Inhibition of SCF attenuates peribronchial remodeling in chronic cockroach allergen-induced asthma

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The progression and severity of chronic asthma likely depends upon the intensity of the damage and remodeling of the tissue. We have developed a chronic model of allergic asthma using multiple cockroach allergen challenges. Using this clinically relevant allergen we have established significant peribronchial fibrosis and mucus overproduction. These remodeling events are accompanied by intense peribronchial inflammation, including lymphocytes and eosinophils. A cytokine that has been identified as having a prominent role in short-term allergic events, stem cell factor (SCF), appears to have a significant role in this late-stage process. Using our polyclonal antibody specific for SCF administered into the airways of mice during the final allergen challenges, we find a significant effect on the chronic peribronchial allergen-induced fibrotic remodeling. This was characterized by reduced inflammation, especially eosinophils, as well as reduced hydroxyproline levels in anti-SCF compared to control antibody-treated animals. In addition, when we examined chemokines associated with the chronic disease and neutralized SCF *in vivo* we observed a corresponding decrease in CCL6 and CCL17. Using an inhibitor, imatinib mesylate, that blocks SCF/c-kit-associated RTK, we find similar results as with anti-SCF for attenuating AHR and fibrotic changes, suggesting that a potential clinical treatment for chronic asthma already exists related to this pathway. These results further support the potential use of SCF/c-kit inhibition for targeting chronic severe asthmatic responses.

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The severity of chronic asthma is governed by the intensity of the inflammation, airway remodeling, airway obstruction, and hyper-responsiveness.^{1,2} Peribronchial leukocyte accumulation is the hallmark of asthma, which affects a significant proportion of the worldwide population. In particular, eosinophils have been reported to be the primary cell associated with induction of bronchial mucosal injury, and are thought to participate in bronchial obstruction and airway hyper-reactivity.^{3–6} Several therapeutic strategies have focused on attenuating airway inflammation, including glucocorticoids and other agents that nonspecifically affect the response. A correlation can be drawn between the intensity of the leukocyte infiltration and the severity of the late-phase response. It is likely that continuous exacerbation and accumulation of eosinophils and other

leukocytes lead to many of the long-term effects of asthma including increasing severity and resistance to conventional therapy. Recent studies have suggested that these latter events correlate and may be attributed to airway remodeling leading to peribronchial thickening and fibrosis. The difficulty in treating any chronic disease, like asthma, will be the ability to attenuate established disease phenotypes.

Although studies have enhanced the understanding of several key mediators that are involved in the progression of asthmatic airway disease, it has been difficult to identify the activation pathways that are responsible for the generation of these factors. Previous publications have allowed our laboratory to identify that stem cell factor (SCF) may be functioning initially as a mast cell activation factor that exacerbates the response and alters the inflammatory cell recruitment during the later stages of the reaction.^{9–12} Importantly, data from a number of laboratories have indicated that SCF has a direct impact on the development of the detrimental pathophysiologic responses in the airway.^{8,13–17} Furthermore, other studies have suggested that by

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blocking SCF during allergen-induced responses an alteration of the severity can be achieved.^{10,18,19} It appears that SCF has an influence directly on the eosinophils. This is supported by data that demonstrated that SCF-stimulated eosinophils had enhanced binding capacity to VCAM-1 and fibronectin,²⁰ but also appears to initiate a degranulation response and induces pro-fibrotic mediators, leukotrienes and chemokines.²¹ Recent data has established increased SCF and c-kit in asthmatic airways²² and in nasal polyps,^{23,24} further supporting a role for SCF/c-kit activation pathways in allergic disease. However, to date no assessment of the role of SCF on chronic allergic disease characterized by severe AHR and peribronchial fibrosis has been performed.

In the present studies we have addressed the role of SCF in chronic allergic responses that lead to detrimental effects including airway inflammation and remodeling. In addition to demonstrating that we could block remodeling using a polyclonal antibody specific for SCF, we were also able to demonstrate that imatinib, which blocks RTK pathways including SCF/c-kit, was able to attenuate airway responses and remodeling in a similar manner as anti-SCF treatment. Altogether, these data begin to highlight our understanding of how a complex chronic response can be governed by an activating cytokine with pleuripotent activity.

Materials and methods

The Animal Model

Balb/c/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used at 6–8 weeks of age. The allergen we used was a clinical-grade skin-test allergen that has little endotoxin contamination (<20 ng/ml). Our chronic model has been set up as outlined below after careful analysis of a number of different protocols (Figure 1). After initial sensitization with cockroach allergen (CRA) on day 0 emulsified in incomplete Freund's adjuvant (IFA), anesthetized mice were given an initial intranasal (i.n.) administration on day 14 using ~1.5 µg of allergen. As mice are obligate nose breathers, the anesthetized animals do not swallow but inhale most of the allergen (we have verified this using Evans blue dye) into the lungs with little going into the esophagus and stomach. Subsequently, the animals are given four additional i.n. allergen challenges 4 days apart. The final two allergen challenges are then given by intratracheal (i.t.) injection by doing a tracheal 'cut-down' surgery and injecting the allergen directly into the airway.

We give a larger allergen challenge at these two time points (~6.0 µg/mouse) and the i.t. injection delivers the allergen directly into the airway. Control unsensitized mice were given the vehicle, saline, over the same period. In the single-challenge responses mice were given a similar systemic sensitization but only given one i.t. challenge of 6 µg using the direct instillation into the trachea as above. We analyzed the animals histologically and biochemically for changes in airway responses as listed below. This methodology provides a chronic and consistent exacerbating airway model that gives us a peribronchial fibrotic response, a difficult aspect to initiate in mice.

Measurement of airway hyper-reactivity. Airway hyper-reactivity was measured using a Buxco mouse plethysmograph which is specifically designed for the low tidal volumes (Buxco, Troy, NY) as previously described. Briefly, the mouse to be tested was anesthetized with sodium pentobarbital and intubated via cannulation of the trachea with an 18-gauge metal tube. The mouse was subsequently ventilated with a Harvard pump ventilator (tidal volume = 0.4 ml, frequency = 120 breaths/min, positive end-expiratory pressure 2.5–3.0 cm H₂O) and the tail vein was cannulated with a 27 G needle for injection of the methacholine challenge. The plethysmograph was sealed and readings monitored by computer. As the box is a closed system, a change in lung volume will be represented by a change in box pressure (P_{box}) that was measured by a differential transducer. Once baseline levels had stabilized and initial readings were taken, a methacholine challenge was given via the cannulated tail vein. After determining a dose–response curve (0.001–0.5 mg), an optimal dose was chosen, 0.250 mg of methacholine. This dose was used throughout the rest of the experiments in this study. After the methacholine challenge, the response was monitored and the peak airway resistance was recorded as a measure of airway hyper-reactivity.

In Vivo Neutralization of SCF

Neutralization of SCF was carried out using a polyclonal rabbit anti-murine SCF antibody developed in our laboratory.¹⁹ The protein A column purified anti-SCF or control antibody was administered i.t. with CRA at the time of the final two i.t. challenges. Paraffin-embedded lung sections were stained and the peribronchial eosinophil accumulation quantitated at the various time points post-challenge. Histological sections were also stained with Masson's trichrome green to visualize collagen deposition intensity.

Figure 1 Sensitization protocol for eliciting the chronic allergen-induced airway remodeling response.

Hydroxyproline Analysis

Total lung collagen levels were determined using a previously described assay.²⁵ Briefly, a sample of lung homogenate was subsequently added to 1 ml of 6 N HCl for 8 h at 120°C. To a 5- μ l sample of the digested lung, 5 μ l of citrate/acetate buffer (5% citric acid, 7.2% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 100 μ l of chloramine-T solution (282 mg chloramine-T, 2 ml of *n*-propanol, 2 ml of distilled water, and 16 ml of citrate/acetate buffer) were added. The resulting samples were then incubated at room temperature for 20 min before 100 μ l of Ehrlich's solution (Aldrich, Milwaukee, WI), were added. These samples were incubated for 15 min at 65°C, and cooled samples were read at 550 nm in a Beckman DU 640 spectrophotometer. Hydroxyproline concentrations were calculated from a standard curve of hydroxyproline (zero to 100 μ g/ml).

ELISAs

Whole lungs were homogenized in 1 ml of high-salt lysis buffer containing protease inhibitors. Debris-free supernatants were isolated and the chemokines measured by ELISA. Antibody pairs from R&D Systems were used for analysis in the ELISAs. The sensitivity of the analyses was ~ 10 pg/ml. No cross-reactivity to any other chemokine or cytokine was detected.

Enumeration of Pulmonary Mast Cells

Lung histology sections of upper airways were deparaffinized and stained for mast cells using Touludene Blue (1%) for 30 s and destained for 1 min with 70% ethanol. Histology slides were dried and coverslipped. The mast cell numbers were determined by counting 10 fields at $\times 200$ magnification around upper airways of the individual sections. The number of mast cells was averaged for each group and compared statistically.

Statistical Analysis

Data were evaluated by one-way analysis of variance and, where appropriate, further evaluated with the parametric Student–Newman–Keuls test for multiple comparisons or the nonparametric Mann–Whitney rank-sum test.

Results

Chronic Airway Cockroach Allergen Administration Induces Severe Peribronchial Remodeling

The model of CRA-induced disease employs multiple airway challenges in sensitized mice to drive an intense airway response that results in substantial

peribronchial inflammation and remodeling. This is depicted in Figure 2, where the BAL images demonstrate intense inflammation including both eosinophilic and mononuclear cell infiltration along with significant mucus-like protein staining (Figure 2a). In addition, using Masson's trichrome green stain we can observe significant peribronchial collagen deposition around the airways (Figure 2b). For comparison, we also examined the level of airway inflammation and remodeling in our single-allergen challenge model that we have previously used extensively to assess the role of cytokine and chemokine-mediated allergic inflammation (Figure 2). As illustrated compared to the single-challenge model in sensitized mice, the chronic challenge induces intense inflammation and significant remodeling responses. Especially prominent are the accumulation of eosinophils. Allergen challenge of naive unsensitized mice demonstrated no eosinophil influx and no mucus (data not shown).

Airway Neutralization of SCF Significantly Reduces Eosinophil Accumulation

The relationship of eosinophil numbers and disease severity is an often observed phenomenon clinically and their role in remodeling has been suggested.^{26–29} We have previously investigated the overproduction of SCF in allergen-sensitized animals after a single i.t. rechallenge of allergen.^{10,12,19,30} Interestingly, one of the clearest effects of the anti-SCF treatment was the reduction of eosinophils. In animals given anti-SCF (100 μ g/mouse) along with the final two airway allergen challenges in the present chronic model we were able to observe a significant reduction in peribronchial eosinophil numbers (Figure 3), reflecting what we have previously observed in single-allergen challenge models. We have also examined other leukocyte populations in the airway and find no significant decrease in either lymphocyte or neutrophil accumulation in chronic allergen challenged animals after anti-SCF compared to control antibody-treated animals (data not shown). We also enumerated mast cells around large airways of chronically challenged mice and found that anti-SCF antibody-treated animals had similar numbers of mast cells compared to control antibody-treated allergen challenged mice (51.6 ± 5.3 and 41.16 ± 4.0 mast cells/10 fields, respectively).

An assessment of the inflammatory mediators associated with accumulation of eosinophils was also assessed in this chronic remodeling response. In the anti-SCF treated animals there was a significant reduction of CCL6 and CCL17 (Figure 4), which have been previously associated with Th2 responses. Examination of other inflammatory chemokines, demonstrated little difference in the magnitude of the responses, including eotaxin (CCL11), RANTES (CCL5), and MIP-1 α (CCL3) (data not shown). This latter aspect of specific chemokine alteration indicates two important

Figure 2 Chronic cockroach allergen challenges leads to significant eosinophil-associated inflammation in the airway (a) as well as peribronchial remodeling (b) compared to a single allergen challenge. BAL and lung histology was analyzed at 24 h post-final allergen challenge and demonstrates intense airway inflammation and mucus production. The remodeling and collagen deposition around the airway was visualized using Masson's trichrome stain and was significantly increased relative to a single allergen challenged allergic animal.

issues: Firstly, the study identified two chemokines that may be closely associated with the chronic allergen responses and remodeling and secondly, chemokines that have been previously linked to eosinophil accumulation, such as eotaxin, were unaltered by the anti-SCF treatment.

Alteration of AHR and Peribronchial Remodeling by Blocking SCF

To further dissect this chronic allergen response we also examined the pathophysiology of the response related by measurement of airway hyper-reactivity (AHR) after a methacholine challenge (Figure 5). The induction of AHR was significantly more severe in the chronic allergen-treated animals than those given only a single-allergen challenge. The animals treated with chronic allergen along with anti-SCF at the time of allergen challenge displayed significant

attenuation in AHR. In addition to using anti-SCF, we also treated mice with an inhibitor of receptor tyrosine kinase associated with c-kit activation, imatinib, which is presently available clinically for use in CML. We have previously demonstrated that this treatment can attenuate AHR in the single-allergen challenge model. These animals treated with imatinib also displayed a significant reduction in AHR similar to that observed with anti-SCF (Figure 5). Thus, by blocking SCF in the airway or its receptor activation pathway we observed similar physiological changes that alleviated the severity of the allergen-induced responses.

As indicated above, the chronic exposure to allergen leads to the deposition of collagen around the airways and may directly impact on airway function. In order to quantitate this further, the lungs from chronically treated animals were removed and processed for hydroxyproline analysis.

Figure 3 Neutralization of SCF or treatment of allergic mice with anti-SCF reduces peribronchial eosinophils accumulation. Chronically sensitized and challenged mice were treated with anti-SCF (100 μ g/mouse) into the airway at the time of the last two airway challenges. Lungs were isolated and processed for histology 24 h after the final challenge. Peribronchial eosinophils were assessed by counting the number of eosinophils in 100 high-power fields (HPF) at $\times 1000$ magnification in five mice/treatment group. Data represents the mean $\pm$ s.e. from five mice/group.

Figure 4 Neutralization of SCF in the airway reduced CCL6 and CCL17 levels in the lungs of allergic mice. Whole-lung homogenates were made from mice 24 h after the final allergen challenge and assessed for chemokine levels using specific ELISAs. Control data were derived from naive animals. The data represents mean $\pm$ s.e. from five mice/group. * $P < 0.05$.

The results displayed in Figure 6 illustrate that when chronically allergen-challenged animals were treated with either the anti-SCF into the airway or with imatinib orally during the final two challenges, there was a significant decrease of hydroxyproline levels indicating that the total lung collagen production and deposition was significantly altered. Ex-

Figure 5 Airway Resistance changes in chronic allergen challenged mice was reduced by anti-SCF or imatinib treatment. Chronically sensitized and challenged mice were treated with anti-SCF (100 μ g/mouse) into the airway or with imatinib (5 mg/kg) orally at the time of the last two airway challenges. Airway hyper-reactivity was assessed in anesthetized animals ventilated by plethysmography using a single optimal dose of methacholine (250 μ g/kg) that elicits minimal increases in airway resistance in naive mice. Data represents mean $\pm$ s.e. from five mice/group. * $P < 0.05$ compared to control ab treated animals.

Figure 6 Hydroxyproline levels in allergic animals is reduced by neutralization of SCF or treatment with imatinib. Chronically sensitized and challenged mice were treated with control antibody (CRA control) or anti-SCF (100 μ g/mouse) into the airway or with imatinib (5 mg/kg) orally at the time of the last two airway challenges. The left lobe of the animals was harvest and assayed for hydroxyproline analysis. Hydroxyproline levels in naive mice range between 15 and 20 μ g in the left lobe. Data represents mean $\pm$ s.e. from five mice/group. * $P < 0.05$ compared to control.

amination of specifically stain tissue sections using Masson's trichrome green indicated a significant decrease in peribronchial remodeling and collagen deposition in animals treated with anti-SCF as well as those treated with imatinib (Figure 7). As previously described,³⁰ it appeared histologically that there was also a significant reduction in mucus accumulation in the airways of both anti-SCF and

Figure 7 Histological examination of Masson's Trichrome stain demonstrates a reduction in peribronchial remodeling and collagen deposition as well as inflammatory cells in anti-SCF and imatinib-treated animals. The right lobe of allergen challenged mice used in Figure 6 to assess hydroxyproline levels were processed for histology and specifically stained for collagen. The pictures are representative of five mice/group.

imatinib-treated animals. Thus, by blocking SCF in the airway for a relatively short period at the end of a chronic allergen challenge, the peribronchial remodeling was significantly reduced.

Discussion

The manifestations of chronic allergic responses in airways can be destructive to lung function. It is

perhaps the restructuring of the airway that becomes the most remarkable and devastating aspect in this chronic disease. These progressively altering responses can be directly linked to the intensity and nature of the inflammation. The combination of Th2 cytokines and eosinophilic inflammatory events both contribute to the responses elicited in the airway during allergic asthma.^{2,5,31,32} Other mediators that are traditionally associated with fibrosis, such as TGF- β , have been difficult to target. Thus,

identification of novel mediators may provide new opportunities to locally target these inflammatory events. In the present studies we have targeted local SCF production in an attempt to interrupt an activation pathway that may have multiple effects. Previous data have demonstrated that local SCF production within the airway has effects on immediate mast cell activation, alters cytokine and chemokine production, and is associated with mucus overexpression.^{9,12,18,30} The results in these studies indicate that by blocking SCF locally during established disease there was an attenuation of the remodeling response around the airways using a chronic allergen model. The ability to deliver an inhibitor into the airway for only a local effect has great appeal for therapeutic intervention to avoid alterations of systemic functions. This latter issue could be especially important as SCF/c-kit has a central role in multiple hematopoietic maturation pathways in bone marrow-derived cells.^{7,8,33}

Blocking SCF within the airway may have multiple effects on this inflammatory system. It has long been known that SCF significantly impacts on mast cell biology and can activate the cells for disease altering effects. Although we observed no alteration in mast cell numbers using a relatively short-term treatment, there may be a reduction in local mast cell numbers if SCF was blocked locally long-term and therefore provide a further benefit. In addition, the activation of other leukocyte populations has also been documented. Especially relevant for the allergic response is the identification of SCF-mediated eosinophil activation. SCF/c-kit-mediated activation of eosinophils has demonstrated that SCF increases the VLA-4-associated adhesion of eosinophils and therefore increased migration to sites of inflammation.²⁰ These original studies were extended by demonstrating that SCF more broadly activates eosinophils for degranulation, mediator release, as well as cytokine and growth factor production, including TGF- β and β -FGF.²¹ This general activation pattern indicates the potential role SCF may play in the activation of local fibroblast and structural cell populations for the remodeling of the airway. In addition, the decrease in key inflammatory mediators such as CCL17 when SCF is blocked might reduce the recruitment and activation of Th2 type lymphocytes via CCR4.^{34–38} It is interesting that only CCL6 and CCL17 are altered and other chemokines were not changed by anti-SCF and might relate to the fact that CCL6 and CCL17 can be induced from eosinophils stimulated with SCF (Kowalski *et al*²¹ and unpublished data) or from other cell populations activated by Th2 cytokines. In support of the latter mechanism are previous results demonstrating that by blocking SCF or inhibiting its production a significant attenuation in local Th2 cytokines can be observed.^{18,30} Thus, there may be both direct and indirect effects of SCF on this chronic Th2-type response.

In addition to blocking SCF in the airway, the feasibility to use an inhibitor of the SCF/c-kit activation pathway was verified using imatinib mesylate, a RTK inhibitor that blocks c-kit activation pathways.³⁹ This inhibitor may also block other RTK pathways, including PDGFR, and therefore is not as specific as the anti-SCF treatment.^{40,41} However, given the fact that imatinib is already used in the clinics, is orally active, and demonstrated a similar effect as anti-SCF, it may have clinical applicability. Several studies have demonstrated the utility of imatinib for other inflammatory diseases including hypereosinophilic syndrome and mastocytosis.^{42–48} We have previously demonstrated the use of imatinib in our short-term single-challenge model to prevent allergen-induced AHR and reduce inflammation.⁴⁹ The present data further support the possibility of using compounds such as imatinib for chronic asthma and inhibiting the remodeling effects of allergic responses. These results are striking.

Over the past several years a number of cell populations have been identified as sources of SCF, including fibroblasts, smooth muscle cells, epithelial cells, macrophages and eosinophils. In addition, the identification of SCF as a transmembrane protein released by enzymatic cleavage during inflammation allows a more complex biology that suggests that significant levels of cytokine may be present before an inflammatory response. A soluble form of SCF is readily released from alveolar macrophages stimulated by either TNF or IL-4,¹⁹ while fibroblast and epithelial cells express a predominant membrane-associated form that is also induced by IL-4 and needs enzymatic cleavage for release (unpublished data). Interestingly, mast cells and eosinophils, potential targets of SCF activation, can also produce and release soluble SCF.^{50,51} Thus, SCF may function to activate cells both through its membrane form during cell-to-cell interactions as well as via its soluble form. The idea that SCF may promote a more fibrotic environment can be gained from studies that have examined expression of SCF in interstitial lung disease and crescentic glomerulonephritis.^{52,53} The present concept is that structural cells, such as epithelial cells and fibroblasts, express SCF and during disease it promotes the local activation of mast cells and/or eosinophils to further exacerbate the production and release of inflammatory and pro-fibrotic factors. The Th2 environment that is established during allergic responses would increase the expression of SCF by the structural and inflammatory cells and further amplify the process. Overall, it may be that by blocking local SCF, a number of interacting inflammatory and differentiation events may be blocked that significantly reduce the progression of chronic allergic disease that leads to a remodeled airway. In the end, the reduction of local SCF levels or blockade of its RTK may serve to attenuate chronic allergic asthma.

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