

GGTI-2133, AN INHIBITOR OF GERANYLGERANYLTRANSFERASE, INHIBITS INFILTRATION OF INFLAMMATORY CELLS INTO AIRWAYS IN MOUSE EXPERIMENTAL ASTHMA

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Statins have been proposed as a novel treatment of respiratory diseases including asthma. Although the mechanism of anti-inflammatory effect of statins is still unclear, an inhibition of protein prenylation by depleting the downstream metabolites of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase might be involved. To test the hypothesis, the effects of GGTI-2133, a direct inhibitor of geranylgeranyltransferase (GGTase), on antigen-induced airway inflammation were investigated in a murine model of allergic bronchial asthma. Mice were sensitized and repeatedly challenged with ovalbumin antigen (OA). Animals were also treated with GGTI-2133 (5 mg/kg/day, i.p.) once a day before and during the antigen inhalation period. Repeated antigen inhalation caused an infiltration of inflammatory cells, especially eosinophils, into airways. Significant increases in interleukin (IL)-4, IL-13, eotaxin, thymus and activation-regulated chemokine (TARC) and leukotriene B₄ (LTB₄) in bronchoalveolar lavage fluids and total and OA-specific IgE in sera were also found in the antigen-exposed animals. The systemic treatments with GGTI-2133 inhibited the antigen-induced eosinophil infiltration into airways almost completely. However, interestingly, the GGTI-2133 treatment did not affect the levels of these chemotactic factors and IgE. These findings suggest that selective inhibition of GGTase is effective for eosinophilic airway inflammation such as asthma.

Allergic bronchial asthma is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness. The response to allergen in the airways involves infiltration of various types of inflammatory cells, such as eosinophils and T-helper 2 (Th2) subset of T lymphocytes (1-3). Both eosinophils and Th2 lymphocytes play a critical role in the pathogenesis of allergic bronchial asthma and produce factors such as cytokines and chemokines. Increased eosinophils and T lymphocytes in the bronchial mucosa and bronchoalveolar lavage (BAL) fluid are distinctive features of the inflammatory response in patients with allergic bronchial asthma

and appear to correlate with the severity of the disease (4-5).

Statins are a class of cholesterol-lowering drugs that decrease mortality from cardiovascular disease (6) and stroke (7). The beneficial effects of statins have been attributed to reduce cholesterol biosynthesis through competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to L-mevalonate. Statins have pleiotropic effects beyond lowering cholesterol levels, such as modulation of innate and/or adaptive immunity (8) and anti-inflammatory actions (9), and have also been proposed as a novel treatment of respiratory diseases

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(10). Although the exact mechanism of action of statins is still unclear, an indirect inhibition of protein prenylation by depleting the HMG-CoA downstream metabolites, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), might be essential for their pleiotropic effects. An importance of inhibition of geranylgeranyl but not farnesyl isoprenoid pathway has also been suggested in their anti-inflammatory effects (11). Thus, selective inhibition of geranylgeranyltransferase (GGTase) may be one of the novel strategies for treatment of respiratory diseases such as bronchial asthma.

To test this hypothesis, the effect of systemic treatment with a GGTase inhibitor, GGTI-2133 (12), on airway inflammation induced by antigen exposure was investigated in mice.

MATERIALS AND METHODS

Animal treatments

Male BALB/c mice were purchased from the Charles River Japan, Inc. (Kanagawa, Japan) and housed in a pathogen-free facility. All animal experiments were approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

Preparation of a murine model of allergic bronchial asthma was performed as described previously (13-16). In brief, mice (8 weeks of age) were actively sensitized by intraperitoneal injections of 8 µg ovalbumin (OA; Seikagaku Co., Tokyo, Japan) with 2 mg Imject Alum (Pierce Biotechnology, Inc., Rockford, IL, USA) on days 0 and 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/mL) for 30 min on days 12, 16 and 20. A control group of mice received the same immunization procedure but inhaled saline aerosol instead of OA challenge. Animals also received intraperitoneal injection with GGTI-2133 (N-[[4-(imidazol-4-yl)methylamino]-2-(1-naphthyl)benzoyl]leucine trifluoroacetate salt; Sigma-Aldrich, St. Louis, MO; 5 mg/kg/day) (16-17) or its vehicle (50% DMSO in PBS) once a day for 10 days from Day 11 to Day 20 (16). The treatment was carried out 1 h before the antigen inhalation on the respective days of the antigen challenge (Days 12, 16 and 20).

Cell counts in bronchoalveolar lavage (BAL) fluids

Twenty-four h after the last OA challenge, mice were sacrificed under urethane (1.6 g/kg, i.p.; Sigma, St. Louis, MO) anesthesia. After thoracotomy, blood samples were collected from the hearts to obtain sera, and bronchoalveolar lavage (BAL) was carried out as described previously (18). The BAL fluid obtained was centrifuged at 500 g for 5 min at

room temperature, and the resultant pellet of the centrifuged BAL fluid was resuspended in 500 µL of 10% formaldehyde and incubated for 10 min at room temperature. The cells were washed with PBS and resuspended in 500 µL of PBS. An aliquot of cell suspension was used for cell counts with a hemocytometer. A 10 µL aliquot of cell suspension was then mounted on silane-coated glass slides and air-dried. Cell types were identified and counted by differential staining microscopy with Diff-Quik (Baxter Healthcare, Miami, FL) under x400 magnification. Eosinophils were defined as cells that had positive staining (red ~ pink granules) with polymorphonuclei. Inflammatory cell populations were determined by first randomly counting 100 cells in duplicate and by multiplying total cell counts per mL of BAL fluid by the percentages of each cell type.

Quantitation of interleukin (IL)-4, IL-13, eotaxin, thymus and activation-regulated chemokine (TARC) and leukotriene B₄ (LTB₄) levels in BAL fluids

The resultant supernatant of the centrifuged BAL fluid was used for cytokines, chemokines and leukotriene B₄ (LTB₄) analyses. The levels of interleukin (IL)-4, IL-13, eotaxin, thymus and activation-regulated chemokine (TARC) and LTB₄ were determined using enzyme-linked immunosorbent assay (ELISA) with Quantikine™ mouse IL-4 immunoassay (M4000B; R&D Systems, Inc., Minneapolis, MN), Quantikine™ mouse IL-13 immunoassay (M1300CB; R&D Systems, Inc.), mouse eotaxin ELISA kit (ELM-Eotaxin-001; RayBiotech, Inc., Norcross GA), Quantikine™ mouse CCL17/TARC immunoassay (MCC170; R&D Systems, Inc.), LTB₄ competitive ELISA kit (EHLTB₄; Pierce Biotechnology, Inc., Rockford, IL), respectively, according to the manufacturer's instructions.

Quantitation of IgE level in serum

Serum was obtained from the blood sample by centrifugation at 3,000 g for 10 min at 4°C. Total immunoglobulin E (IgE) and OA-specific IgE in sera were measured by an ELISA system (Bethyl Laboratories, Inc., Montgomery, TX), as described previously (18).

Statistical analyses

All data were expressed as mean with SEM. Statistical significance of difference was determined by unpaired Student's t-test, Wilcoxon rank-sum test, or Bonferroni/Dunn test. A value of $p < 0.05$ was considered significant.

RESULTS

Effects of systemic treatments with GGTI-2133 on antigen-induced eosinophil infiltration into airways

To determine the effects of GGTI-2133 on the

airway inflammation induced by repeated antigen exposure, different cell counts in BAL fluids were carried out. Total cell counts in BAL fluids of the repeatedly antigen-challenged mice were significantly increased as compared with those of the control group (Fig. 1, Vehicle alone vs. OA-Vehicle groups; $P < 0.001$). Diff-Quik staining of the cells revealed that most of the increased cells were eosinophils (Fig. 1, Vehicle alone vs. OA-Vehicle groups; $P < 0.001$). A slight but significant increase in lymphocytes was also found. Although the GGTI-2133 administration alone had no effect on the number of any cells in BAL fluids of the control animals, both the increases in eosinophils and lymphocytes induced by the repeated antigen exposure were significantly reduced by the systemic treatments with GGTI-2133 (Fig. 1, OA-Vehicle vs.

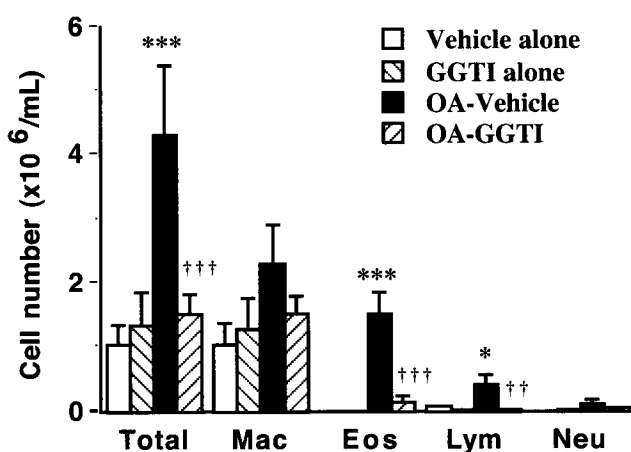


Fig. 1. Effects of systemic treatments with GGTI-2133 on inflammatory cell infiltration into airways induced by repeated antigen challenge in mice. Cell counts were carried out in bronchoalveolar lavage (BAL) fluids obtained from the non-sensitized normal control and repeatedly antigen-challenged (OA) animals that were also pretreated with GGTI-2133 (5 mg/kg/day) or its vehicle (50% DMSO in PBS) intraperitoneally for 10 days as described in the MATERIALS AND METHODS section. Cell types were identified and counted by differential staining microscopy with Diff-Quik under $\times 400$ magnification. Each column represents the mean $\pm$ SE from 5-6 different animals. Total: total cells, Mac: macrophage, Eos: eosinophil, Lym: lymphocyte, and Neu: neutrophil. * $P < 0.05$ and *** $P < 0.001$ vs Vehicle alone group, and †† $P < 0.01$ and ††† $P < 0.001$ vs OA-Vehicle group.

OA-GGTI-2133 groups; $P < 0.001$ in eosinophils and $P < 0.01$ in lymphocytes). Increments of macrophages and neutrophils were also found in the OA-Vehicle group, whereas no statistical significance was observed (Fig. 1).

Effects of systemic treatments with GGTI-2133 on antigen-induced increase in interleukin (IL)-4, IL-13, eotaxin, thymus and activation-regulated chemokine (TARC) and leukotriene B₄ (LTB₄) levels in BAL fluids

As shown in Fig. 2, the levels of IL-4 and IL-13 in BAL fluids of repeatedly antigen-challenged mice (OA-Vehicle group) were significantly increased as compared with those of the control animals (Vehicle alone group; $P < 0.05$, respectively). GGTI-2133 had no effect on the antigen-induced upregulation of these Th2 cytokines (Fig. 2), although there is a tendency to suppress the expression of IL-4. Similarly, GGTI-2133 also had no significant effect on the antigen-induced increase in eotaxin, TARC, or LTB₄ in BAL fluids (Fig. 3).

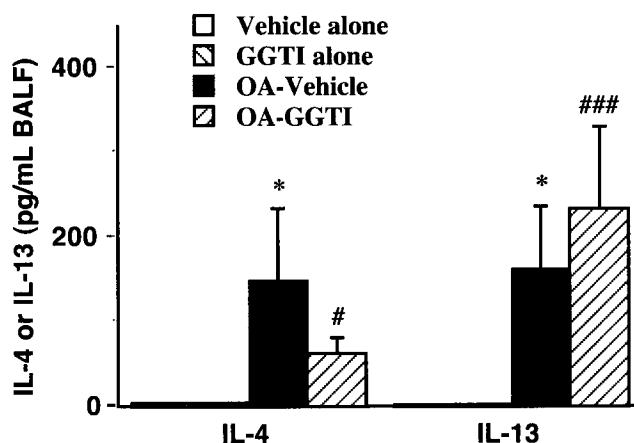


Fig. 2. Effects of systemic treatments with GGTI-2133 on the increased levels of interleukin (IL)-4 (left) and IL-13 (right) in bronchoalveolar lavage (BAL) fluids induced by repeated antigen challenge in mice. BAL fluids obtained from non-sensitized control and repeatedly antigen-challenged (OA) mice were assayed for IL-4 and IL-13 by enzyme-linked immunosorbent assays (ELISAs). GGTI-2133 (5 mg/kg/day) or its vehicle (50% DMSO in PBS) was also pretreated intraperitoneally for 10 days as described in the MATERIALS AND METHODS section. Each column represents the mean $\pm$ SE from at least 5 different animals. * $P < 0.05$ vs. Vehicle alone and # $P < 0.05$ and ### $P < 0.01$ vs. GGTI-2133 alone group.

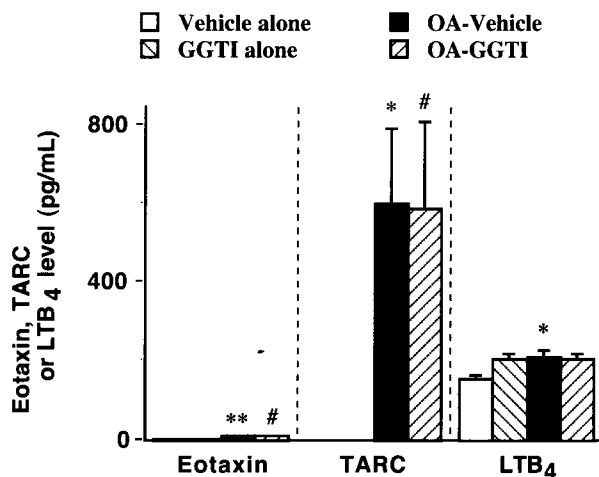


Fig. 3. Effects of systemic treatments with GGTI-2133 on the increased levels of eotaxin (left), thymus and activation-regulated chemokine (TARC; middle) and leukotriene B_4 (LTB_4 ; right) in bronchoalveolar lavage (BAL) fluids induced by repeated antigen challenge in mice. BAL fluids obtained from non-sensitized control and repeatedly antigen-challenged (OA) mice were assayed for eotaxin, TARC and LTB_4 by enzyme-linked immunosorbent assays (ELISAs). GGTI-2133 (5 mg/kg/day) or its vehicle (50% DMSO in PBS) was also pretreated intraperitoneally for 10 days as described in the MATERIALS AND METHODS section. Each column represents the mean $\pm$ SE from at least 5 different animals. * $P < 0.05$ and ** $P < 0.01$ vs. Vehicle alone and # $P < 0.05$ vs. GGTI-2133 alone group.

Effects of systemic treatments with GGTI-2133 on antigen-induced increase in IgE levels in serum

Fig. 4 shows the levels of total and OA-specific IgE in sera of mice. In the non-sensitized normal control mice that were also treated with vehicle for GGTI-2133 (Vehicle alone group), a detectable total IgE level, which is not significantly different from naive animals (data not shown), was found (Fig. 4, left panel). Total IgE level was markedly and significantly increased when animals were sensitized and repeatedly challenged with OA antigen (OA-Vehicle group, $p < 0.01$ vs Vehicle alone group; Fig. 4, left panel). Neither the basal level nor the antigen-induced increment of total IgE was affected by the systemic treatment with GGTI-2133: no significant difference was observed between the Vehicle alone and the GGTI-2133 alone groups or between the OA-Vehicle and the OA-GGTI-2133 groups (Fig. 4,

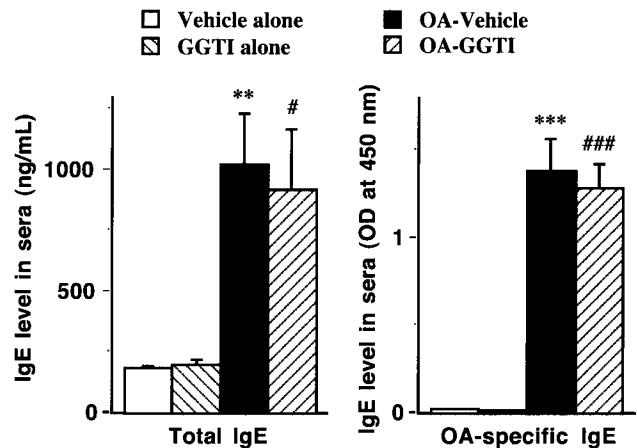


Fig. 4. Effects of systemic treatments with GGTI-2133 on the increased levels of total immunoglobulin E (IgE; left panel) and ovalbumin (OA)-specific IgE (right panel) in sera induced by repeated antigen challenge in mice. Sera obtained from non-sensitized control and repeatedly antigen-challenged mice were assayed for total and OA-specific IgE by enzyme-linked immunosorbent assays (ELISAs). GGTI-2133 (5 mg/kg/day) or its vehicle (50% DMSO in PBS) was also pretreated intraperitoneally for 10 days as described in the Materials and Methods section. Each column represents the mean $\pm$ SE from at least 5 different animals. ** $P < 0.01$ and *** $P < 0.001$ vs. Vehicle alone and # $P < 0.05$ and ### $P < 0.001$ vs. GGTI-2133 alone group.

left panel). Similar results were also obtained in case of the OA-specific IgE (Fig. 4, right panel).

DISCUSSION

There is increasing evidence that statins might have anti-inflammatory properties in animal models of allergic bronchial asthma (9, 20-21). It is possible that one of the mechanisms of action of statins is their indirect inhibitory effect of protein prenylation. Therefore in this study, the effect of GGTI-2133, a direct inhibitor of GGTase, on airway inflammation was tested in a murine model of allergic bronchial asthma. Our previous study revealed that the *in vivo* GGTI-2133 administration protocol used inhibited both geranylgeranylation and activation of RhoA in the mouse airways (16).

The present study clearly demonstrated that

systemic treatments with GGTI-2133 reduced the inflammatory cell infiltration: the increases in BAL cells, mainly eosinophils, induced by antigen exposure were attenuated (Fig. 1). It is unlikely that the inhibitory effects of GGTI-2133 on inflammatory cell infiltration are due to the inhibition of proinflammatory mediator release or IgE production, because the GGTI-2133 treatments did not affect the increased expression of IL-4, IL-13, eotaxin, TARC and LTB₄ in BAL fluids (Figs. 2 and 3) and total and OA-specific IgE in sera (Fig. 4) induced by antigen. Similarly, lovastatin, which is able to inhibit GGTase indirectly, also inhibited the infiltration of inflammatory cells into airways without affecting the production of proinflammatory cytokines and IgE in rat models of allergic asthma (20). Thus, the anti-inflammatory effect of GGTI-2133 might result from the inhibition of GGTases in these cells. Migration of leukocytes is believed to be involved in a coordinated remodeling of the actin cytoskeleton. The monomeric GTP-binding proteins, RhoA, Rac1, and Cdc42, are critical regulators of actin reorganization in fibroblasts and other non-hemopoietic cells (22-23). An involvement of Rho family GTPases in the transmigration across endothelial cell layers has also been demonstrated in leukocytes (23-26). Posttranslational prenylation, especially geranylgeranylation, is required for the activation of these Rho family GTPases.

In addition to the inhibitory effect of GGTI-2133 on airway inflammation currently indicated, the antigen-induced bronchial smooth muscle hyperresponsiveness, one of the causes of airway hyperresponsiveness (27-28), was also attenuated by the identical GGTI-2133 administration protocol used in the present study (16). It is therefore possible that direct inhibitors of GGTase, such as GGTI-2133, might be effective for treatment of allergic bronchial asthma. On the other hand, a slow turnover of isoprenylation has been suggested in smooth muscle tissues (29-30). Moreover, the GGTase inhibitors may be unable to inhibit the activity of proteins that are already isoprenylated. Thus, the GGTase inhibitor may behave like an "asthma controller medicine" such as the inhaled corticosteroids but not a "quick-relief medicine" such as the short-acting β_2 -stimulants. Further detailed studies are required to evaluate the efficacy of GGTase inhibitors on asthma

treatment.

Because the prevalence of asthma is recently rising (31), there is an increased need for the development of new drugs for its treatment, especially for patients who respond poorly to conventional therapy such as glucocorticoids. Recently, statins have been proposed as a novel treatment of respiratory diseases including asthma (21, 32-34). However, their effectiveness to asthma is still controversial (34-35). The contradictory effects of statins may be explained by the inhibition of downstream pathways of HMG-CoA reductase other than GGTase. The current findings suggest that selective inhibition of GGTases is effective for allergic bronchial asthma.

In conclusion, the present study demonstrated that a GGTase inhibitor, GGTI-2133, ameliorates the infiltration of inflammatory cells into airways induced by repeated antigen exposure in mice. Selective GGTase inhibitors such as GGTI-2133 may be beneficial for the treatment of allergic bronchial asthma.

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