

Local administration of antisense phosphorothioate oligonucleotides to the c-kit ligand, stem cell factor, suppresses airway inflammation and IL-4 production in a murine model of asthma

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Background: The c-kit ligand, stem cell factor (SCF), is an important activating and chemotactic factor for both mast cells and eosinophils. These cells are known to play a fundamental role in the pathogenesis of asthma.

Objective: Our goal was to analyze the functional role of SCF in the pathogenesis of asthma.

Methods: The expression of SCF was targeted in fibroblasts, epithelial cells, and locally in a murine model of asthma in mice induced by ovalbumin sensitization with an antisense DNA strategy.

Results: We could suppress SCF expression in NIH 3T3 fibroblasts and SP1 epithelial cells by a specific antisense phosphorothioate oligonucleotide overlapping the translation start site of SCF, whereas control oligonucleotides were virtually inactive. We then focused on the role of SCF in a murine model of asthma associated with late-phase allergic inflammation in ovalbumin-sensitized mice: Local intranasal administration of FITC-labeled SCF antisense oligonucleotides led to strong DNA uptake in interstitial lung cells associated with a striking reduction of intracellular SCF expression. Such intrapulmonary blockade of SCF expression after repeated allergen challenges suppressed various signs of lung inflammation including IL-4 production and infiltration of eosinophils. SCF antisense DNA treatment was at least as effective as corticosteroid treatment.

Conclusion: These data indicate a critical role for SCF in a murine asthma model and suggest that local delivery of SCF antisense oligonucleotides may be a novel approach for the treatment of inflammatory lung disorders such as asthma. (*J Allergy Clin Immunol* 2001;107:279-86.)

Key words: Stem cell factor, mast cells, antisense DNA, asthma, T_H2 cytokines

Mast cells and eosinophils appear to play a fundamental role in the pathogenesis of the asthmatic reaction by producing mediators that promote airway inflammation.^{1,2} For instance, allergen-induced activation of mast cells by IgE-receptor cross-linking leads to histamine release, oxygenation of arachidonic acid by 5-lipoxygenase, and generation of leukotrienes such as LTB₄ in the airways.^{3,4} Such mast cell-derived bronchoconstrictors appear to contribute to the early bronchospastic response in asthma occurring within minutes after allergen challenge.^{5,6} Furthermore, mast cells are believed to participate in several aspects of disease progression through the production of cytokines (eg, IL-4, IL-5), angiogenic and fibrotic factors, and the release of chemokines (eg, RANTES),⁷⁻⁹ thereby favoring infiltration of the airway tissues by eosinophils and lymphocytes.⁹⁻¹³

The growth, differentiation, and activation of connective tissue mast cells is believed to be mainly regulated in the microenvironment by the c-kit ligand, stem cell factor (SCF), which is produced by resident cells such as fibroblasts.¹⁴⁻¹⁹ Withdrawal of SCF from SCF-dependent mast cell cultures in vitro results in apoptosis. Furthermore, administration of recombinant SCF in vivo leads to local and systemic mast cell hyperplasia in both mice and human beings.²⁰⁻²³ In addition, we have reported that the decrease in mast cell number after application of glucocorticoids is associated with a decrease in SCF production,²⁴ suggesting that these agents may at least partially mediate their effects by suppressing SCF activities. This hypothesis is supported by recent studies on the effects of corticosteroids in patients with allergic rhinitis.²⁵

In further extension of the above studies, we hypothesized that direct targeting of SCF production and its subsequent downregulation would inhibit activation of mast cells and eosinophils and the consecutive development of asthmatic inflammation. To explore this hypothesis, we examined the effects of specific SCF blockade in a mouse model of asthma induced by ovalbumin (OVA) sensitization.²⁶

METHODS

Materials

The following reagents were used: Dulbecco modified Eagle medium, RPMI 1640 medium, glutamine, penicillin, and streptomycin (Gibco Laboratories, Grand Island, NY); FBS (Bioproducts

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M. F. N. supported by grants from the Innovationsstiftung Rheinland-Pfalz, the DFG (Ne 490/1-1, Ne 490/2-1), and the Gerhard Hess program of the DFG (Ne 490/3-1).

Received for publication September 19, 2000; revised November 15, 2000; accepted for publication November 15, 2000.

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0091-6749/2001 \$35.00 + 0 1/83/113049

doi:10.1067/mai.2001.113049

Abbreviations used

BAL: Bronchoalveolar lavage
OVA: Ovalbumin
SCF: Stem cell factor

for Science Inc, Indianapolis, Ind); water-soluble dexamethasone (Sigma Chemical Co, St Louis, Mo); crystalline OVA (Pierce Chemical Co, Rockford, Ill); aluminum potassium sulfate (Sigma Chemical Co); polyclonal rabbit anti-mouse SCF (Genzyme Corp, Cambridge, Mass); biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, Calif); streptavidin-conjugated Cy3 (Dianova, Hamburg, Germany); and phosphorothioate oligonucleotides (Roth GmbH, Karlsruhe, Germany).

Cell lines and culture conditions

NIH 3T3 fibroblasts were purchased from American Tissue Culture Collection (Rockville, Md). Fibroblasts were maintained in Dulbecco's modified Eagle medium in 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 50 µg/mL streptomycin. Murine epidermal SP1 keratinocytes, a papilloma-derived cell line, were kindly provided by Prof J. Schweizer (Division of Tumor Cell Regulation, German Cancer Research Center, Heidelberg, Germany). SP1 cells were maintained in 4× minimal essential medium, as previously described.²⁷

Phosphorothioate oligonucleotides

Oligonucleotides were synthesized with a phosphorothioate backbone to improve resistance to endonucleases. The antisense phosphorothioate oligonucleotides consisted of 18-mer analogues to the 5' end of the murine SCF sequence (gene bank accession No. M59915), which spans the translation initiation site. This sequence is highly evolutionarily conserved and identical between mice, rats (gene bank accession No. M59966), and human beings (gene bank accession No. M59964). In addition, several control (mismatched and nonsense) oligonucleotides were prepared. The nonsense oligonucleotides consisted of 18-mers containing the same nucleotide composition as the antisense oligonucleotides. None of the oligonucleotides had a C plus G dinucleotide or a quadruple G sequence element, which are known to cause unspecific effects.^{28,29}

Phosphorothioate oligonucleotides were used in indicated concentrations for coinubation studies with NIH 3T3 and SP1 cells. In addition, antisense phosphorothioate oligonucleotides were used for local administration in mice with late-phase asthmatic responses in vivo, as specified below. The sequences of phosphorothioate oligonucleotides were as listed in Table I.

Coinubation of NIH 3T3 fibroblasts and SP1 epithelial cells with phosphorothioate oligonucleotides

Cells were cultured on coverslips (diameter 12 mm) in 24-well plates in triplicate for each condition as described.²⁴ In brief, 2 × 10⁴ fibroblasts and epithelial cells were grown to a subconfluent state for 24 hours. Cells were then washed twice in PBS and incubated in serum-free medium. For coinubation experiments, phosphorothioate oligonucleotides were incubated for 45 minutes at room temperature with Lipofectamine reagent (Life Technologies, Gibco BRL, Germany) in serum-free medium according to the instructions provided by the manufacturer to improve the cellular uptake of the DNA. Oligonucleotides were then added to the cell cultures at a final concentration of 4 to 12 µmol/L. After coinubation for 24 hours at 37°C, fresh growth medium with 20% FBS was added to the cell culture, and the cells were analyzed after an additional 24 hours for SCF expression. In some experiments, cells were

TABLE I. SCF translation start site (M59964)

CGCGTGCCTT TCCTT	ATGAA GAAGACACAA ACTTG-GATTA
SCF antisense DNA	5'-TGT CTT CTT CAT AAG GAA-3'
SCF nonsense DNA	5'-GTG TAC AGA TTA CTC ATT-3'
SCF mismatched DNA	5'-TGT CTG CTC TAT AAT GAA-3'

Mismatches are *underlined*; SCF translation starts in *boldface*.

treated with dexamethasone (10⁻⁵, 10⁻⁶, and 10⁻⁷ mol/L) instead of antisense DNA for 72 hours, and dexamethasone was replaced at 24 and 48 hours.²⁴

SCF protein detection and localization of FITC-labeled antisense oligonucleotides in fibroblasts and keratinocytes by immunofluorescence

NIH 3T3 and SP1 cells were coincubated with FITC-labeled antisense phosphorothioate oligonucleotides (MWG and Firma Roth, Karlsruhe, Germany). FITC-positive cells were then analyzed by direct immunofluorescence at an excitation wavelength of 490 nm. To determine the efficacy of DNA uptake, we counted FITC-positive cells in 3 randomly selected high-power fields (magnification × 400) on 3 coverslips per cell line.

To determine SCF expression, we fixed cells in 4% paraformaldehyde.²⁴ Coverslips were incubated with rabbit anti-mouse SCF (10 µg/mL) in PBS/0.1% BSA/0.1% Triton X-100. Controls for nonspecific staining were incubated without the primary antibody. Coverslips were incubated with biotinylated goat antirabbit (1:100 dilution; Vector Laboratories) IgG antibody for 1 hour at room temperature. Binding was detected with streptavidin-conjugated Cy3. Coverslips were analyzed with an Olympus UMCB/AX70 microscope for fluorescence.

Allergen sensitization and rechallenge of Balb/c mice

Four to 5 mice per group and experiment were sensitized to OVA and rechallenged with OVA as described.²⁶ In brief, female Balb/c mice (6-8 weeks of age) received an intraperitoneal injection of 100 µg OVA (0.2 mL OVA solution; 500 µg/mL in normal saline solution) complexed with aluminum potassium sulfate on days 0 and 14. On days 14, 25, 26, and 27, the mice were anesthetized with 0.2 mL 2.5% Avertin (1 g/mL of tribromoethanol in tertiary amylalcohol) in PBS injected intraperitoneally before receiving 50 µL of either 100 µg OVA in saline solution (day 14) or 50 µg OVA in saline solution (days 25, 26, and 27) intranasally. Control animals received saline solution with aluminum potassium sulfate intraperitoneally on days 0 and 14 and 50 µL of saline solution intranasally on days 25, 26, and 27 (Table II).

Treatment of OVA-sensitized mice with antisense oligonucleotides and dexamethasone

Mice were given phosphorothioate oligonucleotides by intranasal administration (370 µg) at days 25, 26, and 27 (Table II). To localize antisense oligonucleotides in vivo, we performed intranasal injection of 370 µg FITC-labeled antisense phosphorothioate oligonucleotides in saline solution on day 27 in 2 to 3 mice per experiment. To compare the efficacy of SCF antisense treatment to classic treatment of asthmatic reactions with glucocorticoids, we gave a group of OVA-treated mice 1 mg of water-soluble dexamethasone in 50 µL saline solution intranasally on days 25, 26, and 27, 30 minutes before the OVA injection.

TABLE II. Time points of the administration of OVA, saline solution, and antisense DNA

	Day		
	0	14	25-27
OVA group (50 µg)	IP OVA	IP and IN OVA	IN OVA
Saline	IP saline	IP and IN saline	IN saline
AS-SCF, MM-SCF, or Dex	IP OVA	IP and IN OVA (100 µg)	IN OVA + IN AS-SCF, MM-SCF, or Dex

IP, Intraperitoneal; IN, intranasal; AS-SCF, SCF antisense DNA; MM-SCF, SCF-mismatched DNA; Dex, dexamethasone.

Protein extraction and Western blot analysis

Lung tissue was taken at day 28 and homogenized in the presence of protease inhibitors (Aprotinin, Sigma Chemical Co) to obtain lung proteins. Protein concentrations were determined by spectrophotometry with the Bio-Rad (Hercules, Calif) protein assay. Next, 50 µg of protein was separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. After the membrane had been incubated in blocking solution, 3 µg/mL of antimouse SCF polyclonal antibodies was added, and incubation was performed overnight at 4°C. The next day the membrane was incubated in peroxidase-conjugated antirabbit IgG for 1 hour at room temperature. Finally, enzyme activity was detected with the ECL Western Blotting Detection System (Amersham, Heidelberg, Germany).

Bronchoalveolar lavage and ELISA for IFN-γ and IL-4

Twenty-four hours after the last intranasal challenge with either OVA or saline solution at day 27, bronchoalveolar lavage (BAL) of the right lung was performed after the left lung was tied off at the mainstem bronchus. All BAL fluid (1 mL of saline solution 3 times) was collected, and BAL fluid cells were counted with a 100-µL aliquot. Accordingly, samples were centrifuged at 1200 rpm for 5 minutes at 4°C, and the resulting cell pellets were resuspended in PBS. Cytospins were made by centrifugation at 450 rpm for 5 minutes at room temperature. Eosinophils were then detected by staining of cytopins according to May Grünwald–Giemsa methods. Cytospins were analyzed with an Olympus UMCB/AX70 microscope at 400×. Results are reported as the average percentage of eosinophils ± SEM.

To determine cytokine concentrations, we centrifuged samples at 1200 rpm for 5 minutes at 4°C. Supernatants were analyzed by specific ELISA for content of IFN-γ and IL-4 with commercially available kit systems (Pharmingen, San Diego, Calif). The sensitivity of the ELISA was 5 pg/mL.

Lung histology

Twenty-four hours after the last intranasal challenge with either OVA or saline solution at day 27, the lungs were analyzed by histology. The right lung was removed and frozen in liquid nitrogen for subsequent protein extraction. The left lung was cut in half. One half was fixed overnight in Carnoy's solution at 4°C and then embedded in paraffin. Twelve sections of each lung in 5 mice per treatment group were then stained with toluidine blue (0.5%; Sigma Chemical Co) at pH 0.5, as previously described.³⁰ The second half was immediately frozen in liquid nitrogen and subsequently used for immunocytochemical and histochemical studies.

Immunohistochemistry

Ten-micrometer-thick cryosections from each lung were cut, fixed in 4% paraformaldehyde for 10 minutes, washed in PBS, and stained for SCF as described above. Under these experimental conditions, we could simultaneously localize cells containing FITC-labeled antisense oligonucleotides and SCF protein.

Statistical analyses

Differences were evaluated for significance by the Kruskal-Wallis test (program: Statview 4.5 for Macintosh). All data are reported as the mean values ± SEM.

RESULTS

Suppression of SCF expression in fibroblasts and keratinocytes by a specific antisense phosphorothioate oligonucleotide

In an effort to understand the functional role of SCF in asthma, we sought to directly target SCF expression using an antisense DNA approach. We observed in coin-cubation studies a strong uptake of FITC-labeled antisense oligonucleotides in the cytoplasm and to a lesser extent in the nucleus of cultured NIH 3T3 fibroblasts and SP1 keratinocytes (Fig 1). By morphometric analysis 95.6% ± 6.1% of NIH 3T3 fibroblasts and 93.1% ± 14.7% of SP1 keratinocytes were FITC positive after 24 hours, suggesting a very efficient DNA uptake. This finding was associated with a striking downregulation of SCF expression after 24 and 48 hours in both cell lines (Fig 2). In contrast, neither the mismatched nor the non-sense control oligonucleotides were able to decrease constitutive SCF expression in fibroblasts (Fig 2) or epithelial cell cultures. Antisense-induced downregulation of SCF was dose dependent (4–12 µmol/L) and comparable to that induced by 10⁻⁵ to 10⁻⁷ mol/L dexamethasone in both NIH 3T3 and SP1 cells (data not shown). These data suggest that SCF antisense phosphorothioate oligonucleotides specifically downregulate SCF expression in epithelial cells and fibroblasts and that this effect is comparable to that observed with glucocorticoids.

Intranasal administration of SCF antisense oligonucleotides decreases lung SCF protein expression in a murine model of asthma

Previous studies by several groups^{24,31} demonstrated that local administration of corticosteroids causes a decrease in mast cell number in the dermis due to down-regulation of local SCF production. Therefore, in further studies, we attempted to directly test the effects of a more specific approach to downregulation of local SCF production in mast cell-dependent diseases. We thus focused on the effects of SCF antisense oligonucleotides in a murine model of asthma associated with late phase-allergic pulmonary inflammation in OVA-sensitized mice.²⁶

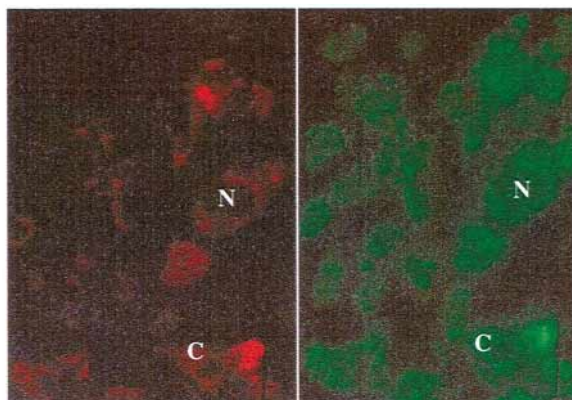


FIG 1. Efficient uptake of SCF antisense oligonucleotides in NIH 3T3 fibroblasts. Cells were coincubated with FITC-labeled anti-sense oligonucleotides for 24 hours followed by immunodetection of SCF (left panel) and FITC-labeled oligonucleotides (right panel) by immunofluorescence. Antisense oligonucleotides were mainly seen in the cytoplasm (C) and nucleus (N) of NIH 3T3 cells. One representative of 3 experiments performed in triplicate is shown. Similar data were obtained in SP1 keratinocytes (not shown). (Original magnification $\times 400$.)



FIG 2. Inhibition of SCF protein expression in NIH 3T3 fibroblasts by SCF antisense phosphorothioate oligonucleotides. Cells were coincubated with SCF antisense oligonucleotides (SCF-AS) or control oligonucleotides (mismatched DNA [SCF-MM], nonsense DNA [SCF-NS]; 10 $\mu\text{mol/L}$ each) for 24 hours as indicated. Antisense but not control mismatched or nonsense oligonucleotides led to decreased SCF expression. Cells were stained with a polyclonal antibody against SCF. Cell viability in separate wells was assessed by trypan blue exclusion and was greater than 90% in all cases, suggesting that there was no direct toxic effect of the antisense DNA. Similar data were obtained in SP1 keratinocytes (not shown). (Original magnification $\times 400$.)

In initial studies on the potential importance of SCF protein production in OVA-immunized mice, we immunostained lung sections from OVA-treated and control mice with anti-SCF antibody. OVA-immunization caused a striking increase in lung SCF protein expression compared with saline treatment (Fig 3, B and A, respectively). Interestingly, SCF protein in OVA-treated mice was mainly expressed in cells of the lung interstitium and the epithelial layers. Next, we assessed the efficacy of DNA uptake in lung cells after local, intranasal delivery of FITC-labeled antisense DNA. It was found that SCF antisense oligonucleotides are mainly taken up by interstitial lung cells (compare Fig 4, A and C with Fig 4, B and D). In contrast, little or no antisense DNA was

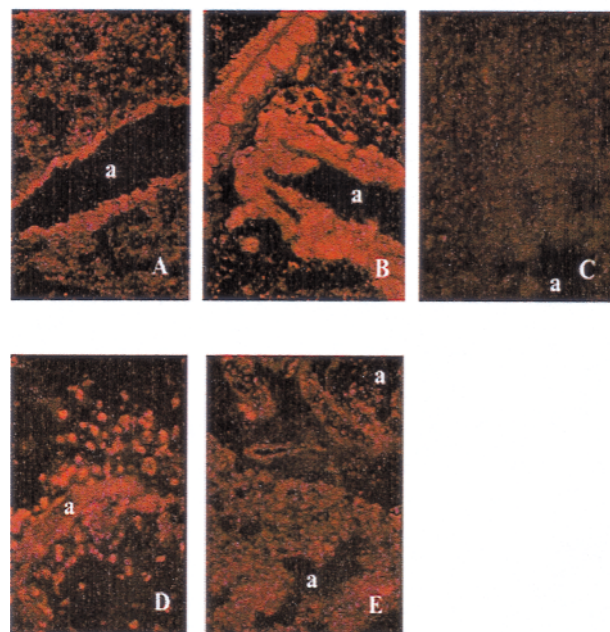


FIG 3. Inhibition of SCF protein expression in the lung after local administration of antisense oligonucleotides. Sections of OVA-immunized mice (B) and saline-treated control mice (A) were stained with an antibody against SCF. OVA immunization caused an upregulation of SCF expression. Intranasal administration of SCF antisense oligonucleotides suppressed SCF expression in the lung interstitium of OVA-immunized mice (C). In contrast, mismatched-treated mice showed high amounts of SCF protein (D). SCF expression in dexamethasone-treated lungs was similar to that in PBS-treated animals (E). a, Airways. (Original magnification $\times 400$.)

observed in the bronchial epithelium. Intranasal delivery of SCF antisense oligonucleotides was associated with a visible decrease of SCF expression (Fig 3, C), whereas control oligonucleotides (mismatched and nonsense DNA) were virtually inactive (Fig 3, D). Similarly to antisense DNA, intranasal administration of dexamethasone caused a decrease in SCF protein expression (Fig 3, E). Western blot analysis of SCF (Fig 5) confirmed downregulation of SCF expression on antisense treatment. Taken together, these results indicate that local administration of specific antisense oligonucleotides leads to a specific downregulation of SCF expression in lung parenchymal cells in vivo that is comparable to that induced by corticosteroids.

Inhibition of allergen-induced airway inflammation by SCF antisense oligonucleotides

To assess the histologic effects of SCF antisense phosphorothioate oligonucleotides on allergen-induced airway inflammation, we obtained lung tissues 24 hours after the last of 3 sequential OVA challenges (days 25-27) on day 28. Lung sections were stained with toluidine blue (Fig 6) and analyzed by light microscopy. In untreated OVA-sensitized mice, an infiltration of the bronchial interstitium with eosinophils was observed. Furthermore, mast cells were seen mainly around the mainstem bronchi of untreated OVA-immunized mice.

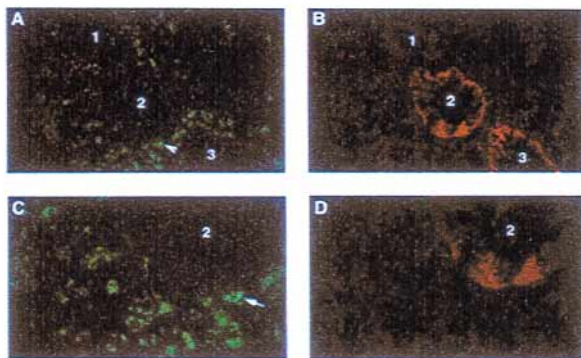


FIG 4. Localization of SCF antisense oligonucleotides in the lung of OVA-sensitized mice on local administration of DNA. There was a strong uptake of FITC-labeled antisense DNA by interstitial lung cells (arrows in **A** and **C**) but not by epithelial cells. A weak staining for SCF after local antisense application was still observed in the airway epithelium (see bronchi No. 1 to 3 in **B** and **D**), suggesting that SCF antisense treatment is more effective in downregulating SCF expression in the interstitium than in the epithelial areas (see Fig 3 for overall reduction of SCF expression on antisense administration).

Treatment of OVA-sensitized mice with mismatched oligonucleotides had no detectable effects. In contrast, no inflammatory signs were seen in OVA-sensitized mice on treatment with corticosteroids and SCF antisense oligonucleotides.

Antisense oligonucleotides to SCF inhibit allergen-induced IL-4 production and the number of eosinophils in the BAL fluid

In further studies on the potential mechanisms of antisense-induced suppression of lung inflammation, we assessed allergen-induced infiltration of eosinophils in the lung.³² The number of eosinophils in the BAL fluid was significantly reduced after administration of SCF antisense oligonucleotides compared with the number found in untreated mice (Fig 7). In contrast, treatment with control mismatched oligonucleotides had no significant effect on the OVA-induced influx of eosinophils in the airways.

Finally, we determined T_H1 and T_H2 cytokine levels in the BAL fluid of OVA-sensitized mice (Fig 8). Neither OVA nor antisense treatment altered IFN- γ levels in the BAL fluid compared with levels in saline-treated mice. However, corticosteroids led to a small decrease of IFN- γ concentration in the BAL fluid of OVA-treated mice. In contrast to IFN- γ levels, the IL-4 concentration in the BAL fluid was significantly suppressed ($P < .05$) by SCF antisense treatment compared with levels in OVA-sensitized mice, suggesting that SCF antisense DNA selectively inhibits IL-4 but not IFN- γ cytokine levels in BAL fluid.

DISCUSSION

In this study we demonstrate a key role for c-kit ligand SCF in the pathogenesis of a murine asthma model induced by OVA. We found that specific blockade of SCF

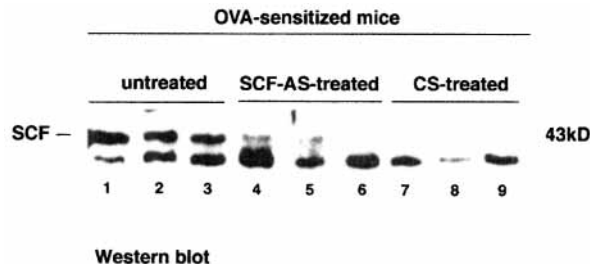


FIG 5. Western blot analysis of SCF protein expression in OVA-sensitized mice after treatment with corticosteroids (CS) or local administration of SCF antisense (AS) DNA. Lung tissue of OVA-sensitized mice was homogenized, and equal amounts of proteins were separated by SDS-PAGE and blotted to a nitrocellulose membrane followed by immunohybridization with SCF antibody (see Methods). Three representative mice per group are shown. There was a striking downregulation of lung SCF protein expression on corticosteroid and antisense DNA treatment.

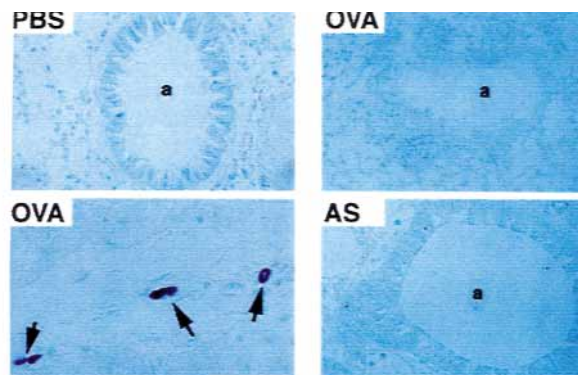


FIG 6. Histologic evidence of airway inflammation in OVA-sensitized and control mice: Effect of SCF antisense treatment. Paraffin-embedded lung sections were stained with toluidine blue. Note the presence of lung inflammation and mast cells (arrows) in OVA-sensitized mice (OVA) compared to antisense-treated mice (AS) and saline-treated control mice (PBS).

expression by antisense oligonucleotides leads to suppression of lung inflammation, IL-4 production, and eosinophilia in OVA-sensitized mice. Taken together with the increased SCF expression in allergic asthma, these data suggest the potential therapeutic utility of SCF antisense oligonucleotides as a novel molecular approach for the treatment of patients with allergic disorders.

Previous studies have shown that SCF critically regulates development and activation of mast cells in vitro and in vivo.³³⁻³⁵ In addition to its regulatory role for mast cells, SCF is a newly recognized ligand for c-kit on eosinophils and has been shown to activate migration of these cells into inflamed tissues through eotaxin and the very late antigen 4/vascular cell adhesion molecule 1 pathway.^{36,37} These data are consistent with a model where local SCF production induces eosinophil migration and IL-4 and TNF production by mast cells with consequent upregulation of endothelial vascular cell adhesion molecule 1 expression that further enhances eosinophil recruitment into inflamed lung tissue.³⁶⁻³⁸

These data prompted us to examine SCF expression in

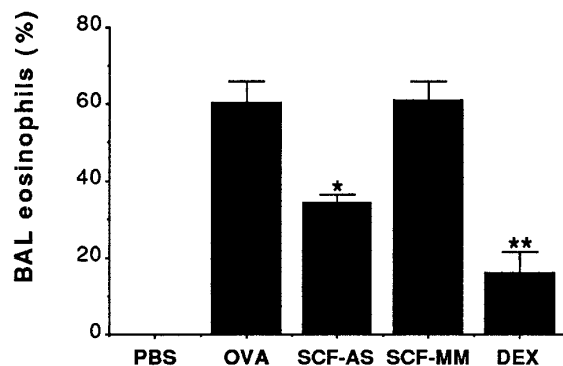


FIG 7. Percentage of eosinophils in the BAL fluid of OVA-sensitized mice. Eosinophils on cytospins were detected by staining according to May Grünwald-Giemsa methods and quantified as specified in the Methods section. Data are reported as the mean percentage of eosinophils in the BAL fluid $\pm$ SEM. Significant differences compared with OVA treatment are indicated (* $P < .05$; ** $P < .01$). SCF-AS, OVA-sensitized mice treated with SCF antisense DNA; SCF-MM, mice treated with mismatched DNA control oligonucleotides; Dex, OVA-sensitized mice treated with dexamethasone.

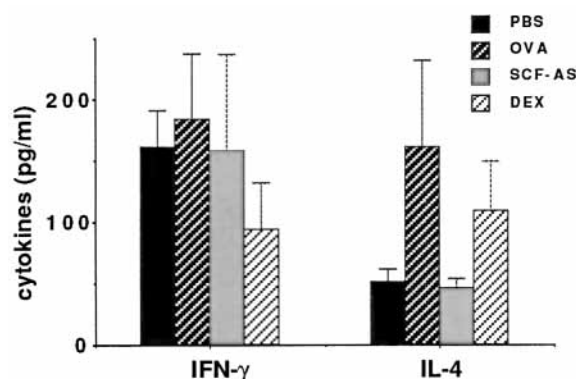


FIG 8. SCF antisense treatment selectively reduces IL-4 cytokine concentration in BAL fluid. Analysis of IFN- γ and IL-4 cytokine concentration in the BAL fluid from lungs of saline-treated mice (PBS; $n = 5$), OVA-sensitized mice (OVA; $n = 5$) and OVA-sensitized mice treated with SCF antisense DNA (SCF-AS; $n = 4$) or dexamethasone (DEX; $n = 4$). Cytokine levels (IFN- γ , IL-4) were determined by specific ELISA and are reported as mean values $\pm$ SEM. IL-4 concentration in BAL fluid was significantly suppressed ($P < .05$) by SCF antisense treatment compared with levels in OVA-sensitized mice.

a murine model of asthma induced by OVA. Consistent with previous observations on the production of SCF in allergic airway inflammation,³⁹ we observed that OVA sensitization of mice caused strong upregulation of local SCF expression. This finding led us to further investigate the functional role of this factor using an antisense DNA strategy. Antisense experiments have been previously used to delineate various important functions of regulatory molecules in vitro and in vivo.^{29,40} For instance, antisense oligonucleotides to IL-4 have revealed an important function of this cytokine in the late allergic response in the airways.⁴¹ Here, we targeted SCF expression in epithelial cells and fibroblasts by coinubation with SCF antisense oligonucleotides. The observed

effects of the SCF antisense oligonucleotides were shown by previously established criteria to be both effective and specific²⁹: (1) immunocytochemistry studies showed that the levels of SCF protein were greatly reduced in cells exposed to the antisense oligonucleotides; (2) treatment of cells with several control oligonucleotides did not affect SCF levels; (3) expression of a nontargeted protein was not affected by antisense oligonucleotides (data not shown); and (4) direct toxic effects of the antisense oligonucleotides were excluded.

Local delivery of antisense DNA has been previously shown to result in efficient DNA uptake in target cells and to reduce the required antisense dosage compared with systemic delivery of antisense DNA.²⁹ Accordingly, we used in vivo studies to test the uptake efficacy of FITC-labeled SCF antisense phosphorothioate oligonucleotides by the cells of the lung on local, intranasal administration. Although it cannot be excluded that some antisense DNA was swallowed or delivered through the nose on local administration, we did not see FITC-positive cells in the gut. However, we observed an efficient uptake of FITC-labeled DNA in interstitial lung cells that expressed large amounts of SCF on OVA sensitization, suggesting that many antisense molecules are delivered directly into lung cells. However, lung epithelial cells did not show high uptake of antisense DNA, possibly because of a destruction of surface receptors for phosphorothioate oligonucleotides during local inflammation. Antisense uptake in lung cells was followed by downregulation of the expression of SCF protein. These data suggest that local administration of SCF antisense DNA leads to an efficient uptake in lung cells with consecutive local blockade of the expression of SCF. Interestingly, antisense-induced suppression of SCF expression was accompanied by a suppression of OVA-induced lung inflammation with attenuation of IL-4 cytokine synthesis and accumulation of eosinophils in the airways.

The inhibition of asthmatic reactions by antisense-induced specific blockade of SCF is a novel approach that has the potential advantage of suppressing allergic asthma by inhibiting the release of mast cell mediators such as histamine, leukotrienes, PGs, platelet-activating factor, and tryptase.^{42,43} Furthermore, mast cells are capable of secreting several proinflammatory cytokines actively involved in the allergic response, including IL-4, IL-5, TNF, lymphotactin, and IL-16, which may be suppressed on administration of SCF antisense. However, our data on the regulatory role of SCF are consistent with those of a previous report³⁹ showing that blockade of SCF function by antibodies leads to attenuation of lung inflammation. Administration of antisense DNA to SCF, however, is a different therapeutic approach that aims at suppressing SCF production intracellularly rather than neutralizing preformed SCF protein extracellularly. Furthermore, local delivery and local suppression of SCF expression by antisense DNA may also prevent several side effects of systemic SCF neutralization because SCF is a well-established pleiotropic factor with important function, for example, in hematopoiesis.^{14,15}

Because mast cell-deficient mice are still susceptible to OVA-induced allergic airway inflammation, it has been suggested that these cells are not required for the induction of chronic airway inflammation.⁴⁴ Thus it is tempting to speculate that the therapeutic effects of SCF antisense oligonucleotides in OVA-induced allergic airway inflammation are at least partially due to inhibition of recruitment and activation of eosinophils. For instance, antisense oligonucleotides might suppress production of the chemokine eotaxin in allergic reactions because eotaxin production in mast cells is upregulated by membrane-bound SCF expressed on fibroblasts.⁴⁵

In agreement with previous observations,²⁶ IL-4 expression was increased in the BAL fluid of OVA-sensitized mice, possibly because of an increased local production by lymphocytes or mast cells.⁴⁴ Interestingly, we observed in this study that local SCF antisense treatment strongly downregulates local IL-4 production induced by OVA-sensitization in the BAL fluid. Such downregulation was even more pronounced than in dexamethasone treatment. Therefore antisense-induced downregulation of local IL-4 production may also contribute to the efficacy of SCF antisense DNA in the treatment of OVA-induced lung inflammation.

Corticosteroids are highly effective in patients with allergic asthma possibly because they inhibit mast cell functions and suppress SCF-dependent growth and differentiation of mast cells.²⁴ In this study we designed a more specific strategy to downregulate SCF expression in allergic asthma in vivo using antisense DNA. Furthermore, we demonstrated that the beneficial effects of SCF antisense treatment on the asthmatic reaction were similar to those found during treatment with corticosteroids. These data indicate a critical role for SCF in a murine asthma model. Furthermore, the data suggest that antisense oligonucleotide to SCF may be a novel molecular approach for the treatment of mast cell-mediated disorders such as asthma in human beings.

We thank Dr Emil Z. Chi (Department of Pathology, University of Washington, Seattle) for his helpful advice on the murine asthma model and Mrs Mechthild Schipp, Raja Atreya, and Brigitte Bartsch (Mainz, Germany) for their excellent technical assistance. We also thank Prof A. Reske-Kunz (Department of Dermatology, University of Mainz), Prof R. Buhl (Medical Clinic III, University of Mainz), and Prof H. A. Lehr (Institute of Pathology, University of Mainz) for helpful discussions, critical reading of the manuscript, and help with the toluidine blue staining analysis.

REFERENCES

1. Broide DH, Gleich GJ, Cuomo AJ, Coburn DA, Federman EC, Schwartz LB, et al. Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *J Allergy Clin Immunol* 1991;88:637-48.
2. Casale TB, Wood D, Richerson HB, Zehr B, Zavala D, Hunninghake GW. Direct evidence for a role for mast cells in the pathogenesis of antigen-induced bronchoconstriction. *J Clin Invest* 1987;80:1507-11.
3. Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serham CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science* 1987;237:1171-6.
4. Dahlen SE, Hansson G, Hedqvist P, Björck T, Granström E, Dahlen B. Allergen challenge of lung tissue from asthmatics elicits bronchial constriction that correlates with release of leukotrienes C4, D4, and E4. *Proc Natl Acad Sci U S A* 1983;80:1712-6.
5. Wenzel SE, Westcott JY, Larsen GL. Bronchoalveolar lavage fluid mediator levels 5 minutes after allergen challenge in atopic subjects with asthma: relationship to development of late asthmatic responses. *J Allergy Clin Immunol* 1991;87:540-8.
6. Henderson WR, Lewis DB, Albert RK, Zhang Y, Lamm WJE, Chiang GKS, et al. The importance of leukotrienes in airway inflammation in a mouse murine model of asthma. *J Exp Med* 1996;184:1483-94.
7. Gordon JR, Burd PR, Galli SJ. Mast cells as a source of multifunctional cytokines. *Immunol Today* 1990;11:458-64.
8. Plaut M, Pierce JH, Watson CJ, Hanley-Hyde J, Nordan RP, Paul WE. Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. *Nature* 1989;339:64-7.
9. Meininger DC, Zetter B. Mast cells and angiogenesis. *Semin Cancer Biol* 1992;3:73-82.
10. Broide DH, Smith CM, Wasserman SI. Mast cells and pulmonary fibrosis: identification of a histamine releasing factor in bronchoalveolar lavage fluid. *J Immunol* 1990;145:1838-47.
11. Bousquet J, Chané P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033-9.
12. Russi EW, Perruchoud AP, Yerger LD, Stevenson JS, Tabak J, Marchette B, et al. Late phase bronchial obstruction following nonimmunologic mast cell degranulation. *J Appl Physiol* 1984;57:1182-9.
13. Denburg JA. Microenvironmental influences on inflammatory cell differentiation. *Allergy* 1995;50:25-8.
14. Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, et al. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* 1990;63:235-43.
15. Zsebo KM, Wypych MJ, McNiece IK, Lu HS, Smith KA, Karkare SB, et al. Identification, purification, and biological characterization of hemopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* 1990;63:195-201.
16. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, et al. Stem cell factor (SCF) is encoded at the S1 locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* 1990;63:213-24.
17. Inemura A, Tsai M, Ando A, Wershil BK, Galli SJ. The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *Am J Pathol* 1994;144:321-9.
18. Godin I, Deed R, Cooke J, Zsebo KM, Dexter M, Wylie CC. Effects of the steel gene production on mouse primordial germ cells in culture. *Nature* 1991;352:807-9.
19. Heinrich MC, Dooley DC, Freed AC, Band L, Hoatlin ME, Keeble WW, et al. Constitutive expression of steel factor gene by human stromal cells. *Blood* 1993;82:771-83.
20. Mekori JA, Oh CK, Metcalfe DD. IL-3-dependent murine mast cells undergo apoptosis on removal of IL-3. Prevention of apoptosis by c-kit ligand. *J Immunol* 1993;151:3775-84.
21. Tsai M, Shih LS, Newlands GFJ, Takeishi T, Langley KE, Zsebo KM, et al. The rat c-kit ligand, stem cell factor, induces development of connective tissue-type and mucosal mast cells in vivo: analysis by anatomical distribution, histochemistry and protease phenotype. *J Exp Med* 1991;174:125-31.
22. Yee NS, Paek J, Besmer P. Role of kit-ligand in proliferation and suppression of apoptosis in mast cells: basis for radiosensitivity of white spotting and steel mutant mice. *J Exp Med* 1994;179:1777-87.
23. Galli SJ, Iemura A, Garlick DS, Gamba-Vitalo C, Zsebo KM, Andrews RG. Reversible expansion of primate mast cell population in vivo by stem cell factor. *J Clin Invest* 1993;91:148-52.
24. Finotto S, Mekori YA, Metcalfe DD. Glucocorticoids decrease tissue mast cell number by reducing the production of the c-kit ligand, stem cell factor, by resident cells. In vitro and in vivo evidence in murine systems. *J Clin Invest* 1997;99:1721-9.
25. Kim YK, Nakagawa N, Nakano K, Sulakvelidze J, Dolovich J, Denburg J. Stem cell factor in nasal polyposis and allergic rhinitis: increased expression by structural cells is suppressed by in vivo topical corticosteroids. *J Allergy Clin Immunol* 1997;100:389-99.
26. Henderson WR, Chi EY, Albert RJK, Chu AJ, Lamm WJE, Rochon Y, et al. Blockade of CD49d (alpha4-integrin) on intrapulmonary but not circulating leukocytes inhibits airway inflammation and hyperresponsiveness in a mouse model. *J Clin Invest* 1997;100:3083-92.
27. Sutter C, Nischt R, Winter H, Schweitzer J. Aberrant in vitro expression

- of keratin K13 induced by Ca²⁺ and vitamin A acid in mouse epidermal cell lines. *Exp Cell Res* 1991;195:183-93.
28. Gura T. Antisense has growing pains. *Science* 1995;270:575-7.
 29. Wagner RW. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science* 1993;260:1510-4.
 30. Finotto S, Dolovich J, Denburg J, Jordana M, Marshall JS. Functional heterogeneity of mast cells isolated from different microenvironments within nasal polyp tissue. *Clin Exp Immunol* 1994;95:343-50.
 31. Pipkorn U, Hammarlund A, Enerback L. Prolonged treatment with topical glucocorticoids results in an inhibition of the allergen-induced wheal-and-flare response and a reduction in skin in mast cells numbers and histamine content. *Clin Exp Allergy* 1989;19:19-25.
 32. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airway hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195-201.
 33. Kunisada T, Lu SZ, Yoshida H, Nishikawa S, Mizoguchi M, Hayashi S, et al. Mastocytosis and epidermal melanocytosis induced by keratinocyte expression of transgenic stem cell factor. *J Exp Med* 1998;187:1565-73.
 34. Wershil BK, Tsai M, Geissler EN, Zsebo KM, Galli SJ. The rat c-kit ligand, stem cell factor, induces c-kit receptor dependent mouse mast cell activation in vivo: evidence that signaling through the c-kit receptor can induce expression of cellular function. *J Exp Med* 1992;175:245-55.
 35. Columbo M, Horowitz EM, Botana LM, MacGlashan DW, Bochner BS, Gillis S, et al. The recombinant human c-kit receptor ligand, rhSCF, induces mediator release from human cutaneous mast cells and enhances IgE-dependent mediator release from both skin mast cells and peripheral blood basophils. *J Immunol* 1992;149:599-608.
 36. Yuan Q, Austen KF, Friend DS, Heidman M, Boyce JA. Human peripheral blood eosinophils express functional c-kit receptor for SCF that stimulates very late antigen 4 (VLA4) mediated cell adhesion, fibronectin and vascular cell adhesion molecule 1 (VCAM-1). *J Exp Med* 1997;186:313-23.
 37. Iademarco MF, Barks JL, Dean DC. Regulation of vascular adhesion molecule-1 expression by IL-4 and TNF-alpha in cultured endothelial cells. *J Clin Invest* 1995;95:264-71.
 38. Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R, et al. IL-4, IL-5, and IL-6 and TNF-alpha in normal and asthmatic airways—evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994;10:471-80.
 39. Lukacs NW, Strieter RM, Lincoln PM, Brownell E, Pullen DM, Schock HJ, et al. Stem cell factor (c-kit ligand) influences eosinophil recruitment and histamine levels in allergic airway inflammation. *J Immunol* 1996;156:3945-51.
 40. Wagner RW. Gene inhibition using antisense oligodeoxynucleotides. *Nature* 1994;372:333-5.
 41. Molet S, Ramos-Barbon D, Martin JG, Hamid Q. Adoptively transferred late allergic response is inhibited by IL-4, but not IL-5, antisense oligonucleotide. *J Allergy Clin Immunol* 1999;104:205-14.
 42. Robinson C, Holgate ST. Mast cell-dependent inflammatory mediators and their putative role in bronchial asthma. *Clin Sci* 1985;68:103-12.
 43. Schleimer RP, MacGlashan DW, Peters SP, Pinkard RN, Adkinson NF, Lichtenstein LM. Characterization of inflammatory mediator release from purified human lung mast cells. *Am Rev Respir Dis* 1986;133:614-7.
 44. Brusselle GG, Knips JC, Tavernier JH, van der Heyden JG, Cuvelier CA, Pauwels RA, et al. Attenuation of allergic airway inflammation in IL-4-deficient mice. *Clin Exp Allergy* 1994;24:73-80.
 45. Hogaboam C, Kunkel SL, Strieter RM, Taub DD, Lincoln PM, Standiford TJ, et al. Novel role of transmembrane SCF for mast cell activation and eotaxin production in mast cell-fibroblast interactions. *J Immunol* 1998;160:6166-71.