The Role of Stem Cell Factor (c-kit Ligand) and Inflammatory Cytokines in Pulmonary Mast Cell Activation

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Mast cells play a critical role in allergic airway responses via IgE-specific activation and release of potent inflammatory mediators. In the present study, we have isolated and characterized primary mast cell lines derived from the upper airways of normal mice. The primary mast cell lines were grown and maintained by incubation with interleukin-3 (IL-3) and stem cell factor (SCF) and shown to be c-kit (SCF receptor) positive by flow cytometry. Subsequently, we examined the proliferation of both airway and bone marrow-derived mast cell lines in response to inflammatory and hematopoietic cytokines, including SCF, IL-1, IL-3, interferon-γ, IL-4, and IL-10. The results from the pulmonary mast cell lines were compared with those from bone marrow-derived mast cells. Pulmonary mast cell lines were capable of proliferating in response to IL-3, IL-4, IL-10, and SCF, whereas the combination of SCF with the other cytokines did not increase the response over SCF alone. In contrast, the bone marrow-derived mast cells proliferated strongest to SCF or IL-3, but only modestly to IL-4 and IL-10. Furthermore, the combination of SCF with IL-3, but not the other cytokines, exhibited an increase in bone marrow-derived mast cell proliferation. Cytokine-specific stimulation of histamine release in the airway-derived and bone marrow-derived mast cells showed parallel results. SCF was the only cytokine shown to induce substantial histamine release. However, when certain nonhistamine releasing cytokines were combined with SCF, a synergistic increase in histamine release was induced in upper airway, but not bone marrow-derived mast cells. The results of these studies suggest that cytokines differentially modulate induction of proliferation and degranulation of bone marrow and upper airway-derived mast cells and may further indicate a cytokine activation cascade in tissue mast cells.

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In the present study, we were interested in the response of mast cells isolated from the upper airway of mice in comparison to bone marrow-derived mast cells. Our results suggest a distinction in the proliferative pathways of upper airway-derived cells compared with bone marrow-derived cells. Interestingly, the upper airway-derived mast cells showed the best proliferative response to IL-4 and IL-10 cytokines. This was in contrast to bone marrow-derived mast cells, which proliferated best to SCF and exhibited only modest responses to IL-4 and IL-10. However, our results indicate that SCF can strongly induce histamine release in both pulmonary and bone marrow-derived mast cells. The histamine release response was synergistically increased when combined with other individual cytokines in pulmonary mast cells but not in bone-marrow-derived mast cells. These results indicate that pulmonary mast cell activation may have distinct pathways that control proliferation and degranulation, as compared with bone marrow-derived mast cells.

MATERIALS AND METHODS

Isolation and expansion of pulmonary mast cells. Pulmonary mast cell lines were derived from upper airways from pathogen-free mice that were dissected from perfused lungs. The upper airways were cut into small pieces and incubated with Dulbecco's modified Eagle's medium (MEM) supplemented with 1 mmol/L L-Glu, 10 mmol/L HEPES, antibiotics, and 10% fetal calf serum with IL-3 (10 ng/mL) and SCF (10 ng/mL). The media was changed every other day and transferred to a new flask at the end of each week. By the end of 2 to 3 weeks, a nonadherent population of large granular cells had grown out. These isolated cells appeared homogeneous in cytospin preparations stained by Diff Quik (Baxter, MacGraw Park, IL) with a typical mast cell granular appearance. The homogeneity of these cell lines were determined by flow cytometric analysis of surface markers, by histamine release assays, and by electron microscopy, as described below. These cell lines were routinely expanded, as described above, for 6 to 9 weeks. Similar procedures were used to grow bone marrow-derived mast cells from the femurs of mice.

Flow cytometry. Flow cytometric analysis of mast cell lines was performed to determine purity of the cultures. The staining procedure...
were grown out of upper airways of mice by isolating and culturing minced tissue in the presence of IL-3 (10 ng/mL) and SCF (10 ng/mL). Cells were then examined by electron microscopy to elucidate the cellular purity and verify mast cell morphology (original magnification: (A) x 4,200; (B) x 15,000).

Fig 1. Pulmonary mast cell line morphology. Mast cells were grown out of upper airways of mice by isolating and culturing minced tissue in the presence of IL-3 (10 ng/mL) and SCF (10 ng/mL). Cells were then examined by electron microscopy to elucidate the cellular purity and verify mast cell morphology (original magnification: (A) x 4,200; (B) x 15,000).

was performed on ice in Dulbecco’s phosphate-buffered saline (D-PBS) with 2% fetal bovine serum and 0.1% sodium azide. A total of 1 x 10^6 cells were stained in 100 μL of buffer. Pelleted cells (5 minutes at 1,400 rpm) were then incubated for 30 minutes on ice with specific antibody, anti-CD3, anti-CD4, anti-CD8, anti-B220, anti-CD23, anti-CD11b, anti-c-kit or a subclass control (Pharmingen, San Diego, CA) directly conjugated with fluorescein isothiocyanate. After incubation, an additional 2 mL of cold D-PBS was added and the cells were pelleted by centrifugation (5 minutes at 1,400 rpm at 4°C). The pelleted cells were washed twice with D-PBS and resuspended in 100 μL of 1% paraformaldehyde for 15 minutes. After incubation, the cells were centrifuged with the addition of 2 mL of D-PBS and stored at 4°C in D-PBS containing 0.1% sodium azide until analyzed by flow cytometry. Cells were analyzed within 24 hours of the staining procedure.

Mast cell proliferation assays. Mast cells were plated in 96-well plates at a concentration of 2 x 10^5/well and assessed for cytokine-specific induction of proliferation. The cytokines used included IL-1β, IL-3, IL-4, IL-10, SCF, and various combinations of these cytokines (0.1 to 10 ng/mL). These cytokines were studied because previous observations showed their ability to induce mast cell proliferation.3,9 The mast cells were then incubated for 24 hours at 37°C in 5% CO2 and pulsed with 3H-thymidine (1 μCi/well) for an additional 8 to 12 hours. The plates were then harvested onto glass fiber filters and the 3H-thymidine uptake was determined using a β-scintillation counter to determine proliferation. Each experiment was performed in duplicate for each cell line.

Histamine release assays. Histamine levels in supernatants were determined by enzyme-linked immunosorbent assay (Amac, Inc, Westboro, MA). The pulmonary mast cell lines (2 x 10^5) were preincubated for 30 minutes with serum from Schistosoma mansoni-infected mice that contain high antigen-specific titers of IgE (10^6). After the incubation with various concentrations of this serum, the cells were exposed to the specific schistosome egg antigens (SEA) for 30 minutes and the supernatants were harvested. As positive controls, mast cells were exposed to compound 48/80 (Sigma, St Louis, MO), a mast cell degranulator, and to cells sonicated to determine total intracellular histamine levels.

Mast cell lines were also exposed alone, or in combination, to various cytokines in an effort to determine the role of cytokine activation on histamine release. The mast cell supernatants were harvested 24 hours after the addition of the cytokines and histamine levels were measured. The cytokines (10 ng/mL) used in these cultures were IL-1β, IL-3, IL-4, IL-10, SCF, and interferon-γ (IFN-γ; R&D, Minneapolis, MN).

Statistical analysis. Proliferation and histamine release assays were analyzed by ANOVA and significance was determined with P values < .05.

RESULTS

Characterization of airway-derived mast cell lines. Airway mast cells are believed to be an important initiating cell for the development of allergic pulmonary reactions. To examine their functions, we isolated and grew airway mast cells in tissue culture. Initially, we separated the upper airways from normal pathogen-free mice and exposed the tissue to IL-3 (10 ng/mL) and SCF (10 ng/mL). After 1 week of culture, a population of nonadherent granular cells appeared in culture. Subsequent cell cultures were restimulated with IL-3 and SCF. Interestingly, the long-term cell viability was dependent on the presence of SCF, because culture with only IL-3 showed a cessation of growth. This latter observation was consistent with recent observations that SCF inhibits mast cell apoptosis.16 After 4 weeks in culture the cell population appeared to be homogeneous by electron microscopy showing cells with classical mast cell morphology (Fig 1).

The cell lines were then analyzed for purity by flow cytometric analysis. The results in Fig 2 indicated that the cell line was T-cell marker negative, CD3, 4, 8, as well as B-cell marker negative, B220 and CD23 and CD11b. All of the cells were strongly positive for c-kit (SCF receptor) surface protein. Similar results were obtained with the bone marrow-derived mast cells, indicating that greater than 95% of the cells were c-kit positive. These results indicated that the cells were neither T or B cells and exhibited a high level of expression of the c-kit protein, a major characteristic of mast
cells. In addition, both mast cell types exhibited IgE + antigen-specific histamine release (data not shown).

**Proliferation of mast cell lines.** The signals that drive mast cell proliferation and expansion may be critical for the development of increased numbers of mast cells at local sites of inflammation. To examine the cytokine-induced proliferation responses of the cell populations, 2 x 10⁶ mast cells/well were incubated in 96-well plates for 24 hours in the presence or absence of various cytokines. At the end of the 24 hours, the cell cultures were pulsed with ³H thymidine for 8 to 12 hours and harvested and the incorporated thymidine uptake was counted. SCF, IL-3, IL-4, and IL-10 were all potent inducers of proliferation in the pulmonary mast cell lines (Fig 3), with IL-4 and IL-10 inducing peak proliferative profiles. When SCF was added in combination with IL-3, the proliferation response compared with the individual cytokines was not increased. In bone marrow-derived mast cell lines, SCF induced the strongest proliferative response, followed by IL-3 (Fig 4). IL-4 and IL-10 induced a modest proliferative response. Furthermore, unlike the pulmonary-derived mast cells, SCF in combination with IL-3 induced an increased proliferative response. Although the latter data were consistent with earlier reports, these results overall suggested altered regulation of the proliferative response of upper airway-derived mast cell populations compared with bone marrow-derived mast cells.

**Histamine release of cytokine-activated mast cell lines.** We have also examined the cytokine-specific histamine releasing activity between the cell populations. Airway and bone marrow-derived mast cells (2 x 10⁶) were activated with individual or combinations of cytokines and histamine release examined (Figs 5 and 6). SCF alone (10 ng/mL) induced histamine release in both pulmonary (Fig 5A) and bone marrow (Fig 6A)-derived mast cell lines, whereas IL-1, IL-3, IL-4, IL-10, and IFN-γ (10 ng/mL) induced only minimal histamine release in pulmonary mast cells. Interestingly, IL-3 induced release of histamine in bone marrow-derived mast cells, whereas the rest of the cytokines did not. The concomitant addition of cytokines (IL-3, IL-4, or IL-10) with SCF to upper airway-derived mast cells resulted in a synergistic increase in histamine release with all of the cytokines tested (Fig 5B). In contrast, when concomitant

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![Fig 2. Flow cytometric analysis of the pulmonary mast cell lines. Pulmonary mast cells (1 x 10⁶ cells) were incubated with fluorescein isothiocyanate conjugated antibodies specific for specific cell surface markers. These included control antibody, CD3, CD4, CD8, B220, CD23, CD11b, and c-kit. Mast cells were incubated with the individual antibodies (1:1,000 dilution) for 30 minutes on ice in PBS buffer containing 0.2% sodium azide. Cells were washed and fixed with 2% paraformaldehyde for 5 minutes. Cells were analyzed by FACS within 18 hours of fixation. Data are representative of similar results from three different mast cell lines.](image_url)
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addition of cytokines with SCF were exposed to bone marrow-derived mast cells, a increased release of histamine was not observed as with the airway-derived mast cells. These results indicate that airway-derived mast cells appeared to be more responsive to multiple cytokine stimuli than did bone marrow-derived mast cells.

DISCUSSION

The initiation of allergic airway inflammation is triggered by the antigen-specific activation of mast cells via FeRc bound IgE. The subsequent degranulation of mast cells can cause airway reactivity due to the release of inflammatory mediators, such as histamine, serotonin, PGD2, LTC/D4, LTB4, and PAF. In the present study, we were interested in assessing the cytokine requirements for proliferation and degranulation of mast cells found locally within the lung as well as with bone marrow-derived mast cells. Pulmonary mast cell lines were established from the upper airways of mice using IL-3 and SCF to grow mast cell populations. Mast cells derived in this manner were c-kit positive and showed antigen-induced histamine release. A novel aspect of these studies was the determination of differences between proliferation profiles of bone marrow-derived mast cells and airway-derived mast cells. The bone marrow-derived mast cells were maximally induced when receiving a dual cytokine stimulus, such as SCF and IL-3, which was consistent with earlier reports. In contrast, the airway-derived mast cells in this study proliferated in response to a single stimulus (SCF, IL-3, IL-4, or IL-10) and did not additionally proliferate when exposed to dual stimuli. It was most interesting that TH2 type cytokines, IL-4 and IL-10, induced the highest level of proliferation in airway-derived mast cell. This latter observation may be very significant in asthmatic responses within the lung, which have been shown to be associated with TH2 type responses. In addition, bone marrow-derived mast cells exhibited an augmented cytokine-induced
histamine release. SCF induced the release of histamine from both pulmonary and bone marrow-derived mast cells and this response could be increased by the addition of other cytokines only with the airway-derived mast cells. SCF-induced histamine release has previously been observed in connective tissue-type mast cells, and the augmented release induced by additional cytokines in the present study may prove pertinent during the pathogenesis of a number of pulmonary diseases.

There may be several reasons for the differences observed in pulmonary mast cells as compared with the bone marrow-derived mast cell line proliferation profiles. The most feasible explanation is that the airway-derived mast cells were presumably established from mature mast cell lineages, whereas bone marrow cell lines are initiated from stem cells and may be comparably immature. An alternative explanation may entail the contact with additional signals of activation in tissue-derived mast cells, such as interactions with other cell types or matrix proteins. These "mature" mast cells may be affected by multiple mechanisms, depending on soluble and cellular signals to which they are exposed. Finally, these "mature" cell types may be stimulated via alternate pathways of activation. These pathways have been exhibited in previous studies showing a requirement for additional signals induced by contact with other cells and/or matrix proteins. The examination of intracellular signaling pathways may elucidate the specific signals induced within mast cells during cellular contact.

The observations made in this study suggest that a number of different cytokines may induce proliferation of mast cells within tissue. Interestingly, the cytokines that consistently induced the best proliferative response with the airway-derived mast cells were IL-4 and IL-10. These TH2 type cytokines have been implicated in allergic diseases, such as asthma, and can be released from mast cells during degranulation events. Attractively, IL-10, which has previously displayed suppressive effects on cytokine production, may be a primary activator of mast cell proliferation and degranulation. This mechanism may contribute to increased mast cell proliferation locally during inflammatory states.

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**Fig 4.** Cytokine-induced proliferation of bone marrow-derived mast cells. Mast cells (2 x 10^5/well) were incubated in 96-well plates with or without various cytokines. IL-3, IL-4, IL-10, SCF, IFN-γ, or IL-1 (0.1 to 10 ng/mL) was used to stimulate the mast cells for proliferation. After 24 hours, cells were pulsed with ^3H-thymidine, harvested after 6 hours, and counted by β-scintillation. Data represent the mean ± SE of a single cell line. Repeat experiments from four other mast cell lines show similar patterns of proliferation. *P < .05; **P < .01.
responses. Asthmatic reactions may be attributed to both the local proliferation of resident mast cells as well as to migration of basophils from the periphery into the inflamed tissue. Furthermore, because the mast cell degranulation can be induced by SCF and augmented by other cytokines, mast cells may contribute to the pathogenesis of a number of pulmonary diseases such as idiopathic pulmonary fibrosis and adult respiratory syndrome. These diseases do not appear to have significantly elevated IgE present for an antigen-specific mast cell activation and have relatively undefined effector pathways. Investigation into the involvement of mast cells in these disease states may yield useful information and new avenues of treatment.

The findings of this manuscript suggest a difference in the activational pattern for activation in pulmonary-derived mast cells compared with bone marrow-derived mast cells. In addition, these studies have outlined a cytokine-mediated mechanism of histamine release that is dependent on SCF-induced activation of mast cells and can be further augmented by additional cytokine stimulation. Data indicate that cytokine-mediated mechanisms of activation lead to mast cell proliferation and degranulation, suggesting mast cells may be involved in a number of nonallergic pulmonary diseases.

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