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Novel Role of Transmembrane SCF for Mast Cell Activation and Eotaxin Production in Mast Cell-Fibroblast Interactions

Cory Hogaboam,* Steven L. Kunkel,* Robert M. Strieter,† Dennis D. Taub,‡ Pam Lincoln,* Theodore J. Standiford,† and Nicholas W. Lukacs* *

Mast cell activation can be induced by multiple mechanisms, including IgE-, complement-, and stem cell factor (SCF)-mediated pathways. In addition, the interaction of mast cells with particular cell populations, such as fibroblasts, have also demonstrated increased mast cell reactivity. In these studies, we have investigated the role of fibroblast-mast cell interaction for induction of histamine release and chemokine production and the specific role of SCF during this interaction. Primary pulmonary fibroblast cell lines were grown in culture and used throughout these studies. Mast cells were grown in parallel with fibroblasts by incubation of bone marrow cells with SCF and IL-3. During mast cell-fibroblast coculture, increased histamine release could be attenuated either by separation of the cell populations using a Trans-Well setup, which did not allow cellular contact, or by specific anti-SCF Ab. In addition, a significant increase in eotaxin, a potent eosinophil-specific C-C chemokine, was also observed during fibroblast-mast cell interaction. The production of eotaxin was cell contact dependent and could be inhibited using an anti-SCF Ab or specific antisense therapy. SCF was constitutively produced from fibroblasts in its transmembrane form and could be induced by TNF. SCF-coated plates induced significant mast cell-derived eotaxin production, whereas soluble SCF induced little or no eotaxin, suggesting a necessity for receptor cross-linking for activation. These studies indicate that fibroblast-mast cell contact plays a role in exacerbation of histamine release and eotaxin production. The Journal of Immunology, 1998, 160: 6166–6171.
production and therefore cause increased leukocyte accumulation at the site of the response. In the present studies, we have used fibroblast-mast cell cocultures to examine the role and production of histamine and eotaxin from the two cell populations. Interestingly, eotaxin was specifically up-regulated during the cocultures by transmembrane SCF, possibly indicating a specific activation role during disease, which relates to eosinophil accumulation.

Materials and Methods

Isolation and expansion of mast cells

Mast cell lines were derived from the bone marrow of pathogen-free CBA/J mice (The Jackson Laboratory, Bar Harbor, ME), which was perfused from femurs (31). The cells were incubated with Dulbecco’s MEM supplemented with 1 mM t-glutamate, 10 mM HEPES, antibiotics, and 10% FCS. After fibroblasts began growing out from the primary lung explants, the tissue pieces were re-suspended in 0.6 M NaCl, 0.26 M H3BO3, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C (in 0.6 M NaCl, 0.26 M H3BO3, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C. The cells were centrifuged (1000 g; 5 min) were coated with 50 μl of a double-ligand method as previously described (32). Briefly, flat-bottomed 96-well plates were coated with 10 μg/ml recombinant mouse SCF and 20 μg/ml anti-SCF Ab for 1 h at 37°C. Nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 1 h at 37°C. Standards were 1/2-log dilutions of recombinant mouse SCF. The specific Ab ELISAs were used at 200 ng/ml or 1000 ng/ml and a nonionic detergent Triton X-100.

Histamine release assays

Histamine levels in supernatants were determined by ELISA (Amac, West- brook, ME). As positive controls, mast cells were exposed to compound 48/80 (Sigma) and a mast cell degranulator, and cells were sonicated to determine total intracellular histamine levels.

Measurement of SCF from pulmonary fibroblasts

Confluent 35-mm plate cultures were coincubated with various cytokines (IL-1, TNF, IL-4, IFN-γ, and IL-10) at various concentrations (0.1, 1.0, and 10 ng/ml). After 24 h, supernatants were harvested, and the adherent fibroblast cells were lifted off the plates using Ca2+- and Mg2+-free HBSS at 4°C for 15 min. Once the fibroblasts were lifted from the culture dishes, the cells were lysed by sonication and the cytoplasmic and membrane portions of the cells were separated by ultracentrifugation (100,000 × g). After separation, the membrane fractions were lysed with PBS containing Triton X-100 (0.1%). The culture supernatant, cytoplasmic, and lysed membrane fractions were all assayed by specific SCF ELISA. No problem was encountered in the ELISA with the nonionic detergent Triton X-100.

mRNA analysis and antisense therapy

Total cellular RNA from the cultures was isolated by homogenizing in a solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-ME. After homogenization, the suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1.0% SDS. The mixture was then extracted twice each with phenol-chloroform and chloroform-isooctanol alcohol. The RNA was alcohol precipitated, and the pellet was dissolved in diethyl pyrocarbonate water.

Histamine levels in supernatants were determined by ELISA (Amac, Westbrook, ME). As positive controls, mast cells were exposed to compound 48/80 (Sigma) and a mast cell degranulator, and cells were sonicated to determine total intracellular histamine levels.

Pulmonary-derived fibroblasts

Whole mouse lungs were excised from a normal CBA/J mouse, cut in 1-mm sections, and washed twice with PBS (pH 7.2). The lung pieces were incubated in 75-mm2 flasks in DMEM supplemented with 1 mM t-glutamine, 10 mM HEPES, antibiotics, and 10% FCS. After fibroblasts began growing out from the primary lung explants, the tissue pieces were removed. The fibroblasts were allowed to grow until near confluency and were then trypsinized (0.25%) off the flask and transferred to a 2 × 150-mm2 culture flask. This was repeated once more before plating the fibroblast cells into 35-mm plates. Fibroblasts were allowed to grow in the culture dishes until they were near confluency. The expansion process required ~28 days, and <1% of passenger cells (i.e., macrophages) were observed by nonspecific esterase stain.

Fibroblast-mast cell cocultures

Cultured mast cell populations (2 × 106) were added to six-well plates with or without confluent primary fibroblast cultures. After 24 h, the culture supernatants were harvested and frozen at -20°C. In some cultures, the cell populations were separated by a 0.45-μm membrane in a Trans-Well culture dish (Costar, Kennebunkport, ME) or coincubated with anti-SCF serum (1:100 dilution). In some experiments, fibroblasts were preincubated with TNF for 6 to 8 h before mast cell coculture.

Production of anti-SCF and anti-eotaxin Abs

Rabbit anti-murine SCF and anti-eotaxin Abs were prepared by multiple-site immunization of New Zealand White rabbits with recombinant murine SCF or eotaxin (Genzyme, Cambridge, MA) in CFA. Polyclonal Abs were titered by direct ELISA and specifically verified by the failure to cross-react with mIL-3, mIL-1α, mTNF, mMIP-1α, hIL-6, hMIF, hMIP-1β, hMCP-1, hIL-8, hRANTES, hMIP-1α, hTNF, and hMIP-1β. The IgG portion of the serum was purified over a protein A column and used in a sandwich ELISA.

ELISAs

Mouse-specific cytokines were quantitated by ELISA using a modification of a double-ligand method as previously described (32). Briefly, flat-bottomed 96-well microtiter plates (ImmuNo-Plate 196-F; Nunc, Roskilde, Denmark) were coated with 50 μl/well of rabbit anti-SCF or anti-eotaxin Ab (in 0.6 M NaCl, 0.26 M H3BO3, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C and then washed with PBS, pH 7.5, and 0.05% Tween-20 (wash buffer). Nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Standards were 1/2-log dilutions of recombinant cytokines from 10 pg/ml to 100 ng/ml. This ELISA method consistently detected cytokine concentrations above 50 pg/ml. The specific Ab ELISAs did not cross-react with each other or with other cytokines or chemokines, including mIL-3, mIL-1α, mTNF, mMIP-1α, IL-6, mIF, mMIP-1β, hMCP-1, hIL-8, hRANTES, hMIP-1α, hTNF, and hMIP-1β.
Degranulation appears to require cell-to-cell contact, as separation of the cell populations with a 0.45 μm Trans-Well culture dish significantly decreased histamine release. Interestingly, there was still a significant histamine release in Trans-Well culture dishes when fibroblasts were present in the lower chamber. Finally, if fibroblasts were incubated with TNF 6 h before the addition of the mast cells and then washed, the histamine release response was significantly augmented. Preactivation of the fibroblasts with other cytokines, including IL-4, IL-1, and IFN-γ, had no effect on histamine release (data not shown).

One of the most important events to occur during infectious diseases is the recruitment of leukocytes. When bone marrow mast cells were combined with the pulmonary fibroblasts, a significant increase in eotaxin, a C-C chemokine family member that can specifically recruit certain leukocyte populations, was observed. The fibroblast-mast cell interaction produced significant increases in levels of eotaxin (Fig. 2) when the cell populations were combined. Preactivation of the fibroblast populations with increasing levels of TNF induced significant increases in the release of eotaxin during cell-to-cell interactions (Fig. 2); however, TNF did not induce eotaxin production in either fibroblasts or mast cells alone (data not shown). Preactivation of the fibroblasts with other cytokines, such as IL-1 and IFN-γ, had no effect on eotaxin production (data not shown). Altogether, these studies verify earlier studies demonstrating that fibroblast-mast cell interaction leads to an activation/degranulation event.

Histamine and eotaxin production is mediated by SCF during fibroblast-mast cell interaction

Previous studies have indicated that fibroblast populations can augment mast cell activation and differentiation. Separation of the two cell populations by a Trans-Well membrane significantly attenuated the eotaxin production (Fig. 3), indicating that cell-to-cell interactions were required. These responses may be exaggerated by the production of a major mast cell growth and survival factor, SCF. Preactivation of the fibroblast with anti-SCF demonstrated that histamine release in 24-h-accumulation cultures could be attenuated (Fig. 1). Likewise, regulation of eotaxin production was

**FIGURE 1.** Induction of histamine release during fibroblast-mast cell interaction is dependent on contact and SCF interaction. Mast cells (1 × 10⁵) were layered onto monolayers of normal lung fibroblasts. Histamine levels were assayed in the 24-h cell-free supernatant. Data represent means ± SE from three repeated experiments. * signifies p < 0.05, and ** signifies p < 0.01 compared with mast cells + fibroblasts.

**FIGURE 2.** Induction of eotaxin during mast cell-fibroblast interaction can be augmented by preincubation of the fibroblasts with TNF-α. Mast cells (1 × 10⁵) were layered onto monolayers of normal lung fibroblasts. Eotaxin levels were measured in 24-h cell-free supernatants using specific ELISAs. Fibroblasts were preincubated overnight with TNF (1.0 or 10 ng/ml) and washed before addition of mast cells. TNF does not up-regulate eotaxin from lung fibroblasts. Data are representative of three repeated experiments. * signifies p < 0.05, and ** signifies p < 0.01.

**FIGURE 3.** Eotaxin production is contact dependent and specifically inhibited by anti-SCF during fibroblast-mast cell interaction. Mast cells (1 × 10⁵) were layered onto monolayers of normal lung fibroblasts. Polyclonal anti-SCF Ab (1:200 dilution) was added to the fibroblast cultures 30 min before the mast cells. Eotaxin was assayed in 24-h culture supernatants. Data represent means ± SE from three different experiments. * signifies p < 0.05 compared with mast cells + fibroblasts + control Ab.
observed by the anti-SCF treatment during the cell-to-cell interaction (Fig. 3). In addition, we have also constructed sense and antisense oligonucleotides specific for SCF and inhibited the production of eotaxin by preincubation (overnight) of fibroblasts with the antisense, but not sense, SCF-specific nucleotides (Fig. 4). The use of the antisense SCF oligonucleotide demonstrated a significant reduction in isolated membrane SCF production by the fibroblasts compared with the sense-treated cells (0.48 ± 0.24 vs 2.0 ± 0.12 ng/ml, respectively). To determine whether SCF on its own was enough to drive eotaxin production, we stimulated mast cells with soluble SCF or by SCF-coated plates to simulate the transmembrane form. The data in Table I indicate that eotaxin was produced only on SCF-coated plates and not by soluble SCF after 24 h of incubation. Interestingly, other degranulating stimuli, such as 48/80 and IgE + Ag, demonstrated no eotaxin production from the mast cells, while they did induce histamine release (15 and 12% of total, respectively) as previously described (34), further demonstrating the importance of SCF-mediated activation in eotaxin production. Interestingly, when soluble SCF was added into the mast cells plated onto the plate-bound cultures, an inhibition of eotaxin production was observed, suggesting a blockage of the cross-linking of c-kit. These data suggest that SCF can drive eotaxin production primarily in its solid phase form and that the different forms of SCF may have specific regulatory functions in chemokine production.

SCF production from fibroblast is predominantly in the transmembrane form

To better define the role of SCF during fibroblast-mast cell interaction, we have examined pulmonary fibroblasts stimulated with various inflammatory stimuli, including IL-1, IL-4, TNF, IFN-γ, and IL-10. After 24 h poststimulation, cell-free culture supernatants were harvested. Sonicated fibroblasts were separated by ultracentrifugation to separate membrane from soluble components. The membrane portion was dispersed with PBS containing 0.1% Triton X-100. Little or no SCF protein was measured in the supernatant fraction of the stimulated fibroblasts (<100 pg/ml). However, nearly all of the detectable SCF was in the membrane fractions of the stimulated fibroblasts. Interestingly, substantial levels of SCF were detected in control fibroblasts (2.0–2.5 ng/ml), while only TNF-stimulated fibroblasts produced increases in membrane SCF (Fig. 5). All of the SCF protein from the sonicated fibroblasts was detected in the membrane fraction, and none was found in the cytoplasmic portion. These data help to explain why cell-to-cell interactions were required above for the SCF-mediated production of eotaxin.

### Table I. Solid phase, but not soluble, SCF induces eotaxin in mast cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Eotaxin</th>
</tr>
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<tbody>
<tr>
<td>Mast cells</td>
<td>&lt;50 pg/ml</td>
</tr>
<tr>
<td>Mast cells + SCF (10 ng/ml)</td>
<td>&lt;50 pg/ml</td>
</tr>
<tr>
<td>Mast cells + SCF (50 ng/ml)</td>
<td>&lt;50 pg/ml</td>
</tr>
<tr>
<td>Mast cells + SCF-coated plates</td>
<td>5.75 ± 2.7 ng/ml</td>
</tr>
<tr>
<td>+ soluble SCF (10 ng/ml)</td>
<td>&lt;50 pg/ml</td>
</tr>
<tr>
<td>Mast cells + IgE + Ag</td>
<td>&lt;50 pg/ml</td>
</tr>
<tr>
<td>Mast cells + 48/80</td>
<td>&lt;50 pg/ml</td>
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![Figure 4](http://www.jimmunol.org)  
**FIGURE 4.** Antisense oligonucleotide specific for SCF blocks eotaxin production during fibroblast-mast cell interaction. Antisense SCF was incubated with fibroblasts 24 h before and during the coculture of mast cells at 20 μM concentration. This concentration demonstrated a >75% reduction in SCF from fibroblasts in these studies. Eotaxin was measured in 24-h culture supernatants. Data represent means ± SE of six repeats. * signifies p < 0.05 compared with mast cells + fibroblasts + sense SCF.

![Figure 5](http://www.jimmunol.org)  
**FIGURE 5.** Transmembrane SCF is up-regulated by TNF-α in pulmonary fibroblasts. Confluent pulmonary fibroblasts were incubated (24 h) with various cytokines (10 ng/ml), and both the culture supernatant and isolated Triton X-100 membrane lysates (see Materials and Methods) were assayed for SCF using a specific ELISA. No SCF has been detected in the culture supernatants from the stimulated pulmonary fibroblasts. Data are from a representative experiment that has been repeated several times with similar results. * signifies p < 0.05.
 mast cell activation, histamine release, and eotaxin production. To verify this aspect, the lung fibroblasts were assayed for SCF-specific mRNA using specific primers that differentially recognize soluble or transmembrane forms of SCF. Both forms of SCF were detectable by reverse transcription-PCR techniques (data not shown). Altogether, it appears that the mast cell activation described above is driven, at least in part, by the transmembrane form of SCF that is found constitutively and can be up-regulated by TNF in normal lung fibroblasts.

Discussion
The persistent activation of mast cells during chronic inflammation may contribute to the pathogenesis of a number of chronic inflammatory disorders. Mast cells participate in several biologic processes, including acute inflammatory and allergic responses, angiogenesis, fibrosis, and leukocyte recruitment. The latter process appears to be one of the most critical events that occur during acute and chronic inflammation. The persistent influx and activation of particular leukocyte populations is thought to promote and maintain chronic allergic and fibrotic disease phenotypes. In the present study, we have examined one possible mechanism of persistent eosinophil recruitment through the production of eotaxin during mast cell-fibroblast interactions. Both of these cell populations have the ability to produce significant levels of eotaxin. However, the fact that these cells may work together to produce synergistic levels of eotaxin during cell-to-cell interaction may help to explain their relationship during disease progression. In addition to the observations that eotaxin and histamine are released during the interaction of the two cell populations, it appears that transmembrane SCF is, in part, responsible for their interaction. This latter observation is supported by earlier investigations that identified SCF as a mediator of mast cell binding to cells and matrix (20, 21). The fact that fibroblast-derived SCF is transmembrane may relate to the close proximity of the two cell populations in tissue and may further suggest an intimate/necessary relationship for mast cell survival. Since SCF is required for long-term mast cell survival (18), the stromal cell populations that surround tissue mast cells may provide a “steady-state” level of transmembrane SCF, which maintains mast cell survival. However, during disease states, when cytokine cascades (TNF) are initiated, the increased expression of SCF (as well as other adhesive interactions) may trigger the mast cell and heighten its activational state. This idea may be indicative of chronic fibrotic diseases in which an increased number of mast cells can be observed within fibrotic lesions (5, 16, 17). Interestingly, we have described a role for SCF in allergic airway disease, in which SCF overproduction appears to augment the response of mast cells and allow persistent and prolonged activation leading to eosinophil accumulation into the airways (36). Eotaxin production, an eosinophil-specific chemotactic factor, was dependent upon SCF during the fibroblast-mast cell interaction in the present studies. Although both cell populations have the ability to produce eotaxin, these studies demonstrate that mast cells can be induced to produce eotaxin by transmembrane SCF. However, previous studies have shown that fibroblasts can also be induced to produce eotaxin only by IL-4 stimulation (our unpublished data). It is conceivable that SCF is activating the mast cell to release products, including IL-4, which conversely activate the fibroblast to increase its production of eotaxin (our studies in progress). Overall, however, these data suggest a regulatory role for SCF in mast cell activation and eotaxin production.

The fact that fibroblast cell populations can produce a distinct form of SCF may indicate a divergence in the function of soluble vs transmembrane SCF. In the present studies, plate-bound, but not soluble, SCF drove eotaxin production. The production of membrane SCF from stromal cell populations may contribute to the normal maintenance and/or to the normal healing process within tissue. The overzealous production of transmembrane SCF may lead to augmented and persistent mast cell activation, eotaxin production, eosinophil recruitment, and tissue pathology. SCF activates mast cells via c-kit, a protein tyrosine kinase-coupled receptor. It appears from the present studies that eotaxin production is dependent upon cross-linking of these receptors in a solid phase. These events would be indicative of the overproduction of eotaxin, leukocyte recruitment and activation, and, finally, tissue pathology. Although there is a paucity of data to support these concepts, the idea of SCF in chronic disease progression has been demonstrated in other investigations. SCF has been linked to eosinophil accumulation not only in allergic models of inflammation (36) but also in infectious parasitic models in which the neutralization of SCF decreased the accumulation of eosinophils, the effector cells in many parasitic infections (37). Thus, although SCF may play a detrimental effect in chronic inflammatory diseases such as asthma, it may be beneficial in infectious parasitic diseases.

The relationship between mast cell activation and eosinophilia repeats itself throughout various types of chronic inflammatory events. In allergic inflammation, the persistent activation of mast cells appears to be a primary event that may directly lead to the accumulation of eosinophils (38). This would be mediated by the level and the type of chemokines that are produced during the mast cell activation. In the most severe cases of allergic asthma, a considerable level of stromal cell proliferation and “airway thickening” is observed within the large airways, where mast cells are predominantly found. The interrelationship between mast cells, transmigrating eosinophils, and the increased numbers of activated stromal cells may contribute to the chronicity and severity of the asthmatic responses. In chronic fibrotic diseases, it has now been reported that a significant influx of eosinophils may participate in the maintenance of the fibrotic response (39, 40). Interestingly, there may be a strong relationship between the severity of the fibrotic event and the number of mast cells and eosinophils that are present. This would be exemplified in these studies by the SCF-dependent expression of eotaxin, a C-C chemokine with potent eosinophil chemoattractant and activating functions (41–43). The persistent activation of mast cells and eotaxin production within these disease states may relate to the constant influx of leukocytes (eosinophils) that appear to mediate the tissue pathology even in the cases in which an Ag stimulus may not be apparent. Although previous studies have not observed eotaxin production from mast cells (44, 45), the transmembrane SCF-induced mechanism was not examined. In addition, there may be an effect due to the maturity of the mast cell population, as studies have shown that a heterogeneous population of mast cells likely exists within any culture of mast cells grown from bone marrow cells (46).

The results from these studies have outlined a novel relationship between mast cell and fibroblast populations. The production of histamine and chemokines during this interaction indicates a possible mechanism for disease progression. Transmembrane SCF may have a role in exacerbation of mast cell activation (histamine release) and eotaxin production, which could subsequently lead to increased eosinophil accumulation and tissue pathology. Future studies will examine the expression of SCF in fibroblast populations from inflammatory and fibrotic lesions, as up-regulated production of SCF may exacerbate and maintain the altered disease status.
References