Long-Term Generation of Human Mast Cells in Serum-Free Cultures of CD34⁺ Cord Blood Cells Stimulated With Stem Cell Factor and Interleukin-3

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The generation of murine mast cells is supported by several cytokines, and mast cell lines are frequently established in long-term cultures of normal murine marrow cells. In contrast, growth of human mast cells was initially dependent on coculture with murine fibroblasts. The growth factor produced by murine fibroblasts and required to observe differentiation of human mast cells is attributable in part to stem cell factor (SCF). However, other factors are likely involved. We have previously shown that the combination of SCF and interleukin-3 (IL-3) efficiently sustains proliferation and differentiation of colony-forming cells (CFCs) from pre-CFC enriched from human umbilical cord blood by CD34⁺ selection. With periodic medium changes and the addition of fresh growth factors, five consecutive cultures of different cord blood samples gave rise to differentiated cells and CFCs for more than 2 months. Although differentiated cells continued to be generated for more than 5 months, CFCs were no longer detectable by day 50 of culture. The cells have the morphology of immature mast cells, are Toluidine blue positive, are karyotypically normal, are CD3⁴⁺, CD3⁴⁻, CD45⁺, c-kit⁻, and c-fms⁻, and die in the absence of either SCF or IL-3. These cells do not form colonies in semisolid culture and are propagated in liquid culture stimulated with SCF and IL-3 at a seeding concentration of no less than 10⁴ cells/mL. At refeddings, the cultures contain a high number (>50%) of dead cells and have a doubling time ranging from 5 to 12 days. This suggests that subsets of the cell population die because of a requirement for a growth factor other than SCF or IL-3. These results indicate that the combination of cord blood progenitor and stem cells, plus a cocktail of growth factors including SCF and IL-3 is capable with high efficiency of giving rise in serum-deprived culture to human mast cells that behave like factor-dependent cell lines. These cells may represent a useful tool for studies of human mast cell differentiation and leukemia.

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MATERIALS AND METHODS

Collection and separation of cord blood cells. Umbilical cord blood samples were collected at the New York Hospital-Cornell Medical Center. Stem cell factor (SCF) and interleukin-3 (IL-3) are required for growth of human mast cells from normal human CD34⁺ cord blood cells in serum-deprived suspension culture stimulated with SCF and IL-3 under serum-deprived conditions.

We have previously shown that the combination of SCF and IL-3 is at least as efficient as a stromal layer in sustaining the proliferation and differentiation of colony-forming cells (CFCs) from pre-CFC in serum-deprived liquid culture. The target cells for our studies were CD34⁺ cells isolated from human umbilical cord blood. In this report, we describe the establishment of long-term cultures of human mast cells from normal human CD34⁺ cord blood cells in serum-free suspension culture stimulated with SCF and IL-3 under serum-deprived conditions.

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The serum-deprived culture medium was composed of granulocyte colony-stimulating factor (G-CSF), SCF (all from Amgen, Thousand Oaks, CA), nonadherent cells were removed and then adherent (defined as CD34+) cells were harvested by mechanical agitation of the flask as described. Alternatively, CD34+ cells were separated directly from the light-density cell fraction by affinity chromatography with the Ceprate device (Cellpro, Bothell, WA), as described by the manufacturer. The frequencies of CFC and of CD34+ cells in the CD34+ cell populations purified according to the two techniques were subsequently analyzed in semisolid cultures (see below), or reanalyzed by flow cytometry after staining with a CD34-specific antibody (Gen Trak, Plymouth Meeting, PA). The frequencies of CD34+ cells as determined by fluorescence-activated cell-sorting analysis were 3% or 30% for the cells purified by panning or by affinity chromatography, respectively, and were comparable with the frequencies of CFC detected in the two populations. Despite the differences in the frequencies of CD34+ cells and CFC in cells purified by either of the two techniques, similar results were obtained in liquid culture.

Hematopoietic growth factors. The purified recombinant human hematopoietic growth factors used included erythropoietin (Epo), granulocyte colony-stimulating factor (G-CSF), SCF (all from Amgen, Thousand Oaks, CA), IL-3 (Genetics Institute, Cambridge, MA), G-CSF, IL-3, and SCF were used at concentrations that induced the optimal response in fetal bovine serum (FBS)-deprived cultures of human marrow cells. These concentrations are 2 x 10^-10 mol/L of G-CSF, 2 x 10^-10 mol/L of IL-3, 100 ng SCF/mL, and 1.5 U Epo/mL per culture.

Establishment of mast cell cultures from CD34+ cord blood cells. Harvested CD34+ cord blood cells (2.5 x 10^6 purified cells/flask) were incubated at 37°C in liquid culture under serum-deprived conditions in the presence of recombinant human IL-3 (2 x 10^-10 mol/L; Genetics Institute, Cambridge, MA) and recombinant human SCF (100 ng/mL; Amgen, Thousand Oaks, CA). The concentrations of IL-3 and SCF used in this paper were previously shown to induce optimal proliferation of human progenitors and mast cells. The serum-deprived culture medium was composed of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 100 U of penicillin (250 ng of amphotericin B, and 100 μg of streptomycin), deionized bovine serum albumin (BSA; 2 x 10^-4 mol/L), BSA-adsorbed cholesterol (4 μg/mL), and soybean lecithin (12 μg/mL), iron-saturated human transferrin (5 x 10^-7 mol/L), insulin (1.7 x 10^-6 mol/L), nucleosides (10 μg/mL each), inorganic salts, sodium pyruvate (10^-4 mol/L), and L-glutamine (2 x 10^-3 mol/L). All the chemicals were obtained from Sigma Chemical Co (St Louis, MO). Cell growth was monitored periodically with an inverted microscope. When the cell concentration in the flasks appeared to reach more than 0.5 x 10^6/mL, the cultures were semi-depopulated by replacing 50% of the medium with fresh medium and growth factors. The removed cells were counted and immunophenotyped and their content of CFCs was evaluated in semisolid cultures.

Colony assays. The CFC content of the harvested cells was evaluated in a standard methylcellulose assay. Briefly, each 1-mL dish contained PBS (Hyclone, Logan, UT; 30% vol/vol), BSA (0.9%, wt/vol), β-mercaptoethanol (7.5 x 10^-5 mol/L), antibiotics (100 U of penicillin, 250 ng of amphotericin B and 100 μg of streptomycin), and methylcellulose (0.8%, wt/vol, final concentration) in IMDM.

Table 1. Antigenic Markers of Long-term Cultures Derived From CD34+ Cord Blood Cells

<table>
<thead>
<tr>
<th>Mast Cell Cultures</th>
<th>No. 38</th>
<th>No. 41</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34 (Gen Trak Inc, Plymouth Meeting, MA)</td>
<td>-</td>
<td>-</td>
<td>pre-CFCs, CFCs</td>
</tr>
<tr>
<td>CD33 (Gen Trak Inc)</td>
<td>+</td>
<td>+</td>
<td>gp 67, CFCs, monocytes, mast cells</td>
</tr>
<tr>
<td>HLA-DR (Gen Trak Inc)</td>
<td>-</td>
<td>-</td>
<td>B cells, monocytes, activated T cells</td>
</tr>
<tr>
<td>CD5 (Gen Trak Inc)</td>
<td>-</td>
<td>-</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>CD45 (Gen Trak Inc)</td>
<td>±</td>
<td>±</td>
<td>Leukocyte common antigen</td>
</tr>
<tr>
<td>CD14 (Gen Trak Inc)</td>
<td>-</td>
<td>-</td>
<td>Monocytes, macrophages, granulocytes</td>
</tr>
<tr>
<td>CD16 (Gen Trak Inc)</td>
<td>-</td>
<td>-</td>
<td>NK cells, granulocytes</td>
</tr>
<tr>
<td>CD11b (Gen Trak Inc)</td>
<td>-</td>
<td>-</td>
<td>B cells</td>
</tr>
<tr>
<td>CD 42b (Armac Inc, Westbrooke, ME)</td>
<td>-</td>
<td>-</td>
<td>Platelet gplb</td>
</tr>
<tr>
<td>CD W64 (Harlen Inc, Indianapolis, IN)</td>
<td>-</td>
<td>-</td>
<td>Monocytes</td>
</tr>
<tr>
<td>c-kit (Dr A. Ulrich or Amac Inc)</td>
<td>+</td>
<td>+</td>
<td>CFCs, mast cells</td>
</tr>
<tr>
<td>c-fms/CSF-1 receptor (Oncogene Science Inc, Manhasset, NY)</td>
<td>-</td>
<td>-</td>
<td>Monocytes</td>
</tr>
</tbody>
</table>

Abbreviation: NK, natural killer.

Colony growth was stimulated with combinations of growth factors at appropriate concentrations, including Epo (1.5 U/mL), IL-3 (2 x 10^-10 mol/L), and SCF (100 ng/mL) for erythroid burst-forming cell (BFU-E) growth and mixed-cell CFC growth and G-CSF (2 x 10^-10 mol/L), IL-3 (2 x 10^-10 mol/L), and SCF (100 ng/mL) for granulocyte-macrophage CFC (GM-CFC) growth. Colonies were identified by their characteristic features after 12 to 14 days in culture and enumerated as described.

Characterization of the cell cultures. Cell-surface phenotype was determined by cytofluorimetric analysis on FACScan (Becton Dickinson, Mountain View, CA) of cells incubated with several antibodies specific for antigens expressed on hematopoietic cells. The source of the antibodies is specified in Table 1. Cytotoxic analysis was performed with specific kits provided by Sigma or with Toluidine blue (1% wt/vol in McIlvaine buffer, pH 4.0). For electron microscopy studies, the cells were fixed in suspension with phosphate-buffered 3% glutaraldehyde, osmicated, and embedded in PolyBed 812. Thin sections were examined with a Philips EM 410 electron microscope. Karyotypic analysis was performed in the Laboratory of Human Genetics of the Lindsley F. Kimball Research Institute by Dr James German.

RNA preparation and Northern blot analysis. RNA was extracted with phenol-chloroform from acid guanidinium-isothiocyanate cell lysates. RNA was size-fractionated by electrophoresis on agarose (1%) gel under denaturing conditions and blotted onto nylon membranes (Bio-Rad Laboratories, Richmond, CA) that were subsequently hybridized with the human c-kit (American Tissue Culture Collection depository), myeloperoxidase (a gift of Dr G. Rovera, Wistar Institute, Philadelphia, PA), β-globin, the α chain of the F,E receptor (Dr C. Wood, Genetics Institute, Cambridge, MA), or glyceraldehyde-3-phosphate dehydrogenase (G3PD) probe, as indicated. Each probe was radiolabeled by random oligonucleotide
priming (Amersham International, Amersham, UK) to a specific activity of 4 to 8 x 10^5 dpm/mg. After probing, the membranes were washed as recommended by the manufacturer and exposed for appropriate lengths of time with X-Omat film (Sigma) in cassettes for autoradiography (Amersham).

DNA preparation and Southern blot analysis. The possibility of Epstein-Barr virus (EBV) infection was investigated by Southern analysis. High molecular-weight genomic DNA was prepared by the procedure of Herrman and Frischauf. DNA (10 mg) was digested with Pst I and HindIII (New England Biclals, Beverly, MA), separated by electrophoresis on 0.8% agarose gel, and transferred to a nylon membrane. Membranes were baked at 80°C for 30 minutes in a vacuum oven and hybridized with cDNA radiolabeled by random oligonucleotide priming (Amersham). The probe represented a fragment of the virus genome (p 107.5) and was kindly provided by Dr Riccardo Dalla Favera (Columbia University, New York, NY).

RESULTS

Long-term generation of cord blood cells. The growth pattern of CD34+ cord blood cells in liquid culture stimulated with IL-3 and SCF is shown in Fig 1. During the first 2 to 3 months, large numbers of differentiated cells including erythroid as well as myelomonocytic cells and CFCs of all types (BFU-E, GM-CFC, and mixed-cell CFC) were generated. CFC were no longer detectable after 50 days of culture, but differentiated cells continued to accumulate. The morphology of these cells became more uniform over time (Fig 2A). Beyond 3 months, cell proliferation was maintained with medium changes and the addition of fresh growth factors every 3 to 4 days.

Fig 1. Total cell number (top) and progenitors of all types (bottom) in a typical long-term culture of CD34+ cord blood cells stimulated with SCF and IL-3. At each time point, the cultures were semi-depopulated as indicated by the double values connected with a vertical line. The values have not been corrected for semi-depopulation. The number of CFCs decreased to levels below detection by day 50, whereas large numbers of cells continued to be generated throughout the culture period.

The different mast cell cultures obtained are summarized in Table 2. The oldest cultures are now 5 to 8 months old and the cells have a doubling time ranging from 5 to 12 days. Furthermore, if cryopreserved, these cells can be thawed and cultured again for another prolonged period of time (>4 months) with no change in morphology or growth parameters; therefore, these cells behave like growth-factor–dependent cell lines. The long doubling time results from the fact that the cultures contain a high number (>50%) of trypan blue-positive (nonviable) cells.

The proliferation of these cells is dependent on the presence of SCF in combination with IL-3. Cells cultured in the absence of growth factor or in the presence of either SCF or IL-3 alone died within a few days. The growth of these cultures is also cell concentration-dependent since cultures were lost when initiated with a cell concentration less than 10^5/mL. No proliferation was observed if FBS (up to 20% vol/vol) was added to the liquid cultures. The cells failed to proliferate in semisolid culture at any cell concentration—even in FBS-deprived conditions.

Morphology, cell surface phenotype, and cytochemical analysis. To identify and characterize the cells generated in culture, two of the long-term cultures, #38 and #41, were examined for cell morphology, immunophenotype, and cytochemical markers at months 7 and 4, respectively. As observed by light microscopy, May-Grünwald-Giems-stained cells were mononuclear, filled with cytoplasmic granules, and had a mast cell-like morphology (Fig 2A). Cell-surface expression of CD33 and c-kit further indicated that the cultures contained mast cells (Table 1 and Fig 3). These findings were consistent with the detection of metachromatic granules in the cytoplasm of Toluidine blue-stained cells and more than 90% of all viable cells were Toluidine blue-positive (Fig 2B and Table 3). However, although the cells did not express myeloperoxidase or β-globin (Fig 4), they were positive for markers specific for other cell types, including tartric acid-sensitive acid phosphatase, tartric acid-resistant acid phosphatase, and naphthol AS-D chloroacetate esterase (Fig 5 and Table 3). This raised the possibility that the in vitro-derived mast cells are immature. In agreement with this, a representative cell from the generated cultures exhibited ultrastructural features of immature mast cells as shown by electron microscopy. The micrograph in Fig 6 showed that, except for small indentations, the nucleus was oval and unsegmented, it displayed a dispensed chromatin pattern, and there were numerous cytoplasmic granules that had a heterogeneous content. Further indication that the mast cells were immature came from the fact that the granules were negative for safranin staining and the cells did not express detectable levels of the a chain of the high affinity F,E receptor (not shown), another marker of mature mast cells.

Expression of c-kit protein/RNA and downmodulation by SCF. To determine the homogeneity of the long-term cultured mast cells with regard to cell surface expression of c-kit receptor, a human c-kit–specific monoclonal antibody was used for immunofluorescence analysis and it is reason-
able to assume that the fluorescence intensity correlates with the level of c-kit protein. As shown in Fig 3, almost all the cells expressed c-kit protein on the cell surface with a unimodal distribution. This indicates that the mast cell culture generated represents a relatively homogeneous population.

Because SCF and IL-3 are constantly present in the medium, they may influence the steady-state level of c-kit protein on these cells. Removal of both SCF and IL-3 from the culture medium for 6 hours elevated the mean fluorescence intensity (MFI) on the cell surface twofold ($79.8 \pm 13.6 \div 39.1 \pm 4.4, P < .01$). The increase in the level of c-kit protein
was rapid and dependent on the duration of deprivation of the growth factors, such that an increase in relative intensity of 20% was observed as early as 1 hour after withdrawal of SCF from the culture. The c-kit–specific fluorescence intensity (40 ± 5) for cells maintained in the presence of SCF alone for 6 hours was similar to that for cells incubated with both SCF and IL-3 (39.1 ± 4.4), indicating that SCF is the predominant modulator of c-kit expression. This is further supported by the finding that the increases in c-kit–specific fluorescence intensities were similar whether the cells were maintained in the presence of IL-3 alone or in the absence of both SCF and IL-3. The upregulation of c-kit receptor level in these human mast cells upon deprivation of SCF is in agreement with reports that kit ligand downregulates the cell surface expression of c-kit protein on murine bone marrow–derived mast cells by accelerating receptor internalization and receptor ubiquitination/ degradation.34,35
Two c-kit RNA species were detected in the long-term cultures of cord blood cells (Fig 4A). Yarden et al report that a single c-kit transcript of 5 kb was detected in Northern blots with human placental poly(A)+ RNA. It is unclear whether the lower–molecular-weight RNA observed here is c-kit mRNA or if it represents an alternatively spliced c-kit RNA. However, both of these RNA species increased proportionately over time in the cultures with SCF and IL-3.

**Karyotypic analysis.** Culture 37 was diploid, with the karyotype 46,XY. High-resolution G-banding of 20 cells showed the absence of gross deletions and rearrangements.

**Analysis for the presence of EBV in cells.** Southern analysis of the cell lines showed no evidence for EBV infection.

**DISCUSSION**

Previously, we and others have reported on the long-term suspension culture of CD34+ cord blood mononuclear cells. We showed the potential for such suspension cultures to generate large numbers of CFCs from pre-CFCs as well as differentiated cells of a variety of lineages, including macrophages, mast cells, neutrophils, and, in the presence of Epo, erythroblasts.

SCF and other growth factors, such as Epo or G-CSF, gave rise to short-term (up to 15 days) cultures containing large numbers of erythroblasts or neutrophils, respectively, whereas long-term (up to 1 month) maintenance of these cultures under serum-deprived conditions requires the presence of SCF and IL-3. We have now extended these studies to show that with refeeding of serum-deprived cultures and the addition of fresh growth factors, including SCF and IL-3, the culture can be maintained indefinitely. However, the morphology of the cells generated in culture changes over time. Whereas cells expressing β-globin and myeloperoxidase prevail at early time points (day 15), cells expressing myeloperoxidase prevail from day 15 to the end of the third month. At this point, the cell population generated in culture became homogeneous in morphology and myeloperoxidase negative, expressed high levels of c-kit, and had the ultra-structural morphology consistent with that of immature mast cells. In our study, continuously growing cultures have been maintained for up to 8 months in the presence of SCF and IL-3 and in the absence of a source of serum. Interestingly, the colony-forming ability of the cells was lost by the 50th day of culture, but differentiated cells continued to accumulate. The cells could be frozen and thawed repeatedly without change in morphology or growth characteristics. Because progenitor cells were not detectable by this time and the culture could be propagated with as few as 10^5 cells/mL, these mast cells have the potential to self-replicate and, therefore, behave as cell lines. However, we did not formally prove these are cell lines because we have been unable to clone them. They would not grow in semisolid culture or in liquid culture under limiting dilution conditions. It is possible that normal human mast cells might retain a limited self-replication potential if stimulated with the appropriate growth factor combinations.

The long-term mast cell cultures were established with a high degree of efficiency from CD34+ cord blood cells (5 of 5 cultures). Similar mast cell cultures were also established from CD34+ cells purified from fetal blood or from purified murine adult stem cells (results not shown).

Previous studies showed that the in vitro growth of human mast cells required a murine stromal cell line for support. Recently, Mitsu et al showed that the growth factor produced by the murine stroma and primarily responsible for mast cell development is SCF. In fact, mononuclear cord blood cells growing in FBS with the addition of SCF would give rise by day 15 to differentiated cells which were shown functionally to be immature mast cells. The mast cells proliferated for up to 2 months, after which bromodeoxyuridine incorporation was no longer detectable. Because the mast cells grown under these conditions remained immature in appearance and had a low proliferative index after several weeks in culture, it was speculated that SCF is primarily a maintenance factor. In murine mast cells, IL-3 and SCF upregulate the expression of two different genes involved in the prevention of apoptosis: IL-3 upregulates the expression of bcl-2 and SCF upregulates the expression of p53. These results would suggest that SCF and IL-3 are required at two different steps of the transduction pathway, which prevents apoptosis in murine mast cells.

In contrast with the work of Mitsu et al, we did not detect mast cells before 2 months of culture, and culture maintenance required both SCF and IL-3. One explanation of this difference could be that mast cell differentiation was triggered by two different progenitor cell populations in the
two studies. Light-density cells were used by Mitsui et al, and these cells contained progenitors that had the ability to rapidly differentiate and mature into mast cells. Our studies, which were performed with CD34+ cells, may have been relatively enriched for primitive progenitor cells that required additional time in culture to express the mast cell phenotype.

We have now established long-term maintenance (>8 months) of human mast cells in serum-deprived cultures that was dependent on the presence of both SCF and IL-3. If either of these growth factors was removed from culture, the cultures could not be maintained. But even under these conditions, a high proportion of nonviable cells was found at each refeeding of the cultures, leading to a long (5- to 12-day) doubling time for the cultures and suggesting that additional growth factors are necessary for maintaining the viability of the cells or for permitting maturation in vitro. An IL-3-like factor and a factor capable of maintaining a higher proportion of mast cells might be produced by accessory cells or provided by the FBS in the culture system used by Mitsui et al. Two possible candidates for an autocrine mast cell growth factor are nerve growth factor (NGF) and IL-4. In fact, murine mast cells functionally express NGF and IL-4 receptors. Furthermore, rat mast cells produce NGF in an autocrine fashion (R. Montalcini, personal communication) and human mast cells produce IL-4. We are currently planning to evaluate the possibility of an autocrine loop involving IL-4 or NGF in the growth of human mast cells.

The results of our study have several implications. First, the fact that cord blood stem cells gave rise with high efficiency to mast cells that are not able to form colonies in semisolid medium suggests that immortalization, but not full transformation of these cells, had occurred in vitro. Furthermore, these cells are clearly growth factor dependent and, in fact, require multiple growth factors for maintenance. However, the fact that we were able to establish these long-term mast cell cultures with high efficiency raises a cautionary note about the potential long-term clinical use of SCF or strategies to expand stem cells in vitro using combinations of growth factors including SCF.

Second, the fact that many of the cells die under serum-deprived conditions, even in the presence of SCF and IL-3, suggests that other factors are necessary for the maturation and maintenance of viability of human mast cells. Thus, these cells may provide a tool to identify such growth factors.

Finally, it will be of interest to determine what the evolution of these human long-term cultures is after more time and whether changes in growth factor dependence (or the ability to grow in the absence of growth factors) is seen, suggesting further progression along the transformation pathway. To date, we have not seen such changes, but because these cells have an apparent normal karyotype and are not infected with the EBV, they could be a useful model for study of human tumorigenesis.

To our knowledge, these are the first human long-term mast cell cultures established and they should provide a useful tool for studies of mast cell differentiation as well as the biology of cord blood stem cells and progenitor cells that appear to be so easily maintained under these conditions.

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