

Original article

Inhibitory effects of sunitinib on ovalbumin-induced chronic experimental asthma in mice

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Keywords: asthma; airway remodeling; tyrosine kinase inhibitor; sunitinib

Background Tyrosine kinase signaling cascades play a critical role in the pathogenesis of allergic airway inflammation. Sunitinib, a multitargeted receptor tyrosine kinase inhibitor, has been reported to exert potent immunoregulatory, anti-inflammatory and anti-fibrosis effects. We investigated whether sunitinib could suppress the progression of airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling in a murine model of chronic asthma.

Methods Ovalbumin (OVA)-sensitized mice were chronically challenged with aerosolized OVA for 8 weeks. Some mice were intragastrically administered with sunitinib (40 mg/kg) daily during the period of OVA challenge. Twelve hours after the last OVA challenge, mice were evaluated for the development of airway inflammation, AHR and airway remodeling. The levels of total serum immunoglobulin E (IgE) and Th2 cytokines (interleukin (IL)-4 and IL-13) in bronchoalveolar lavage fluid (BALF) were measured by ELISA. The expression of phosphorylated c-kit protein in the lungs was detected by immunoprecipitation/Western blotting (IP/WB) analysis.

Results Sunitinib significantly inhibited eosinophilic airway inflammation, persistent AHR and airway remodeling in chronic experimental asthma. It reduced levels of total serum IgE and BALF Th2 cytokines and also lowered the expression of phosphorylated c-kit protein in remodelled airways.

Conclusions Sunitinib may inhibit the development of airway inflammation, AHR and airway remodeling. It is potentially beneficial to the prevention or treatment of asthma.

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Asthma is a chronic eosinophilic inflammatory disease of the airways associated with pathologic structural changes such as subepithelial fibrosis, hyperplasia of mucus glands, myofibroblast and smooth muscle proliferation, and vascular changes,¹ which are termed airway remodeling. Changes of remodeling occur in response to persistent inflammation and correlate with the development of airway hyperresponsiveness (AHR).^{2,3} Airway remodeling has been recently one of the main goals in asthma research because it has been implicated to influence airway behavior and evolution of asthma.

Current asthma therapies are effective in reducing airway inflammation and hyperresponsiveness but their effects on airway remodeling are not as evident. Inhaled glucocorticoids remain the most effective long-term treatment for asthma. However, some asthmatic patients still exhibit AHR following a prolonged treatment with steroids. Moreover, patients with chronic asthma treated with inhaled glucocorticoids, particularly in the long-term may develop serious side effects.⁴ Thus there is a need for new or alternative approaches to prevent airway remodeling.

Sunitinib, a novel oral multitargeted receptor tyrosine kinase inhibitor, has been identified as a potent inhibitor of vascular endothelial growth factor receptors (VEGFRs), the platelet-derived growth factor receptors (PDGFR and PDGFR- β), the stem cell factor (SCF) receptor c-kit, and fetal liver tyrosine kinase receptor 3 (FLT3) in both biochemical and cellular assays.^{5,6} Recent studies have demonstrated that sunitinib exerts potent immuno-

regulatory, anti-inflammatory and anti-fibrotic effects.⁷⁻⁹ Thus sunitinib may have a therapeutic potential for the treatment of asthma where airway structural changes occur in response to persistent inflammation.

METHODS

Antigen sensitization, challenge and treatment

Female SPF BALB/c mice (8–10 weeks old) were obtained from Shanghai Slac Laboratory Animal Co. Ltd and raised in the SPF Laboratory Animal Center of Nanjing Medical University. This study was performed under a protocol approved by the Institution of Animal Ethics Committee. Thirty-six BALB/c mice were randomly assigned to a control group, an ovalbumin (OVA) group, and a sunitinib group. Every group contained 12 mice, 6 for pulmonary resistance and 6 for bronchoalveolar lavage (BAL). The protocol for sensitization and inhalational challenge has previously been described in detail.^{10,11}

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Briefly, mice in the OVA group and sunitinib group were sensitized on days 0, 7 and 21 by intraperitoneal injection of 50 µg chicken egg ovalbumin (OVA, Grade V, Sigma, USA) emulsified in 1 mg of aluminium hydroxide in a total volume of 200 µl. One week after the last sensitization, the mice were exposed to 1% aerosolized OVA for 30 min/d, 3 days per week for 8 weeks. For aerosolized challenges, the mice were placed in a Plexiglas box (29 cm × 22 cm × 8 cm) and underwent aerosolized OVA using an ultrasonic nebulizer (NE-U11B, Omuron Co., Tokyo, Japan). During the last 8 weeks, the mice in the sunitinib group were administered intragastrically with sunitinib at a dose of 40 mg/kg.^{9,12} The control group was injected and nebulized with normal saline instead of OVA.

Evaluation of airway responsiveness

Airway responsiveness to acetylcholine chloride was measured with AniRes 2005 animal lung function analysis systems (SYNOL High-Tech Co. Ltd, Beijing, China). The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (80 mg/kg) 12 hours after the last OVA challenge. A plastic tube of 2 mm in internal diameter was inserted into the trachea via tracheotomy for mechanical ventilation, and a 27-gauge needle was inserted into the caudal vein for drug administration. The mice were then placed in a whole body plethysmography chamber and ventilated mechanically at a rate of 90 breaths per minute with a tidal volume of 6 ml/kg. After establishment of a stable airway pressure recording, acetylcholine chloride of progressive doses (5 µg/kg, 15 µg/kg, 45 µg/kg, 135 µg/kg, 405 µg/kg) was administered intravenously with a microinfusion pump. After each dose, the data were continuously collected for 5 seconds to 5 minutes and maximum values of lung resistance (RL) were taken to express changes in murine airway function.^{13,14}

Analysis of bronchoalveolar lavage fluid (BALF) and serum

The mice were bled 12 hours after the last OVA exposure by retroorbital puncture using heparinized capillary tubes. Blood samples were centrifuged (10 minutes, 4°C, 1000 × *g*), and plasma was stored at -70°C until use. The lungs were washed three times with 0.5 ml saline to collect BALF. The BALF was centrifuged (10 minutes, 4°C, 1000 × *g*), and the total number of inflammatory cells in BALF was counted with a hemocytometer. Differential cell counts were conducted using cytopspin techniques and Wright's staining by counting at least 200 cells. The levels of IL-4 (Jingmei Biotech Company, Shanghai, China) and IL-13 (R&D Systems, Minneapolis, USA) in BALF and total serum IgE (Bionewtrans Pharmaceutical Biotechnology Co. Ltd., Franklin, USA) were determined by ELISA according to the manufacturer's protocol.

Histological and immunohistochemical analysis of lung tissue

The left lungs taken from sacrificed mice were immersed in 10% formaldehyde overnight, and then embedded in

paraffin and sections (5 µm thickness) were stained with either hematoxylin & eosin to assess the inflammatory cell infiltrates, periodic acid schiff (PAS) to quantify airway goblet cells, and Masson's trichrome to determine collagen deposition in the lungs. Results were expressed as the areas occupied by periodic acid (purple color) stained mucin, and aniline blue (blue color) stained collagen per micrometer length of bronchiolar basement membrane with 150–200 µm internal diameter by counting at least 10 bronchioles in each slide. The mass of airway smooth muscle (ASM) was also estimated from the measurement of the area of α -smooth muscle specific actin (α -SMA) immunoreactivity as previously described.¹⁵ Results were expressed as the area of α -SMA immunostaining per mm length of basement membrane of bronchioles. For quantitative or semiquantitative analysis, images of these sections were examined using an image-analysis system (Image-Pro Plus; Media Cybernetics, Inc., Georgia, USA) calibrated with a reference measurement slide.

Immunoprecipitation/Western blotting (IP/WB) analysis

The right lung tissues were weighed and diced into very small pieces. Then the tissues were pulverized in liquid nitrogen, and lysed in cold HTNG buffer (50 mmol HEPES pH 7.5, 150 mmol NaCl, 1.5 mmol MgCl₂, 10% (v/v) glycerol, 1% Triton X-114, 1% sodium orthovanadate, 2 mmol NaF, 1 mmol phenylmethyl sulfonylfluoride). The mixture was incubated on ice for 60 minutes transferred to a microcentrifuge tube, and centrifuged at 10 000 × *g* for 20 minutes at 4°C. Protein concentration in the lysates was determined with a BCA assay kit (P0012, Beyotime Institute of Biotechnology). One mg total protein from each sample was immunoprecipitated overnight at 4°C with an anti-c-kit antibody (sc-1494, Santa Cruz, USA) and Protein A/G-agarose (sc-2003, Santa Cruz). The immunocomplexes were washed with cold HNTG lysis buffer containing protease inhibitor cocktail. The eluted proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with phosphatase-free defatted milk (P1622, Applygen Technologies Inc.) at 4°C overnight, before being incubated for 2 hours at room temperature with an anti-phosphotyrosine antibody (sc-7020, Santa Cruz) at a 1:300 dilution, and then incubated with peroxidase conjugated goat anti-rabbit IgG for 1 hour. Proteins were detected by enhanced chemiluminescence (Santa Cruz). Membranes were then stripped for 20 minutes at room temperature with RestoreTM Western Blot Stripping Buffer (P1650, Applygen Technologies Inc.). To detect total c-kit levels, the membranes were reprobed with the same anti-ckit antibody (1:300) that was used for the immunoprecipitation.

Statistical analysis

Data were presented as mean ± standard error (SE). Statistical analysis was performed by one-way analysis of variance (ANOVA) and least significant difference (LSD)

test with SPSS 13.0 software (SPSS Inc., Chicago, USA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Sunitinib reduced AHR in chronic experimental asthma

Airway responsiveness was assessed as the airway resistance in response to increasing doses of methacholine. The airway resistance generated by methacholine administration (at doses from 5 µg/kg to 405 µg/kg) increased significantly in the OVA-sensitized and OVA-challenged mice as compared with the controls. Treatment with sunitinib, however, significantly reduced AHR compared with that in the OVA group (*P* < 0.05) (Figure 1a).

Sunitinib inhibited chronic airway inflammation

In the murine asthma model, OVA sensitization and challenge resulted in a substantial increase in the number of total leukocytes in the BALF. Total inflammatory cells and eosinophils counts in the BALF were significantly decreased in sunitinib-treated mice compared with OVA-challenged mice (Figure 1b). The results of histological examination of lung tissues paralleled the cell numbers in the BALF. Marked influxes of inflammatory cells into the airway and around the blood vessels were detected in the OVA-sensitized/challenged mice, but not in the control mice. Sunitinib-treated mice resulted in a marked reduction of the inflammatory cell infiltration into the peribronchiolar and perivascular regions (Figure 2a–c).

Sunitinib decreased levels of Th2 cytokines in BALF and total serum IgE

The levels of Th2 cytokines IL-4 and IL-13 in the BALF and total serum IgE were increased significantly by airway challenge with OVA when compared with those of the control group. The administration of sunitinib reduced the concentrations of Th2 cytokines and total serum IgE (Table).

Sunitinib alleviated airway remodeling in chronic experimental asthma

In order to evaluate goblet cell hyperplasia, lung sections were stained with PAS staining. Overproduction of mucus and goblet cell hyperplasia were observed in the OVA-sensitized/challenged mice, but not in the control mice (Figure 2d–f). There was a greater reduction in the proportion of goblet cells in the airway of mice treated with sunitinib compared with that in the OVA-sensitized/challenged mice (Figure 2g). The areas of Masson's trichrome-positive peribronchiolar collagen layer and α-smooth muscle (α-SMA)-stained smooth muscle layer in the mice airways were obviously increased after chronic allergen exposure (Figure 3a–f). Treatment with sunitinib significantly inhibited the collagen deposition/fibrosis surrounding the airways in bronchioles and limited the increase in the thickness of the smooth muscle layer in all

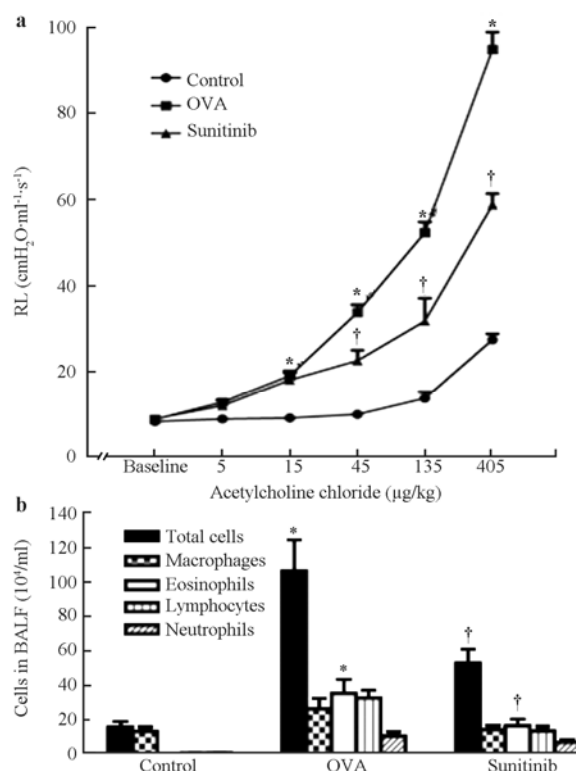


Figure 1. Treatment with sunitinib decreases the development of AHR and inflammatory cell accumulation in BALF in a murine model of chronic asthma. Airway responsiveness (a) and inflammatory cell accumulation (b) in BALF were determined as described in the Methods section. Data are the mean ± SE (*n* = 6 per group). **P* < 0.01 compared with the control group. †*P* < 0.05 compared with the OVA group.

Table. Levels of IL-4 and IL-13 in BALF as well as total serum IgE in the three groups

Groups	BALF (pg/ml)		Serum (ng/ml)
	IL-4	IL-13	Total IgE
Control	17.6±3.72	89.96±4.00	0.60±0.16
OVA	84.18±12.09*	129.14±5.97*	2.21±0.30*
Sunitinib	36.72±7.57†	96.39±1.70†	0.86±0.08†

Treatment with sunitinib reduces the levels of Th2-type cytokines IL-4 and IL-13 in BALF and total serum IgE in a murine model of chronic asthma. Data are the mean ± SE (*n* = 6 per group). **P* < 0.01 compared with the control group. †*P* < 0.05 compared with the OVA group.

bronchioles compared with the OVA-sensitized/challenged mice (Figure 2 g and h).

Sunitinib decreased the protein level of c-kit phosphorylation

To investigate the ability of sunitinib to inhibit c-kit kinase activity, the tyrosine phosphorylation of c-kit protein was monitored by IP/WB analysis. As shown in Figure 4, the expression of c-kit phosphotyrosine protein in the OVA mice was increased compared to that in the control mice. Sunitinib significantly reduced the protein level of phosphotyrosine c-kit.

DISCUSSION

Airway inflammation and remodeling are important pathophysiologic features of chronic asthma. The model

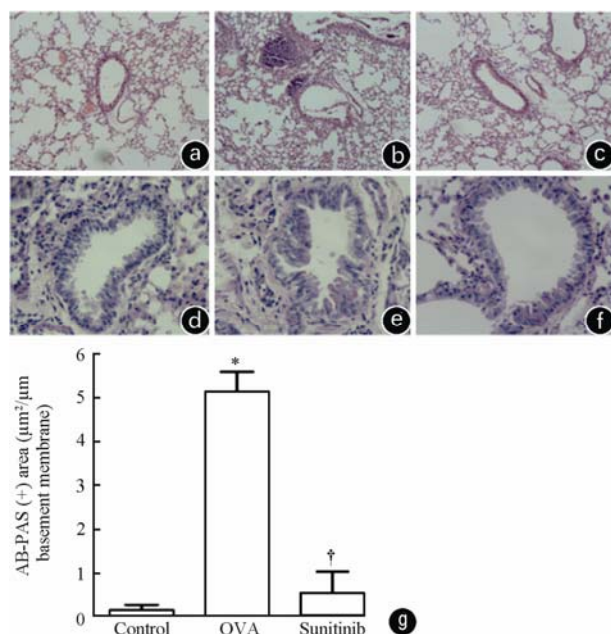


Figure 2. Sunitinib attenuates chronic airway inflammation and antigen induced mucus production in a murine model of chronic asthma. Hematoxylin and eosin staining (original magnification $\times 100$): (a) control group, (b) OVA group, (c) sunitinib group. Periodic acid Schiff (PAS) staining (original magnification $\times 200$): (d) control group, (e) OVA group, (f) sunitinib group. The area occupied by periodic acid stained mucin per micrometer length of bronchiolar basement membrane (g) was determined as described in the Methods section. Data are the mean $\pm$ SE ($n=6$ per group). * $P < 0.01$ compared with the control group. † $P < 0.05$ compared with the OVA group.

of chronic asthma used in the present study has been reported to reproduce many characteristic features of human asthma such as eosinophilic infiltration, goblet cell hyperplasia and basement membrane thickening.^{10,14,16} Here we demonstrate for the first time that sunitinib is effective in inhibiting eosinophilic airway inflammation and airway remodeling in parallel with a marked reduction of AHR in a murine model of chronic asthma.

It is clear that airway inflammation is a fundamental characteristic of asthma, and over time may lead to airway remodeling. In this study, administration of sunitinib daily significantly reduced the total inflammatory cell number in BALF. In addition, treatment with sunitinib also decreased the size and extent of leukocyte infiltration into the lung. Moreover, administration of sunitinib significantly reduced the number of mucus producing goblet cells within the bronchiolar epithelium. The observed reduction in pulmonary inflammation and mucus production might result from the concomitant reduction in BALF levels of Th2 type cytokines, IL-4 and IL-13.^{17,18} Eosinophils are implicated in airway remodeling by the release of cytokines and fibrogenic factors such as transforming growth factor (TGF)- β , vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP9) and IL-13.^{19,20} It is conceivable that sunitinib mediates its anti-remodeling effect at least partly through modulation of eosinophilic airway inflammation.

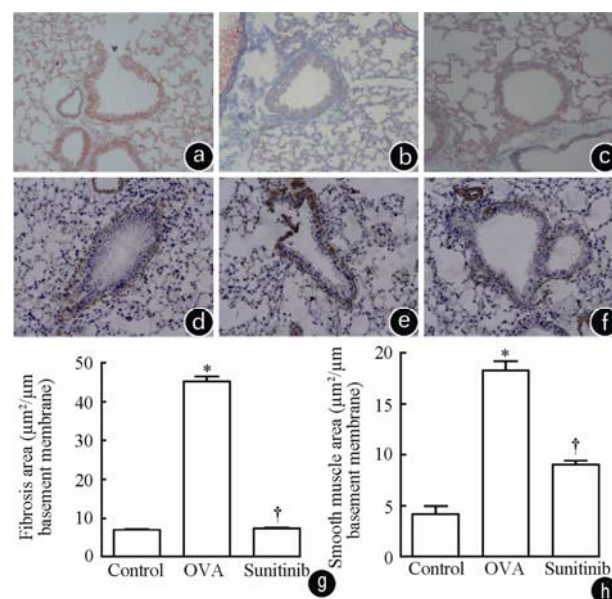


Figure 3. Sunitinib inhibits peribronchial fibrosis and areas of α -smooth muscle actin (SMA)-stained smooth muscle in chronic experimental asthma. Masson's trichrome-positive peribronchiolar collagen layer (original magnification $\times 200$): (a) control group, (b) OVA group, (c) sunitinib group. Peribronchial α -SMA immunostaining (immunohistochemistry, original magnification $\times 200$) of lung tissue from control (d), OVA (e) and sunitinib-treated mice (f). Mean area of airway fibrosis (g), and immunostaining for peribronchial α -SMA (h) were determined as described in the Methods section ($n=6$ per group). Data are the mean $\pm$ SE ($n=6$ per group). * $P < 0.01$ compared with the control group. † $P < 0.05$ compared with the OVA group.

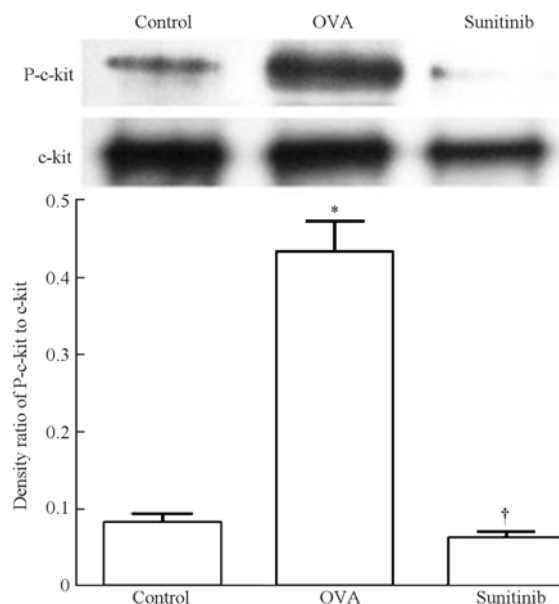


Figure 4. Sunitinib decreases the level of c-kit phosphorylation. Immunoprecipitation/Western blotting (IP/WB) analysis for phosphorylated c-kit (P-c-kit) and c-kit in the lungs of three group mice. Data are the mean $\pm$ SE ($n=6$ per group). * $P < 0.01$ compared with the control group. † $P < 0.01$ compared with the OVA group.

Both airway inflammation and airway remodeling can contribute to airway hyperreactivity. We observed a significant increase in AHR in the chronic model which

was associated with marked airway fibrosis and increased thickness of the peribronchial smooth muscle layer. Subepithelial fibrosis is a key feature of airway remodeling in association with the assessment of asthma severity. IL-4 and IL-13 are regarded as key mediators of tissue fibrosis in the pathophysiology of asthma. IL-4 and IL-13 are known to be able to influence matrix production and proliferation of fibroblasts mainly through the production of TGF- β from bronchial epithelial cells.²⁰⁻²² Moreover, Th2 cytokines IL-4 and IL-13 increase bronchial smooth muscle cell proliferation and potently induce the production of numerous cytokines that participate in airway inflammation and remodeling of asthma.²³⁻²⁵ Our results suggested that treatment with sunitinib significantly inhibited this fibrosis, and we found an about 80% decrease in the subepithelial collagen thickness in treated mice. In addition, administration of sunitinib also significantly decreased the smooth muscle area of bronchioles in α -SMA stained lung tissue sections. In this study, sunitinib treatment resulted in a significant reduction in BALF IL-13, which is thought to be critical in the establishment of AHR.^{18,26} Thus, the beneficial effect of sunitinib in AHR could also be derived from the observed reduction in pulmonary inflammation and airway remodeling.

Recently, significant increases in SCF and its c-kit receptor expression have been found in the epithelium and subepithelium of asthmatic patients.^{27,28} Inhibition of SCF attenuates peribronchial inflammation and remodeling in chronic experiment asthma,²⁹ suggesting an important role for SCF/c-kit pathway in the pathophysiology of chronic asthma. In the present study, the expression of c-kit protein was significantly increased in the remodeled airways. Treated with sunitinib inhibited the c-kit protein expression which may conceivably address its anti-inflammation and anti-remodeling actions.

In summary, this study demonstrates that treatment with sunitinib can prevent the development of the pulmonary eosinophilic inflammation, sustained AHR, and airway remodeling in a murine model of chronic asthma. In addition, sunitinib inhibits the levels of Th2-type cytokine IL-4 and IL-13 in BALF and the protein level of c-kit phosphorylation in the lung. Therefore, the use of sunitinib may be a promising approach towards a novel asthma therapy.

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