Eosinophil Activation of Fibroblasts from Chronic Allergen-Induced Disease Utilizes Stem Cell Factor for Phenotypic Changes

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In the present studies the role of stem cell factor (SCF) in mediating eosinophil and fibroblast activation during their interaction was investigated. SCF was significantly higher in fibroblasts grown from lungs of chronic allergen-challenged mice compared to fibroblasts grown from normal mice. When eosinophils were layered onto fibroblasts from allergic mice, a significant increase in SCF was detected compared to fibroblasts from nonallergic mice. The interaction of fibroblasts with eosinophils also increased the production of asthma-associated chemokines, CCL5 and CCL6, was dependent on cell-to-cell interaction, and was observed only with fibroblasts derived from lungs of chronic allergen-challenged mice and not from those derived from unchallenged normal mice. Chemokine production was significantly decreased when anti-SCF antibodies were added during eosinophil-fibroblast interaction. The interaction of fibroblasts from chronic allergen-challenged mice with eosinophils also increased α-smooth muscle cell actin and procollagen I expression as well as induced transforming growth factor-β. The changes in myofibroblast activation were dependent on SCF-mediated pathways because anti-SCF antibody treatment reduced the expression of all three of these latter fibrosis-associated markers. Thus, our data suggest that SCF mediates an important activation pathway for fibroblasts during chronic allergic responses on interaction with recruited eosinophils and suggest a potential mechanism of airway remodeling during chronic disease. (Am J Pathol 2008, 172:68–76; DOI: 10.2353/ajpath.2008.070082)
which were deficient in both SCF and pulmonary mast cells, demonstrated significant reduction in the allergen-induced airway hyperresponsiveness responses. Neutralization of SCF in vivo was very beneficial in murine models of asthma, attenuating Th2 responses, eosinophilia, mucus production, airway remodeling, and collagen deposition. In the present study we investigated SCF-dependent cross talk between eosinophils and lung fibroblasts derived from chronic allergen-challenged animals (CRA fibroblasts) compared to those from control animals (naïve fibroblasts). The results from the present studies indicate that SCF has an important role in eosinophil-induced fibroblast activation.

**Materials and Methods**

**Animals**

Female BALB/c mice, 6 to 8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained under standard pathogen-free conditions. All experiments involving the use of animals were approved by the University of Michigan care and use of animals committee.

**Mouse Chronic Cockroach Allergen (CRA) Asthma Model**

Normal female BALB/c mice were sensitized intraperitoneally and subcutaneously with 1000 protein nitrogen units of CRA (Holister Stier, Toronto, Canada) 1/1 in IFA (Sigma-Aldrich, St. Louis, MO). Then mice were challenged intranasally with 150 protein nitrogen units of CRA on days 14, 18, 22, and 26 after initial sensitization to localize the response to the lung. The final two allergen challenges were given by intratracheal injection 4 days apart on days 30 and 34. On day 38, 4 days after the final allergen challenge, animals were sacrificed and lungs were removed.

**Mouse Lung Fibroblast Isolation and Culture**

Mouse lung fibroblasts were isolated from lung tissue by mincing and enzymatic digestion with 0.2% collagenase IV in RPMI 1640 (Mediatech, Inc., Herndon, VA) supplied with 2% fetal calf serum (Atlas, Fort Collins, CO) for 30 minutes at 37°C with continuous agitation. After digestion with collagenase, isolated cells were centrifuged, washed, and cultured in complete medium composed of Dulbecco’s modified Eagle’s medium (Mediatech, Inc.) supplement with 15% fetal calf serum, 1% of L-glutamine, and 100 U/ml of penicillin, streptomycin, and fungizone (Cambrex, Walkersville, MD). Cells were used for experiments after three passages.

**Antigen-Elicited Peritoneal Eosinophil Purification**

Eosinophils were elicited by injection of thioglycollate plus soluble egg antigen into the peritoneum of Schistosoma mansoni-infected mice. Soluble egg antigen was prepared in our laboratory by grinding isolated eggs from heavily infected S. mansoni mice as previously described. The injection of soluble egg antigen into infected mice induces a pool of circulating eosinophils recruited into the peritoneum in an antigen-specific manner. After 48 hours the mice were peritoneal lavaged and the cells collected. The initial population that was isolated from the peritoneum was ~50% eosinophils with only 2 to 5% neutrophils and ~35 to 45% mononuclear cells (lymphocytes and macrophages). Adherent cell populations were removed from the population by plastic adherence in tissue culture dishes for 1.5 hours using standard media (RPMI 1640 supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum). The nonadherent cells were washed and resuspended in phosphate-buffered saline (PBS)/bovine serum albumin (90 μl of PBS/bovine serum albumin per 107 cells), and eosinophils were purified by negative immunomagnetic bead-coupled antibodies to exclude contaminating immune cells using the MACS system (Miltenyi Biotec, Auburn, CA). The antibodies used were anti-Thy1 (for T cells), anti-B220 (for B cells), and anti-MHC class II (for APCs) (Miltenyi Biotec). After the plate adherence and MACS separation, the population of cells contained >97% eosinophils contaminated with neutrophils (~1%) and mononuclear cells (1 to 2%).

**Culture of Purified Eosinophils**

Freshly isolated murine eosinophils (3 x 10⁶ per well) were cultivated overnight in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (BioWhittaker, Walkersville, MD), and 10% heat-inactivated fetal bovine serum (Atlas) with 5 ng/ml of IL-5.

**Fibroblast-Eosinophil Cell Co-Culture**

Lung fibroblasts cultured to 60% confluence in six-well plates were overlaid with 1 x 10⁶ murine eosinophils in our standard media (RPMI 1640 supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum). After 24 hours, eosinophils were pelleted, and supernatants were harvested and frozen at ~20°C. In some cultures, cell populations were separated by a 3-μm membrane in a trans-well culture dish (Costar, Kennebunkport, ME) or co-incubated with 50 μg of anti-SCF antibodies or whole rabbit serum control antibodies. To determine whether there were more eosinophils adherent to fibroblasts from allergic mice compared to those from naïve animals, we performed co-culture experiments using an eight-well Lab-Tek (Nalge Nunc International, Rochester, NY) cell culture system. Fibroblasts were grown to ~60% confluence, and eosinophils (1 x 10⁵/well) were added to the fibroblasts. After 24 hours slides were washed to remove nonadherent cells, stained using Diff-Quick stain (Dade Behring Inc., Newark, DE), and eosinophils adhered to fibroblasts counted.
by light microscopy. The data were collected using 40 high-power fields (×400 magnification) per group and counted in a blinded manner.

Western Blot

Similar protocols to the ones described here were reported elsewhere.16,17 Cells were lysed in 1% Nonidet P-40 buffer containing complete protease inhibitor cocktail (Roche, Indianapolis, IN) for 30 minutes on ice with frequent vortexing and centrifuged (1200 × g, 5 minutes at 4°C). Equal amounts of protein from a detergent-soluble fraction were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred onto polyvinylidene difluoride membranes and probed with monoclonal anti-α-smooth muscle actin (α-SMA) antibodies (1:1000; Sigma, St. Louis, MO) and corresponding horseradish peroxidase-conjugated secondary IgG antibodies (1:2000). Proteins were visualized by chemiluminescence (Super Signal West Pico chemiluminescent substrate; Pierce, Rockford, IL) using a Kodak photo imager (Eastman-Kodak, Rochester, NY). GAPDH (1:2000; Cell Signaling Technology, Inc., Danvers, MA) was detected as a loading control.

Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNA was purified from isolated eosinophils using TRIzol reagent (Life Technologies, Inc., Grand Island, NY) and chloroform. RNA was quantified by measuring absorbance at 260 nm. Samples were then standardized to 5 μg/ml with diethyl pyrocarbonate water. RNA was then reverse-transcribed to cDNA and 2 μl of this cDNA was used in the TaqMan reaction mixture as we described before.13 The specific primer/probe sets for real-time PCR were predeveloped by Applied Biosystems, Foster City, CA, except mouse procollagen I and SCF primers. The sequence of the primers used are as follows: 5’-TCGTTGACGCAGCCTGGA-3’; reverse: 5’-G-AGGGCAGAGGCTGATGAG-3’ for procollagen, 5’-GTGGGTCGCCAGGCAACAC-3’ (sense) and 5’-GTCCTGCCCGTGTGGTGAAAGC-3’ (antisense) for β-actin (350 bp), 5’-CCTCAGCCTGGACTACTCTTT-3’ (sense) and 5’-GTCATTCTCAAGGGAGCTGG-3’ (antisense) for soluble SCF (358 bp), and 5’-CCTCAGCCTGGACTACTCTTT-3’ (sense) and 5’-TTGGGGCCTTCCCTTCTTG-3’ (antisense) for transmembrane SCF (302 bp). The mixture was first incubated for 5 minutes at 94°C and then cycled 40 times at 95°C for 30 seconds, followed by 58°C for 45 seconds, and elongated at 72°C for 75 seconds, as described.18 This format allowed optimal amplification with little or no nonspecific amplification of contaminating DNA as checked using nonreverse-transcribed mRNA. After amplification, the sample (20 μl) was separated on a 2% agarose gel containing 0.3 mg/ml (0.003%) of ethidium bromide. The bands were visualized and quantified using a Molecular Imager FX scanner and Quantity One software (Bio-Rad, Hercules, CA).

Enzyme-Linked Immunosorbent Assays

Cytokines were quantified from supernatants of overnight cultured cells using a sandwich enzyme-linked immunosorbent assay system, as we described before.13 The murine enzyme-linked immunosorbent assays were designed using standardized antibodies that were purchased from R&D Systems (Minneapolis, MN) that detect protein at concentrations greater than 10 pg/ml, are specific, and do not cross-react with any other cytokines.

Measurement of SCF from Pulmonary Fibroblasts

After co-culture of fibroblasts and eosinophils for 24 hours, eosinophils were washed out and fibroblasts lysed in 0.1% Nonidet P-40 containing complete protease inhibitors cocktail (Roche Diagnostics Corp., Indianapolis, IN) to allow quantitation of all SCF, membrane and soluble. To clear out nonadherent cells, collected supernatants were centrifuged at 400 × g for 5 minutes at + 4°C and transferred to a fresh tube. In the case of soluble SCF measurements in the supernatant, we did not add the Nonidet P-40 before collection of the supernatant.

Statistical Analyses

Statistical significance was determined using analysis of variance with P values less than 0.05 followed by Student-Neuman-Keuls post-test when appropriate.

Results

Fibroblasts and Eosinophil Interaction Activates Membrane SCF Production

Previous studies demonstrated that neutralization of SCF during chronic allergen responses reduced peribronchial remodeling and collagen deposition.14 Although lung fibroblasts produce significant levels of the transmembrane form of SCF (mSCF),18 soluble SCF may also contribute to the cellular activation profile.19,20 However, when investigating the expression of the soluble form of SCF in our studies, no significant level of soluble SCF expression (mRNA or protein) in fibroblasts cultured alone or with eosinophils was found (data not shown). Thus our studies have focused on the transmembrane form of SCF. Figure 1 illustrates that fibroblasts from chronic allergen-challenged animals (CRA fibroblasts) express significantly higher mSCF mRNA levels compared to naïve fibroblasts (Figure 1). Because previous studies have demonstrated altered fibroblast function when they interact with eosinophils,21–24 we were interested in how eosinophils influenced mSCF production from fibroblasts. Increased mSCF production was observed when eosinophils were combined with CRA fibroblasts compared to naïve fibroblasts, which displayed no increase in SCF mRNA or protein when combined with eosinophils (Figure 1). Although the levels of SCF mRNA
and protein appeared to be additive in the co-cultures with CRA fibroblasts, the difference compared to the naive fibroblast cultures was significant and noteworthy.

Because earlier studies indicated that SCF contributes to mast cell and eosinophil adherence, the numbers of eosinophils adhered to the fibroblasts were examined. To determine whether there was a general increase in eosinophil adherence to the fibroblasts from lungs of allergic mice, co-cultures were washed free of nonadherent eosinophils, fixed, and differentially stained. The morphometric enumeration of adhered eosinophils in the co-cultures demonstrated an increase in the number of eosinophils adhered to the fibroblasts in allergic versus naive cultures (CRA fibroblasts 178 ± 28 versus naive fibroblasts 128 ± 10), but this was not statistically significant. In additional studies when cultures were treated with anti-SCF, no reduction in eosinophil adherence could be observed compared to control antibody-treated fibroblasts from allergic mice (control Ab, 169 ± 58 versus anti-SCF, 152 ± 38). Thus, although SCF may contribute to cellular adhesion, its role in these static cultures was not evident.

CCL5 and CCL6 Production Requires Contact between Fibroblasts and Eosinophils and Is Dependent on SCF

SCF is an eosinophil degranulator and activator that may play a number of roles during an inflammatory/immune response, including production of CC chemokines from eosinophils. In particular, CCL6 can be produced at significant levels by eosinophils, whereas CCL5 levels are more modest but significantly increased. When the two populations were combined, a significant increase in both CCL5 and CCL6 could be observed in the production of both chemokines (Figure 2, A and B). To determine whether cell-to-cell contact was required for production of chemokines, we used transwell barrier plates (3 μm pores) to perform co-cultures of naive or CRA fibroblasts with eosinophils. The transwell separation of the two cell populations inhibited CCL5 and CCL6 production (Figure 2, A and B).
We conclude that the observed increase in chemokine production in fibroblast and eosinophil co-cultures requires cell-to-cell contact. In the next set of experiments, we examined whether cell-to-cell contact-induced chemokine production involved SCF-mediated activation. CRA fibroblasts produced significantly higher levels of CCL6 compared to naive fibroblasts (Figure 3A). After eosinophils were overlaid onto naive or CRA fibroblasts, CCL6 production was significantly elevated. The increase in CCL6 production was much more dramatic in CRA fibroblast-eosinophil co-cultures, and anti-SCF antibodies significantly reduced CCL6 production in only the CRA fibroblast-eosinophil co-cultures. The CCL5 level was also significantly increased after naive and CRA fibroblasts were combined with eosinophils (Figure 3B). Similar to CCL6 production, anti-SCF antibody treatment was not effective in blocking CCL5 production in naive fibroblast-eosinophil co-cultures, whereas the treatment significantly reduced CCL5 in CRA fibroblast-eosinophil co-culture. The antibody treatments had no effect in cultures with eosinophils alone. Together, these data suggest that lung fibroblasts from chronic CRA-challenged animals induced higher chemokine production during eosinophil interaction and depended on SCF-mediated mechanisms.

A final aspect of these investigations was whether the interactions of eosinophils with fibroblasts led to a degranulation event by the eosinophils. To this end, we assessed the level of eosinophil peroxidase in supernatants of 4-hour cultures as previously performed. The eosinophil peroxidase assay demonstrated that a significant degranulation event occurred equally as well when eosinophils were incubated with fibroblasts either from nonallergic mice or from mice that had received chronic allergen exposures (Figure 3C). When anti-SCF antibody was added to the co-culture, there was a slight but insignificant reduction in the amount of eosinophil peroxidase released into the culture supernatant. Thus, a general degranulation event could be observed in the co-cultures, but this was neither dependent on the source of the fibroblast nor the level of SCF expressed.

Eosinophil-Induced Fibroblast Activation and Phenotypic Change Correlates with TGF-β Expression

In addition to chemokine production, eosinophils have also been suggested to induce development of myofibroblasts. To examine this aspect we assessed α-SMA, which is the primary indication for myofibroblast differentiation. At baseline CRA fibroblasts exhibited significantly higher α-SMA mRNA expression level (Figure 4A) and protein production (Figure 4B) compared to fibroblasts from naive animals. We did not observe any change in mRNA expression or protein production in fibroblasts from naive mice when cultured with eosinophils. In contrast, CRA fibroblasts co-cultured with eosinophils resulted in significantly increased α-SMA mRNA expression (Figure 4A) and protein production (Figure 4B). Administration of anti-SCF antibodies inhibited eosinophil-induced α-SMA mRNA expression and protein production in fibroblasts from chronic allergen-challenged animals.

A second aspect of myofibroblast activation is collagen production. To investigate the regulation of collagen production during the fibroblast-eosinophil interaction, we examined procollagen type I expression. CRA fibroblasts maintained a significantly higher level of procollagen type I expression compared to naive fibroblasts (Figure 5). When eosinophils were added to the fibroblast co-cultures, CRA fibroblasts were much more responsive to eosinophil-induced activation and demonstrated significantly higher procollagen type I expression than the naive fibroblasts (Figure 5). Addition of anti-SCF antibodies significantly suppressed collagen I mRNA expression in the fibroblast-eosinophil co-cultures (Figure 5A). We also assessed the protein expression using Western blot analysis and found a similar up-regulation of protein in only the CRA fibroblast co-cultures and a dependence on SCF, as addition of specific antibodies reduced the amount of type I collagen protein (Figure 5B). These
observations indicate that SCF is involved in the activation process during fibroblast-eosinophil interaction.

As indicated above, fibroblasts from chronic allergen-challenged lungs displayed a myofibroblast phenotype with increased collagen and α-SMA when combined with eosinophils. TGF-β is a primary stimulus for differentiation of fibroblasts into myofibroblasts.1,29 When we assessed the expression of TGF-β during the cellular interaction, we observed an increased expression when eosinophils were co-cultured with CRA fibroblasts but not with naive fibroblasts (Figure 6). Treatment of fibroblast only with anti-SCF had no effect on the level of α-SMA produced.

Discussion

Multiple mediators have been implicated and targeted during chronic allergic disease. Increases in SCF and c-Kit mRNA have been identified in the airway epithelial cell layer of asthmatic patients compared with controls.30–32 In addition, significant differences in the number of SCF- and c-Kit-expressing cells was observed by in situ hybridization and immunohistochemistry, suggesting a coordinated role for SCF in the pathophysiological changes that occur in asthma.32 Evidence for a role of SCF in human airway disease was supported by studies demonstrating increased SCF expression in nasal polyps of asthmatics and allergic rhinitis patients.33,34 Additionally, SCF has been implicated as a potential mediator in fibrotic diseases in the kidney and intestine.34–37 Recently, the role of SCF has been verified using a chronic
mouse model of allergic disease characterized by airway hyperresponsiveness, mucus overproduction, eosinophil accumulation, and peribronchial collagen deposition. Thus, SCF is up-regulated in inflammatory conditions and appears to be associated with chronic remodeling disease.

Numerous cell populations have been shown to express SCF, including epithelial cells, smooth muscle cells, macrophages, mast cells, eosinophils, and fibroblasts. SCF production by pulmonary fibroblasts has previously been characterized in several end-stage diseases. SCF activates eosinophils to promote adhesion to matrix and VCAM-1 via VLA4, while also having the ability to promote degranulation and production of inflammatory and profibrotic factors. Furthermore, a number of publications have outlined that fibroblasts express SCF and can promote the activation, adhesion, and survival of mast cells, which also have a detrimental role in the development of allergic disease. By evaluating mouse lung fibroblasts derived from chronic allergen-treated animals (CRA fibroblasts) or those from control, unsensitized animals, we demonstrate that fibroblasts from chronic disease have elevated SCF expression and protein production. Fibroblasts grown from the lungs of chronically challenged mice also produced additional mediators, including CCL5 and CCL6, that were further up-regulated on contact with eosinophils. The increased chemokines were abrogated by treatment with anti-SCF antibodies during the co-culture period. These observations correspond with previous data that demonstrated that SCF can promote chemokines from mast cells and eosinophils and suggest a potential mechanism for SCF activation during development of chronic allergic disease. These findings at least in part explain the beneficial role of anti-SCF treatment in vivo that resulted in reduced airway inflammation and remodeling during chronic CRA-induced mouse asthma.

One of the primary considerations in our studies was to assess the impact of eosinophils on fibroblast maturation/differentiation to a myofibroblast phenotype. Previous studies have indicated that eosinophils can contribute significantly to myofibroblast differentiation. Eosinophils store and release several fibrogenic factors, such as...
as TGF-β, that can stimulate fibroblast activation and collagen synthesis. In the present study CRA fibroblasts displayed significantly enhanced expression of type I collagen and α-SMA, two important markers of myofibroblast differentiation. In addition, the expression of TGF-β was also markedly increased when eosinophils were combined with fibroblasts, and this was SCF-dependent. The data indicate that fibroblasts from chronic allergen-challenged animals have an altered phenotype compared to that observed in fibroblasts from unchallenged animals. The data further indicate that interaction with eosinophils via an SCF-mediated mechanism enhances the inflammatory response and myofibroblast phenotype. Our overall hypothesis, illustrated in Figure 7, suggests that 1) the increased expression of SCF by fibroblasts in allergic airway provides a significant stimulus for eosinophil activation during an allergic response and 2) this interaction leads to TGF-β production that 3) subsequently causes myofibroblast differentiation. At the same time the overexpression of chemokines during the allergic response increases inflammatory cell influx. We would suggest that targeting the SCF/c-kit activation pathway in chronic inflammatory diseases would have a beneficial effect on the control of the disease process. This has recently been supported by studies using a c-kit tyrosine kinase inhibitor, imatinib, that by itself can block the development of chronic allergen responses in animal models. Thus, local airway application of SCF- and/or c-kit-specific inhibitors may provide significant attenuation of clinical disease during periods of chronic inflammation and remodeling.

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