

# The expression of stem cell factor and c-kit receptor in human asthmatic airways

S. Z. Al-Muhsen\*†, G. Shablovsky\*, R. Olivenstein\*‡, B. Mazer\*† and Q. Hamid\*‡

\*Meakins-Christie Laboratories, †Montreal Children Hospital and ‡The Montreal Chest Research Institute, Royal Victoria Hospital, McGill University, Montreal, Quebec, Canada

## Summary

**Background** Asthmatic airways are characterized by infiltration with a variety of inflammatory cells such as mast cells and eosinophils. Stem cell factor (SCF) is an important activating and chemotactic factor for both mast cells and eosinophils. In addition, it is a critical growth and differentiation factor for mast cells.

**Objectives** To investigate the contribution of SCF to the pathogenesis of asthma, we examined the expression of SCF and its receptor c-kit in bronchial biopsies and bronchoalveolar lavage (BAL) specimens obtained from asthmatic subjects ( $n = 13$ ) and non-asthmatic control subjects ( $n = 10$ ).

**Methods** SCF and c-kit were detected by *in situ* hybridization (ISH) and immunocytochemistry (ICC). In order to phenotype the cells expressing SCF and c-kit in asthmatic tissue and BAL cells, combined ISH and ICC were also performed.

**Results** There was a significant difference ( $P < 0.001$ ) in the SCF mRNA expression in asthmatic airway epithelium ( $70.38 \pm 12.33\%$  positive cells) compared with controls ( $12.7 \pm 17.21\%$  positive cells). There was also a significant difference in subepithelial SCF-mRNA expression, being higher in asthmatics ( $P < 0.001$ ). A significant difference was also found in c-kit receptor mRNA expression in asthmatic biopsies both in epithelium ( $P < 0.001$ ) and subepithelium ( $P < 0.05$ ) compared with controls. ICC results were consistent with the ISH for both SCF and c-kit receptor from asthmatics and controls. The SCF and c-kit receptor mRNA and immunoreactivity in cells recovered from bronchial washing were also significantly higher in asthmatics compared with controls ( $P < 0.05$ ). While SCF expression was localized predominantly in the epithelial layer in bronchial biopsy tissues, alveolar macrophages were found to be the major source of SCF in bronchial washing from asthmatic subjects.

**Conclusion** The results of this study demonstrate the increased expression of SCF and its receptor, c-kit within human asthmatic airways, which suggests an important role of this cytokine in the pathophysiology of asthma.

**Keywords** asthma, atopy, c-kit, eosinophils, mast cells, SCF

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## Introduction

Asthma is a chronic inflammatory disease characterized by episodic airway obstruction, bronchial hyper-responsiveness, and infiltration of the airways by activated inflammatory cells, particularly eosinophils, T lymphocytes, and mast cells [1–3]. Mast cells appear to play an important role in the orchestration of asthmatic airway inflammation [4]. Cross-linking of allergen-specific IgE bound to high-affinity IgE receptors on mast cells leads to cell activation and release of potent mediators. These mediators are involved in the widely accepted role of mast cells in the early asthmatic response [5, 6]. In addition, mast cells appear to be involved in disease progression and perpetuation of the late asthmatic response [7, 8]. This important role of mast cells is achieved by virtue of

sustained synthesis and release of cytokines (e.g. IL-4, IL-5, IL-13), and growth factors [9–11]. Mast cells could also be involved in inflammation in asthmatic airway, through the release of a number of chemotactic factors (e.g. regulated upon activation, normal T cells expressed and secreted, monocyte chemoattractant protein-1) that have the ability to recruit inflammatory cells such as eosinophils and lymphocytes [12].

Stem cell factor (SCF), also called mast cell growth factor, or kit ligand, is a multi-potent growth factor involved in the early stages of haematopoiesis [13, 14]. It is produced *in vitro* by a variety of cells, but mainly fibroblasts [15], epithelial cells [16], endothelial cells [17] and by mast cells themselves [18]. SCF protein is expressed in two forms: soluble (sSCF), and membrane bound (mSCF), and the relative importance of either form during haematopoiesis or disease is not known [19, 20]. As indicated by its name, SCF is a critical growth and differentiation factor for human mast cells [21, 22]. It is also essential for their activation, adhesion and chemotaxis to the site of inflammation [23, 24]. In addition, it prevents their

Correspondence: Dr Qutayba Hamid, Meakins-Christie Laboratories, McGill University, 3626 St Urbain Street, Montreal, Quebec, Canada H2X 2P2. E-mail: Hamid@meakins.lan.mcgill.ca

apoptosis [25]. SCF was shown to induce mast cell degranulation directly, and via enhancing antigen-induced (human lung-derived) mast cell degranulation [26, 27]. Furthermore, SCF is necessary for IL-4 expression by activated human lung mast cells [28]. It also has been shown to enhance eosinophil adhesion, which further supports its role in the pathophysiology of asthma [29]. Recent data in mice demonstrated that SCF levels are high in allergic asthma [30]. In addition, intra-tracheal administration of SCF in allergic and normal mice induced airway hyper-responsiveness (AHR), which corresponds to mast cell activation [31].

SCF expression has been described in allergic rhinitis and nasal polyposis [32, 33]. However, to date the expression of SCF and its receptor, c-kit, has not been described in the human asthmatic airways. Therefore, in the current study, we examined SCF and c-kit mRNA expression and immunoreactivity in human-allergic asthmatic tissue compared with control subjects. In addition, we studied their cellular origin and localization within the airways.

## Materials and methods

### Subjects

Thirteen patients fulfilling the American Thoracic Society criteria for asthma [34], having documented airways reversibility to inhaled  $\beta_2$ -agonists and increased airways responsiveness to methacholine ( $PC_{20}$ ), were recruited from the asthma clinic at the Montreal Chest Institute, McGill University. These asthmatic patients were classified as mild to moderate, and had their symptoms controlled by regular usage of inhaled selective  $\beta_2$ -agonists. The subjects' clinical characteristics are given in Table 1. Subjects were non- or ex-smokers who had stopped smoking for at least 12 months and had smoked less than 5 pack-years in their lifetime. Subjects using inhaled or oral corticosteroids, non-steroidal anti-inflammatory medications (cromolyn, ketotifen), theophylline, long-acting  $\beta_2$ -agonists, leukotriene antagonists, or antihistamines within 3 months prior to the study were excluded. None of the subjects had a history of respiratory tract infection within the previous 6 weeks, or immunotherapy within the previous 12 months. As control subjects, 10 non-asthmatic individuals volunteered to participate in the study. None were current smokers, or had taken corticosteroids in the year preceding the study. Within the study groups, none had any evidence of other pulmonary disease.

### Study design

The study was approved by the Ethics Committee of the Montreal Chest Hospital and all subjects gave written informed consent.

Patients and normal controls were screened by a questionnaire, spirometry, and a methacholine inhalation test (if forced expiratory volume in 1 s ( $FEV_1$ ) > 70% predicted) [35]. Allergy prick skin tests were performed and atopy was defined as a > 3 mm skin weal response to one or more of 13 common allergens. Blood was drawn for complete and differential blood count, total serum IgE and coagulation studies. Endoscopic bronchial wash and bronchial biopsies were obtained from each subject.

**Fibreoptic bronchoscopy, bronchial biopsies and bronchial wash fluid processing** Fibreoptic bronchoscopy was performed according to recommended guidelines. Bronchial washings were performed by gently wedging the bronchoscope in the right middle lobe, rapidly instilling 50 mL of saline into a single subsegment and then aspirating a mean volume of 30 mL. The bronchial wash fluid was transferred into sterile polypropylene tubes and centrifuged at 300 g for 7 min at 4 °C. The cell pellet was then suspended in RPMI 1640 at a concentration of  $1 \times 10^6$  cells/mL and cytopsin preparations of the bronchial wash (100  $\mu$ L aliquots) were made. The cells were cytopsin onto either poly-L-lysine-coated (for *in situ* hybridization (ISH)) or untreated slides (for immunocytochemistry (ICC)). For ISH, the cytopsin were fixed in 4% freshly prepared paraformaldehyde, washed twice in diethyl pyrocarbonate-treated phosphate-buffered saline (PBS), baked overnight at 37 °C and stored at -80 °C until further use. For ICC, the cytopsin were briefly fixed in a solution of acetone : methanol (40 : 60), air dried and stored at -80 °C.

After fixation in paraformaldehyde, the bronchial biopsies were washed in 15% PBS-sucrose and blocked in optimal cutting temperature media using isopentane cooled to -70 °C in liquid nitrogen. The tissues were subsequently sectioned (10  $\mu$ m thick), then baked overnight at 37 °C and stored at -80 °C until further use.

**In situ hybridization (ISH)** To detect SCF and *c-kit* mRNA in cytopsin bronchial biopsies and bronchial wash fluid cells, digoxigenin complementary riboprobes (cRNA antisense) were used. The cDNA sequences for SCF *c-kit*, were inserted into pGEM vectors, grown in *Escherichia coli* and linearized with the appropriate enzymes. Prior to ISH, *in vitro*

**Table 1.** Clinical and demographic characteristics of asthmatics and normal control subjects

	Asthmatic patients	Normal subjects
Number of subjects	13	10
Age in years (mean, range)	(29.9, 19–60)	(31.8, 31–54)
Sex (males : females)	7 : 6	7 : 3
Total IgE in kU/mL (median, range)	(97.2, <32.7–4240)	(32.7–230)
Allergen prick skin test (positive : negative)	(12 : 1)	(6 : 4)
Eosinophils in ( $10^6$ /L) (mean, range)	(300, 71–570)	(137, 54–400)
$FEV_1$ (% predicted) (mean, range)	(92, 70–128)	(101, 77–121)
$PC_{20}$ (mean, range)	(1.9, 0.3–6.0)	(15.2, 8.2 to > 16)

$FEV_1$ , forced expiratory volume in 1 s.

transcription to generate sense and antisense probes was performed in the presence of DIG-UTP and the appropriate T7 or SP6 polymerases as previously reported [36]. There were no significant hybridization signals observed when sections were treated with RNase prior to hybridization, nor when sections were treated with a sense probe identical to the cytokine receptor mRNA. Cells positive for SCF and c-kit immunoreactivity were detected with a chromogen.

**Immunocytochemistry (ICC)** To detect the phenotype of inflammatory cells recovered by bronchial wash and bronchial biopsies, we used specific immunohistochemical markers for eosinophils (major basic protein (MBP)), macrophages (CD68), mast cells (tryptase), and antibodies for SCF and c-kit. ICC was performed using a modified alkaline phosphate anti-alkaline phosphatase (APAAP) method which we have previously reported [37]. A negative control slide using the appropriately diluted isotype control was included in each ICC experiment.

**Combined ICC and ISH** To phenotype the cells expressing SCF and c-kit receptor mRNA in cytopins from bronchial wash fluid and bronchial biopsies, the technique of combined ICC/ISH was used as previously described [38]. Briefly, cytopsin preparations on poly-L-lysine coated slides were immunostained with monoclonal antibodies to CD68 (macrophages), tryptase (mast cells) and MBP (eosinophils). Following visualization of the positive immunostaining using the APAAP technique, the cytopins underwent ISH for SCF and c-kit receptor mRNA.

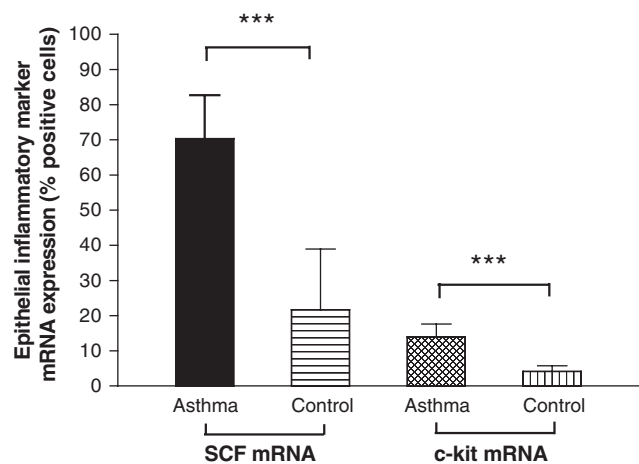
**Data analysis** The slides were coded and counted blindly using  $\times 100$  magnification with an eyepiece graticule. Two to six fields per section were counted depending on the size of the biopsy and the patterns of the alignment covering an intact area of epithelial and subepithelial tissue. The results from bronchial biopsies are expressed as both the percentage of mRNA- or immunoreactive-positive cells within the epithelial cell layer, and the mean number of positive cells in the subepithelium per millimetre of basement membrane of the airway. A minimum of 1000 bronchial wash cells were counted and the number of positive cells presented as a percentage of the total cells. For the co-localization studies, the results were expressed as a percentage of the SCF and c-kit receptor mRNA positive cells which co-expressed CD68, tryptase, or MBP. Graphic and statistical analysis was performed with GraphPad Prism and InStat softwares (GraphPad Software Inc, San Diego, CA, USA). All data are reported as means  $\pm$  SD. Significance testing was carried out by use of the non-parametric Mann-Whitney *U*-test. A *P*-value of less than 0.05 was considered significant.

## Results

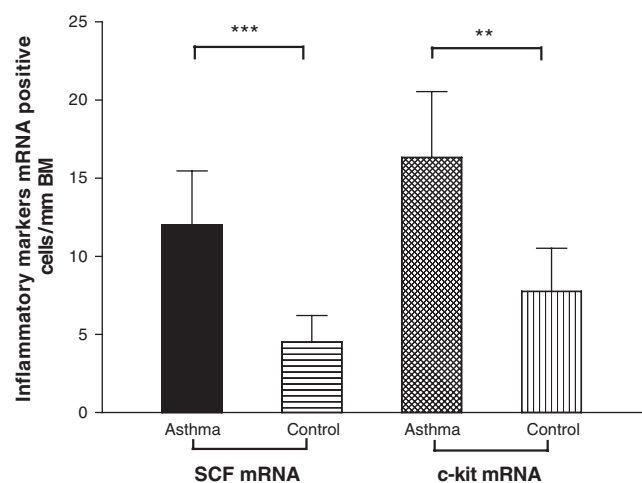
### SCF and c-kit mRNA and immunoreactivity in bronchial biopsies

In bronchial biopsies from asthmatic and control individuals, cells positive for SCF mRNA were localized to the airway epithelium, as well as the subepithelial cell layer. Within the epithelial cell layer, there was a significant increase in the percentage of cells expressing SCF mRNA in asthmatic subjects compared with normal controls (SCF mRNA-

positive cells; asthmatics,  $70.38 \pm 12.33\%$ ; controls,  $12.7 \pm 17.21\%$ ;  $P < 0.001$ ; Fig. 1). Similarly, there was a significant increase in the numbers of SCF mRNA-positive cells in the subepithelial layer when asthmatics were compared with normal controls (SCF mRNA-positive cells per millimetre of basement membrane; asthmatics,  $12 \pm 3.46$ ; normal controls,  $4.5 \pm 1.7$ ;  $P < 0.001$ ; Fig. 2). c-kit receptor mRNA-positive cells were also significantly elevated in the epithelial cell layer of bronchial biopsies from asthmatics compared with normal control individuals (c-kit receptor mRNA-positive cells; asthmatics,  $13.88 \pm 3.72$ ; controls,  $4.13 \pm 1.64$ ;  $P < 0.001$ ; Fig. 1). Similarly, there was a significant increase of c-kit receptor mRNA-positive cells in the subepithelial layer of specimen from asthmatic subjects when



**Fig. 1.** Stem cell factor (SCF) and c-kit receptor mRNA expression in bronchial epithelium in allergic asthmatics and control subjects. Significantly higher percent of epithelial cells are positive for SCF mRNA in asthmatic subjects compared with normal control ( $***P < 0.001$ ). Similar statistically significant difference in the percentage of epithelial cells positive for SCF receptor, c-kit mRNA, in asthmatics compared with control ( $***P < 0.001$ ). Vertical bars represent the mean  $\pm$  SD.



**Fig. 2.** Expression of stem cell factor (SCF) and c-kit receptor mRNA in subepithelium in allergic asthmatics and control subjects. Numbers of cells expressing SCF mRNA per millimetre basement membrane are significantly higher in asthmatic compared with normal controls ( $***P < 0.001$ ). Similarly, there was significant statistical difference in the number of subepithelial cells expressing the SCF receptor, c-kit mRNA, per millimetre basement membrane in asthmatics compared with controls ( $**P < 0.05$ ). Vertical bars represent the mean  $\pm$  SD.

compared with normal controls (c-kit receptor mRNA-positive cells per millimetre of basement membrane; asthmatics,  $16.33 \pm 4.21$ ; normal controls,  $7.75 \pm 2.76$ ;  $P < 0.05$ ; Fig. 2). ICC data were consistent with the ISH results for both SCF and c-kit immunoreactivity in bronchial biopsies of asthmatics and control subjects. Figure 3 demonstrates a representative example of SCF and c-kit immunostaining in bronchial biopsies and bronchial wash samples from asthmatic subjects and controls.

#### SCF and c-kit receptor mRNA and immunoreactivity in cells recovered by bronchial wash

Cells recovered from bronchial washing fluid were examined in asthmatic and control subjects. There were greater numbers of SCF mRNA and SCF-immunoreactive cells recovered from the bronchial wash fluid of asthmatic individuals than from normal controls (SCF mRNA-positive cells per 1000 cells; asthmatics,  $54.37 \pm 17.41$ ; controls,  $15.13 \pm 4.49$ ;  $P < 0.001$ ; Fig. 4; SCF-immunoreactive cells/1000 cells; asthmatics,  $75.0 \pm 27.12$ ; controls,  $18.75 \pm 8.54$ ;  $P < 0.05$ ; Fig. 4). Similarly, c-kit receptor mRNA-positive cells and c-kit-immunoreactive cells were significantly greater in bronchial wash fluid from asthmatics compared with normal controls (c-kit receptor mRNA-positive cells per 1000 cells; asthmatics,  $46.88 \pm 21.03$ ; controls,  $16.0 \pm 4.50$ ;  $P < 0.05$ ; Fig. 4; c-kit receptor-immunoreactive cells per 1000 cells; asthmatics,  $60.88 \pm 23.40$ ; controls,  $24 \pm 9.09$ ;  $P < 0.05$ ; Fig. 4).

#### Inflammatory cell numbers in bronchial biopsies

The bronchial biopsies of the asthmatic subjects exhibited significantly greater number of MBP-positive cells compared with the normal controls [MBP-positive cells per millimetre basement membrane (BM); asthmatics,  $12.70 \pm 3.56$ ; controls,  $0.87 \pm 0.83$ ;  $P < 0.001$ ], while tryptase-positive cells did not significantly differ between these groups (tryptase-positive cells per millimetre BM; asthmatics,  $10.4 \pm 1.5$ ; controls,  $8.13 \pm 2.64$ ;  $P < 0.08$ ). There was a strong correlation between c-kit expression and MBP-positive cells ( $r = 0.72$ ).

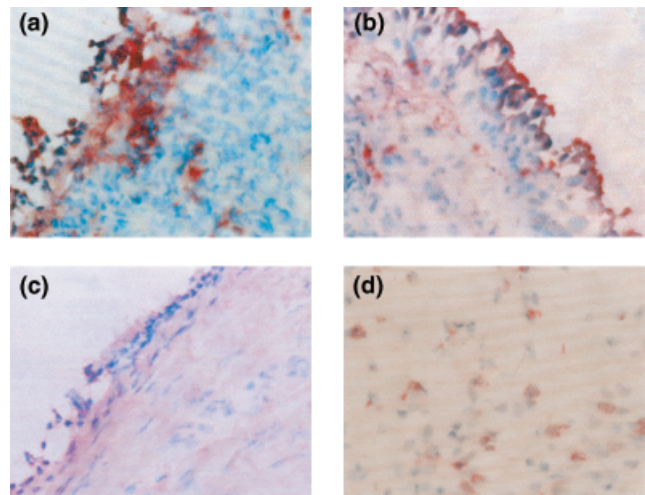
#### Co-localization of SCF to monocytes/macrophages

Using double ICC with anti-SCF and anti-CD68 antibodies, the SCF-immunoreactivity was co-localized preferentially to alveolar macrophages in the bronchial washings.

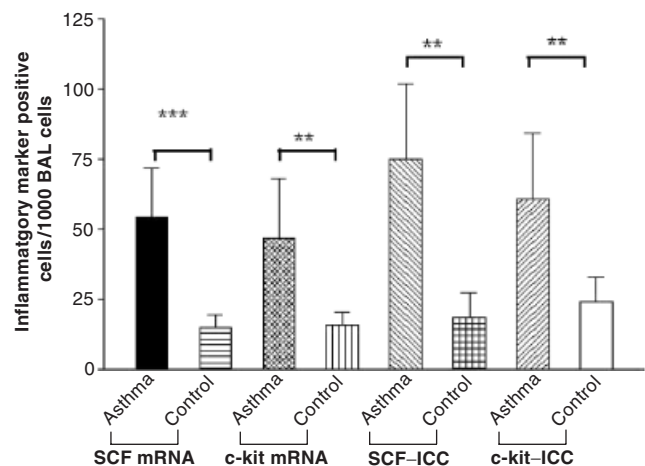
We examined the percentage of SCF+c-kit receptor expressing cells in the individual subjects to determine if these parameters would correlate with asthma severity. There was no correlation between FEV<sub>1</sub> and PC<sub>20</sub> and the percentage of cells expressing SCF or c-kit mRNA or protein (data not shown).

## Discussion

The *in vivo* importance of SCF in the pathophysiology of asthma has been suggested mainly by animal models of airway inflammation, and there have been no detailed studies published to date examining the expression of SCF or its



**Fig. 3.** Immunocytochemistry for stem cell factor (SCF) (a) and c-kit (b) in bronchial biopsies from asthmatic individuals, showing immunoreactivity localized primarily to the epithelium. It is also noted to be present in a number of inflammatory cells in the subepithelial tissue. (c) A representative example of SCF immunostaining from a normal control subject. (d) SCF immunostaining in the bronchial wash fluid of an asthmatic subject.



**Fig. 4.** Stem cell factor (SCF) and its receptor mRNA expression and immunoreactivity in the bronchial washings from allergic asthmatics and normal controls. Number of SCF mRNA-positive cells per 1000 cells is significantly higher in asthmatics compared with normal controls ( $***P < 0.001$ ). Increased number of c-kit mRNA-positive cells per 1000 cells are found in allergic asthmatic compared with normal subjects ( $**P < 0.05$ ). Consistently, the SCF and c-kit immunoreactive cells recovered from the bronchial wash are significantly higher in asthmatics compared with controls ( $**P < 0.05$ ). Vertical bars represent the mean  $\pm$  SD.

receptor c-kit in human asthmatic airways. In this study, we investigated the *in vivo* expression of SCF and its receptor, *c-kit*, in human bronchial tissue obtained from atopic asthmatic patients and control subjects. Using ISH and ICC (staining techniques), our results provide direct evidence that SCF is expressed within human airways. Its expression, however, was found to be up-regulated significantly in bronchial biopsy specimens and bronchial wash fluid obtained from subjects with asthma compared with those from controls. SCF mRNA was predominantly localized to the airway epithelium and was associated with macrophages within the submucosa. We also demonstrated that the number of cells expressing mRNA

for the SCF receptor, c-kit, was increased within the bronchial mucosa of asthmatic individuals compared with controls. Our experiments extend previous preliminary observations in human and animal models of an association between SCF and supports the concept that SCF may be a critical factor involved in the pathogenesis of atopic asthma.

In a mouse model of AHR, expression of SCF was significantly increased after allergen challenge, compared with vehicle challenge. Inversed expression of SCF was found in alveolar macrophages and bronchial epithelium [39]. In similar studies, allergen challenge elicited an increase in SCF production in both serum and lung, which correlated with an increase in AHR and mast cell activation [30, 40]. Treatment of mice with neutralizing anti-SCF antibody during allergen challenge inhibited mast cell activation, AHR and bronchial inflammation [30, 31, 39]. Bronchial alveolar lavage fluid taken from atopic asthmatic subjects has been shown to activate mast cells *in vitro*. The additions of a neutralizing antibody against SCF abrogate mast cell activation [40]. Glucocorticoids were shown to decrease SCF production and mast cell numbers, an effect reversed by administration of SCF [41, 42]. Recent data have demonstrated that local delivery of SCF antisense oligonucleotides in a murine asthma model was as effective as steroids in suppressing lung inflammation, eosinophils infiltration, and IL-4 production [43].

SCF is a critical factor for the growth, survival, differentiation, and activation of mast cells. SCF primes mast cells to respond to FcεRI cross-linking for augmented exocytosis [26] and cytokine production [28]. SCF can act directly on mast cells to induce histamine release [44]. Furthermore, it is essential for adhesion and chemotaxis of mast cells. Therefore, increased expression of SCF may contribute to the increased mast cell activation in allergic asthma, augmenting the response to allergen and facilitating persistent mast cell activation.

Using the technique of ISH and ICC, we localized the expression of SCF to airway epithelium. The percentages of epithelial cells positive for SCF mRNA were significantly higher in asthmatics compared with control subjects. Airway epithelial cells are an important source of a variety of cytokines, as well as chemotactic and growth factors, including SCF [45]. Our data support the evidence for the contribution of epithelial cells to asthma pathophysiology.

In addition, SCF was expressed in the subepithelium of bronchial biopsies taken from asthmatic subjects. Using the technique of double ICC, we found that the macrophages are the major source of SCF in the bronchial wash from asthmatic patients. Since a higher number of macrophages were shown to express SCF in asthmatics as compared with normal controls in our study, it is possible that activated macrophages produce SCF in higher amounts.

SCF exerts its multiple effects through interaction with its surface receptor, c-kit, a member of the receptor tyrosine kinase family [46]. Previous studies identified c-kit receptor protein on eosinophils from asthmatic subjects and normal controls [29, 47]. SCF was shown to activate the adhesion of eosinophils to fibronectin and vascular cell adhesion molecule *in vitro*. In addition, SCF was shown to be a potent chemotactic factor attracting eosinophils to the site of allergic inflammation [29]. More recent data demonstrated a role for

SCF in eosinophil development, chemotaxis, activation, and mediator release [48, 49].

Our data demonstrated an increased expression of c-kit (SCF receptor) in allergic asthmatics as compared with normal controls. Furthermore, the expression of c-kit strongly correlated with the eosinophils numbers in bronchial biopsies (Fig. 4). The finding of significant suppression of eosinophilic infiltration, histamine release and AHR in response to local administration of SCF neutralizing antibody, or SCF-antisense oligonucleotides, suggests that inhibition of SCF may be a promising novel therapeutic approach in asthma [39, 50, 43].

In conclusion, we have demonstrated a significantly higher expression of SCF and c-kit receptor in human airways from atopic asthmatic subjects in bronchial biopsy tissue and bronchial wash fluid. These data support the significant role of SCF in pathogenesis of asthma. To gain further insight into that role, it is important to elucidate which SCF transcript(s), either sSCF or mSCF, predominate in the tissue from allergic asthmatics. It may also be of interest to explore the effect of blocking SCF as a potential therapeutic option.

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