Stem Cell Factor Influences Mast Cell Mediator Release in Response to Eosinophil-Derived Granule Major Basic Protein

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Stem cell factor (SCF) is an important mast cell growth, differentiation, and survival factor. We investigated whether SCF influenced the response of mouse mast cells to an IgE-independent stimulus, eosinophil-derived granule major basic protein (MBP). Mouse bone marrow cultured mast cells (BMCMC) were derived in either concanavalin-stimulated mouse spleen conditioned medium (CM) or SCF. The cloned growth, factor-independent mast cell line Cl.MC/C57.1 was also studied. BMCMC in SCF exhibited cytochemical staining properties, pro tease and histamine content, and increased serotonin uptake consistent with more mature differentiated mast cells as compared with BMCMC in CM or Cl.MC/C57.1 cells. BMCMC in SCF released serotonin, 3H-labeled arachidonic acid metabolites and tumor necrosis factor-α (TNF-α) on stimulation with MBP, while no response was seen from either BMCMC in CM or Cl.MC/C57.1 cells. All three mast cell populations released mediators on stimulation with the cationic MBP analog, poly-L-arginine, indicating that the cationic charge did not explain the selective response of BMCMC in SCF to eosinophil-derived granule MBP. These findings show that SCF significantly influences mast cell differentiation and the responsiveness of mast cells to eosinophil-derived granule MBP.

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MATERIALS AND METHODS

Cells. Studies were conducted with three different populations of murine mast cells. A cloned, growth factor–independent mast cell line (CLM/C57.1) that was originally derived from BMCMC isolated from BALB/c mice13 was maintained in Dulbecco’s modified Eagle’s medium (GIBCO, Grand Island, NY) with 10% heat-inactivated fetal calf serum (Intergen, Purchase, NY), 50 µmol/L 2-mercaptoethanol, and 2 mmol/L L-glutamine at 37°C in 5% CO2. In addition, femoral bone marrow cells were obtained from BALB/c mice and maintained in medium as above, but supplemented with either Concanavalin A–stimulated mouse spleen cell-conditioned medium (BMCMC in conditioned medium [CM]), as previously described,14 or recombinant rat SCF15 (50 ng/mL). Mast cells derived in 20% WEHI–3-conditioned medium (a source of IL-3) were also examined. After 4 to 6 weeks, mast cells represented more than 98% of the total cells as determined by neutral red staining.

Histochemical analysis and histamine content of different mast cell populations. The different mast cell populations were washed and suspended at 3 × 106 cells/mL in their respective media. Cytocentrifuge slides were prepared from each mast cell population and stained in 0.5% alcian blue (Rowley Biochemical Institute, Rowley, MA), pH 0.1 at 60°C for 10 minutes. Slides were then immediately counterstained in 0.1% safranin (Rowley Biochemical Institute) for 5 minutes at room temperature. The slides were then rinsed in water, 95% ethanol, 100% ethanol, and xylene.

One-milliliter aliquots (3 × 106 mast cells) were taken and centrifuged at 800 rpm for 10 minutes. Pellets and supernatants were taken for determination of specific histamine release as previously described.15

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mast cell protease expression. Total RNA was isolated from mast cells using UltraScript RNA (Biotech Laboratories Inc, Houston, TX) and treated with 1 U of Heparinase I (Sigma Chemical Co, St Louis, MO) per microgram RNA and 40 U of RNase inhibitor (Sigma Chemical Co) at 25°C for 2 hours. One microgram of total RNA from each sample was reverse transcribed with oligo dT and then 1/20 of this product was amplified with 100 pmol of primers for individual mast cell proteases in 50 µL of PCR Supermix (GIBCO-BRL, Grand Island, NY). The primer sequences for murine mast cell protease 2 (mMCP2), murine mast cell protease 4 (mMCP4), and murine mast cell carboxypeptidase A (mMCCPA) were as follows: 5'-GTGATGACTGCTGGGACTCTG, 5'-GTAATCTCTCCTGCTCTCCT, 5'-ACCCAGGGTTACTCAGAGCTC, 5'-CTTGAAGAGTCTGACTCAGG, 5'-ACACAGGATCGAA TG TGGAG; 3'-GGTCTTTCTTCTACACATGC, 3'-GGTCTTTCTTCTACACATGC, 3'-GGTCTTTCTTCTACACATGC, respectively. PCR was performed as follows: 94°C, 3 minutes; 30 cycles of 94°C, 1 minute; 52°C, 2 minutes; 72°C, 2 minutes; 72°C, 8 minutes. The PCR products were identified by electrophoresis in a 1.5% agarose gel and verified by molecular size.

Isolation of human eosinophils and purification of MBP. Human eosinophils and eosinophil granule proteins were isolated as previously described.16 Briefly, whole blood was obtained from healthy and hyper eosinophilic donors in the Clinical Research Center at the Beth Israel Deaconess Medical Center (Boston, MA). Venipuncture procedures were approved by and performed within the guidelines of the Institutional Care and Use Committee at the Beth Israel Deaconess Medical Center. Theuffy coat was collected and separated by density centrifugation in Ficoll-Paque (Pharmacia, Piscataway, NJ). Red blood cells were lysed in NH4Cl, and the remaining granulocytes were washed in phosphate-buffered saline (PBS) with 2% fetal bovine serum, and counted. Eosinophils were then isolated by negative selection using the magnetic cell separation system.17 The granulocytes were incubated with CD 16 antibodies conjugated to magnetic particles (50 µL of particles per 5 × 107 cells) for 30 minutes at 6°C, then passed through a separation syringe containing iron fibers. Eosinophil number and purity were assessed by Randolph’s stain with eosinophil preparations of greater than 95% purity typically obtained. The eosinophils were lysed in hypertonic sucrose (0.25 mol/L) and centrifuged at 400g for 10 minutes. The cellular lysates were then centrifuged at 13,000g for 20 minutes to pellet eosinophil granules. The granules were lysed in 10 mmol/L HCl and the lysates were fractionated by column chromatography using Sephadex G-50 (Pharmacia) equilibrated with 0.025 mol/L acetate buffer (pH 4.3), 0.15 mol/L NaCl. MBP was identified by its distinctive chromatographic filtration pattern. The purity of MBP was assessed by protein gel electrophoresis and the concentration determined by spectrophotometric absorbance at 277 nm.16

The effects of MBP or poly-L-arginine on the release of 3H-5-hydroxytryptamine from murine mast cells. The effect of either MBP or poly-L-arginine, a highly charged synthetic analog of eosinophil-derived granule proteins,18 on mast cell mediator release was determined using a 3H-5-hydroxytryptamine (3H-SHT) release assay, as previously described.19 Briefly, mast cells were incubated with 1 mCi/mL of [5,12-3H](N)-hydroxytryptamine creatine sulfate (NEN Dupont, Boston, MA) for 2 hours. The cells were washed three times and then challenged with their respective media alone (unstimulated) or different concentrations of either MBP or poly-L-arginine (Sigma Chemical Co) for 10 minutes at 37°C. Each mast cell population was maintained in its respective medium throughout the incubation, wash, and challenge periods. The cells were then centrifuged at 1,000 rpm for 10 minutes. Radioactive counts were measured in the supernatants and pellets, and the percentage of specific 3H-SHT release was calculated as follows: % Specific Release = 100 × [(cpm Stimulated Supernatants/cpm Stimulated Cells + Stimulated Supernatants) − (cpm Unstimulated Supernatants/cpm Unstimulated Cells + Unstimulated Supernatants)]. In some experiments after 3H-SHT incubation, mast cells were centrifuged and radioactive counts determined as a measure of total serotonin uptake.

Effects of MBP, poly-L-arginine, or IgE/antigen on arachidonic acid mediator release from BMCMC in SCF. We also examined the effects of MBP or poly-L-arginine on the release of 14C-labeled arachidonic acid from BMC MC in SCF as previously described.20 The mast cells were incubated with 14C arachidonic acid (1 mCi/mL) for 12 hours at 37°C. In some experiments, murine monoclonal IgE antidi nitrophenyl (DNP) antibodies (3 µg/mL) were added for the final 2 hours of incubation. The cells were washed three times, then incubated with different concentrations of either MBP, poly-arginine, 20 ng/mL DNP30-40-human serum albumin (Sigma Chemical Co), or calcium ionophore (Sigma Chemical Co) for 10 minutes. Radioactive counts were measured in the supernatants and pellets and the percent specific release of 14C-labeled arachidonic acid was calculated as follows: % Specific Release = 100 × [(cpm Stimulated Supernatants/cpm Stimulated Cells + Stimulated Supernatants) − (cpm Unstimulated Supernatants/cpm Unstimulated Cells + Unstimulated Supernatants)].

Effects of MBP or poly-L-arginine on the production of TNF-α by mast cells. Mast cells were incubated with medium alone or different concentrations of poly-L-arginine or MBP for 30 minutes at 37°C. The supernatants were collected and immediately stored at −80°C. Immuno reactive TNF-α protein was measured by enzyme-linked immunosorbent assay (ELISA) (Endogen, Woburn, MA).

Statistical analysis. The results of differences in TNF-α production between the MBP or poly-L-arginine and unstimulated cells or specific 3H-SHT release from different mast cell populations were analyzed for statistical significance (defined as P < .05) by the Student’s t-test (two-tailed).

RESULTS

BMCMC in SCF exhibit positive staining with safranin, mCCP4, mCCP-2, and mCCP-4 protease expression, increased histamine content, and increased uptake of 3H- serotonin. We initially compared the phenotypic characteristics of BMCMC grown in SCF as the only exogenous growth factor

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BMCMC in SCF) with BMCMC derived in Concanavalin A-stimulated spleen conditioned medium, a source of multiple growth factors (BMCMC in CM), or a cloned, growth factor-independent mast cell line, Cl.MC/C57.1. We used a histochemical approach for defining mast cell heterogeneity by comparing the staining characteristics with alcian blue and safranin. Such an approach can identify mucosal-type mast cells by their cytoplasmatic staining with alcian blue and their absence of cytoplasmatic staining with safranin, while connective tissue-type mast cells exhibited strong cytoplasmatic staining with safranin. We found that BMCMC in CM (Fig 1A) or conditioned medium and Cl.MC/C57.1 mast cells (not shown) exhibited cytoplasmatic staining with alcian blue, but failed to stain with safranin. In contrast, BMCMC in SCF exhibited heterogeneous cytoplasmatic staining characteristics (Fig 1B). The majority of cells exhibited mixed alcian blue and safranin staining of their cytoplasmic granules (55% ± 6% of total mast cells), while cells with either predominantly alcian blue cytoplasmatic staining cells or predominantly safranin staining accounted for the remainder of the cells (25% ± 5% and 20% ± 4%, respectively).

Mast cell heterogeneity has also been defined by the expression of various neutral proteases. Therefore, we examined whether the differences in histochemical staining characteristics between BMCMC in SCF and BMCMC in CM were accompanied by changes in protease gene expression. We found that BMCMC in CM expressed mCCPA and mMCP-2, but not mMCP-4 (Fig 2A), consistent with previous reports. In contrast, BMCMC in SCF expressed mCCPA, mMCP-2, and mMCP-4 (Fig 2A). Thus, in addition to the changes in histochemical staining properties, BMCMC in SCF also exhibited a change in protease gene expression.

We also determined the histamine content of these mast cell populations and the ability of the different mast cell populations to take up exogenous 5-hydroxytryptamine (5-HT). BMCMC in CM and the Cl.MC/C57.1 line contained approximately 0.1 pg of histamine per cell, which is consistent with previous reports, but the BMCMC in SCF contained approximately 25 times more histamine on a per cell basis (Fig 2B). In addition, we found that BMCMC in SCF took up approximately 10 times the amount of 5-HT compared with either BMCMC in CM or the Cl.MC/C57.1 line (Fig 2C). The increased histamine content and uptake of exogenous 5-HT exhibited by BMCMC in SCF are consistent with a maturational/phenotypical change in the cells, as previously reported for BMCMC in CM that had been changed and maintained in medium with SCF as the only exogenous growth factor for 4 to 6 weeks.

MBP induces 5-HT release from mouse mast cells grown in SCF. We next examined the ability of eosinophil granule MBP to induce mast cell degranulation and preformed mediator release from the different mast cell populations. MBP elicited a dose-dependent release of 5-HT from BMCMC in SCF, but had no effect on either the Cl.MC/C57.1 line, BMCMC in CM (Fig 3A), or BMCMC derived in WEHI-3-conditioned medium, a source of IL-3 (not shown). Cell viability, as determined by Trypan blue exclusion, was not significantly changed in BMCMC in SCF challenged with MBP compared with cells challenged with a similar dilution of column elution buffer (98% vs 96%, respectively, at the highest concentration of MBP). Thus, MBP, at the concentrations used, had no inherent cytotoxic effects on any of the mast cell populations examined.

Poly-L-arginine stimulates the release of 5-HT from mouse mast cells. MBP has a high content of arginine residues (15%) in its amino acid sequence that results in a highly charged cationic protein. Several laboratories have reported that some of the stimulatory effects of MBP are related to its charge and these effects can be imitated by poly-L-arginine, a highly charged cationic analog of MBP. Therefore, we examined the effect of poly-L-arginine on mast cell mediator release.

In contrast to the response with MBP, all three populations of
murine mast cells examined released [3H]-5HT after stimulation with poly-L-arginine (Fig 3B), and there was no adverse effect on mast cell viability (data not shown). The concentration of poly-L-arginine necessary to elicit 5-HT release was high (10^{-2} mol/L) and there appeared to be a threshold effect in that almost no specific serotonin release occurred at lower concentrations. Nevertheless, this effect of poly-L-arginine was seen in each of the mast cell populations tested (Fig 3B).

MBP and poly-L-arginine stimulates the release of arachidonic acid metabolites and TNF-α from mast cells grown in SCF. In addition to preformed mediators such as serotonin and histamine, immunologically stimulated murine mast cells can generate products of arachidonic acid metabolism and a number of multifunctional cytokines. Therefore, we investigated the effects of MBP on the release of newly synthesized arachidonic acid mediators and cytokine production. Like the histochemical and biochemical heterogeneity exhibited by mast cells, the pathways of arachidonic acid metabolism used by mast cells is characteristic of different mast cell populations. Mucosal mast cells use the lipoxygenase pathway and produce mainly leukotriene C_4, while the cyclooxygenase pathway predominates in connective tissue-type mast cells, resulting in the production of prostaglandin D_2. To define whether arachidonic acid metabolites derived from either pathway were produced, we prelabeled BMCMC with [14C]-arachidonic acid and determined if MBP or poly-L-arginine elicited the release of arachidonic acid mediators. We found that MBP induced a dose-dependent release of [14C]-arachidonic acid from BMCMC in SCF (Fig 4). MBP was more effective than poly-L-arginine in eliciting [14C]-arachidonic acid release (Fig 4). The release of [14C]-arachidonic acid from BMCMC in SCF by MBP at 10^{-7} mol/L was similar to that seen when the cells were sensitized with IgE anti-DNP antibodies and challenged with 20 ng/mL of DNP-30-40-human serum albumin (25.5% ± 0.2% specific release) or stimulated with calcium ionophore at 4 µmol/L (28.1% ± 2% specific release).

We also examined the effect of MBP or poly-L-arginine on the production of TNF-α in all three mast cell populations. We found that MBP induced significant TNF-α release from BMCMC in SCF, but had little effect on TNF-α production in either BMCMC in CM or the Cl.MC/C57.1 cell line (Fig 5A). In contrast, poly-L-arginine induced the production of TNF-α in all three mast cell populations (Fig 5B).

**DISCUSSION**

The mechanisms involved in mast cell activation by stimuli other than IgE antibodies have not been completely defined. We found that SCF is an important factor in the development of mast cell responsiveness to the eosinophil granule-associated protein MBP. Now, in addition to its role as a growth and survival factor for mast cells in vitro and in vivo, our study shows that SCF also influences mast cell/eosinophil interactions.

The population of mast cells derived in SCF from the bone marrow of BALB/c mice had several phenotypical characteristics of mature connective tissue-type mast cells. The mast cells exhibited positive staining for safranin, increased histamine content, increased uptake of 5-HT compared with the negative safranin staining, low histamine and 5-HT uptake typically seen in BMCMC in CM. These findings are similar to a previous report in which BMCMC in CM were transferred into medium containing SCF as the only exogenous growth factor. After 4 weeks in culture, the mast cells exhibited alterations in their phenotype similar to the BMCMC in CM, but not identical to peritoneal mast cells. However, in this study, mast cell function was not examined.

As well, we found that BMCMC in SCF expressed the protease mMCP-4, which is typically found in connective tissue-type mast cells and not mucosal-type mast cells. Thus, phenotypically mature mast cells could be derived directly from mouse bone marrow cells using soluble SCF as the only exogenous growth factor. However, it is important to
note that while these cells had characteristics similar to mature connective tissue-type mast cells, BMCMC in SCF represent a heterogeneous population of mast cells. Thus, BMCMC in SCF are similar, but not identical to connective tissue mast cells derived from the peritoneal cavity of mice.

We found that MBP elicited the release of a mast cell granule-associated mediator (serotonin) and newly synthesized mediators (arachidonic acid metabolites) from BMCMC in SCF. In addition, MBP induced significant TNF-α production by BMCMC in SCF. These responses did not occur in BMCMC in CM. Thus, in addition to the biochemical and morphological changes seen in BMCMC in SCF, the mast cells also underwent a functional change in their responsiveness to MBP. Ogasawara, et al. reported that the coculture of BM-derived mast cells with Swiss 3T3 fibroblasts for 4 to 6 days resulted in the cells acquiring responsiveness to polycationic activators, such as compound 48/80 or substance P, but that short-term culture with soluble SCF (6 days) did not confer responsiveness to compound 48/80. We have found that BMCMC in CM that were switched into SCF-containing medium and maintained for 6 weeks acquired responsiveness to substance P also release mediators in response to challenge with substance P (manuscript in preparation). Thus, SCF either as a soluble dimer and, presumably in its membrane bound form associated with fibroblasts or smooth muscle cells, can alter mast cell responses to certain agents that activate mast cells.

MBP is thought to damage cell membranes and is a toxin to helminths and mammalian cells in vitro. But in our study, MBP did not affect mast cell viability. This is similar to other reports demonstrating noncytolytic mediator release from resident mast cells and human basophils by MBP. It has been suggested that the effects of MBP are the result, at least in part, of the high cationic charge of MBP. However, cationic charge alone cannot explain the effects of MBP on mast cells. If cationic charge only was responsible for mast cell activation, then MBP would be predicted to elicit mediator release from each of the different mast cell populations examined. However, we found that MBP induced mediator release only from BMCMC in SCF and not BMCMC in CM or C1.MC/C57.1 cells. This is in contrast to a synthetic MBP analog, poly-L-arginine, which elicited mediator release from all populations. This suggests that poly-L-arginine is not an ideal alternative to

**Fig 3.** Effect of MBP on [³H-5HT] release in different populations of murine mast cells. BMCMC grown in stem cell factor (BMCMC in SCF), BMCMC grown in Concanavalin A stimulated spleen conditioned medium (BMCMC in CM), or the growth factor independent cloned mast cell line, C1.MC/C57.1, was incubated with [³H-5HT] for 2 hours, washed and then challenged with different concentrations of MBP (A) or poly-L-arginine (B) for 10 minutes. Specific 5-HT release was calculated as described in Materials and Methods. Results are expressed as mean ± SD (n = 3). ***P < .001 by the two-tailed paired Student’s t-test. Similar results were obtained in a repeat experiment.

**Fig 4.** Effect of MBP or poly-L-arginine on [¹⁴C arachidonic acid release from BMCMC maintained in SCF. BMCMC grown in SCF (BMCMC in SCF) were incubated with [¹⁴C arachidonic acid for 12 hours. BMCMC in SCF were incubated with different concentrations of MBP or poly-L-arginine for 10 minutes. The data are expressed as mean ± SD (n = 3). Specific arachidonic acid release was calculated as described in Materials and Methods.
The two-tailed paired Student's t-test.

The data are expressed as mean ± SD (n = 3). ***P < 0.05 by the two-tailed paired Student's t-test.

Previous studies have shown that MBP stimulates the preformed mediator release from connective tissue-type mast cells isolated from the peritoneal cavity of rats. Our findings show that SCF is an important factor in the development of mast cell responsiveness to MBP. At this time, it is not clear whether SCF induces the growth of a population of mast cells that respond to MBP or, alternatively, induces a direct change in MBP unresponsive mast cells that leads to responsiveness, such as by the induction of an as yet unidentified MBP receptor or receptor-like complex. We favor the former hypothesis, because BMCMC in SCF after 3 weeks in culture (mast cells representing >75% of the total cell population) did not release serotonin to challenge with MBP (data not shown). These findings suggest that SCF is not simply facilitating the action of MBP, but may, in fact, be driving the proliferation and differentiation of a population of mast cells that have the capacity to respond to MBP.

The findings of our study may have important implications regarding mast cell/ eosinophil interactions in vivo. As previously discussed, SCF has important effects on mast cell growth and differentiation. The responsiveness of mast cell populations under the influence of SCF to eosinophil-derived granule proteins may be important in inflammatory and allergic reactions. For example, MBP-induced mast cell degranulation may lead to an amplification of the inflammatory response through the generation of preformed and newly synthesized mediators by mast cells. As well, granule release by mast cells may promote the downregulation of the inflammatory response by the release of heparin from connective tissue-type mast cells, which has been shown to neutralize the biological activity of MBP. Thus, the ability of mast cells to release mediators in response to MBP may have multiple, complex biological consequences.

In conclusion, our study shows that BMCMC in SCF express phenotypic characteristics similar to mature connective tissue mast cells as defined by histochemical staining, protease expression, histamine content, and 5-HT uptake. BMCMC in SCF release preformed 5-HT, products of arachidonic acid metabolism, and TNF-α in response to MBP. These findings suggest that SCF is an important factor in the development of a functional response of mast cells to eosinophil-derived granule MBP.

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