A Novel CC-Chemokine Receptor 3 Antagonist, Ki19003, Inhibits Airway Eosinophilia and Subepithelial/Peribronchial Fibrosis Induced by Repeated Antigen Challenge in Mice

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Abstract. CC-chemokine receptor 3 (CCR3) is a chemokine receptor for which major ligands, CC-chemokine ligand (CCL) 11, CCL24, and CCL26, are known to be involved in chemotaxis for eosinophils. In the present study, we evaluated the effect of a low molecular weight CCR3-receptor antagonist, Ki19003 (4-[[5-(2,4-dichlorobenzylureido)pentyl][1-(4-chlorophenyl)ethyl]amino]butanoic acid), on airway remodeling in a mouse model of allergic asthma. BALB/c mice were sensitized twice by intraperitoneal injection of ovalbumin (OA) and exposed daily to 1% OA for 3 weeks. Twenty-four hours after the final antigen challenge, bronchoalveolar lavage and histological examinations were carried out. Ki19003 clearly inhibited antigen-induced increase in the number of eosinophils in bronchoalveolar lavage fluid (BALF), but did not affect the number of other cell types examined in this study. Ki19003 also inhibited the increased production of transforming growth factor-β1 in BALF and the amount of hydroxyproline in the lungs in a dose-dependent manner. Furthermore, Ki19003 significantly attenuated allergen-induced subepithelial and peribronchial fibrosis. These findings indicate that CCR3 antagonism prevents not only the infiltration of eosinophils into the airways but also the development of allergen-induced subepithelial and peribronchial fibrosis. Therefore, a CCR3 antagonist may be useful in the treatment of airway remodeling, especially subepithelial and peribronchial fibrosis, in allergic asthma.

Keywords: asthma, eosinophil, eotaxin, subepithelial fibrosis, transforming growth factor-β1

Introduction

Bronchial asthma is a chronic inflammatory disorder, characterized by variable and reversible airway obstruction, airway eosinophilic inflammation, and bronchial hyperresponsiveness (1); however, chronic asthmatic patients develop irreversible alterations of pulmonary function despite appropriate and aggressive anti-inflammatory therapy (2, 3). These alterations result from structural changes of the airways, known as airway remodeling, characterized by goblet cell hyperplasia/hyperplasia, subepithelial fibrosis, and smooth muscle hyperplasia/hypertrophy (4). The precise mechanisms leading to airway remodeling are still unknown, but are thought to result from an injury–repair response driven by several mediators derived from inflammatory and resident cells.

Recently, we have established a mouse model of allergic asthma in which sensitized animals are exposed daily to allergen aerosol for 3 consecutive weeks (5 – 7).
As a result, mice develop a typical T helper type 2 (Th2) response leading to bronchial hyperresponsiveness to cholinergic stimuli, eosinophilic inflammation, goblet cell hyperplasia/hypertrrophy, and subepithelial/peribronchial fibrosis (5 – 7). Allergen-induced airway remodeling was revealed to be Th2-dependent and closely associated with the intensity of airway eosinophil infiltration (5, 6). Moreover, there was a clear correlation between subepithelial fibrosis and both the level of transforming growth factor-β1 (TGF-β1) and the number of eosinophils in bronchoalveolar lavage fluid (BALF) (5).

Chemokines are chemotactic cytokines that play an important role in orchestrating immune responses by directing the migration of leukocytes into inflamed tissues (8, 9). CC chemokine receptor 3 (CCR3) has been shown to belong to a family of 7 transmembrane-spanning G protein–coupled receptors and to be the principal receptor for CC chemokine ligand (CCL) 11 (eotaxin-1), CCL24 (eotaxin-2), and CCL26 (eotaxin-3) (10 – 12). CCR3 is primarily expressed on eosinophils and is involved in eosinophil infiltration (13, 14). It is also reported that CCR3 is expressed on a subset of Th2 cells (15), mast cells (16), airway smooth muscle cells (17), and airway epithelial cells (18) in humans, but little is known about the expression of CCR3 on cells except for eosinophils in mice. The association of CCR3, its ligand, and eosinophils with asthma has been widely studied (19 – 22); for example, eotaxin and CCR3 mRNA are expressed and co-localized in the bronchial mucosa of asthmatics. Moreover, the intensity of their expression correlates with increases in airway responsiveness in patients with atopic asthma (19).

In the present study, we investigated the effects of a novel low molecular weight CCR3-receptor antagonist, Ki19003, on airway eosinophil infiltration and airway remodeling in a mouse model of allergic asthma. Here we showed that a selective and orally active CCR3-receptor antagonist inhibited not only eosinophilic inflammation, but also subepithelial/peribronchial fibrosis in the airways. These findings suggest that eosinophils play an important role in the development of airway remodeling and that a CCR3 antagonist could be useful for the treatment of subepithelial/peribronchial fibrosis in allergic asthma.

**Materials and Methods**

**Animals**

Seven-week-old female BALB/c mice (Japan SLC, Shizuoka) were used and housed in plastic cages in an air-conditioned room at 22 ± 1°C with relative humidity of 60 ± 5%. The mice were fed a standard laboratory diet and given water ad libitum. All experiments were carried out following the rules and regulations for the care and use of experimental animals as stipulated by Gifu Pharmaceutical University in 2008.

**Agents**

Ki19003 (4-[5-(2,4-dichlorobenzylureido)pentyl][1-(4-chlorophenyl)ethyl]amino]butanoic acid) (Fig. 1) was synthesized in the Research Laboratories of Kyowa Hakko Kirin Co., Ltd. Ki19003 was suspended in 0.2% NaHCO₃ solution and was orally administered. Prednisolone was suspended in 0.5% methylcellulose solution and was orally administered.

The following drugs and chemicals were purchased commercially: ovalbumin (OA) and bovine serum albumin (BSA) (Seikagaku Kogyo, Tokyo); Türk solution (Wako Pure Chemical Industries, Ltd., Osaka); sodium pentobarbitone (Abbott Lab., Chicago, IL, USA); EDTA-2Na, hydroxy-L-proline, and acetylcholine chloride (ACh) (Nacalai Tesque, Kyoto); Diff-Quick solution (International Reagent Corp., Ltd., Kobe); pancuronium bromide (Sigma, St. Louis, MO, USA); prednisolone acetate (Shionogi, Osaka); monoclonal anti-mouse IgE antibody (LO-ME-3; Serotec, Oxford, UK); peroxidase-conjugated streptavidin (Dakopatts a/s, Glostrup, Denmark); recombinant murine (rm) CCL2, rm CCL11, rm CCL19, rm IL-3, rm IL-5, and rm GM-CSF (PeproTech, London, UK).

**Transfectants**

Murine pre-B cells (L1.2) expressing murine CCR3 or CCR7 were used (12, 23). Murine CCR2-expressing cells (U937) were obtained as described previously (24).

**Murine eosinophilic granulocytes**

Murine bone marrow–derived eosinophilic granulocytes were obtained as described previously with minor modifications (25). Briefly, bone marrow was flushed from femurs of 10 – 12-week-old BALB/c mice using a syringe filled with Hank’s buffer solution. Bone marrow cells were washed with RPMI1640 medium and cultured in RPMI1640 medium containing 10% fetal bovine serum, rm IL-3 (3 ng/ml), rm IL-5 (2 ng/ml), and rm GM-CSF (1 ng/ml) for the first period of 7 days. The culture
was continued without rm IL-3 or rm GM-CSF for the next 7 days. Medium was changed on day 2, 5, 7, 9, and 12. Murine eosinophilic granulocytes were morphologically confirmed by hematoxylin-eosin staining, and purity was more than 90%.

Migration assay
The chemotaxis assay was performed using a 24-well microchemotaxis chamber as described previously with minor modifications (26). In brief, chemoattractants were diluted in RPMI 1640 medium supplemented with 1 mg/ml BSA and placed in the lower wells (250 μl/well). Cells were suspended in RPMI 1640 medium containing 1% BSA at 1 × 10^7 cells/ml and added to the upper wells (100 μl/well), which were separated from the lower wells by a polycarbonate filter with 5-μm pores. The chamber was incubated for 1 h at 37°C in 5% CO2 and 95% air. Filters were removed and migrated cells were counted by a cell counter (Coulter Electronics, Luton, UK). All assays were performed in triplicate.

CCL11- or antigen-induced eosinophilic migration in the airway
Mice were anesthetized with an i.p. injection of sodium pentobarbitone (60 mg/kg). CCL11 was diluted with PBS containing 0.1% BSA, and the solution of CCL11 (1.2 μg/body) was administered intratracheally. BAL was performed 2 h after CCL11 administration. Ki19003 (3 and 10 mg/kg) was orally administered 1 h before CCL11 administration.

Mice were actively sensitized by intraperitoneal injections of 10 μg OA with 2 mg alum on day 0 and 5. On day 12, they were exposed to OA (0.5% diluted in sterile physiological saline) for 30 min. Ki19003 (10, 30, and 100 mg/kg) and prednisolone (5 mg/kg) were orally administered 0.5 h before and 10 h after antigen challenge. BAL was performed 24 h after the final antigen challenge according to previously described methods (27). BALF was collected and sera were obtained by centrifugation and stored at −80°C. Antigen-specific IgE in the mouse serum was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (28). Briefly, serum OA-specific IgE was measured by coating monoclonal rat anti-mouse IgE antibody (LO-ME-3) on a 96-well flat culture plate (Nunc Immuno-Plate I 96-F; Nunc, Roskilde, Denmark) at a concentration of 5 μg/ml. After blocking with 1% w/v BSA, serum dilutions were incubated for 1 h followed by biotinylated-OA and peroxidase-conjugated streptavidin. Sequentially diluted monoclonal anti-OA IgE (provided by Dr. Kiniwa, Taiho Pharmaceutical Co., Ltd., Saitama) was used as a standard. Optical densities of the enzymatic reactions were read using an automatic ELISA plate reader (Titertek Multiscan MCC/340; Lab Systems Oy, Helsinki, Finland) at 450 nm (reference 690 nm). The detection limit was 1 ng/ml.

Model of airway remodeling
Experiments were performed according to previous reports (6, 7). Mice were actively sensitized by intraperitoneal injections of 50 μg OA with 1 mg alum on day 0 and 12. Starting on day 22, they were exposed to OA (1% diluted in sterile physiological saline) for 30 min every day for 3 consecutive weeks. As a negative control, animals were injected with OA plus alum and exposed to saline in a similar manner. Ki19003 (100 and 200 mg/kg) and prednisolone (5 mg/kg) were orally administered 1 h before antigen challenge twice a day. BAL, biochemical, and histological examinations were performed 24 h after the final antigen challenge.

BAL was performed according to previously described methods (6, 7). Mice were killed with an i.p. injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and the left bronchi were tied for histological examination. The right air lumen was then washed 4 times with 0.5 ml calcium- and magnesium-free PBS containing 0.1% BSA and 0.05 mM EDTA-2Na. This procedure was repeated three times (total volume: 1.3 ml, recovery >85%). BALF from each animal was pooled in a plastic tube, cooled on ice, and centrifuged (150 × g) at 4°C for 10 min. Cell pellets were resuspended in the same buffer (0.5 ml). BALF was stained with Türk solution and the number of nucleated cells was counted in a Burker chamber. A differential count was made on a smear prepared with a cytocentrifuge (Cytospin II; Shandon, Cheshire, UK) and stained with Diff-Quick solution (based on standard morphologic criteria) of at least 300 cells. The supernatant of BALF was stored at −30°C for determination of cytokine production.

Measurement of OA-specific IgE level in serum
Immediately prior to the final antigen challenge, blood was collected and sera were obtained by centrifugation and stored at −80°C. Antibody-specific IgE in the mouse serum was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (28). Briefly, serum OA-specific IgE was measured by coating monoclonal rat anti-mouse IgE antibody (LO-ME-3) on a 96-well flat culture plate (Nunc Immuno-Plate I 96-F; Nunc, Roskilde, Denmark) at a concentration of 5 μg/ml. After blocking with 1% w/v BSA, serum dilutions were incubated for 1 h followed by biotinylated-OA and peroxidase-conjugated streptavidin. Sequentially diluted monoclonal anti-OA IgE (provided by Dr. Kiniwa, Taiho Pharmaceutical Co., Ltd., Saitama) was used as a standard. Optical densities of the enzymatic reactions were read using an automatic ELISA plate reader (Titertek Multiscan MCC/340; Lab Systems Oy, Helsinki, Finland) at 450 nm (reference 690 nm). The detection limit was 1 ng/ml.

Measurement of cytokine levels in BALF
The amount of cytokines in the supernatant of BALF
was measured by enzyme immunoassay: IL-13 (R&D Systems, Minneapolis, MN, USA) and interferon-γ (IFN-γ) (Endogen, Woburn, MA, USA). TGF-β1 content in BALF was measured by ELISA (Genzyme Tecne, Minneapolis, MN, USA), which can detect mouse TGF-β1 protein due to the high homology of TGF-β1 across species. The assay detects only the active form of TGF-β1. Each sample was activated before measuring according to the manufacturer’s recommendations. The detection limit of each kit is 1.5 pg/ml for IL-13, 10 pg/ml for IFN-γ, or 7 pg/ml for TGF-β1.

Measurement of hydroxyproline content in the right lungs

Whole collagen content of the right lung was evaluated by determining hydroxyproline content as described previously (6, 7). Briefly, after recovery of BALF, the right lung lobes were removed and sliced into 1-mm-thick sections. The sliced lung samples dried with acetone were hydrolyzed with 2 ml of 6 N HCl at 120°C for 24 h in sealed glass tubes. The amount of hydroxyproline in the hydrolysate was measured according to Kivirikko et al. (29). Authentic hydroxyproline (hydroxy-L-proline) was used to establish a standard curve.

Histopathological study

The left lungs were distended with 10% buffered formalin via the trachea (10 cmH₂O) for 30 min and then excised and immersed in fresh fixative for 24 h. Tissues were sliced and embedded in paraffin, and 6-μm sections were stained with Masson-Trichrome for light microscopic examination. Section analyses, described below in detail, were performed in a blind fashion, and slides were presented in random order for each examination.

Masson-trichrome-stained sections were used for assessment of subepithelial fibrosis using a Leica image analysis system (Leica, Cambridge, UK) as described previously (7). Briefly, using a 10-fold magnification objective, two to four specimens of the Masson-trichrome-stained histological preparations of the left lobe, in which the total length of the epithelial basement membrane of the bronchioles was 1.0 – 2.5 mm, were chosen, avoiding the selection of the furcation of the bronchus and the surrounding blood vessels in the largest airway. Subsequently, with a 40-fold magnification, the fibrotic area (stained in blue) at 20-μm depth beneath the basement membrane was measured. The means of the fibrotic area divided by basement membrane length in 2 – 4 preparations of one mouse were calculated, and then the mean scores of subepithelial fibrosis were calculated in each group.

Measurement of airway function

Measurements of bronchial responsiveness to intravenous ACh were performed as previously described (30). Briefly, to measure bronchial responsiveness to ACh, mice were anesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and the jugular vein was cannulated for intravenous injection of ACh. Mice were injected with pancuronium bromide (0.1 mg/kg, i.v.) to stop spontaneous respiration and then ventilated with a rodent ventilator (New England Medical Instruments Inc., Medway, MA, USA) with oxygen supplemented air at 120 strokes/min, at a stroke volume of 0.3 ml/animal. Bronchoconstriction was measured according to the overflow method, using a bronchospasm transducer (model 7020; Ugo Basil, Milan, Italy) connected to the tracheal cannula. To measure bronchial responsiveness to ACh, changes in respiratory overflow volume were measured using increasing doses of ACh. The increase in respiratory overflow volume induced by ACh was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. The bronchial responsiveness to ACh was evaluated as the provocative dose of ACh required to cause 50% bronchoconstriction (PD₅₀), which was calculated from each dose–response curve.

Statistical analyses

Results are presented as the mean ± S.E.M. Statistical analysis was performed using the SAS software program (Release 9.1.3; SAS Institute, Cary, NC, USA). When comparing two groups, if the P value of variance calculated by the F-test was less than 0.05, significant difference was analyzed by the Aspin-Welch test. If not, significant difference was analyzed by Student’s t-test. When comparing more than three groups, if the P value of variance calculated by the Bartlett test was less than 0.05, significant difference was analyzed by Steel’s test. If not, significant difference was analyzed by Dunnett’s test. In all tests, P values less than 0.05 were considered significant.

Results

Effect of Ki19003 on chemokine-induced migration of CCR2-, CCR3-, or CCR7-expressing cells in vitro

As shown in Table 1, Ki19003 inhibited the CCL11-induced migration of CCR3-expressing L1.2 cells with IC₅₀ values of 0.02 μM. In contrast, Ki19003 did not inhibit CCL2- or CCL19-induced migration of CCR2- or CCR7-receptor–expressing cells even at 10 μM. Ki19003 showed no cytotoxicity against these transfectants at 10 μM (data not shown).
Effect of Ki19003 on CCL11-induced migration of bone marrow–derived eosinophilic granulocytes

Ki19003 inhibited CCL11-induced migration of bone marrow–derived eosinophilic granulocytes in a concentration-dependent manner (Fig. 2). Ki19003 showed no cytotoxicity against the cells at 10 μM (data not shown).

Effect of Ki19003 on CCL11-induced increase in the number of eosinophils in BALF

We confirmed the effects of Ki19003 on eosinophil infiltration induced by CCL11 in vivo (Fig. 3). Intratracheal administration of CCL11 into the trachea resulted in an increased number of eosinophils in BALF. Ki19003 at an oral dose of 3 or 10 mg/kg significantly inhibited the increase in the number of eosinophils in BALF in a dose-dependent manner.

Effect of Ki19003 on allergen-induced increase in the number of eosinophils in BALF in sensitized BALB/c mice

To determine the doses of Ki19003 used in a model of airway remodeling, we examined the effect of Ki19003 on allergen-induced increase in the number of eosinophils in BALF in sensitized BALB/c mice (Fig. 4). Single OA exposure to sensitized mice resulted in an increase in the number of eosinophils in BALF. Ki19003 at an oral dose of 100 mg/kg significantly and clearly inhibited the increase in the number of eosinophils in BALF. Prednisolone also significantly inhibited airway eosinophilia by single antigen challenge.
Effects of Ki19003 on leukocyte infiltration, cytokine levels in BALF, and OA-specific IgE level in serum

We first examined the effect of Ki19003 on the numbers of inflammatory cells in BALF. In OA-inhaled mice, the numbers of eosinophils (Fig. 5A) and lymphocytes (Fig. 5B) were significantly increased compared with those in saline-inhaled mice. As expected, Ki19003 selectively and significantly inhibited allergen-induced airway eosinophilia at an oral dose of 200 mg/kg. In contrast, Ki19003 did not affect the increase in the numbers of lymphocytes. Ki19003 also showed no effects on the numbers of macrophages (control: $1.558 \pm 0.142 \times 10^5$; Ki19003 at 100 mg/kg: $1.606 \pm 0.118 \times 10^5$; Ki19003 at 200 mg/kg: $1.839 \pm 0.093 \times 10^5$) and neutrophils (control: $0.017 \pm 0.009 \times 10^5$; Ki19003 at 100 mg/kg: $0.016 \pm 0.005 \times 10^5$; Ki19003 at 200 mg/kg: $0.009 \pm 0.003 \times 10^5$) in BALF. Prednisolone at a dose of 5 mg/kg significantly prevented increases in the numbers of eo-
sinophils and lymphocytes in BALF. In contrast, prednisolone showed a tendency to inhibit increases in the numbers of macrophages (control: 1.558 ± 0.142 × 10^5; prednisolone at 5 mg/kg: 1.306 ± 0.095 × 10^5), but it did not reach significant differences in this study due to experimental variation. Next, we evaluated the effect of Ki19003 on IL-13 (Fig. 5C) and IFN-γ (Fig. 5D) levels in BALF. In OA-exposed mice, the level of IL-13 was significantly increased compared with that in saline-exposed mice. Ki19003 did not affect increases in the level of IL-13 or decreases in the level of IFN-γ in the BALF, whereas prednisolone significantly prevented increases in the level of IL-13 and tended to prevent decreases in the level of IFN-γ in the BALF. We also evaluated the effect of Ki19003 on antigen-specific IgE (Fig. 5E) level in serum. In OA-exposed mice, the level of antigen-specific IgE was increased compared with that in saline-exposed mice. Treatment with Ki19003 did not affect the increase in the level of OA-specific IgE, although prednisolone significantly prevented it.

Effect of Ki19003 on the level of TGF-β1 in BALF and the amount of hydroxyproline in the lung

To investigate the effect of Ki19003 on fibrogenic responses, we examined the production of a fibrogenic cytokine, TGF-β1 (Fig. 6A), in BALF, and the amount of hydroxyproline (Fig. 6B), which is a collagen-specific amino acid, in right lung tissues. Oral dose of Ki19003 at 200 mg/kg prevented increases in the level of TGF-β1 in BALF and the amount of hydroxyproline in the lung. Prednisolone also significantly inhibited these responses at an oral dose of 5 mg/kg.

Effect of Ki19003 on subepithelial and peribronchial fibrosis

We examined the effect of Ki19003 on airway subepithelial and peribronchial fibrosis using morphological and quantitative assessment. Figure 7 shows representative sections of each group stained with Masson-trichrome to detect connective tissue. OA-exposed mice (Fig. 7B) showed increased deposition of connective tissue compared with saline-exposed mice (Fig. 7A). Oral administration of Ki19003 at a dose of 200 mg/kg significantly prevented the increased deposition of connective tissue around the Airways (Fig. 7C). Prednisolone also significantly inhibited these fibrotic changes at 5 mg/kg (Fig. 7D). Figure 7E shows the quantitative analysis of histological examination. Repeated allergen inhalation increased the fibrotic area beneath the basement membrane of the bronchi. Orally administered Ki19003 at 200 mg/kg significantly inhibited increases in the fibrotic area, which was comparable to that in prednisolone-treated mice.

Effect of Ki19003 on airway function

We finally evaluated the effect of Ki19003 on airway responsiveness to ACh (Fig. 8). Repeated antigen challenge resulted in increased airway responsiveness to ACh. Ki19003 at a dose of 200 mg/kg slightly but significantly inhibited the increase in airway responsiveness, whereas prednisolone clearly inhibited this response.

Discussion

In the present study, we evaluated the effects of Ki19003, a newly synthesized, orally active, and CCR3-selective antagonist, on airway remodeling induced by repeated antigen challenge in mice. First, we confirmed the selectivity of this compound for the CCR3 receptor using transfectants expressing murine CCR3 and mouse
bone marrow–derived eosinophilic granulocytes. Ki19003 selectively inhibited CCL11-induced infiltration of the transfectants in vitro and the migration of eosinophils in vitro and in vivo. Second, we showed the efficacy of this compound on allergen-induced airway eosinophilia in an acute model of allergic asthma. Finally, we demonstrated that Ki19003 significantly inhibited the increased level of TGF-β1, which is a fibrogenic cytokine, and the amount of hydroxyproline as a marker of airway fibrosis, associated with inhibition of the number of eosinophils in the BALF after chronic exposure to allergen. Furthermore, Ki19003 clearly inhibited subepithelial/peribronchial fibrosis in histological analysis. These results demonstrate that CCR3 antagonism alleviates the development of airway remodeling by preventing eosinophil infiltration into the airways; therefore, a CCR3 antagonist could be a new candidate to treat airway remodeling, especially subepithelial fibrosis, in allergic asthma.

Subepithelial fibrosis in the basement membrane region in the airways is a typical feature of airway remodeling in asthmatic patients (31). In this study, a CCR3-receptor antagonist prevented eosinophilic inflammation and subepithelial/peribronchial fibrosis in the airways. These results suggest that eosinophils are involved in the development of allergen-induced subepithelial and peribronchial fibrosis in the airways. The role of eosinophils in the development of airway remodeling has been reported in human and animal models. Flood-Page et al. demonstrated that mild asthmatic patients with anti–IL-5 antibody, which specifically decreased the number of airway eosinophils, significantly reduced the deposition of tenascin, lumican, and procollagen III, which are extracellular matrix proteins, in the bronchial mucosal reticular basement membrane when compared with a placebo (32). We have reported the importance of eosinophils in the development of airway subepithelial fibrosis using a mouse model of airway remodeling (33). Eosinophil infiltration and subepithelial and peribronchial fibrosis in the airways are abolished in sensitized and allergen-exposed IL-5 receptor–null mice or in mice treated with a neutralizing antibody against IL-5, whereas they are markedly increased in IL-5 transgenic animals. It is known that IL-5 is mainly involved in the maturation of

Fig. 7. Ki19003 significantly and dose-dependently inhibited allergen-induced subepithelial and peribronchial fibrosis after 3 weeks of allergen challenge. Histological analysis of lung sections stained with Masson-trichrome 24 h after the final antigen challenge in sensitized BALB/c mice (A – D). A, vehicle-treated saline-exposed animal; B, vehicle-treated ovalbumin-exposed animal; C, Ki19003 (200 mg/kg)–treated ovalbumin-exposed animal; D, prednisolone (5 mg/kg)–treated ovalbumin-exposed animal. Fibrotic area beneath the basement membrane of the bronchi was evaluated (panel. E). Values represent the mean ± S.E.M. of 8 – 9 mice in each group. OA, ovalbumin-exposed; Pred, prednisolone; S, sensitized; Sal, saline-exposed. ***P < 0.001, ***p < 0.001 [vs. vehicle-treated OA-exposed animal (S-OA)]; †P < 0.05 [vs. vehicle-treated OA-exposed animal (S-OA)].

Fig. 8. Ki19003 partially prevented airway hyperresponsiveness to ACh after 3 weeks of allergen challenge. Values represent the mean ± S.E.M. of 8 – 9 mice in each group. OA, ovalbumin-exposed; Pred, prednisolone; S, sensitized; Sal, saline-exposed; Veh, vehicle. ***p < 0.001 (vs. vehicle-treated OA-exposed animal); †P < 0.05 (vs. vehicle-treated OA-exposed animal).
eosinophils in bone marrow (34), while CCR3 is predominantly related to the infiltration of eosinophils into the tissue (35). In the present study, we confirmed the same phenotypes, namely, the reduction of eosinophil infiltration and subepithelial/peribronchial fibrosis in the airways in mice treated with a CCR3-receptor antagonist, as observed in mice lacking IL-5 activity. Recently, Humbles et al. reported that in mice with complete deletion of the eosinophil lineage, peribronchiolar collagen deposition is decreased compared with that in wild-type mice (36). These findings strongly suggest that eosinophils play a critical role in the development of allergen-induced airway subepithelial fibrosis.

TGF-β1 is one of the most important cytokines involved in the development of tissue fibrosis (37); it is produced from various cells, including eosinophils, macrophages, and epithelium in the airways (37). TGF-β1 is a potent profibrotic cytokine that can stimulate fibroblasts to promote the synthesis and secretion of extracellular matrix proteins (37). It is also reported that TGF-β1 decreases the synthesis of enzymes, such as matrix metalloproteinase, and increases the synthesis of inhibitors, including tissue inhibitor of metalloproteinase 1 (37). In asthmatics, the levels of TGF-β1 are increased, and the expression of TGF-β1 is correlated with the severity of the disease and the degree of subepithelial fibrosis (38). In the present study, a CCR3-receptor antagonist prevented collagen deposition, associated with decreases in the TGF-β1 level in the BALF. Our observations suggest that the reduction of TGF-β1 by CCR3 antagonism may lead to the prevention of subepithelial fibrosis induced by repeated allergen challenge.

It is known that myofibroblasts contribute to tissue fibrosis through the production of extracellular matrix proteins (37). Myofibroblast hyperplasia is an important pathological feature of bronchial asthma and its intensity is correlated with the amount of subepithelial collagen deposition (39). In our previous report using this model, within the first week of allergen challenge, TGF-β1 was mainly produced by eosinophils, whereas after 2 – 3 weeks, myofibroblasts were the major source of this factor (33), suggesting that contribution to the development of subepithelial fibrosis shifts from eosinophils to myofibroblasts in our model. TGF-β1 is a chemoattractant for fibroblasts and myofibroblasts that can be found in areas of developing fibrosis (40 – 42). TGF-β1 can stimulate fibroblast differentiation into the myofibroblast phenotype and suppress myofibroblast apoptosis (43, 44). Recently, eosinophil and fibroblast interaction in the development of tissue fibrosis has been reported (45, 46). From these findings, we speculate that in this model, TGF-β1 produced from eosinophils may play an important role in the initiation of airway fibrosis by affecting chemotaxis and differentiation of fibroblasts. Therefore, the decrease of TGF-β1 produced from eosinophils and/or myofibroblasts in the airways as a result of CCR3 antagonism may lead to the prevention of subepithelial/peribronchial fibrosis in this study. More recently, it was reported that CCR3 is constitutively expressed in cultured lung and primary bronchial fibroblasts, and CCL11 shows a direct and selective profibrogenic effect on lung and bronchial fibroblasts in humans (47). Thus, further study will be needed to demonstrate the direct effect of the CCR3-receptor antagonist on fibroblasts and myofibroblasts.

In the present study, Ki19003 slightly inhibited hyperresponsiveness to ACh in spite of the clear inhibition of airway eosinophilia. In contrast, anti-CCR3 antibody (48) and a CCR3 antagonist (49) strongly inhibited airway eosinophilia and airway hyperresponsiveness to inhaled methacholine in a mouse model of allergic asthma. The discrepancies between these results may be due to differences in the protocols used in each study. The role of CCR3 in the development of airway hyperresponsiveness in a mouse model of allergic asthma remains controversial. It is therefore suggested that the absence or presence of airway hyperresponsiveness observed in CCR3-deficient mice may depend on the sensitization and challenge protocol (50, 51).

Recently, a monoclonal antibody to human IL-5, mepolizumab, has been used in a clinical trial. The antibody caused a significant reduction in both blood and induced sputum eosinophils, but there were no changes in either the late asthmatic reaction or airway hyperresponsiveness (52). However, very recently, Haldar et al. reported that mepolizumab inhibited exacerbation and improved QOL scores in patients with refractory eosinophilic asthma. Moreover, they also reported that airway thickness and the total wall area were inhibited in subjects treated with mepolizumab compared with in the placebo group (53). Although further investigations are awaited regarding the relevance of clinical responses and changes in airway thickness, the prevention of airway eosinophilia has proved to be beneficial for asthma control. In terms of healthcare economics, a low molecular weight antagonist seems to be important in the treatment of asthma; therefore, a low molecular weight CCR3 antagonist could be a promising drug for asthma therapy.

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