IL-10 gene transfer showed increased mortality associated with high circulating IL-10 protein over 10–100 ng/ml (unpublished data). As BO is localized at the lower conducting airway, airway-directed gene transfer can be considered in attempts to avoid systemic effects. However, the low rate of airway gene transfer using currently available vectors, including adenovirus, is hazardous to deliver IL-10 gene directly to the airway epithelium.

We recently reported that recombinant Sendai virus (SeV), a new gene transfer agent, produce log-orders-

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high gene transfer efficiency throughout the respiratory tract, as compared to currently available vectors, including adenovirus.¹¹ In the present study, we determined if SeV-IL-10 would inhibit luminal occlusion in a murine post-transplant fibrous airway obliteration model by airway-directed gene transfer of IL-10 without increasing blood levels.

Results

Natural course of epithelial lining in tracheal allografts

To clarify if allogenic responses in tracheal explants might differ among strains of mice, we histologically checked six different syngenic (C57BL/6J to C57BL/6J, $n=5$, Balb/c to Balb/c, $n=5$, and C3H to C3H, $n=5$) or allogenic (C57BL/6J to Balb/c, $n=5$, Balb/c to C57BL/6J, $n=5$, C57BL/6J to C3H, $n=5$, C3H to C57BL/6J, $n=5$, Balb/c to C3H, $n=5$, and C3H to Balb/c, $n=5$, respectively) donor-recipient pairs on 3, 7, 28 days after tracheal implantation. All tracheal tissues for syngenic

and allogenic implantation showed a complete loss of epithelial lining on day 3 (data not shown), probably due to ischemic damage. On day 7, isografts showed almost complete coverage of the regenerative squamous-type epithelium (Figure 1a-A), while epithelial lining of allografts was extensively lost or was partly replaced by squamous epithelium-like regenerative epithelium (Figure 1a-B). In the later phase on day 28, isografts showed complete recovery of the differentiated respiratory epithelium and were filled with mucinous materials (Figure 1a-C). On the other hand, allografts showed complete loss of the luminal space which was occupied with proliferation of spindle-shaped cells associated with loose connective tissue (Figure 1a-D). These histological findings were consistent in all donor-recipient pairs, suggesting that tissue response to allogenic grafts did not largely depend on the strains of mice; therefore, the following experiments were done using C57BL/6J to Balb/c (allogenic) and Balb/c to Balb/c (syngenic).

Figure 1 (a) Early (A and B) and late (C and D) change in histopathology of tracheal isograft (donor and recipient: Balb/c) and allograft (donor: C57BL/6; recipient: Balb/c). Lower panels are a high-powered view of the squared area of each upper panel. Lu: lumen; Ca: cartilage; E: epithelial layer. Hematoxylin-eosin staining. Original magnification: upper panels, $\times 26.5$; lower panels, $\times 106$. A. Tracheal isograft 7 days after transplantation. Note that the luminal surface is lined with squamous-type regenerative epithelium. B. Tracheal allograft 7 days after transplantation. Note that the lining epithelium is lost extensively. C. Tracheal isograft 28 days after transplantation. The luminal space is completely opened and filled with secretion produced by respiratory epithelium. D. Tracheal allograft 28 days after transplantation. The lining epithelium is completely lost and the lumen is occupied with proliferation of spindle-shaped cells associated with loose connective tissue. (b) Effect of short-term intraperitoneal administration of cyclosporine A (CsA: 25 mg/kg/day for 8 days) on histopathology (A: PBS injected control; B: CsA) and on SeV-mediated IL-10 gene transfer efficiency (C). A and B. Histological findings of lining epithelium in allograft 7 days after transplantation. Lower panels are a high-powered view of the squared area of each upper panel. A PBS-treated allograft shows extensive loss of tracheal epithelium, except for partial coverage of fragmented epithelial cells (arrows). B. Histopathological findings of CsA-treated allograft. More than 90% of the luminal surface is covered by the squamous-type regenerative epithelium. Lu: lumen; Ca: cartilage; E: epithelial layer. Hematoxylin-eosin staining. Original magnification: upper panels, $\times 26.5$, lower panels, $\times 106$.

Short-term treatment of cyclosporine A accelerates epithelial lining as well as airway-directed SeV-mediated gene transfer efficiency

Next, we tested the effect of the short-term treatment of cyclosporine A (CsA) on the epithelial lining and SeV-mediated gene transfer efficiency. CsA treatment (25 mg/kg/day) initiated 1 day prior to tracheal implantation was continued daily for 8 days and then withdrawn on the day before virus administration (day 7).

Untreated allografts showed extensive loss of epithelium (Figure 1b-A), as seen in a representative result shown in Figure 1a-B, while CsA-treated allografts had an over 90% epithelial lining (Figure 1b-B). We then assessed the effect of short-term treatment of CsA on SeV-IL-10 gene transfer. As shown in Table 1, IL-10 expression was markedly increased by CsA treatment which suggested that CsA-mediated acceleration of epithelial lining dramatically enhanced SeV-mediated gene transfer. Under condition of CsA treatment, a dose-dependent increase of IL-10 expression was also apparent, as shown in Table 1.

Time course of SeV-mediated IL-10 expression and its effect on endogenous IFN- γ expression

As a subsequent experiment, we examined murine IL-10 protein levels in allografts and serum on 2, 7, and 14 days following the luminal administration of SeV-IL-10.

Table 1 Intratracheal IL-10 protein expression of CsA-treated or CsA-untreated allografts 2 days after intratracheal injection of SeV-IL-10, and significant dose-dependent increase of gene transfer efficiency

CsA treatment	No. of animals(n)	Virus titer (pfu/50 μ l)	Murine IL-10 level (pg/graft)
No	3	0	<5
Yes	3	0	<5
No	6	5×10^7	228 ± 78.3
Yes	6	2×10^6	12.3 ± 5.4
Yes	6	1×10^7	401.9 ± 255.3
Yes	6	5×10^7	3626.7 ± 1371.9^a

^a $P < 0.01$ versus other groups.

As shown in Figure 2a, large amounts of intratracheal IL-10 protein in allografts transfected with SeV-IL-10 (5×10^7 pfu) were evident 2 days after gene transfer ($n=9$, 3517.66 ± 951.76 pg/graft) and values decreased 7 days after gene transfer ($n=5$, 5.3 ± 2.3 pfu/graft). On the other hand, intratracheal IL-10 protein in allografts treated mice with PBS or SeV-lacZ was not significantly detected. Serum IL-10 protein in all mice was also not detected at all time points (data not shown).

Intratracheal expression of Th1-cytokine, IFN- γ , was subsequently assessed using the same samples (Figure 2b). On day 2 after gene transfer, both allografts which injected SeV-IL-10 and SeV-lacZ showed significantly elevated IFN- γ level (IL-10: 17.3 ± 6.5 ng/graft, $n=3$, and lacZ: 51.9 ± 28.9 ng/graft, $n=3$, respectively), while no significant expression was found in grafts treated with PBS, which suggested that the cause of early elevation of IFN- γ might be related to vector-mediated induction of the Th1 response and not to allogeneic antigens. The allogeneic induction of IFN- γ in PBS-treated grafts was significant on day 7, and allografts treated with SeV-lacZ also showed high IFN- γ levels. At this time, IL-10 gene transfer completely diminished the expression of endogenous IFN- γ , thereby indicating successful inhibition of Th1 response due to both allogeneic and vector infection.

Effects of IL-10 gene transfer on the obstructive pathology of post-transplant fibrous airway obliteration in mice

Finally, we tested the effects of airway-directed IL-10 gene transfer. The experimental protocol is shown in Figure 3, typical histological findings are given in Figure 4, and the results are summarized in Figure 5.

All allografts with PBS or transfected with SeV-lacZ showed complete luminal occlusion with associated myofibrous tissue, regardless of short-term CsA treatment. On the other hand, allografts transfected with SeV-IL-10 showed a preserved lining of squamous-type epithelium and significant inhibition of luminal occlusion, as compared with findings in other groups ($P < 0.05$). This effect was significantly enhanced by CsA treatment.

Figure 2 Bar graphs indicating time course of intratracheal IL-10 (a) and IFN- γ protein expression in isografts or allografts after PBS (50 μ l), SeV-lacZ (5×10^7 pfu/50 μ l), or SeV-IL-10 (5×10^7 pfu/50 μ l) injection. At each time point, the grafts were harvested and subjected to enzyme-linked immunosorbent assay.

Figure 3 Experimental protocol.

Figure 4 Histological findings of allografts 28 days after transplantation. A and B Histopathological findings of CsA-treated tracheal allografts injected with PBS (A) or SeV-lacZ (B). Both allografts show complete luminal occlusion with myofibrous tissue. C. Histopathological findings of CsA-treated tracheal allografts injected with SeV-IL-10. The luminal loss is well prevented and the luminal surface is lined with squamous-type regenerative epithelium (arrows). Lower panels are a high-powered view of the squared area of each upper panel. Lu: lumen, Ca: cartilage; E: epithelial layer. Hematoxylin-eosin staining. Original magnification: upper panels, $\times 26.5$, lower panels, $\times 106$.

Figure 5 Average of luminal opening in each group. Bar graphs indicating average of the luminal opening in each group. In allografts, the group that was SeV-IL-10 (5×10^7 pfu) injected after CsA treatment showed significant inhibition of luminal occlusion compared with other groups. (* $P < 0.05$).

Discussion

Few experimental studies on gene therapies for orthotopic lung transplant have been documented. As

technical difficulty may be one reason, a heterotopic tracheal transplantation model is used for research on transplant fibrous airway obliteration after lung transplantation. Using this model, we investigated if local SeV-mediated IL-10 gene transfer targeted to the luminal airway epithelium might prevent luminal obstruction in murine heterotopic tracheal allografts. Key findings obtained in this study were: (1) short-term administration of CsA accelerated not only regeneration of the airway epithelium, but also SeV-mediated gene transfer efficiency (2) exogenously transduced IL-10 completely diminished local Th1 cytokine INF- γ expression, without any significant leakage to the systemic circulation; and (3) airway-directed IL-10 gene transfer prevented luminal loss of allografts, this effect being significantly enhanced by short-term CsA administration. To our knowledge, this is the first report indicating the significant therapeutic effects of airway-directed IL-10 gene transfer regarding development of post-transplantation fibrous obliteration, and suggests the usefulness of our novel vector; recombinant SeV can be considered for use in a clinical setting.

Because of the requirement of a simple technique and histological findings similar to the case of human disease, a heterotopic tracheal transplantation model in rodents has been frequently used in laboratory studies

on BO. Although the relevance of this model has been discussed, immune response-mediated development of the post-transplant fibrous airway obliteration in this model is considered worthwhile of study. Boehler *et al* reported that Th1 cytokines were up-regulated in rat model,⁶ and in a subsequent study they also showed that intramuscular administration of adenovirus encoding IL-10 (Ad-IL-10) inhibited the luminal occlusion of allografts via increased protein levels in the bloodstream.¹⁰ Although these studies are important to show the 'proof of principle' regarding the role of Th1 cytokines and IL-10 in the development of BO, possible safety concerns in a clinical setting may arise because of increased levels of IL-10 in the systemic circulation. IL-10, an immunosuppressive cytokine, promotes Th2-type cytokines and inversely inhibits Th1-type cytokines and inversely inhibits Th1-type cytokines, including IFN- γ .^{8,9} IL-10 also has a significant role down-regulating major histocompatibility complex class II molecules and inhibits antigen-presenting cell functions and production of tumor necrosis factor (TNF)- α and - β , IL-12, B7 expression and intercellular adhesion molecule-1 (ICAM-1) gene transcription.^{12–16} These broad immunosuppressive effects of IL-10 might be systemically harmful for recipients, with respect to infectious diseases. In this regard, airway-targeted local gene delivery seems more likely to be relevant in preventing BO in a clinical setting.

We have previously reported the dramatically high gene transfer potential of SeV vector to airway epithelium *in vivo*,¹¹ and this characteristic was considered to be useful for local IL-10 gene delivery to treat BO. At that time, however, requirement of airway epithelium in allografts was not known concerning efficient gene transfer. Since King *et al* reported that immunosuppression using CsA significantly reduced development of obliteration in tracheal allografts,³ we used CsA in the present study and found that CsA treatment had a beneficial effect not only for the epithelial lining but also on SeV-mediated gene transfer efficiency. These findings show that CsA preserved the lining epithelium that is needed for efficient transfection of SeV-IL-10. Since CsA and other immunosuppressants are clinically available drugs, the current protocol can be applied to clinical use with minor modification.

A question raised in this study is why IFN- γ was markedly diminished on day 7 when IL-10 was already down-regulated, as shown in Figure 3b, while suppression of IFN- γ was not evident at the highest IL-10 level on day 2. One possible explanation is that 2 days was too early for IL-10 to suppress functions of inflammatory cells, including natural killer cells, monocytes/macrophages, and T-lymphocytes, all of which could produce IFN- γ and transmigrate against virus-infected cells. It may be that accumulation of expressed IL-10 suppressed the activity of these cells later, resulting in down-regulation of IFN- γ on day 7.

Limitations of the current study are the technical difficulty assessing the long-term effect of locally delivered IL-10. Tracheal allografts collapsed in 2 months due to destruction of tracheal cartilage in this mouse model, even where sufficient CsA was given (unpublished observation). Also, as shown in Figure 3, the current SeV system produces a foreign gene in only 2

weeks. Future studies using different models and improved vectors persistently express a foreign gene.

In summary, we here demonstrate that airway-directed expression of IL-10 protein could efficiently prevent luminal occlusion of tracheal allografts, and this effect was markedly enhanced by short-term administration of CsA, suggesting a clinically relevant regimen. We propose that SeV-mediated gene transfer can be developed as a safe and effective therapeutic method relevant to clinical use.

Materials and methods

Gene transfer vectors

Recombinant SeVs were constructed, as described.^{17–20} In brief, 18 bp of spacer sequence 5'-(G)-CGGCCGCA-GATCTTCACG-3' with a NotI restriction site were inserted between the 5' nontranslated region and the initiation codon of the nucleoprotein (N) gene. This cloned SeV genome also contained a self-cleaving ribosome site from the antigenomic strand of the hepatitis delta virus. The entire cDNA-coding *E. coli* lacZ with a nuclear localizing signal (for SeV-nls-lacZ) and murine IL-10 (for SeV-IL-10) were amplified by PCR, using primers with a NotI site and new sets of SeV E and S signal sequence tags for an exogenous gene, and then inserted into the NotI site of the cloned genome. The entire length of the template SeV genomes, including exogenous genes, was arranged in multiples of six nucleotides (so-called 'rule of six').²¹ Template SeV genomes with an exogenous gene and plasmids encoding N, P, and L proteins (plasmid pGEM-N, pGEM-P, and pGEM-L) were complexed with commercially available cationic lipids, and then co-transfected with vaccinia virus vT7-3 into CV-1 or LLMCK cells. Forty hours later, the cells were disrupted by three cycles of freezing and thawing and injected into the chorioallantoic cavity of 10-day-old embryonated chicken eggs. Subsequently, the virus was recovered and the vaccinia virus eliminated by a second propagation in eggs. Virus titer was determined by hemagglutination assay, using chicken red blood cells,²² and viruses were stored at -80°C until use. The murine IL-10 cDNA for constructing SeV-IL-10 was obtained from American Type Culture Collections (clone 68027, Manassas, VA, USA).

Animals

A total of 229 pathogen-free male C57BL/6J, Balb/c, and C3H mice (6–8 weeks old, Charles River grade) were purchased from KBT Oriental (Tosu, Saga, Japan). All animal experiments were done using approved protocols and in accordance with recommendations for the proper care and use of laboratory animals by the Committee for Animal and Recombinant DNA Experiments at Kyushu University.

Experiment protocol

Experimental groups and experimental protocols are summarized in Figure 3. Untreated control ($n=20$) and some of the treated groups were daily given cyclosporine A (CSA) intraperitoneally at 25 mg/kg/day for 8 days, as started from day 1 before transplantation. On day 0, the tracheae from donor mice were transplanted into the subcutaneous tissue of recipient mice, as described.³

Seven days after transplantation, subcutaneous tissue was incised, grafts were exposed and these lumens were filled with 50 μ l of PBS or of various titers of vectors (Figure 3). Two 7, 14 and 21 days after gene transfer, mice were killed and grafts and sera were collected.

IL-10 and IFN- γ protein analysis

2, 7, and 14 days after gene transfer, grafts and serum samples from each group were analyzed using commercially available ELISA kits and as instructed by the manufacturer (R&D Systems, INC., USA).

Histochemistry

Samples of grafts were fixed in buffered 10% neutral formalin solution for 24 h. After embedding in paraffin, samples were cut at 5 μ m thickness and stained with hematoxylin and eosin (H&E).

Evaluation of luminal occlusion of grafts

Luminal occlusion was evaluated as described.⁵ In brief, luminal occlusion was defined as the area containing tissue inside of a cartilage ring divided by the total inner area of a cartilage ring. The inner area of cartilage ring includes not only tracheal lumen but the submucosal and epithelial tissue. Luminal occlusion in the isograft measured using this method was 26.7%; therefore we determined the % opening by (luminal occlusion in isograft/luminal occlusion in samples) $\times$ 100.

Statistical analysis

All values are expressed as the mean $\pm$ s.e.m. Mann-Whitney *U*-test was used to analyze data and a value of *P* < 0.05 was considered to have statistical significance.

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