Introduction

Lung transplantation can be a successful clinical therapy for end-stage pulmonary disease, through the improvement of surgical techniques and immunosuppressive regimens. However, bronchiolitis obliterans (BO), a form of chronic rejection, affects up to 65% of all lung transplant recipients and is the leading cause of late mortality after lung transplantation. This commonly occurs six or more months after transplantation, and may present with cough, a decrease of more than 15% in FEV1.0%, and hyperinflation on chest X-ray films. Histologically, epithelial cell desquamation with luminal fibrosis, as well as graft arteriosclerosis affecting both large elastic and small muscular arterioles of the pulmonary circulation, characterize chronic rejection.

Chemokines are small cytokines that mediate cell...
chemotaxis and activation. Spec1f2 specific chemokines may play significant roles in mononuclear cell recruitment during chronic rejection. In murine models of BO, regulated-on-activation, normal T cells expressed and secreted (RANTES)/CCL5 play important roles.

The activity of chemokine-chemokine receptor (CCR) interaction may be reduced in vivo by neutralization of ligands or blockade of chemokine receptors. In this study, we sought to determine the effect of RANTES/CCL5 receptors CCR1 and CCR5 blockade in BO development. Met-RANTES, an amino-terminal modified derivative of RANTES/CCL5, antagonizes the RANTES/CCL5 receptors CCR1 and CCR5 attenuates tissue damage associated with acute kidney rejection and cardiac allograft vasculopathy (CAV).

There has been recent interest in characterizing the expression and determining the function of the mitogen-activated protein kinases (MAPK). Three major subtypes of MAPK have been identified to date: extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal protein kinase (JNK), and p38 kinase. MAPK plays a crucial role in the response of cells to various external stimuli. A recent study has revealed that ERK is essential for mediating chemokine-triggered chemotaxis. We also have already reported on CCL5–CCR5 interaction and ERK expression.

Hertz and colleagues developed a mouse heterotopic airway transplant model to study the pathogenesis of OB, and found that allografts demonstrated subepithelial inflammation, epithelial necrosis, and early fibroproliferation reproducing human BO after 21 days as opposed to isografts, which did not show these effects.

We used this model to study the role of chemokine RANTES/CCL5 and receptor CCR1–CCR5 interaction and ERK activation in a murine model of bronchiolitis obliterans.

Materials and Methods

Animals and drugs
BALB/c (H2-d) and C57BL/6 (H2-b) mice were obtained from pathogen-free inbred colonies and housed in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of Tokyo Medical University. Met-RANTES were purchased from RD Systems, Inc (Minneapolis, MN). MAPK family antibodies were purchased from Cell Signaling Technology, Inc (Danvers, MA). A mouse CCL5/RANTES Quantikine ELISA Kit was purchased from RD Systems, Inc (Minneapolis, MN).

Heterotopic tracheal transplantation model

(a) Donor procedure
Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine 80/16 mg/kg and then were exsanguinated. Using a sterile technique, the trachea was transected distally to the larynx and proximally to the carina after a midline incision and blunt dissection. Adherent tissue was carefully removed and the trachea was soaked in PBS solution prior to the transplantation.

(b) Recipient procedure
Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine 80/16 mg/kg. The graft was placed in a small subcutaneous pocket on the dorsum, between the scapulae of the anesthetized recipient mice. The wound was closed with Prolene sutures. At the time of graft removal, mice were anesthetized as described earlier.

Experimental groups
Balb/c strain donor tracheas (allografts) were transplanted into C57BL/6 recipient mice. The allograft recipients received either Met-RANTES (20 μg intraperitoneally (IP) daily in 0.5 ml of PBS) or vehicle (0.5 ml of PBS IP daily) beginning on postoperative day 1. The donor tracheas were harvested on day 21 after transplantation (n=5 in each group). No immunosuppression was given. C57BL/6 strain donor tracheas (isografts) were also transplanted in C57BL/6 recipients and harvested on day 21 (n=5) after transplantation.

Measurement of RANTES in allografts and isografts
For protein measurements in grafts, grafts were homogenized in a lysis buffer. Homogenates were centrifuged, and protein concentrations were determined. RANTES concentrations were measured using a general enzyme-linked immunosorbent assay (ELISA) procedure.

Histological evaluation and morphometry
After harvesting at 21 days, cross-sectional allograft specimens were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned at 5-μm thickness and stained with HE. Luminal occlusion was evaluated by determining the reduction in luminal area using NIH Image program version 1.59 (National Technical Information Service, Springfield, VA).

Analysis of epithelial loss
HE stained sections of transplanted airway were exam-
ined with the same image analysis software. The original inner circumference of the airway was determined, and a cursor was then used to trace areas of intact, normal, abnormal, and absent or necrotic respiratory epithelium. A percentage of airway circumference that was still lined by epithelium was then determined.

**Western blotting**

Tracheal grafts were homogenized in a lysis buffer (10 mmol/L Tris Cl [ph7.6], 150 mmol/L NaCl, 1% [w/v] sodium deoxycholate, 1% [v/v] Triton X-100, 0.1% [w/v] sodium dodecyl sulfate, 1% [v/v] aprotinin, 2 mmol/L Na3vo4, to which was added leupeptin [1 μg/mL], pepstatin [1 μg/mL], and 1 mmol/L phenylmethylsulfonylfluoride). Homogenates were centrifuged, and protein concentrations were determined. Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. ERK was detected by incubating with primary antibodies diluted 1:1,000. Primary incubation was followed by washes of Tris-buffered saline with 0.005% Tween20. The blot was then incubated with secondary antibody, and antibody-reactive protein was detected with chemiluminescence. Densitometric analysis was performed with Gelexpert software. Optical densities (mean±standard error of the mean (SEM) ) were obtained in all animals after three determinations for each band. ERK expression was described throughout as the proportional (-fold) increase or decrease relative to the levels observed in the T-ERK.

**Statistical analysis**

Results are reported as means±SEM of several experiments. One-way ANOVA or Student’s t-test were used where appropriate to compare the difference between experimental groups. A p-value less than 0.05 was considered to indicate a statistically significant difference.

**Results**

**RANTES/CCL5 protein levels in grafted tracheal tissue (ELISA)**

The RANTES/CCL5 protein levels in allografts are significantly higher than in isografts at day 21 after transplantation (Fig. 1).

**Met-RANTES ameliorates fibrous airway obliteration**

Although vehicle-treated allografts averaged 96%±2% occlusion at 21 days, there was a significant reduction (p<0.05) of luminal obliteration noted in the 21 day Met-RANTES treated allografts (Fig. 2). In the isografts, the airway displayed a normal architecture without luminal narrowing.

**Epithelial loss**

Vehicle-treated allografts and Met-RANTES treated allografts demonstrated very little remaining respiratory epithelium at 21 days (Fig. 3). There was no significant difference between these two groups.

**Western blots**

*ERK expression in isografts and allografts with or without Met-RANTES treatment:* Allografts with vehicle treatment showed high expression of phosph-ERK (4-fold) compared to isografts and allografts with Met-RANTES treatment (Fig. 4, A and B).

**Discussion**

BO is the major cause of morbidity and mortality in long term survivors of heart-lung and lung transplantation, afflicting up to 65% of survivors.1,2) Augmented immuno-
suppression has been effective in inhibiting disease progression in only a small number of patients.

Suga and Maclean reported that specific chemokines may play significant roles in mononuclear cell recruitment during chronic rejection in a mouse model of BO, in which RANTES/CCL5 played an important role. First of all, we examined RANTES/CCL5 expression of transplanted tracheal grafts. We found increased level of Met-RANTES Ameliorates Fibrous Airway Obliteration

Fig. 2. Airway obliteration. Percentage airway obstruction (means±SEM) are shown as bar graphs for each group. At day 21, although vehicle-treated allografts averaged 96±2% occlusion, there was significantly less (*p<0.05) luminal obliteration noted in the Met-RANTES treated allografts.

Fig. 3. Vehicle-treated allografts and Met-RANTES treated allografts demonstrated very little remaining respiratory epithelium at 21 days. There was no significant difference between these two groups.

Fig. 4. A: Representative immunoblots of ERK in transplanted trachea at 21 days after transplantation. Allografts with vehicle treatment showed high expression of phosph-ERK compared to isografts and allografts with Met-RANTES treatment.

B: Quantitative analysis of ERK expression by densitometry. Allografts with vehicle treatment showed 4 fold higher expression of phosph-ERK compared to isografts and allografts with Met-RANTES treatment (*p<0.05).
RANTES/CCL5 in allografts with vehicle treatment as compared with isografts.

Next, we used Met-RANTES for blocking of RANTES/CCL5 signalling. Treatment with Met-RANTES, a chemokine receptor antagonist, has been demonstrated to prevent acute renal transplant rejection and chronic rejection by blocking the recruitment of mononuclear cells.\textsuperscript{10} However, whether Met-RANTES treatment can affect the progression of airway obliteration remained unclear. In the present study, we showed that CCR1 and CCR5 blockade by Met-RANTES ameliorates the development of obliterative airway disease in mouse tracheal allografts.

The MAPK-signaling pathway is an important molecular component for tissue injury and chemotaxis.\textsuperscript{7} Some chemokines including RANTES stimulate ERK activation. In our experiment, RANTES stimulated ERK activation in tracheal epithelial cells and immune cells.\textsuperscript{8} It has been hypothesized that Met-RANTES blocks RANTES/CCL5 signalling and also decrease ERK phosphorylation.\textsuperscript{8} In our experiments, allografts with vehicle treatment showed high expression of phospho-ERK (4-fold) compared to isografts and allografts with Met-RANTES treatment.

Chemokine receptor blockade may be of therapeutic benefit in controlling the development of chronic rejection and ERK may be a new molecular target for chronic rejection.

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References