Human embryonic stem cells: a source of mast cells for the study of allergic and inflammatory diseases

Short title: Human embryonic stem cell-derived mast cells

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Category: Immunobiology
Abstract:

Human mast cells are tissue resident cells with a principal role in allergic disorders. Cross-linking of the high-affinity receptor for IgE (FcεRI) results in release of inflammatory mediators initiating the clinical symptoms of allergy and anaphylaxis. Much of our knowledge regarding the mechanisms of mast cell activation comes from studies of mouse bone marrow derived mast cells. However, clear differences have been identified between human and mouse mast cells. Studies of human mast cells are hampered by the limited sources available for their isolation, the resistance of these cells to genetic manipulation, and differences between cultures established from different individuals. To address this limitation, we developed a simple co-culture-free method for obtaining mast cells from human embryonic stem cells (hES). These hES-derived mast cells respond to antigen by releasing mast cell mediators. Moreover, the cells can be generated in numbers sufficient for studies of the pathways involved in their effector functions. Genetically modified mast cells, such as GFP expressing cells, can be obtained by introduction and selection for modification in hES cells prior to differentiation. This direct co-culture-free differentiation of hES cells represents a new and unique model to analyze the function and development of human mast cells.
Introduction:

Mast cell activation plays a critical role in the protective response to certain parasites and in the pathogenesis of allergic diseases. Mast cells are derived from hematopoietic precursors that migrate from the bone marrow and complete their differentiation in the microenvironment of peripheral tissues under the influence of stem cell factor and other cytokines derived from resident cells\(^1\). Mast cell effector functions depend on their capacity to bind antigen specific IgE via high affinity IgE receptors (Fc\(\varepsilon\)RI) and subsequent cross-linking of these receptors with multivalent antigen. Cross-linking of Fc\(\varepsilon\)RI initiates a series of signaling events including phosphorylation of intracellular proteins and intracellular calcium mobilization, leading to mast cell degranulation and release of preformed proteases, biogenic amines, and the biosynthesis of cytokines, chemokines, and lipid mediators. The importance of this effector cell in allergic disease makes the understanding of mast cell function essential for the development of new therapeutics for these disorders\(^2\).

Much of our understanding of mast cell biology comes from mouse models because of the ease with which these cells can be cultured from mouse bone marrow (bone marrow-derived mast cells, BMMC), and the ability to use these BMMC, especially populations obtained from genetically manipulated mice, to reconstitute mast cell deficient mouse lines. However, many differences have been noted between mouse and human mast cells, including differential cytokine requirements for development and proliferation\(^3\), regulation of Fc\(\varepsilon\)RI expression by Th2 cytokines\(^4\), the ability of mediators such as prostaglandins to regulate mast cell function\(^5,6\), and response to anti-allergic drugs\(^7\). Human mast cells can be isolated in their mature form from a few human tissues including lung and skin\(^8,9\). Alternatively, human mast cells can be derived from isolated CD34+ hematopoietic precursors from bone marrow, cord blood or peripheral blood. CD34+ positive cells are cultured in medium supplemented with recombinant
human stem cell factor (rhSCF) and recombinant human interleukin 6 (rhIL-6)\textsuperscript{10-12}. While human mast cells isolated using this approach are valuable sources for many studies, there are a number of limitations. First, mast cells cannot be cultured indefinitely, thus a continuous source of primary tissue/blood is required. Second, genetic differences are present between each population, as they are isolated from different individuals. Finally, primary mast cells cannot be easily genetically manipulated, and therefore, studies with these cultured mast cells are generally limited to the use of pharmacological approaches. Together, these limitations have proven an obstacle in the study of human mast cell function, development, and biology.

Human embryonic stem (hES) cells are capable of both self-renewal and differentiation into cells of germ layers, i.e., ectoderm, endoderm, and mesoderm. hES cells, therefore, offer an attractive alternative for establishing human mast cell cultures. If a reliable method for obtaining functional mast cell populations can be established, the genetic makeup of the cells will remain consistent between experiments and genetic manipulations could be carried out in the hES cells, a cell type far more amenable to these maneuvers. Previous work has shown that many cell lineages, including hematopoietic progenitors, can be generated from hES cells \textit{in vitro}, and furthermore, that hES cell-derived hematopoietic progenitors can be differentiated into T-cells, neutrophils, macrophages, and dendritic cells\textsuperscript{13}.

In general, differentiation of hES cells into hematopoietic progenitors requires the co-culture of hES cells with cell lines derived from aorta-gonad-mesonephros or cell lines such as S17 or OP9\textsuperscript{14-17}. Alternatively, hematopoietic precursors have also been isolated from hES cell derived embryoid bodies (EB), structures composed of all three germ layers, growing in a complex mixture of cytokines\textsuperscript{18-20}. These approaches have been successfully used to induce the differentiation of mouse, human and primate
embryonic stem cells into hematopoietic CD34+ cells. However, hematopoietic precursors are rare and the establishment of primary cultures of mature immune cells using these strategies has required isolation of the CD34+ hematopoietic precursors prior to differentiation into specific lineages. It is also not clear if this methodology will yield precursors capable of differentiating into mature human mast cells at a frequency sufficient for experimental studies.

A number of reports, however, suggest that it should be possible to identify conditions optimal for supporting the generation of human mast cells from ES cells. First, previous studies have reported isolation of mast cells from mouse ES cells. Evidence suggesting that similar differentiation may be possible for human cells comes from a study in which cells expressing tryptase and chymase, enzymes found in mast cells, were identified after co-culture of hES cells with murine fetal liver-derived stromal cells. More recently, co-culture of cynomolgus monkey embryonic stem cells with the murine aorta-gonad-mesonephros-derived stromal cell line AGM-S1 yielded clusters of hematopoietic progenitors which, when mechanically separated from stromal cells, could be differentiated into cells with a number of mast cell characteristics.

Here we report two different approaches for the generation of mast cells from hES cells. We compare these methods both in terms of the yield and the morphological, biochemical and functional characteristics of the human mast cells generated. We show that hES cells can be differentiated into hematopoietic progenitors by direct differentiation of immobilized EB in the absence of supporting cell lines. Subsequent differentiation of the progenitors resulted in hES-derived mast cells (ESMC) expressing tryptase, chymase and functional FcεRI. Activation of those ESMC by multiple ligands led to phosphorylation of intracellular substrates, intracellular calcium mobilization and histamine release after
antigen stimulation. Furthermore, we show that these cells can be generated not only from the original hES cells lines, but also from genetically modified hES populations and clonal isolates.

**Methods:**

**Cell cultures**

hES cell line H1 and H9 (WA01, WA09, National Stem Cell Bank, USA) were maintained on irradiated mouse embryonic fibroblasts at 37°C, 5% CO2 in hES medium (Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM/F12), 20% KO-Serum Replacement, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen GIBCO), 0.055 mM β-mercaptoethanol, 8 ng/mL basic fibroblast growth factor (Peprotech)). hES were passaged every 5-6 days. For culture conditions of OP9 cells and cord blood derived mast cells see supplementary methods.

**Differentiation of hES cells to hematopoietic progenitors**

EB were obtained by dispase (Invitrogen GIBCO) treatment of hES cells for 10 min, aggregates of hES cells were transferred to 60 mm anti-adhesive-treated plates in differentiation medium (Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen GIBCO) 15% FCS, 2 mM L-glutamine, 4 x 10^-4 methyl thioglycolate, 300 μg/ml Transferin (Roche), 50 μg/ml ascorbic acid (Sigma), 5% Protein-free hybrydoma medium (Invitrogen GIBCO)). Four days later 20 to 50 of the EBs that formed were transferred to a Matrigel treated (BD Bioscience) 60mm plate with 3 mL of mast cell medium I (Stem Pro-34 SMF supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen GIBCO), 100 ng/mL SCF, 5 ng/mL IL-3, 100 ng/mL IL-6, 25 ng/mL...
Flt-3L (Amgen). Medium was replaced every 5 to 7 days and non adherent cells were collected after 3 weeks. For differentiation of progenitors by OP9 co-culture see supplementary methods.

Differentiation of hematopoietic progenitors to mast cells

CD34+CD43+ cells were cultured in IMDM medium supplemented with 10% FBS, 1% nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from GIBCO, Invitrogen), SCF (100ng/ml) and IL-6 (50 ng/ml)(Amgen).

Mast cell characterization

For detailed methods of mast cell characterization including histochemistry, flow cytometry, gene expression and functional assays see supplementary methods.

Genetic modification of hES

Lentiviral transfection and electroporation are described in supplementary methods

Results:

Differentiation of hES cells to hematopoietic progenitors

We first compared the ability of different culture conditions, namely co-culture with OP9 cells and differentiation to EB, to direct the hES cell differentiation towards the hematopoietic lineage, as assessed by the appearance of CD34+ cells. In both cases, hES cell cultures that had achieved confluency were removed from mouse fibroblasts and basic FGF, factors essential for maintaining ES cells pluripotency. To test the ability of OP9 cells to stimulate differentiation to CD34+ cells, hES cells were transferred to
monolayers of OP9 cells and cultured for 8-16 days (Figure 1A). CD34+ cells were recovered from each culture using magnetic beads labeled with anti-CD34 antibody. The number of CD34+ cells in the culture was dependent on the length of exposure to the OP9, with maximal yields observed at 12 days (data not shown). Interestingly, as shown in Figure 1B, only 1/3 of the CD34+ cells also expressed CD43, another marker for hematopoietic progenitors. Average yield of CD34+ CD43+ cells after 12 days of co-culture with OP9 cells was $5 \times 10^5 + 3 \times 10^5$ per $1 \times 10^6$ of hES cells (n=4).

To induce differentiation of hematopoietic progenitors in co-culture-free conditions through formation of EB, large aggregates of hES cell colonies were cultured in suspension (Figure 1C). The EBs were then transferred to plates coated with laminin-rich Matrigel and cultured in serum-free medium supplemented with hrIL-6, hrIL-3, hrSCF and hrFlt-3L. Clusters of spherical cells appeared 12 days later. Four days after their appearance in culture, these cells transitioned into non-adherent populations that could be collected from the culture supernatant (Figure 1D). Cells continued to be released into the medium for more than 6 weeks. Morphological analysis of these cells indicated a very homogenous population (Figure 1E). This was consistent with fluorescence-activated cell sorting (FACS) analysis showing that the cells displayed uniform cell scatter properties (Figure 1F) and a uniform CD34 profile (Figure 1G). Unlike the CD34+ cells derived by co-culture, the majority of these cells also expressed CD43. Consistent with their hematopoietic lineage, CD45 expression was abundant, whereas the cells did not express the monocyte marker CD14. (Figure 1H and data not shown). The purity of these cells, determined by CD43 and CD45 double-staining, was 89.8% ± 2.1% (mean ± SEM, n = 6). Calculation of the number of CD34+ cells obtained via this route of differentiation showed that it was approximately 10 fold higher than that obtained by co-culture with OP-9 cells. Average hematopoietic progenitor yield was $4.5 \times 10^6 \pm 2.0 \times 10^6$ cells per $1.0 \times 10^6$ hES cells (n = 6). Thus while CD34+ cells can be obtained using
both methods, the intermediate differentiation of the cells first to embryoid bodies had both qualitative and quantitative impact on the hematopoietic cells collected from the cultures. To determine whether co-culture independent differentiation into CD34+ cells was unique to the H1 ES cell line, we examined the ability of a second line, the H9 hES cell line, to form embryoid bodies and release hematopoietic progenitors. As can be seen in supplementary Figure S1, a CD34+ cells were also obtained from the H9 hES cell line under these growth conditions.

**Differentiation of human mast cells from hES cell derived progenitors**

We next determined the ability of the hES H1 derived CD34+ cells to differentiate into mast cells by comparing the derived cells directly with those obtained from cord blood CD34+ cells. We included in this analysis of both CD34+ populations isolated from hES/OP9 co-cultures and those harvested from the supernatant of cultures derived from EB. All CD34+ cells were placed in culture conditions previously defined and shown to differentiate CD34+ human cord blood cells to mast cells\(^{11}\), essentially medium rich in I-L6 and SCF. Differentiation of these cells to mast cells was evaluated using a number of criteria. The presence of granules containing tryptase and chymase is unique to mast cells\(^{25}\), therefore, we first stained cells from both types of cultures with anti-tryptase and anti-chymase antibodies. As can be seen in Figure 2A, tryptase and chymase positive cells were present in both cord blood mast cells (CBMC) and ESMC cultures. In both cases >80% of the cells were positive after 8 weeks in culture. Staining of cells with DAPI revealed that similar to CBMC, ESMC have poly-lobed nucleus\(^{26,27}\). To determine whether the method used to obtain CD34+ cells from hES cells impacted the subsequent differentiation into tryptase/chymase positive cells, a more extensive comparison of the appearance of these cells was carried out. In both cultures tryptase/chymase positive cells became apparent four weeks
after the initiation of differentiation and by week 8 more than 90% of cells expressed both chymase and tryptase (Figure 2A,B, S2). We did not find any qualitative differences in tryptase and chymase expression between mast cells differentiated from OP9 cell co-culture or by differentiation from EBs, although the overall number of positive staining cells was higher from CD34+ cells differentiated from EBs: the average hES derived mast cells (ESMC) yield of chymase/tryptase staining was $1.2 \times 10^6 + 0.8 \times 10^6$ per $1.0 \times 10^6$ of CD34+ progenitors isolated from OP9 co-culture ($n=4$) while $3.2 \times 10^6 \pm 1.1 \times 10^6$ positively stained cells were obtained from $1.0 \times 10^6$ hematopoietic progenitors differentiated by EB formation ($n = 6$).

To further compare this aspect of mast cell differentiation, we evaluated the tryptase activity in lysates prepared from CBMC and ESMC. The level of activity did not differ between the two cell populations, indicating similar level of enzyme in the CBMCs and ESMC (Figure 2C). Tryptase activity is dependant on heparin sensitive enzyme assembly. Inhibition of tryptase activity by the heparin antagonist Polybrene provides an indirect means of evaluation of heparin levels in mast cell populations. Dose dependant inhibition of tryptase activity was observed in samples prepared from ESMC and CBMC (Figure 2D). Mast cell proteoglycans heparin and chondroitin sulfate stored in mast cell granules can be stained by Toluidine blue. As shown in Figures 2A and S2, granules in mast cells derived by both methods from hES cells, as well as CBMC all stained with Toluidine blue. Taken together, these results show that ESMC derived from hematopoietic progenitors have granules containing the mast cell specific proteins tryptase and chymase, and the proteoglycans, heparin and chondroitin sulfate. However, similar to CBMC these cells do not develop the large granules and the high levels of proteolytic enzymes characteristic of tissue mast cells.
Expression and function of the high affinity IgE receptor on hES derived ES cells.

The high affinity IgE receptor, FcεRI is a heterotetramer or heterotrimer composed of one α-chain, one β-chain and two γ-chains or one α-chain and two γ-chains, respectively. Cross-linking of surface expressed FcεRI receptors leads to mast cell activation during allergic reactions. While expression of the human α chain and the γ chain is not specific to mast cells and can be found at low levels on other hematopoietic cells, the expression of the β chain, an amplifier of signaling through this receptor, is a unique marker for this population. To evaluate expression of FcεRI in ESMC we first analyzed mRNA levels of the three FcεRI chains by real time PCR. The expression of the gene for each subunit was compared to expression of the genes in CBMC. As shown in Table 1, ESMC derived by either co-culture with OP9 or by EB formation expressed similar level of FcεRIβ and FcεRIγ as CBMC. Surprisingly, expression of mRNA encoding the FcεRIα was not detected in ESMC differentiated by OP9/hES cells co-culture. In contrast, FcεRIα transcripts were consistently detected in cultures derived from hES cells that were differentiated into EB. The level of expression was however, lower than that detected on mast cells derived from cord blood (Table 1).

We next determined whether the expression of the FcεRIα by the hES derived mast cells was sufficient to direct expression of the high affinity IgE receptor to the cell surface. Cell surface expression of FcεRI was evaluated by staining of human IgE loaded ESMC with FITC-labeled anti-IgE antibody and FACS analysis. To assess uniformity of mast cell populations, cells were also stained with anti-c-Kit antibody. Consistent with our failure to detect FcεRIα transcripts, we did not detect FcεRI on the surface of ESMC derived by co-culture with OP9 cells (Figure 3A). However, ESMC derived from EB and CBMC do express FcεRI on the cell surface. Surprisingly, similar levels of FcεRI were observed on the
ESMC and CBMC (Figure 3B,C). As shown in Figure 3D,E, more than 95% of ESMC differentiated by EBs formation expressed both FcεRI and c-Kit.

To determine if the FcεRI receptor expressed by the ESMC was functional, we stimulated ESMC exposed to 5 μg/ml hIgE by cross-linking of FcεRI receptor with anti-IgE antibody, and assessed two well-characterized consequences of activation of mast cells by FcεRI cross-linking: changes in MAPK phosphorylation and intracellular Ca++ mobilization. Because ESMC differentiated by OP9 co-culture do not express FcεRI, they were not included in this analysis.

p42/44 ERK1/2 MAPK kinase was shown to be phosphorylated after activation of FcεRI by receptor cross-linking. The extent of ERK1/2 phosphorylation was assessed by phosphospecific staining with antibody recognizing the phosphorylated form of Erk1/2 at T202 and Y204, followed by FACS analysis. Activation of ESMC by FcεRI receptor cross-linking resulted in increased binding of phosphospecific antibody compared to unstimulated control (Figure 4A). The increase was similar to that observed in the CBMC population (Figure 4B).

Changes in intracellular Ca++ is an essential event in mast cell activation with many downstream signaling events dependent upon the sufficient release of Ca++ from intracellular stores. Therefore we next evaluated the intracellular Ca++ release by ratiometric measurement of fluorescence at 340 and 380 nm after stimulation of ESMC loaded with the Ca++ selective fluorescent indicator Fura-2. As shown in Figure 4C, ESMC exposed to 5 μg/mL hIgE for 5 days and cross-linked with 2 μg/mL anti-IgE antibody resulted in the mobilization of intracellular Ca++ similar to CBMC. Taken together, these results show that hES cell derived mast cells express functional FcεRI which is able to stimulate signaling pathways after activation by cross-linking.
Expression and function of PGE$_2$ pathways in hES cell derived mast cells

The activity of the Fc$\varepsilon$RI receptor can be modulated by numerous pathways, including those activated by G-protein coupled receptors found on these cells. Among these are the pathways activated by PGE$_2$, a lipid mediator released at the site of inflammation. The role of this lipid mediator in modulating mast cell function has been particularly difficult to study because of reported differences between species in the effects of PGE$_2$ on mast cells$^{5,6}$. We therefore asked whether hES derived mast cells express these receptors, and if so, whether these cells could provide a model system for studying the impact of PGE$_2$ signaling on mast cell function. PGE$_2$ stimulates four subtypes of prostanoid EP receptors: EP1 which couples to intracellular Ca$^{++}$ mobilization, EP2 and EP4 which couple to Gs, and EP3 which couples primarily to G$\iota/o$ proteins. As shown in Table 1 ESMC as well as CBMC expressed EP2, EP3 and EP4 receptors. We did not observe differences in the level of expression of these receptors between ESMC and CBMC. To determine whether these receptors were functional and have the potential to regulate mast cell activation, we activated ESMC via EP receptors by PGE$_2$$^5$. Similar to Fc$\varepsilon$RI cross-linking, activation of mast cells by PGE$_2$ resulted in both phosphorylation of MAPK kinase Erk1/2 and intracellular Ca$^{++}$ mobilization (Figure 4A, C). PGE$_2$ phosphorylation of MAPK kinase ERK1/2, driven by ESMC activation by 1 $\mu$M PGE$_2$ and assessed by phosphospecific antibody, showed similar level of phosphorylation compared to CBMC. Stimulation by Fc$\varepsilon$RI cross-linking, together with PGE$_2$ stimulation, led to further increase in Erk1/2 phosphorylation (Figure 4A,B). Elevations of intracellular calcium are one of the most significant responses after activation of human mast cells by PGE$_2$. Stimulation of Fura2-loaded ESMC with 1$\mu$M PGE$_2$ resulted in an increase in intracellular calcium release as measured by ratios of fluorescence at 340 and 380 nm (Figure 4C). The increase and kinetics of intracellular calcium mobilization induced by PGE$_2$ was similar to the calcium response in CBMC.
**Evaluation of effector functions in hES cell derived mast cells**

Activation of mast cells by FcεRI results to the release of granules, which contain chemical mediators such as histamine, and also leads to increases in cytokine synthesis\(^2\). We next asked whether the pathways and biochemical machinery necessary for this response were present in the ES cell derived mast cells. To assess the ability of ESMC to degranulate after stimulation, we measured histamine release after activation by either FcεRI cross-linking or PGE\(_2\) treatment. ESMC were primed with hIgE and cross-linked with various concentration of anti-IgE antibody. As shown in Figure 4D, histamine release was dose dependent and reached maximum at a concentration 3 \(\mu\)g of anti-IgE antibody per mL. In contrast, PGE\(_2\) alone was unable to degranulate ESMC at concentrations up to \(10^{-5}\) M (data not shown). Similar to what has been reported for CBMC, PGE\(_2\) (10\(^{-6}\)M) did not augment nor inhibited IgE-stimulated histamine release. Total histamine level for ESMC and CBMC are shown in Table 2. One of the major cytokines released from human mast cells is TNF-\(\alpha\). To assess its release from ESMC we stimulated cells sensitized with hIgE with anti-IgE antibody. TNF-\(\alpha\) was synthesized and released by ESMC following activation by anti-IgE, but not following stimulation with PGE\(_2\) (Figure 4E). PGE\(_2\) was however, capable of completely inhibiting FcεRI-mediated TNF-\(\alpha\) production by these cells. Similar levels of TNF-\(\alpha\) release was seen in CBMC.

One of the major arachidonic acid metabolites produced by FcεRI-activated human mast cells is prostaglandin D\(_2\) (PGD\(_2\)). PGD\(_2\) was produced by both ESMCs and CBMC after stimulation with PGE\(_2\) and activation of the IgE receptor (Figure 4F). However, some differences were observed. ESMCs displayed slightly higher constitutive release of this prostaglandin whereas maximal release by CBMC after stimulation was slightly higher than that observed in ESMC cultures. Similar to histamine release, simultaneous activation of human mast cells by PGE\(_2\) and anti-IgE was not additive in either population.
Derivation of mast cells from genetically modified hES

Genetic modification of mast cells is difficult. In contrast, genetic modification of mouse ES cells is common and methods are becoming established for carrying out similar experimental maneuvers with hES cells. In theory, genetic changes desirable in the mast cell could be introduced in hES cells prior to their differentiation. It was, therefore, of interest to determine whether procedures required for such genetic manipulation of hES cells including growth in selective media and colony isolation would compromise the ability of these cells to produce CD34+ progenitors or to differentiate into the mast cell populations.

To test this, we introduced a GFP gene under the control of the EF-1P alpha promoter into the H1 hES cells using a lentiviral expression system. Two days after transfection >85% cells in culture expressed GFP, and the majority of them remained positive for pluripotent marker TRA-1-81 (Figure 5A). Double positive cells were sorted and used for establishment of hES cells expressing GFP (Figure 5B). EB were generated and further differentiated to CD43+ hematopoietic progenitors (Figure 5D,E), which were subsequently differentiated into mast cells. Differentiated mast cells expressed c-Kit and mast cells specific tryptase together with GFP (Figure 5F,G).

The generation of GFP positive mast cells did not require isolation and expansion of individual hES cell colonies, a process which requires extensive time in tissue cultures and thus is more likely to result in acquisition of mutations that could compromise the pluripotency of hES cells. However, many genetic maneuvers including introduction of mutations by homologous recombination require expansion of clones after growth in selective media. To determine if drug resistant hES cell isolates would retain the ability to differentiate into mast cells, hES cells were transfected by electroporation with a neomycin gene driven by the PGK promoter. G418 resistant hES clones were individually isolated (Figure S3A) and
expanded and the ability of two of these clones to generate CD34+ hematopoietic precursors and mast cells was determined. As shown in Figure S3B, both clones retained the parental ability to differentiate into EBs and produce cultures which released CD34+ CD43+ cells. In both cases these CD34+ cells, upon exposure to IL6 and SCF, differentiated into c-Kit positive populations (Figure S3C). This data demonstrates that individual hES cell clones carrying desirable genetic events can be selected, isolated, expanded and a stock of this cell line maintained. As needed mast cells carrying the same genetic modification can be generated for study.

Discussion:

Here we report that hematopoietic precursors capable of giving rise to mast cells can be isolated from human ES cells and furthermore that they can be obtained without the co-culture of the ES cells with stromal cells. Consistent with previous reports, we found that co-culture of hES cells with OP9 cells yielded CD34+ cells. This protocol generally requires the mechanical separation of the progenitor cells from the stromal cells. To do this, we carried out positive selection of CD34+ cells. Further analysis showed that this yielded a population of cells that can be further divided based on CD43 expression. Previous studies have demonstrated that CD43 reliably separates CD34+ hematopoietic cells from CD34+CD43-KDR+CD31+ endothelial cells. This raises the possibility that only about one third of the cells isolated were hematopoietic precursors, while the remaining cells represent precursor poised for differentiation into endothelial cells. We cannot rule out the possibility that, in part, the greater yield of CD34+ CD43+ precursor cells by direct culture reflects the ease with which these cells could be collected from the EB derived cultures. The release of these cells into the culture media had two important consequences. First, loss of cells during mechanical separations was avoided. Second, the cells could be
harvested for an extended period of time, presumably reflecting the continuous self renewal and
differentiation of an even earlier progenitor cell present in the complex embryo derived culture into the
CD34+CD43+ cell.

To date, the successful generation of large numbers of mast cells from human ES cell derived
progenitors has not been described. We show that both CD34+ populations generated by co-culture and
those generated directly by formation of EB can be differentiated into cells with the well established
characteristics of primary human mast cells, including morphology and expression of proteases and c-Kit.
Furthermore, the time line and cytokine requirements were similar to derivation of mast cells from cord
blood hematopoietic progenitors. However, similar to CBMC, the ESMC have smaller granules, less
heparin and lower levels of proteolytic enzymes than mature tissue mast cells The number of cells
obtained per 1 x 10^6 starting CD34+ cells was higher in direct differentiation compared to OP9 co-culture.
Both populations also yield cells similar in expression of c-kit, chymase, tryptase and proteoglycans.
However, an important difference was noted in FcεRI expression by the mast cells derived by the two
methods. Mast cells differentiated by co-culture with OP9 cells failed to expressed FcεRI on the cell
surface due to lack of expression of the α chain of this receptor. Critical architectural changes at the
FcεRI locus may precede the expression of this gene in mature mast cells, dendritic cells and
macrophages and these early events occur prior to differentiation of the CD34+ cells to mast cells. It is
possible that the OP9 culture conditions failed to send the appropriate signal required for altering the
structure of the locus in a manner amenable for the transcription factors that later would regulate gene
expression. Our data suggest that OP9 may only be able to support lineage-restricted progenitors or be
able to support hESC-derived hematopoietic progenitors without conferring their stem cell function.
Previous studies have shown that PGE2 is an important modulator of mast cell-dependent allergic responses. While in general PGE2 blocks allergic responses in humans and up-regulates the response in mouse mast cells \textit{in vitro}, PGE2 did not suppress exocytosis in CBMC\textsuperscript{6}. Similar to BMMC and CBMC cells, PGE2 stimulated phosphorylation of Erk1/2 in ESMC as well as intracellular calcium mobilization and PGD\textsubscript{2} production. When both stimuli were combined together, histamine release and PGD\textsubscript{2} production was not significantly changed, but production of TNF-\textit{\alpha} was almost totally inhibited. These findings suggest that signaling responses are similar between ESMC and CBMC. The demonstration that the hES derived mast cells express EP receptors opens the door for further investigation of the function of each EP receptor on the mast cell using siRNA vectors. These vectors can be introduced into the hES cells, stable transfectants isolated, and then differentiated into mast cells for study. As a first step toward carrying out such studies, we demonstrated that genetically manipulated hES cells retain their pluripotency, specifically their ability to generate CD34+ progenitor cells and that this can be achieved using a simple co-culture free protocol. Furthermore, these CD34+ cells retain their capacity to differentiate into mast cell populations. This approach should provide genetic tools for human mast cells studies, tools which until now have been limited to the mouse.

In summary, we report here a simple and robust protocol for the generation of human mast cells from hES cells, defined both by the expression of lineage specific markers and by the response of these cells to PGE\textsubscript{2} and antigen. Human mast cells can be obtained by this method in quantities sufficient for biochemical and physiological studies, and therefore will provide a novel tool for both identification of pathways critical to the function of mast cells in immune responses, as well as a uniform source of mast cells for testing novel therapeutic compounds for the treatment of asthma and other allergic diseases.
Acknowledgments

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Authorship contribution:

M.K. designed research, performed experiments, wrote manuscript, A.M.L performed experiments, K.D.C, S.L.T provided CBMC, B.H.K designed research, wrote manuscript.

Conflict-of- interest disclosure: The authors declare no competing financial interests.

References:


Table 1: Receptor expression in ESMC measured by real time PCR:

Table legend: Receptor expression in ESMC and CBMC

Expression pattern, relative to HPRT gene, of several receptors in mast cells derived by co-culture with OP-9 cells and from embryoid bodies compare to cord blood derived mast cells (CBMC) assayed by real time PCR. – expression< 0.1, + expression 0.1-1.0, ++ expression 1.0-10.0, +++ expression >10.0

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Table 2: Intracellular histamine level in human mast cells:

**Table legend:** 1x10^3 ESMC or CBMC 12 weeks in culture were lysed and the cell supernatant analyzed by an EIA histamine assay kit. (n=3).

<table>
<thead>
<tr>
<th>Type of mast cells</th>
<th>Histamine content (μg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESMC</td>
<td>9.32 ± 2.6</td>
</tr>
<tr>
<td>CBMC</td>
<td>8.8 ± 1.9</td>
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</tbody>
</table>
Figure legend:

**Figure 1: Differentiation of human embryonic stem cells (hES) to hematopoietic progenitors.**

A) hES cells were removed from mouse embryonic fibroblasts by treating with colagenase IV and co-cultured for 10 days with a monolayer of OP9 in α-MEM medium containing 15% FCS. (B) Fluorescence activated cell sort analysis (FACS) of a single cell suspension after 12 days of hES cell co-culture with OP9 cells; staining for CD34 and CD43. (C) Embryoid body (EB). hES cells were removed from mouse embryonic fibroblasts by treating with dispase and cultured in EB-differentiating medium for 4 days. (D) Phase contrast image of adherent EBs differentiated in IL-6, IL-3, SCF and FLT-3L for 3 weeks with hematopoietic progenitors releasing to medium, (small arrows show progenitors, large arrow shows differentiated embryoid bodies). (E) Phase contrast image of homogenous population of hematopoietic progenitors collected from medium 4 weeks after EBs differentiation. (F) Forward-side scatter plot of hematopoietic progenitors. (G) CD34 surface expression on hematopoietic progenitors. Histogram shows antibody staining (in dark) relative to isotype-matched control (transparent). (H) CD45 and CD43 surface expression. Microscopic figures (A, C, D, E) were capture using Olympus IX81 inverted fluorescence microscope (Olympus Imaging, PA) with objectives 4x (A, C, D) and 10x (E) with camera Hamamatsu ORCA RC (Hamamatsu Corporation, NJ), operated by software Velocity (PerkinElmer, MA). Bars in insets equal 100 μm. Data shown are representative from at least 3 experiments. (I) Schema showing co-culture free differentiation of human mast cells from hES cells.

**Figure 2: Phenotype of embryonic stem cell derived mast cells:**

Human mast cells differentiated from hES cells by embryonic bodies formation were stained with anti-trypase antibody, anti-chymase antibody, and Toluidine blue, and compared with human mast cells
derived from cord blood (CBMC). Nuclei were stained with DAPI. Figures were captured by Olympus BX61 upright fluorescence microscope (Olympus Imaging, PA) with objectives 40x (anti-chymase, anti-tryptase staining) and 60x (Toluidine blue staining) with camera Hamamatsu ORCA RC (Hamamatsu Corporation, NJ), operated by software Velocity (PerkinElmer, MA). Bars in insets equal 10 μm. Controls represent cells stained without anti-tryptase, anti-chymase antibody. Figures represent 1 of at least 4 independent experiments (B) Quantification of tryptase- and chymase- positive cells in culture during mast cells differentiation. (C) Activity of tryptase measured as a change in fluorescence activity of tosyl-Gly-Pro-Arg-pNA in mast cell lysates after 20 min incubation at 37˚C. Unit is defined as the amount of enzymatic activity that induces an 0.001 change in optical density at 405 nm/min (n=3) (D) Inhibition of tryptase activity by tryptase antagonist Polybrene, activity measured as in C, n=3.

Figure 3: Expression of FcεRI and c-Kit on ESMC:
Fluorescence activated cell sort analysis of ESMC. Staining of cells with anti-IgE antibody after exposure of cells with human IgE for 5 days (black), without IgE pretreatment (empty), and isotype control (empty dotted line). (A) ESMC differentiated by co-culture with OP-9, (B) ESMC differentiated by EBs formation, (C) CBMC. (D) Expression of c-Kit in ESMC differentiated by EBs formation, Isotype control (black), and anti-c-Kit antibody (gray). (E) Double staining of ESMC by anti-IgE and anti-c-Kit antibody. Results shown are representative of 6 experiments.

Figure 4: Activation of ESMC by cross-linking of FcεRI and PGE2:
(A) Phosphorylation of p42/44 ERK assayed by FACS using a phosphospecific antibody that recognizes phosphorylation of T202 and Y204. ESMC (left panel) or CBMC (right panel) were activated by FcεRI
cross-linking or by PGE2 activation. (black-unactivated, green-PGE2, red-anti-IgE, blue-PGE2 and anti-IgE) (B) Quantitative analysis (n=6) of T202/Y204 phosphorylation in ESMC stimulated by PGE2 (white bar), FcεRI (hatched bar) and FcεRI and PGE2 together (dark bar). (C) Intracellular calcium release in ESMC (left panel) or CBMC (right panel) and activated by FcεRI cross-linking 3 μg/ml anti-IgE antibody (blue) or 1 μM PGE2 (red). Point of stimulation is shown by arrow. Calcium mobilization is expressed as the ratio of fluorescence of Fura-2 measured at 340 and 380 nm. Typical results from at least 2 experiments are shown (D) Histamine release in ESMC (left panel) or CBMC (right panel) pre-incubated with hIgE and cross-linked with various concentration of anti-IgE antibody alone or together with 10^{-6}M PGE2. Histamine release is presented as a percentage of total histamine assayed by lysis of cells by Triton X100, n=3 asterisks represent significant differences from 0 μg/ml anti-IgE, p<0.05). (E) Release of TNF-α was evaluated in ESMC (left panel) and CBMC (right panel) pre-incubated with hIgE and cross-linked with 3μg/ml anti-IgE antibody alone or together with 10^{-6}M PGE2, n=3. (F) PGD2 release from ESMC (left panel) or CBMC (right panel) measured using PGD2 Methoxime EIA kit, n=3. Asterisks indicate values significantly different from untreated cells, p<0.05). Results shown in right panels in C and E were obtained under identical condition to those in left panels but on different days.

**Figure 5: Human mast cells differentiated from genetically modified hES cells.**

(A) A reporter gene expressing GFP under EF-1 alpha promoter was expressed in hES cell clone H1 by lentiviral transfection. hES cells double positive for GFP and pluripotent marker TRA-1-81 (region R5) were sorted by MoFlow sorter. Left panel: non-transfected cells, isotype control, Right panel: transfected cells stained with TRA-1-81. (B) hES cell lines expressing GFP were established. (C) Differentiating embryoid bodies formed from GFP-hES cells (D) GFP expressing mast cells progenitors releasing from
differentiated embryoid bodies to medium. Images were capture using Olympus IX81 inverted fluorescence microscope (Olympus Imaging, PA) with objectives 4x (E) Mast cell progenitors express GFP and CD43, left panel isotype control, right panel CD43 antibody. (F) Mast cell progenitors differentiated to mast cells expressing c-Kit, anti-c-kit-APC (gray), isotype control (empty). (G) Mast cells derived from GFP-hES express tryptase. Upper panel: GFP-hES cells, lower panels: mast cells derived from nontransfected H1 clone. Figures were captured by Olympus BX61 upright fluorescence microscope with (Olympus Immaging, PA) objectives 40x with camera Hamamatsu ORCA RC (Hamamatsu Corporation, NJ), operated by software Velocity (PerkinElmer, MA).
Figure 1

A-C: Micrographs showing different stages of cell differentiation.

B-H: Flow cytometry plots for CD34 (PE), CD43 (APC), SS Area, CD34 (PE), and CD45 (FITC).

I: Schematic diagram of the process:
- hES cells on MEFs monolayer
- Embryoid bodies
- Differentiating EBs
- Differentiating mast cells

Timeline:
- 5 days
- 3-12 weeks
- 10-14 weeks
Figure 2

A

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<th>Chymase</th>
<th>DAPI</th>
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B

![Graph B](graphB.png)

C

![Graph C](graphC.png)

D

![Graph D](graphD.png)
Figure 3

A

B

C

D

c-Kit (APC)

E

c-Kit (APC)

FcεRI (FITC)
Figure 4

A

phospho-Erk (PE)

B

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<th>anti-IgE</th>
<th>PGE₂ + anti-IgE</th>
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<tr>
<td>CBMC</td>
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C

PGE₂

anti-IgE

Ratio 340/390 nm

0 100 200 300 400

Time (s)

D

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<td>% of total histamine</td>
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<tr>
<td>0 µg of anti-IgE per ml</td>
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Figure 4 cont.
Human embryonic stem cells: a source of mast cells for the study of allergic and inflammatory diseases

Martina Kovarova, Anne M. Latour, Kelly D. Chason, Stephen L. Tilley and Beverly H. Koller