Non-IgE dependent activation of human lung and cord blood-derived mast cells is induced by eosinophil major basic protein and modulated by the membrane form of stem cell factor

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Running title: Major basic protein induces human mast cell activation

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Abstract

The allergic reaction begins with the antigen-induced aggregation of occupied high affinity IgE receptors expressed on mast cell surface, their activation, and the release of pro-inflammatory mediators that cause the “early phase” of this process. In addition, mast cell activation induces the onset of a “late phase” reaction characterized by the tissue infiltration of inflammatory cells, mainly eosinophils. We have hypothesized that during the late phase mast cells interact with and are activated by eosinophils. Here we report that highly purified human lung mast cells became responsive to eosinophil major basic protein (MBP) when in co-culture with human lung fibroblasts. In addition, cord blood derived-mast cells maintained in co-culture with 3T3 fibroblasts released more histamine and prostaglandin (PG)-D₂ in comparison to cells maintained in suspension. The fibroblast-derived membrane form of stem cell factor (SCF) was found to be involved in the mast cell increased responsiveness to MBP. In fact, cord blood-derived mast cells cocultured with 3T3 in the presence of antisense for SCF or co-cultured with fibroblasts that do not express the membrane form of SCF were inhibited in their histamine releasing activity towards MBP. In addition, this form of SCF induced the expression of a pertussis toxin (Ptx)-sensitive G_i protein, G_i3 that interacts with MBP to trigger mast cell non-IgE dependent activation in a manner similar to other cationic compounds such as compound 48/80. Mast cell responsiveness to eosinophil mediators is potentially novel evidence for an alternative pathway of allergen-independent activation able to contribute to the perpetuation of allergy.
Introduction

The allergic process is initiated when antigen causes the aggregation of occupied high affinity IgE receptors (FcεRI) expressed on the mast cell surface. This event is followed by the immediate release of pro-inflammatory mediators that contribute to the early phase of the allergic process. In addition, mast cell degranulation induces the onset of a second “late phase” reaction, four to eight hours later, characterized by the tissue infiltration of inflammatory cells, mainly eosinophils. We have hypothesized that during the late phase of the allergic reaction mast cells interact with and are activated by eosinophils. In fact, we have shown that antigen-challenged rat peritoneal mast cells release histamine following incubation with the eosinophil mediator MBP by a mechanism similar to that of IgE-independent stimuli induced by cationic compounds such as compound 48/80 and substance P. The importance of mast cell activation induced by eosinophil mediators has been underappreciated because isolated human lung and skin-derived mast cells do not release histamine upon incubation with MBP. According to their protease content, human lung mast cells belong to the tryptase positive subtype of mast cells (MC\textsubscript{T}). In contrast to the tryptase and chymase positive subtype of mast cells (MC\textsubscript{TC}) found for example in the skin and intestinal submucosa, MC\textsubscript{T} are well-known for their unresponsiveness to non-IgE dependent activation. However, mast cell populations can express significant variation in numbers, phenotype and/or function, in different anatomical locations and even in the same tissue under the microenvironment influence, particularly during the course of inflammatory responses. Therefore, we believe that analysis of a particular mast cell population \textit{in vitro}
may not necessarily reflect its behavior in vivo. Indeed, we have demonstrated that the co-culture of rat or human lung mast cells with 3T3 fibroblasts, a system that mimics mast cell microenvironment in vivo, prolongs mast cell survival and increases mast cell responsiveness to IgE-dependent activation.\textsuperscript{8,9} In addition, mouse bone marrow-derived mast cells, the rodent counterpart of human MC\textsubscript{T}, acquired responsiveness to IgE-independent stimuli when in co-culture with 3T3 fibroblasts.\textsuperscript{10} Moreover, under these conditions, bone marrow-derived mast cells changed their phenotype towards connective tissue mast cells as exemplified by the increase in their histamine content and a marked increase in proteoglycans biosynthesis that bear heparin.\textsuperscript{11}

In the present study we investigated whether highly purified human lung mast cells in co-culture with human lung fibroblasts become responsive to MBP. Mast cell responsiveness to eosinophil mediators is potentially novel evidence for an alternative pathway of allergen-independent activation able to contribute to disease. We also compared the susceptibility of cord blood-derived mast cells to IgE-independent activation in suspension and in co-culture conditions, and we defined a novel role for the fibroblast-derived membrane SCF in this effect.
Materials and methods

Human cord blood-derived mast cells

Mononuclear cells were isolated from umbilical cord blood as previously described. Cells were seeded at $10^6$ cells/ml of MEM-α medium containing 10% v/v heat inactivated fetal calf serum (FCS), 100 u/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 10 µg/ml ribonucleases (Biological Industries, Beith Haemek, Israel), 100 ng/ml SCF (a generous gift from Amgen, Thousand Oaks, CA), 10 ng/ml IL-6 (PeproTech, Rocky Hill, NJ), and $3 \times 10^{-7}$ M PGE$_2$ (Sigma Chemicals, St Louis, MO). Half of the culture medium was replaced every week. Cord blood-derived mast cells were harvested for the experiments between 6-9 wk of culture when more than 95% of the cells were stained metachromatically with toluidine blue and positive stained for both tryptase and chymase as assessed by flow cytometry.

Human lung mast cell isolation and purification

Human lung mast cells were isolated from normal tissue of patients undergoing surgery for lung emphysema and carcinoma using an enzymatic method. Human lung mast cells were purified up to 90% via selection of c-kit positive cells by magnetic cell separation (Dynalbeads, Oslo, Norway). Human lung mast cells were cultured at a concentration of $10^6$/ml in Dulbecco’s Modified Eagle Medium (DMEM) (Biological Industries) containing 10% v/v FCS, 100 u/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (DMEM+) and supplemented with 100 ng/ml SCF.

Written consent was obtained from all the volunteers according to the
guidelines established by the Tel Hashomer (cord blood) and Assaf Harofeh (lung biopsies) Medical Center Human Experimentation Helsinki Committees, Israel.

**Rat peritoneal mast cell isolation and purification**

Rat peritoneal mast cells were obtained from Sabra rats by peritoneal lavage and purified as previously described (metrizamide gradient, 22.5%, >96% purity). The rats were cared for according to the Guidelines of the Animal Committee of the Hebrew University of Jerusalem, Israel.

**Human mast cell-fibroblast co-cultures**

Human lung and cord blood-derived mast cells were seeded on confluent monolayers of the human lung fibroblast cell line (MRC 5) and Swiss albino mouse embryonic 3T3 fibroblast cell line (3T3) (American Type Culture Collection, Rockville, MD), respectively, in 24 wells at a density of 5 x 10^4/0.5 ml DMEM+. For some experiments cord blood-derived mast cells were seeded on either Transwell membranes (0.4-μm pore size; Nunc) to separate them from 3T3 fibroblasts; or embryonic fibroblasts from Sl/Sld mutant mice (provided by Dr. T. Jippo, Dept. of Pathology, Osaka University Medical School Japan), or 3T3 fibroblasts treated o.n with SCF sense and antisense (see below). Culture media of cord blood-derived mast cells was always supplemented with 100 ng/ml SCF. Human mast cells were maintained in co-culture for 4 days prior to activation. This co-culture time was determined as optimal in preliminary kinetics experiments.
SCF antisense treatment of 3T3 fibroblasts and human mast cell-fibroblast co-cultures

Oligodeoxynucleotides end-capped with 2'- O -methyl RNA substitutions at three 3'-terminal positions were used at 0.1 pM by pre-incubating 3T3 fibroblast o.n before and during cord blood-derived mast cell co-culture. This antisense concentration was shown to significantly inhibit the level of membrane SCF protein as assessed by confocal microscopy. Sense oligonucleotide at the same concentration did not affect SCF expression. Sequences for sense and antisense oligodeoxynucleotides have been previously published elsewhere. During antisense therapy, 100 ng/ml SCF was added to cord blood-derived mast cells cocultured with 3T3 fibroblasts to support cord blood-derived mast cell viability. Therefore, in these cultures only the influence of the membrane form of SCF on cord blood-derived mast cells was selectively suppressed.

Purification of human eosinophil major basic protein

MBP was purified from eosinophils obtained from patients with marked eosinophilia as described. The purified protein was stored at -70° C, and samples were thawed immediately before use. MBP was judged pure by its banding pattern on SDS-PAGE after staining with Coomassie brilliant blue R. Protein concentrations were determined using the appropriate E277 values.

Human mast cell activation and mediator assays

Cultures were incubated in Tyrode’s buffer containing 0.1 % gelatin (Tg), 1.8 mM CaCl₂, 0.9 mM MgCl₂ (Tg++) for 30 min with one of the following: 2 μg/ml
anti-FcεRI-α chain (22E7) (kindly provided by Dr. J.P. Kochan, Hoffman La Roche, Nutley, NJ); 2, 5 and 10 µg/ml compound 48/80 (Sigma Chemicals, Israel); MBP at 0.001, 0.01, 0.1, 0.1 and 10 µM for human lung mast cells, and at 0.001, 0.01, 0.1, 1, 2 and 5 µM for cord blood-derived mast cells. To assess the effects of Ptx, cultures were incubated for 2 hrs in Tg++ containing 1 µg/ml Ptx (Sigma) and then activated as described. Histamine was measured in the culture supernatants and in sonicated cells by a radioenzymatic assay. Histamine release was calculated as a percentage as follows: histamine in supernatant/(histamine in supernatant + histamine in cells) x 100. Cysteinyl-leukotrienes (cys-LT) generation in supernatants was measured with an ELISA for LTC4/D4/E4 (Amersham Pharmacia Biotech, Buckinghamshire, UK). For this ELISA, the cross-reactivity with LTC4 is 100%, LTD4 is 100%, LTE4 is 70% and LTB4 is 0.3%. PGD2 generation in the supernatants was measured with an ELISA for PGD2 (Cayman Chemical, Ann Arbor, MI). For the PGD2 ELISA, the cross-reactivity with TXB2, PGF2a, or PGE2 is <0.01%. Cys-LT and PGD2 concentrations were calculated according to manufacturer’s instructions and the results expressed in ng/10^6 cells.

**Detection of mast cell viability**

Mast cell viability was assessed by Trypan blue exclusion test. Mast cells were examined blindly 10 min, 60 min and 24 hrs after incubation with the different activators immediately following addition of 0.1 ml Trypan Blue (0.4%) (Sigma, Israel). The percentage of viable cells was calculated as follows: percentage of viable mast cells = (no. of trypan blue-negative cells/no. of total cells) x 100. Viability of mast cells was always >85% along the
experiments.

**SDS-PAGE immunoblot analysis**

Lysates were prepared from 3 x 10^6 cells as described. After protein concentration was determined by Bradford method, samples were resolved in 10% SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes. G_i3α purified from bacterial lysates was used as positive control (Santa Cruz Biotechnology, CA). The membranes were incubated for 1 h at room temperature with 1 µg/ml rabbit anti-human G_i3α (Santa Cruz Biotechnology). After the membranes were washed, the proteins were detected with secondary immunopure goat anti-rabbit antibodies conjugated with horseradish peroxidase (1:5000) (Pierce, Rockford, IL) followed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Bands were scanned and their density calculated as follows: band area x (band intensity of the sample-background intensity).

**Confocal laser microscopy**

Fibroblasts were seeded on 12-mm cover glasses until confluency. Human lung and cord blood-derived mast cells (10^6 cells/0.5 ml) were seeded on these fibroblasts and maintained in co-culture as described above. Control cells consisted of rat peritoneal mast cells that constitutively express G_i3α. Cells were fixed and permeabilized as previously described. Staining was performed by incubating the cells first with 5 µg/ml rabbit anti-human G_i3α (Santa Cruz Biotechnology) for 1 h at room temperature. After washings with PBS, slides were incubated with 20 µg/ml FITC-conjugated goat anti-rabbit
IgG antibodies (Chemicon, Temecula, CA) for 1 h at room temperature. Negative controls consisted of slides in which only the second antibody was added. Slides were examined using a 63 x objective under a Zeiss LSM 410 confocal laser scanning system attached to the Zeiss Axiovert 135 M inverted microscope with 63 x /1.2 C-Apochromat water immersion lens (Carl Zeiss, Thornwood, NY).

**Statistical analysis**

Data are expressed as mean ± SEM of at least three independent experiments. Parametric analysis (ANOVA, followed by Tukey-Kramer post hoc test) was used to compare the effects. In both cases, a probability of <0.05 was considered statistically significant.

**Results**

**Human lung mast cells in co-culture with fibroblasts become responsive to eosinophil major basic protein**

To evaluate whether human lung mast cells can be activated by MBP under fibroblast influence, human lung mast cells were cocultured for 4 days with human lung fibroblasts. Human lung mast cells cocultured with human lung fibroblasts released histamine in a concentration-dependent fashion with a maximum effect at 10 µM MBP (51.4 ± 5.5% histamine release, \( P < 0.001 \), \( n = 5 \)) (Figure 1).
Figure 1. Human lung mast cells cocultured with human lung fibroblasts are activated by MBP with a dose-dependent pattern. Human lung mast cells (90-99% mast cells) were maintained in co-culture with human lung fibroblasts for 4 days before activation. Histamine release was assessed after 30 min incubation with MBP at the concentrations indicated. Data are the mean of ± SEM of five experiments performed in triplicates. $P < 0.001$ compared with cells incubated in buffer alone.

At the same range of MBP concentrations, human lung mast cells maintained in suspension in the presence of SCF (100 ng/ml) did not release significant levels of histamine in comparison with buffer-incubated cells (data not shown). The increased reactivity of cocultured human lung mast cells towards MBP cannot be related to a mast cell change in phenotype towards MC$_{TC}$. In fact, as assessed by confocal microscopy, after co-culture the majority of human lung mast cells were still tryptase rather than tryptase chymase containing cells (data not shown). This finding is in agreement with a previous study carried out by Dvorak, A.M. et al.$^{21}$
Human cord blood-derived mast cells in co-culture with 3T3 fibroblasts are more responsive to IgE-independent stimuli than when cultured in suspension

The addition of either MBP (1 µM) or compound 48/80 (5 µg/ml) to cord blood-derived mast cell cultures induced the release of relatively low percentages of histamine i.e. 16.1 ± 1.4% and 19.7 ± 1.9%, respectively (P < 0.05, n = 26) (data not shown). Therefore, the modest effect of MBP and 48/80 on mast cell histamine release, prompted us to search for culture conditions in which cord blood-derived mast cell susceptibility to IgE-independent activation could be increased. Cord blood-derived mast cells cocultured with 3T3 fibroblasts released two times more histamine (60.8 ± 6.7%) than cord blood-derived mast cells maintained in suspension (31.8 ± 3.6%) when activated by MBP (1 µM) (P < 0.05, n = 5). Cord blood-derived mast cells cocultured with 3T3 released even higher percentages of histamine when the MBP concentration was increased to 2 µM (28.6 ± 4.5% histamine release for cord blood-derived mast cells maintained in suspension vs. 73.7 ± 5.6% for cord blood-derived mast cells cocultured with 3T3 (P < 0.0001, n = 3). Similarly, compound 48/80 (5 µg/ml) induced a 2.9-fold increase in histamine release from cord blood-derived mast cells cocultured with 3T3 (P < 0.05, n = 5) (Figure 2A). In contrast to these effects, no differences in histamine release were obtained when cord blood-derived mast cells cocultured with 3T3 were activated immunologically by the anti-FcεRI-α chain antibody, 22E7 (2 µg/ml).

PGD₂ release was also enhanced following co-culture with 3T3 fibroblasts. In fact, cord blood-derived mast cells from two different donors activated with MBP (1 µM) generated 7.7 ± 1.1 and 88.9 ± 39.8 ng PGD₂/10⁶ cells (P <
0.001), while the same cord blood-derived mast cells maintained in suspension produced only $1.0 \pm 0.6$ and $10.5 \pm 0.9$ ng PGD$_2$/10$^6$ cells, respectively (Figures 2B and 2).

Figure 2. Cord blood-derived mast cells cocultured with 3T3 fibroblasts increased their responsiveness to MBP. Cord blood-derived mast cells (> 90% mast cells) were maintained in suspension (□) or in co-culture with 3T3 fibroblasts (■) in the presence of soluble SCF (100 ng/ml) for 4 days before activation. (A) Histamine release was assessed after 30 min incubation with the activators at the concentrations indicated. Data are the mean of ± SEM of five experiments performed in triplicates. (B and C) PGD$_2$ production and
release was assessed after 30 min incubation with the activators at the concentrations indicated. Data are the mean ± SEM of two experiments performed with cord blood-derived mast cells from two different donors. \( P < 0.05 \) compared with cells maintained in suspension.

In contrast to PGD\(_2\) production, cord blood-derived mast cells activated by MBP did not generate a significant amount of cys-LT both in suspension and in co-culture conditions (data not shown). In control experiments, no cys-LT or PGD\(_2\) were released from 3T3 fibroblasts that had been incubated with 22E7 antibodies (2 µg/ml), 48/80 (5 µg/ml) or MBP (1 µM).

**The fibroblast-derived membrane form of SCF is responsible for priming human mast cells to IgE-independent activation**

To assess whether a soluble fibroblast-derived factor might be responsible for the high mast cell susceptibility to IgE-independent activation, cord blood-derived mast cells were incubated with conditioned media obtained from cord blood-derived mast cells cocultured with 3T3 fibroblasts or were separated from 3T3 fibroblasts using Transwell membranes. Under both culture conditions, mast cell responsiveness to MBP (1 µM) was significantly lower than that of cord blood-derived mast cells cocultured directly on 3T3 (Figure 3A). This suggests that a fibroblast membrane-associated factor(s) rather than a soluble one(s) is responsible for the fibroblast influences on cord blood-derived mast cells. The membrane-bound form of SCF has shown to partially mediate the effects of murine lung fibroblasts and human endothelial cells on murine bone marrow-derived mast cell responsiveness to IgE-
dependent activation and human intestinal mast cell survival and proliferation, respectively. 14, 22 Therefore, we investigated whether membrane SCF expressed on the fibroblasts during co-culture is responsible for the increased susceptibility of human mast cells towards non-IgE dependent activation. For this purpose, the fibroblast expression of the membrane form of SCF was modulated using antisense oligonucleotides for SCF. Cord blood-derived mast cells cocultured with 3T3 and treated with antisense SCF oligonucleotides (0.1 pM) released 83.3% less histamine than sense-treated cells when activated by MBP (1 μM) (P < 0.05, n = 3) (Figure 3B). Antisense-treated cells were also completely inhibited in their production of PGD2 after stimulation with MBP (1 μM) (data not shown). Similar results were obtained when cord blood-derived mast cells were cocultured with embryonic fibroblasts from Sl/Sfδ mutant mice that produce the soluble but not the membrane form of SCF (Figure 3C). 23
Figure 3. Effects of the membrane form of SCF on mast cell histamine release induced by MBP. Cord blood-derived mast cells were maintained in the following conditions in the presence of soluble SCF (100 ng/ml) for 4 days before activation: (A) with conditioned medium of cord blood-derived mast cells and 3T3 fibroblast co-cultures ( ), in Transwell membranes to separate them from 3T3 fibroblasts ( ), in co-culture with 3T3 fibroblasts ( ); (B) in co-culture with 3T3 fibroblasts previously treated o.n with 0.1 pM SCF sense
or SCF antisense (□); (C) in co-culture with embryonic fibroblasts from
Sl/Sl d mutant mice (□) or with 3T3 fibroblasts (■).

Histamine release was assessed after 30 min incubation with the activators at
the concentrations indicated. Data are the mean of ± SEM of three
experiments performed in duplicates. \( P < 0.05 \) compared with cord blood-
derived mast cells cocultured with 3T3 fibroblasts (A, C) or with sense-treated
cells (B).

The fibroblast-derived membrane form of SCF induces \( G_{\text{i3}} \) expression in
human mast cells

We next determined one of the possible mechanism by which mast cells in
co-culture with fibroblasts increase their responsiveness to IgE-independent
activation. It is known that IgE-independent secretagogues stimulate rat
peritoneal mast cells by directly activating \( G_{\text{i3}} \), a Ptx-sensitive \( G_{\text{i}} \) protein that
leads to mast cell exocytosis and eicosanoid generation.\(^{24, 25}\) Therefore, we
investigated the expression of \( G_{\text{i3}} \) protein in cord blood-derived mast cells in
suspension and after co-culture. As shown in Figure 4A, co-culture of cord
blood-derived mast cells with 3T3 fibroblasts resulted in a marked increase
(6.2-folds) in the expression of the alpha subunit of \( G_{\text{i3}} \) in comparison with
cord blood-derived mast cells maintained in suspension as determined by
immunoblotting. Pre-incubation with Ptx significantly inhibited histamine and
PGD\(_2\) released by MBP-activated cord blood-derived mast cells (75.4 and
94.4% inhibition, respectively).

In confocal microscopy images, \( G_{\text{i3,}} \) expression appeared to be highly
localized in the plasma membrane of the cord blood-derived mast cells
cocultured with 3T3 fibroblasts (Figure 4B). Similar results of increased presence of G\textsubscript{i3\alpha} were obtained in human lung mast cells cocultured with human lung fibroblasts (data not shown). Immunoblotting analysis of cord blood-derived mast cells maintained in suspension did not reveal any expression in G\textsubscript{i3\alpha} (Figure 4C). Freshly isolated rat peritoneal mast cells, as expected, constitutively expressed G\textsubscript{i3\alpha}\textsuperscript{24} (Figure 4D).

**Figure 4.** Cord blood-derived mast cells cocultured with 3T3 fibroblasts increased their expression of G\textsubscript{i3\alpha} protein.

(A) SDS-PAGE immunoblot was performed with lysates of cord blood-derived mast cells (CBMC) maintained in suspension or cocultured with 3T3
fibroblasts for 4 days in the presence of soluble SCF (100 ng/ml). G\textsubscript{i3a} purified from bacterial lysates was used as positive control. Confocal microscopy images of G\textsubscript{i3a} protein under orange fluorescence (FITC) showing: (B) membrane localization in cord blood-derived mast cells maintained in coculture with 3T3 fibroblasts for 4 days in the presence of SCF (100 ng/ml), (C) no expression in cord blood-derived mast cells maintained in suspension for 4 days in the presence of SCF (100 ng/ml) and (D) perinuclear localization in freshly isolated rat peritoneal mast cells.

The displayed figures are from a representative single experiment out of three.

As assessed by immunoblotting and further densitometric analysis, cord blood-derived mast cells cocultured with 3T3 and treated with antisense for SCF, expressed less G\textsubscript{i3a} than the sense-treated co-cultures (2.4-folds, Figure 5A) and than the untreated cocultured mast cells (3.7-folds, Figure 4A). Similar decrease in G\textsubscript{i3a} expression was observed in confocal microscopy images of antisense (Figure 5B) in comparison with sense-treated co-cultures (Figure 5C). Furthermore, the same co-cultures treated with antisense for
SCF were unresponsive to MBP (Figure 3B).

**Figure 5.** Effects of the membrane form of SCF on the mast cell expression of \( G_{i3} \) protein. SDS-PAGE immunoblot (A) and confocal microscopy images of \( G_{i3} \) protein under orange fluorescence (FITC) were performed with cord blood-derived mast cells maintained in the presence of soluble SCF (100 ng/ml) and cocultured for 4 days with 3T3 fibroblasts previously treated o.n with 0.1 pM SCF sense (B) or SCF antisense (C). The displayed figures are from a representative single experiment out of three.

**Discussion**

In this work, we have demonstrated for the first time that highly purified human lung mast cells are responsive to the eosinophil mediator MBP when in co-culture with fibroblasts. Co-culture with fibroblasts is an *in vitro* culture system that is more closely to resemble *in vivo* conditions than the *in vitro*
usually used, i.e. keeping the cells in suspension. Furthermore, cord blood-derived mast cells cocultured with fibroblasts were more responsive to MBP and compound 48/80 in comparison to the same cell population maintained in suspension.

It has been shown that incubation of rat peritoneal mast cells with native MBP and eosinophil cationic protein (ECP) but not eosinophil derived-neurotoxin (EDN), results in a concentration-dependent histamine release that requires both [Ca\(^{2+}\)] and metabolic energy.\(^{26}\) In addition, we have recently demonstrated that IgE-desensitized rat peritoneal mast cells release histamine following incubation with MBP. The mechanism causing this release is similar to that of IgE-independent stimuli induced by cationic compounds such as compound 48/80 and substance P.\(^{4}\) However, human mast cell responsiveness towards eosinophil mediators remains not extensively investigated as yet. A previous study has shown that only partially purified human heart mast cells, but not human lung and skin mast cells, released histamine, tryptase and PGD\(_2\) when incubated with ECP and MBP.\(^{5}\) In the present study, we took into account that mast cell functionality/phenotype can be dramatically affected by the microenvironment.\(^{1}\) For example, murine bone marrow-derived mast cells that are mucosal type mast cells, in co-culture with 3T3 fibroblasts changed their phenotype towards connective tissue mast cells, the rodent counterpart of MC\(_{TC}\), and acquired responsiveness to IgE-independent stimuli in these culture conditions.\(^{10,11}\) Importantly, mast cells lost their responsiveness to IgE-independent activation when the co-cultures are dispersed.\(^{27}\) This observation suggests that mast cell functionality and/or phenotype may be rapidly altered once the cells are isolated from the tissue
influence. This might be in line with the lack of responsiveness of freshly isolated human lung mast cells towards IgE-independent activation. In the present study, we found that human lung mast cells respond to MBP after co-culture with human lung fibroblasts whereas freshly isolated and highly purified human lung mast cells are unresponsive to the same stimulus. Moreover, cord blood-derived mast cells that were only slightly responsive to MBP when challenged in suspension, upon co-culture with 3T3 fibroblasts became more responsive to this activation and released more histamine and PGD₂, but not cys-LT. Similar results were found with another cationic secretagogue, compound 48/80.

The preferential production of PGD₂ over cys-LT by mast cells when activated by IgE-independent stimuli can be attributed to the larger requirement for Ca²⁺ by the lipoxygenase than by the cyclooxygenase pathway to exert their enzymatic activities. In fact, it is known that the increase in intracellular Ca²⁺ concentration, after mast cell IgE-independent activation, derived mainly from intracellular stores and is lower than that induced after IgE-dependent activation, derived from both extracellular and intracellular stores. In agreement with this, we observed that cord blood-derived mast cells cocultured with 3T3 fibroblasts produced and released more cys-LT than PGD₂ after IgE-dependent activation (130 ± 43.0 pg/ml cys-LT in comparison to cells maintained in activation buffer that released only 20.0 ± 5.0 pg/ml cys-LT). Therefore, the increase in PGD₂ generation during mast cell co-culture with fibroblasts is not compensating a possible down-regulation of the signaling pathways that lead to cys-LT production.

Besides the low calcium requirements of PGD₂ production, and up-
regulation in the cyclooxygenase (COX) expression may occur during mast cell co-culture with fibroblasts. Indeed, it has been recently shown that murine bone marrow-derived mast cells in co-culture with fibroblasts express higher levels of prostaglandin endoperoxide synthase mRNA and protein after stimulation with compound 48/80.\textsuperscript{10} Similar change in arachidonic acid profile due to an induction of leukotriene C\textsubscript{4} synthase expression has been recently reported in cord blood-derived mast cells exposed to IL-4.\textsuperscript{30} However, in preliminary experiments we could not detect COX-2 up-regulation in cord blood-derived mast cells cocultured with 3T3 fibroblasts (data not shown).

Some of the fibroblast influences on mast cells such as survival, proliferation, maturation and IgE-dependent activation are known to be mediated by SCF.\textsuperscript{31-34} Furthermore, SCF induces eosinophil recruitment in the murine model of allergic airway inflammation by priming mast cells to synthesize higher levels of eotaxin, a strong eosinophil chemoattractant factor.\textsuperscript{35} This together with our observation of SCF produced by eosinophils,\textsuperscript{20} are strong evidence of the importance of SCF in allergic inflammation.

SCF is normally found in both soluble and membrane forms as a result of differential splicing and proteolytic cleavage.\textsuperscript{36} The differential effects of the soluble and membrane form of SCF on mast cells have not yet been extensively studied. However, the involvement of a fibroblast-membrane-derived factor, rather than a soluble one, prompted us to consider SCF in its membrane form as the possible regulator of mast cell responsiveness to IgE-independent stimulus. Indeed, mast cells cocultured with fibroblasts in the presence of the soluble, but not the membrane form of SCF, released much less histamine and PGD\textsubscript{2} when incubated with MBP. In contrast, mast cell
survival and adhesion to the fibroblasts in the same culture conditions were not affected by the absence of the membrane form of SCF (data not shown). Similarly, it has been shown that administration of exogenous soluble SCF to Sl/Sld mice increased mast cell numbers indicating differential effects of the two forms of SCF on mast cell responses. SCF triggers its biologic effects by binding to its receptor, c-kit, a member of the type III receptor tyrosine kinase family. The existence of different isoforms of c-kit for soluble and membrane forms of SCF has not been reported. However, several works have provided evidence that the membrane forms of some growth factors are involved in signaling events distinct from those mediated by the diffusible forms even when acting on the same receptor. For example, growth and survival regulation have been attributed specifically to the membrane form of the heparin-binding epidermal growth factor (EGF)-like growth factor (proHB-EGF), rather than to the diffusible, processed HB-EGF isoform. Moreover, the membrane form of SCF has been shown to induce a prolonged activation of c-kit, inducing more efficient signals for the survival and differentiation of the factor-dependent myeloid cell line MO7e. In accordance with this, we observed that the membrane form of SCF upregulates the expression of the alpha subunit of G\textsubscript{i3}. This G\textsubscript{i3} over-expression may contribute to mast cell responsiveness to the IgE-independent activator, MBP. Indeed, similar correlation between G\textsubscript{i3} expression and responsiveness to compound 48/80 was found in quercetin-treated rat peritoneal mast cells. G\textsubscript{i3} is expressed both in the Golgi and in the plasma membrane. It is the plasma membrane-bound form of G\textsubscript{i3} that appears to facilitate regulated exocytosis since it is more accessible to
interact with cationic secretagogues.\textsuperscript{24} In correlation with this, we found that $G_{i3\alpha}$ expression appeared to be highly localized in the plasma membrane of cord blood-derived mast cells. Besides this observation, it is possible that other changes in IgE-independent signaling can be induced during mast cell-fibroblast co-culture as well, for example a decrease in the rate of $G\alpha$ GTPase activity that regulates $G_{i3}$-mediated exocytosis.\textsuperscript{43}

In conclusion, we demonstrated that human lung and human cord blood-derived mast cells can be activated by an allergen-independent eosinophil-derived stimulus. This event can feasibly take place during the late phase of the allergic process when mast cells encounter the infiltrated and activated eosinophils. Mast cell responsiveness to eosinophil mediators is specifically influenced by the membrane form of SCF.

Together these findings are important in the understanding of microenvironment influences on mast cell functionality that dictate either the resolution or the aggravation of a pathological condition through the interaction with other inflammatory cells such as eosinophils or structural cells such as fibroblasts.
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