

Effect of Imatinib on Airway Smooth Muscle Thickening in a Murine Model of Chronic Asthma

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Key Words

Asthma • Imatinib • Remodeling • Smooth muscle •
Transforming growth factor- β 1 • Stem cell factor

Abstract

Background: Asthma is characterized by airway hyperresponsiveness (AHR), inflammation and remodeling. The tyrosine kinase inhibitor imatinib mesylate was developed to inhibit BCR-ABL kinase activity; however, it also has potent inhibitory activity against the c-Kit and platelet-derived growth factor receptors. The present study aimed to determine whether imatinib suppresses airway smooth muscle (ASM) remodeling and whether its effect is associated with growth factors such as transforming growth factor (TGF)- β 1 and stem cell factor (SCF). **Methods:** We developed a mouse model of airway remodeling, which includes smooth muscle thickening, in which ovalbumin (OVA)-sensitized mice were repeatedly exposed to intranasal OVA administration twice a week for 3 months. Mice were treated with imatinib during the OVA challenge. **Results:** Mice chronically exposed to OVA developed sustained eosinophilic airway inflammation and AHR compared with control mice. In addition, the mice chronically exposed to OVA developed features of airway re-

modeling, including thickening of the peribronchial smooth muscle layer. Administration of imatinib significantly inhibited the development of AHR, eosinophilic inflammation and, importantly, ASM remodeling in mice chronically exposed to OVA. Imatinib treatment significantly reduced the levels of interleukin-4, -5 and -13. In addition, TGF- β 1 and SCF were significantly reduced in the imatinib-treated animals. **Conclusions:** These results suggest that imatinib administration can prevent not only airway inflammation, but also airway remodeling associated with chronic allergen challenge. Imatinib may provide a clinically attractive therapy for chronic severe asthma.

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Introduction

Asthma is a chronic inflammatory disorder characterized by reversible airflow obstruction and airway hyperresponsiveness (AHR) [1]. Alterations in the structure of

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the airway wall, defined as airway remodeling, have also been recognized as regular features of asthma. The structural remodeling changes noted in the asthmatic airway include subepithelial fibrosis, increased smooth muscle mass, angiogenesis and an increase in the number of mucous glands. Due to these changes, it is considered difficult to treat chronic asthma patients [2]. Traditionally, airway smooth muscle (ASM) cells were seen as the main effector cells of airway narrowing. However, new evidence suggests that ASM cells are not only involved in airway narrowing, but also play a role in airway remodeling and inflammation in asthma [3]. ASM cells are also able to secrete immunomodulatory cytokines and chemokines [4–6] and to express surface receptors that are important for cell adhesion and leukocyte activation [7–9].

Transforming growth factor (TGF)- β 1 is a profibrotic cytokine thought to play an important role in promoting the structural changes of airway remodeling in asthma [10]. Stem cell factor (SCF), also known as c-Kit ligand, is a cytokine that induces mast cells to degranulate and has an important role in the maintenance of mast cell survival. It also induces eosinophil adhesion to extracellular matrix components and induces eosinophil activation and degranulation [11].

The tyrosine kinase inhibitor imatinib mesylate (STI571, Gleevec®/Glivec®, Novartis, Basel, Switzerland) was developed to inhibit BCR-ABL kinase activity. Imatinib has shown significant activity in patients with chronic myeloid leukemia (CML) [12, 13]. However, imatinib also has potent inhibitory activity against the c-Kit, discoidin domain and platelet-derived growth factor receptors [14, 15]. In addition to being an attractive and well-tolerated therapy for a number of cancerous diseases, such as Kit-driven gastrointestinal stromal tumors [16], imatinib has demonstrated efficacy in hypereosinophilic syndrome [17–19]. Recently, imatinib mesylate showed efficacy in the treatment of an asthma model [20, 21]. Treatment of mice with imatinib significantly attenuated airway hyperreactivity [20] and peribronchial allergen-induced fibrotic remodeling [21]. However, to date, no assessment of the effect of imatinib on smooth muscle remodeling in a murine model of chronic asthma has been conducted.

The present study aimed to determine whether imatinib suppresses ASM remodeling and whether its effect on ASM is associated with growth factors such as TGF- β 1 and SCF.

Material and Methods

Animals

Female BALB/c mice (n = 14–16 mice/group; Dae-Han Experimental Animal Center, Daejeon, Korea), 8–10 weeks of age were used. The animals were assigned to 1 of the following 4 treatment groups: (1) control; (2) ovalbumin (OVA) challenge; (3) OVA challenge plus 10 mg/kg imatinib, and (4) OVA challenge plus 200 mg/kg imatinib.

Sensitization and Antigen Challenge Protocol

Mice were immunized by subcutaneous injection on days 0, 7, 14 and 21 with 25 μ g of OVA (grade V; Sigma-Aldrich, St. Louis, Mo., USA) adsorbed to 1 mg of aluminum hydroxide (Aldrich, Milwaukee, Wisc., USA) in 200 μ l of phosphate-buffered saline (PBS). Intranasal OVA challenges (20 μ g/50 μ l in PBS) were administered on days 27, 29 and 31 under isoflurane (Vedco, St. Joseph, Mo., USA) anesthesia. Intranasal OVA challenges were then repeated twice a week for 3 months. Age- and sex-matched control mice were treated in the same way with PBS but without OVA. Mice were killed 24 h after the final OVA challenge, and bronchoalveolar lavage (BAL) fluid and lung tissues were obtained. All animal experimental protocols were approved by the Animal Subjects Committee of the Catholic University of Korea.

Administration of Imatinib

Imatinib was generously provided by Novartis Pharmaceuticals (Basel, Switzerland). Dry imatinib powder was dissolved in sterile normal saline. Allergen-sensitized mice were treated by oral gavage with 0.2 ml of saline containing imatinib (10 or 200 mg/kg). Imatinib was given once a day starting on day 35, and then during the OVA challenges for 3 months. The control mice were treated in the same way with normal saline.

Measurement of AHR

AHR was assessed 24 h after the final OVA inhalation using a single-chamber whole-body plethysmograph obtained from All-medicus (An Yang, Korea). The enhanced pause (Penh), a dimensionless value that correlates well with pulmonary resistance measured by conventional two-chamber plethysmography in ventilated mice, was used to monitor airway responsiveness. During plethysmography, mice were exposed for 3 min to nebulized PBS to establish baseline Penh values and were subsequently exposed to increasing concentrations of nebulized methacholine (Mch; 6–100 mg/ml; Sigma-Aldrich) in PBS using an Aerosonic ultrasonic nebulizer (DeVilbiss, Somerset, Pa., USA). Following each exposure, recordings were taken for 3 min. The Penh values measured during each 3-min sequence were averaged and are expressed for each Mch concentration.

Bronchoalveolar Lavage

Mice were killed by CO₂ asphyxiation after measuring airway responsiveness. The trachea was exposed and cannulated with silicone tubing attached to a 23-gauge needle on an 800- μ l tuberculin syringe. BAL fluid was withdrawn after instillation of 1 ml of sterile PBS through the trachea into the lung. The total number of cells in BAL fluid was counted using a hemacytometer. The BAL fluid was cytospun (7 min at 2,000 rpm) onto microscope slides and stained with Diff-Quick (Sysmax, Japan). The percentages of macrophages, eosinophils, lymphocytes and neutrophils

in the BAL fluid were obtained by counting 400 leukocytes on randomly selected areas of the slide using light microscopy. Supernatants were stored at -70°C .

ELISA

The concentrations of interleukin (IL)-4, -5, -10 and -13 as well as TGF- β 1 were measured in the BAL fluid with an ELISA kit (R&D Systems, Minneapolis, Minn., USA). The acid-activated latent form of TGF- β 1 was measured. The protocol followed was according to the manufacturer's instructions. The sensitivity of the ELISA was as follows: IL-4, 2 pg/ml; IL-5, 7 pg/ml; IL-10, 4 pg/ml; IL-13, 1.5 pg/ml, and TGF- β 1, 15.6 pg/ml.

Western Blot

To measure SCF expression, the separated lung tissues immediately frozen in liquid nitrogen were disrupted using a Polytron homogenizer (Tissue TearorTM, Biospec Products Inc., Bartlesville, Okla., USA) and centrifuged. The proteins were purified from the supernatant, and the concentration was assessed using the Bradford method. The protein samples were separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). After blocking with 10% skimmed milk (Difco, BD), the membrane was incubated with anti-SCF antibody (1:200, goat polyclonal; Santa Cruz Biotech, Santa Cruz, Calif., USA) overnight. The membrane was washed 3 times with PBS and incubated with secondary antibody for 2 h. Horseradish peroxidase-conjugated donkey-antigoat IgG (1:1,000; Santa Cruz Biotech) was used as the secondary antibody. The target protein was detected using the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech) and X-ray film.

Hydroxyproline Analysis

Sixty milligrams of lung tissue from each mouse was used for the hydroxyproline assay. A sample of lung homogenate was subsequently added to 250 μl of 12 N HCl for 16 h at 110°C . After centrifugation, 25 μl of each supernatant was assayed. To a 25- μl sample of the digested lung, 25 μl of citrate/acetate buffer (5% citric acid, 7.2% sodium acetate, 3.4% sodium hydroxide and 1.2% glacial acetic acid) and 500 μl of chloramine T solution (1.41 g of chloramine T, 26 ml of n-propanol, 20.7 ml of distilled water and 53.3 ml of citrate/acetate buffer) were added. The resulting samples were then incubated at room temperature for 20 min before 500 μl of Ehrlich's solution (4.5 g of *p*-dimethylaminobenzaldehyde, 18.6 ml of n-propanol and 7.8 ml of 70% perchloric acid) were added. These samples were incubated for 15 min at 65°C , and cooled samples were read at 550 nm in a spectrophotometer. Hydroxyproline concentrations were calculated from a standard curve of hydroxyproline.

Immunohistochemistry

Six-micrometer-thick sections of lung from each paraffin block were deparaffinized with xylene and hydrated in ethanol. For the immunohistochemical detection of α -smooth muscle actin, the lung sections were incubated overnight at 4°C either with a primary mouse monoclonal antibody directed against α -smooth muscle actin (Sigma-Aldrich) or, as a negative control, mouse serum instead of the primary antibody. Immunoreactivity was detected by sequential incubations of lung sections with a biotinylated secondary antibody, followed by peroxidase reagent and 3-amino-9-ethylcarbazole chromogen.

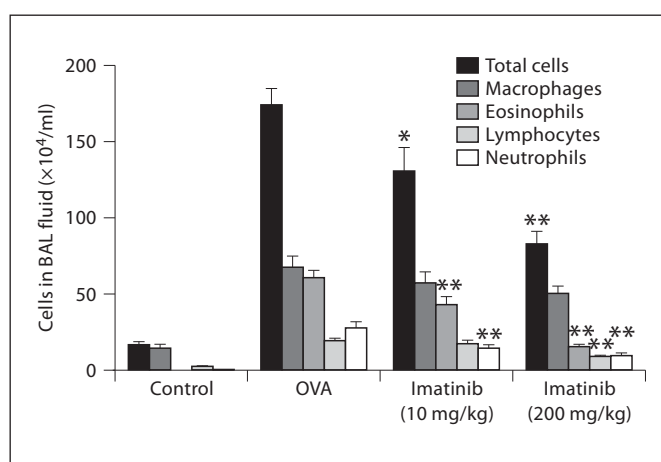


Fig. 1. Effect of imatinib on total and differential cell counts in BAL fluid. Mice were sacrificed 24 h after the final OVA challenge, and BAL cells were isolated. Values are expressed as means $\pm$ SEM ($n = 14$ – 16 /group). * $p < 0.05$, ** $p < 0.01$ compared with the OVA group.

Measurement of Smooth Muscle Area

The immunostained area of α -smooth muscle actin in each paraffin-embedded lung was outlined and quantified using a light microscope attached to an image analysis system (BX50, Olympus, Tokyo, Japan). Results are expressed as the area immunostained per micrometer length of basement membrane of bronchioles (internal diameter 650–750 μm). At least 10 bronchioles were counted in each slide.

Data Analysis

Results from each group were compared by ANOVA with the nonparametric Kruskal-Wallis test, followed by post hoc testing with Dunn's multiple comparison of means. A statistical software package (Graph Pad Prism, San Diego, Calif., USA) was used for the analysis. A p value of <0.05 was considered statistically significant. All results are given as means $\pm$ SEM.

Results

Effect of Imatinib on Airway Inflammation

Repetitive OVA challenge for 3 months in untreated mice induced a significant increase in the number of total cells and eosinophils in BAL fluid. Imatinib therapy in OVA-challenged mice significantly reduced the total number of cells, eosinophils and neutrophils in BAL fluid (fig. 1).

Effect of Imatinib on AHR

Mice in the OVA group showed bronchial hyperresponsiveness compared with the control group. Mice

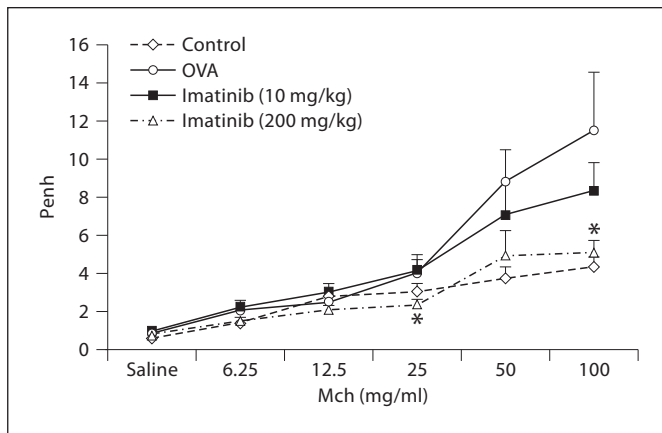


Fig. 2. Effect of imatinib on AHR to Mch. AHR was measured 24 h after the final OVA challenge using an Allmedicus system in which mice were exposed to increasing concentrations of Mch (6.25–100 mg/ml). Values are expressed as means $\pm$ SEM (n = 6–9/group). * p < 0.05 compared with the OVA group.

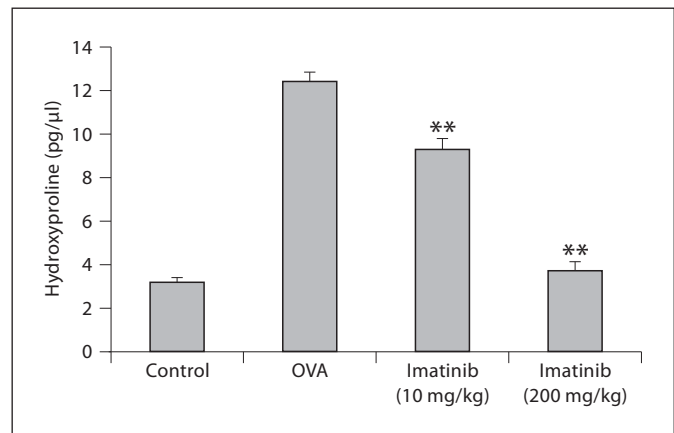


Fig. 3. Effect of imatinib on total lung collagen levels. Sixty milligrams of lung tissue was collected from each mouse for the hydroxyproline assay. Values are expressed as means $\pm$ SEM (n = 6–9/group). ** p < 0.01 compared with the OVA group.

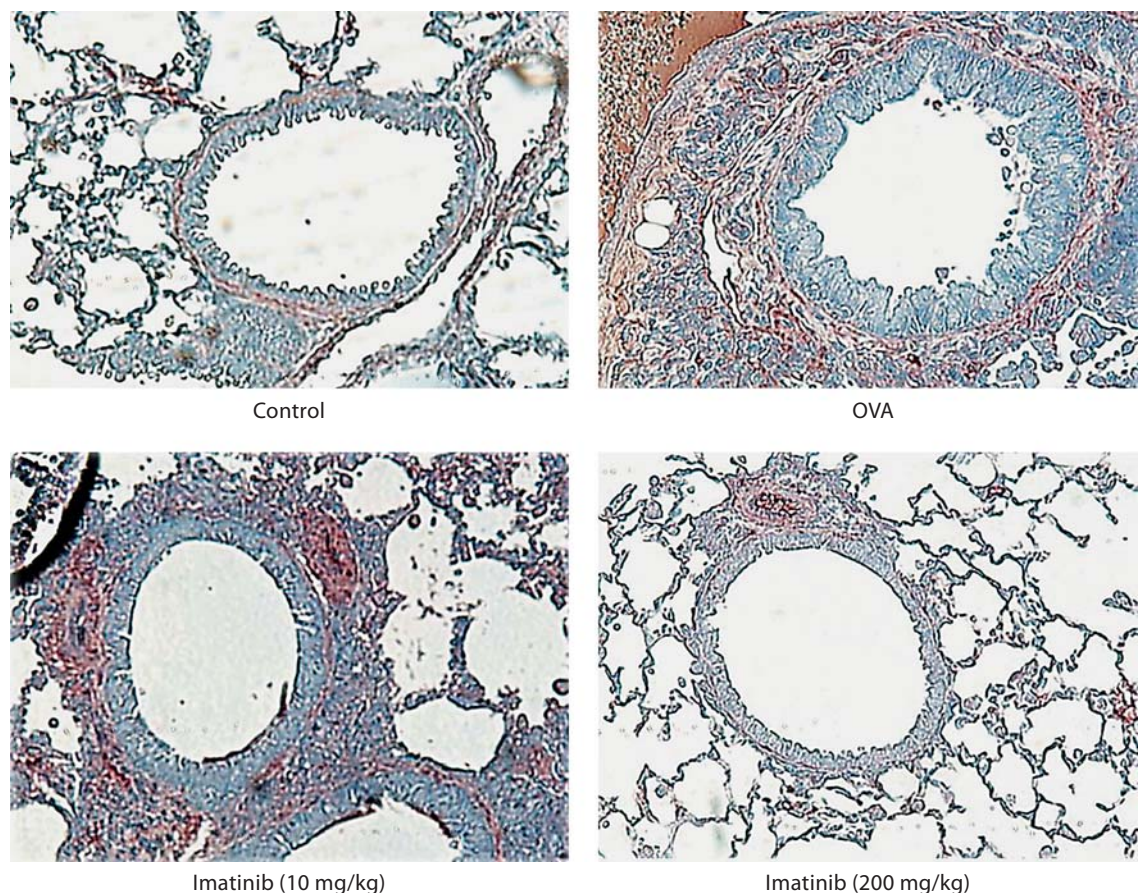


Fig. 4. Immunostaining of peribronchial smooth muscle. Control bronchi derived from mice not challenged with OVA exhibited peribronchial immunostaining (red). Repetitive OVA challenge for 3 months induced an increase in the immunostained area, which was inhibited by imatinib therapy.

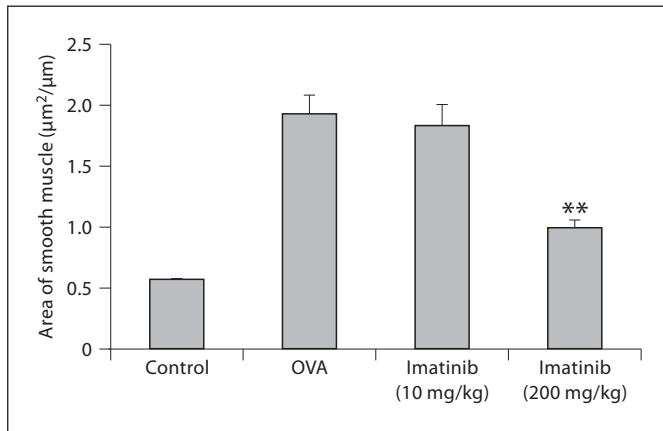


Fig. 5. Effect of imatinib on ASM area. Smooth muscle area is expressed as the immunostained area per micron length of basement membrane of the bronchioles using an image analyzer. Values are expressed as means $\pm$ SEM (n = 14–16/group). ** p < 0.01 compared with the OVA group.

treated with 200 mg/kg imatinib showed a decreased Penh value after receiving 6.25 mg/ml Mch compared with that in the OVA group (p < 0.05). This finding was also observed with 25 and 100 mg/ml Mch (fig. 2).

Effect of Imatinib on Collagen Levels

The total lung collagen levels were measured by hydroxyproline analysis. Repetitive OVA challenge for 3 months induced a significant increase in the level of hydroxyproline (OVA vs. control: 12.42 ± 0.42 vs. 3.15 ± 0.26 pg/ μ l; p < 0.01). Imatinib therapy significantly reduced the level of hydroxyproline in mice subjected to repetitive OVA challenge (10 mg/kg imatinib vs. OVA: 9.28 ± 0.50 vs. 12.42 ± 0.42 pg/ μ l, p < 0.01; 200 mg/kg imatinib vs. OVA: 3.68 ± 0.44 vs. 12.42 ± 0.42 pg/ μ l, p < 0.01; fig. 3).

Effect of Imatinib on the Area of the ASM Layer

The immunostaining area of peribronchial α -smooth muscle actin was quantified by imaging (fig. 4). Repetitive OVA challenge for 3 months induced a significant increase in the area of peribronchial α -smooth muscle actin immunostaining (OVA vs. control: 1.92 ± 0.16 vs. 0.56 ± 0.01 μ m²/ μ m; p < 0.01). Imatinib (200 mg/kg) therapy significantly reduced the area of peribronchial α -smooth muscle actin staining in mice subjected to repetitive OVA challenge (imatinib vs. OVA: 0.98 ± 0.07 vs. 1.92 ± 0.16 μ m²/ μ m; p < 0.01; fig. 5).

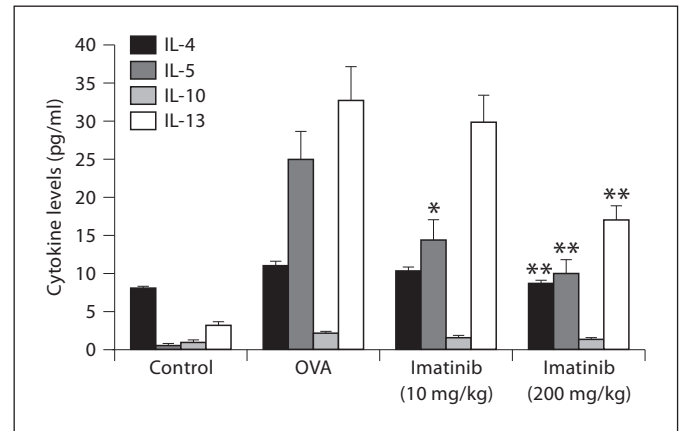


Fig. 6. Effect of imatinib on cytokine levels in BAL fluid. Values are expressed as means $\pm$ SEM (n = 14–16/group). * p < 0.05, ** p < 0.01 compared with the OVA group.

Effect of Imatinib on IL-4, -5, -10 and -13 Levels

Levels of IL-4, -5, -10 and -13 were significantly higher in the OVA group than in the control group. Imatinib treatment reduced the levels of IL-4, -5 and -13 in BAL fluid compared with the OVA group (p < 0.05). The level of IL-10 showed no significant difference between OVA and imatinib groups (fig. 6).

Effect of Imatinib on TGF- β 1 Level

The level of TGF- β 1 was significantly higher in the OVA group than in the control group (OVA vs. control: 445.59 ± 68.59 vs. 29.61 ± 7.78 pg/ml; p < 0.01). Imatinib (200 mg/kg) treatment significantly reduced the level of TGF- β 1 in BAL fluid compared with the OVA group (imatinib vs. OVA: 222.87 ± 33.31 vs. 445.59 ± 68.59 pg/ml; p < 0.01; fig. 7).

Effect of Imatinib on the Expression Level of SCF

SCF expression was significantly higher in the OVA group than in the control group (p < 0.01). Imatinib (200 mg/kg) treatment significantly reduced the level of SCF (p < 0.01; fig. 8).

Discussion

We confirmed that therapeutic administration of imatinib is able to reduce AHR and inflammation. We also demonstrated that imatinib administration can amelio-

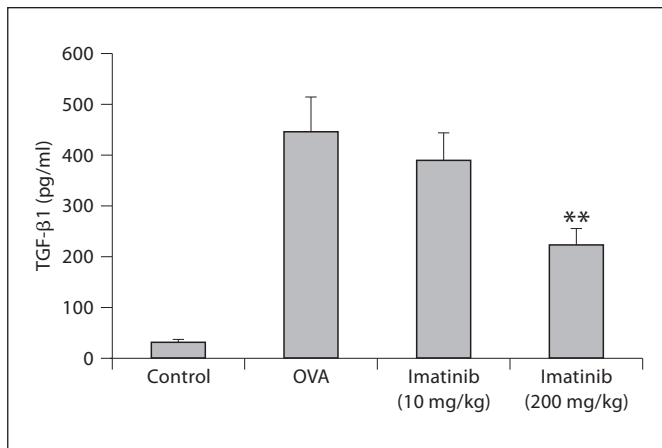


Fig. 7. Effect of imatinib on TGF-β1. Mice were sacrificed 24 h after the final OVA challenge. The TGF-β1 level in BAL fluid was measured with an ELISA. Values are expressed as means $\pm$ SEM (n = 14–16/group). ** p < 0.01 compared with the OVA group.

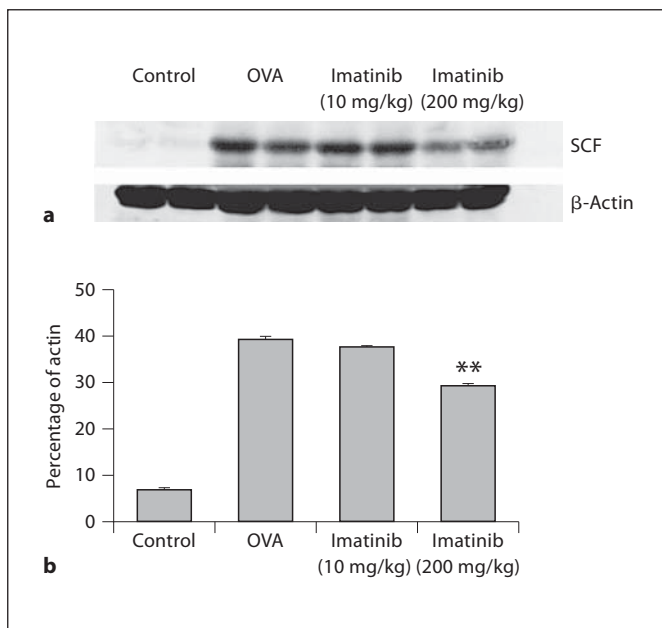


Fig. 8. Effect of imatinib on SCF expression in lung tissue. Repetitive OVA challenge for 3 months induced SCF expression, which was inhibited by imatinib (200 mg/kg) therapy. **a** Western blotting for SCF. **b** The SCF expression ratio. Values are expressed as means $\pm$ SEM (n = 4/group). ** p < 0.01 compared with the OVA group.

rate airway remodeling through inhibition of collagen deposition and smooth muscle layer thickening in asthma.

Airway remodeling is defined as changes in the composition, organization and quantity of cells and extracellular constituents of the airway wall [22]. Accumulated evidence strongly suggests that airway remodeling results in the progressive loss of lung function [23]. Airway remodeling is associated with poorer clinical outcome among patients with asthma. Prevention of airway remodeling has the potential to decrease disease severity, improve control and prevent disease expression [24]. Current asthma therapies, e.g. corticosteroids, are successful for treating allergic inflammation but do not specifically target the remodeling process. Remodeling changes progress despite optimal control of inflammation [25]. Recent data suggest that early use of inhaled glucocorticoids before the age of 2 in children with asthma has no effect on current asthma 8 years later [26], implying that the effects of corticosteroids on structural remodeling are minor. New treatments that attenuate not only inflammation but also remodeling are needed.

Contraction of ASM causes airway obstruction in asthma. However, the precise role of ASM in the pathogenesis of asthma is not fully understood. ASM cells are a source of a wide variety of inflammatory mediators [27, 28]. In addition to their secretory function, ASM cells also express many cell surface molecules, indicating that they may directly interact with immune cells or may have inherent immune functions of their own. Recent studies have shown that ASM also participates in the remodeling process [3], so targeting smooth muscle remodeling could be of great value for treating asthma.

Imatinib is a small-molecule tyrosine kinase inhibitor that binds competitively to the ATP-binding pocket of abelson kinase (c-Abl) with an attractive profile and also inhibits the c-Kit, discoidin domain and platelet-derived growth factor receptors. However, it is devoid of significant activity against most other kinases. Imatinib possesses favorable pharmacokinetic properties, as it is absorbed well after oral administration and must be taken only once daily [14]. Clinical experience in patients with CML demonstrates that imatinib is relatively well tolerated [29]. Imatinib shows efficacy in the treatment of gastrointestinal stromal tumors, whose molecular hallmark is mutation of the Kit gene [30]. This indicates that the utility of imatinib may extend well beyond CML.

TGF-β is a potent fibrotic factor whose expression is increased in asthmatic airways and that is a prime candidate for the initiation and persistence of airway remodel-

ing in asthma [10]. TGF- β stimulates fibroblasts to produce extracellular matrix proteins [31]. It has been shown that the subepithelial fibrosis component of airway remodeling in asthma is mediated through induction of TGF- β expression [32]. TGF- β also plays an important role in ASM proliferation. Xie et al. [33] showed that TGF- β 1 induces ASM hyperplasia through paracrine as well as potential autocrine pathways. It has been proposed that TGF- β 1 is responsible for the conversion of myofibroblasts into smooth muscle cells [10]. Many studies further substantiate this association by showing that therapeutic strategies that reduce or prevent TGF- β 1 overexpression/signaling lead to a parallel decrease in or prevention of ASM enlargement [34]. Some reports show that imatinib decreases the level of TGF- β . Treatment of rats with imatinib after bleomycin administration results in a strong reduction in TGF- β 1 gene expression [35]. Bizario et al. [36] showed that imatinib treatment reduces the levels of TGF- β cytokine and mRNA in *mdx* mice. In a murine lupus model, renal TGF- β expression was reduced by imatinib treatment [37]. Our results are compatible with those results. Imatinib effectively attenuated ASM hyperplasia, and an accompanying decrease in TGF- β 1 levels was observed in the imatinib group. This result suggests that imatinib attenuates ASM by blocking TGF- β 1.

SCF is also involved in many important functions in the pathogenesis of asthma. Lukacs et al. [38] showed that SCF plays a significant role in histamine release and eosinophil accumulation during allergic airway responses. Administration of SCF into the airways increases AHR. [39]. In murine asthma models, *in vivo* neutralization of SCF attenuates airway remodeling [21, 40]; however, few data are available about the effect of imatinib on SCF levels.

Our study is unique in that we have demonstrated the effect of imatinib on smooth muscle thickening in an asthma model. Because ASM cells are the most important cells involved in AHR and are involved in remodeling and inflammation [3], we propose that these cells should be targeted. How imatinib attenuates smooth muscle thickening in asthma is not yet known, but we suggest possible mechanisms. Firstly, imatinib could inhibit ASM remodeling via downregulation of the inflammatory reaction. Remodeling is thought to be the consequence of an aberration of the dynamic process of wound repair that includes matrix production and degradation, leading to tissue reconstruction [3]. The anti-inflammatory effect of imatinib has been documented in some reports [41–44]. In our experiment, imatinib effectively inhibited airway inflammation, which was demonstrated by the

BAL fluid analysis. This inhibition could lead to less secretion of growth factors and cytokines, less damage to epithelial cells and less remodeling. Secondly, imatinib could directly attenuate smooth muscle thickening by inhibiting platelet-derived growth factor receptor and c-Abl tyrosine kinase. Both pathways are involved in the growth of smooth muscle [45, 46] and are also major targets of imatinib. The blocking of these pathways by imatinib could result in attenuation of ASM remodeling.

Several groups have developed an allergen administration method that induces chronic airway inflammation and airway remodeling in mice [47–51]. Associated with the development of chronic eosinophilic inflammation, these repetitively challenged mice develop sustained AHR, increased collagen synthesis and ASM thickening. Compared with other asthma models, the pathological features of this chronic model may be more similar to the patient with asthma. Using this antigen sensitization model and airway challenge, we previously demonstrated the effects of corticosteroids and a peroxisome proliferator-activated receptor- γ agonist on asthma [52, 53].

The selectivity profile for a specific kinase inhibitor may differ depending on the therapeutic needs of the disease and the intended use of the drug. Thus, for chronic inflammatory diseases in which lifelong therapy may be required, a much greater degree of selectivity may be needed to achieve an acceptable therapeutic window. In asthma, for example, it would be necessary to inhibit the target kinase while not inhibiting off-target kinases, which may cause potentially debilitating adverse side effects [54]. To date, corticosteroids have been the major therapeutic agent in asthma. However, corticosteroids block broad pathways and thus have side effects. Imatinib may also block other unnecessary tyrosine kinase pathways, so it may not be a perfect specific therapeutic agent for asthma. However, given that imatinib is already used in clinics, is orally active and is efficacious, it may have clinical applicability [21].

The limitation of this study was the use of Penh to measure AHR. Penh can be influenced by any factor that alters the breathing pattern [55]. Although this includes changes in airway function, it can also include many other factors, including changes in respiratory drive [56, 57]. Thus, it may be unreasonable to state that we measured AHR in this study and that imatinib is effective in inhibiting AHR. Further imatinib studies using invasive plethysmography are needed to confirm the observations obtained with Penh. Another limitation is that this study is descriptive in character. Although we showed that imatinib attenuates airway remodeling with concomitant de-

creased levels of TGF- β 1 and SCF, no mechanistic data were provided. Further studies on the mechanism of imatinib are also needed.

In conclusion, this study has demonstrated that imatinib treatment reduced airway inflammation and smooth muscle remodeling in an asthma model. Our results provide evidence for the therapeutic potential of imatinib in the treatment of chronic severe asthma. Further investigations into the mechanisms through which imatinib inhibits ASM remodeling are needed.

Acknowledgments

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