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+, 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 10^4 cells/mL. At refeedings, 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.

© 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Collection and separation of cord blood cells. Umbilical cord blood samples were collected at the New York Hospital-Cornell Medical Center. From the Laboratory of Hematopoietic Growth Factors, The New York Blood Center, New York, NY; Dipartimento di Biologia Cellulare, Istituto Superiore di Sanità, Rome, Italy; Molecular Biology Program, Sloan-Kettering Institute; Cornell University Graduate School of Medical Sciences; and the Division of Maternal Fetal Medicine, The New York Hospital-Cornell Medical Center, New York, NY.


Supported by research Grant No. HL-46524 from the National Institutes of Health, Department of Health and Human Services; institutional funds of the Lindsley F. Kimball Research Institute of the New York Blood Center; Progetto Finalizzato CNR "Ingegneria Genetica" and "Applicazioni Cliniche Ricerca sul Cancro"; and the Robert Wood Johnson Charitable Trust (to N.S.Y.).

Address reprint requests to Giovanni Migliaccio, PhD, The New York Blood Center, 310 E 67th St, New York, NY 10021.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.

0005-4971/94/8411-0002$3.00/0

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

By Brigitte Durand, Giovanni Migliaccio, Nelson S. Yee, Keith Eddleman, Tellervo Huima-Byron, Anna Rita Migliaccio, and John W. Adamson

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 stroma-free suspension culture stimulated with SCF and IL-3 under serum-deprived conditions.

Proliferation of murine mast cells is sustained by several cytokines including interleukin-3 (IL-3), IL-4, IL-9, and IL-10, as well as stem cell factor (SCF), which is also termed mast cell growth factor or kit ligand. Furthermore, in long-term cultures of normal murine marrow cells, cell lines have frequently been established based on their dependence on IL-3 for growth. Although they retain the capacity to differentiate along other hematopoietic lineages when stimulated with appropriate growth factors, most of these cell lines have a mast cell phenotype. The cells are karyotypically normal by light microscopy and do not induce tumors when injected into syngeneic recipients. The molecular events that result in immortalization of these cell lines are unknown.

In contrast, several human cytokines, including IL-3, have failed to sustain proliferation of human mast cells and, to date, no cell lines have been established from normal human long-term marrow cultures. However, human stromal cells supporting long-term hematopoiesis are less efficient than their murine counterparts. In vitro, growth and differentiation of human mast cells have been reported in cocultures of mononuclear cord blood cells and Swiss albino/3T3 fibroblasts. The growth factor produced by the murine fibroblasts and responsible, at least in part, for proliferation and differentiation of the human mast cells, recently has been identified as SCF, the ligand for the receptor encoded by the proto-oncogene c-kit. In fact, several independent reports have shown human mast cell differentiation from unfractionated cord blood mononuclear cells or adult blood and marrow in serum-supplemented cultures stimulated with SCF. Cells with a mast cell phenotype were first detected after 4 weeks of culture and became the predominant cell population by week 13. The cultures were not maintained beyond that point. However, the establishment of rat mast cell lines has been reported in cultures stimulated with SCF.
The serum-deprived culture medium was composed of granulocyte colony-stimulating factor (G-CSF), SCF (all from Amgen, Thousand Oaks, CA) and soybean lecithin (100 ng/mL), iron-saturated human transferrin (5 µg/mL), insulin (1.7 µg/mL), BSA-adsorbed human SCF (100 ng/mL; Amgen, Thousand Oaks, CA), the concentrations of 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 × 10^(-10) mol/L of G-CSF, 2 × 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 × 10⁶ purified cells/flask) were incubated at 37°C in liquid culture under serum-deprived conditions in the presence of recombinant human IL-3 (2 × 10^(-10) mol/L; Genetics Institute, Cambridge, MA), G-CSF, IL-3, and SCF and were used at concentrations that induced the optimal response in fetal bovine serum (FBS)-deprived cultures of human marrow cells. These concentrations are 2 × 10^(-10) mol/L of G-CSF, 2 × 10^(-10) mol/L of IL-3, 100 ng SCF/mL, and 1.5 U Epo/mL per culture.

Characterization of the cell cultures. Cell-surface phenotype was determined by cytofluorimetric analysis on FACSscan (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. Cytochemical 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 lysis. RNA was size-fractionated by electrophoresis on agarose (1%) gel under denaturing conditions and blotted onto nylon membranes (Biorad 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

| Table 1. Antigenic Markers of Long-term Cultures Derived From CD34⁺ Cord Blood Cells |
|-----------------------------------------------|---------------|-----------|
| Mast Cell Cultures                           | No. 38 No. 41 | Specificity               |
| CD34 (Gen Trak Inc, Plymouth Meeting, MA)    | – – c-kit    | pre-CFCs, CFCs             |
| CD33 (Gen Trak Inc)                          | + +           | gp 67, CFCs, monocytes, mast cells |
| HLA-DR (Gen Trak Inc)                        | – –           | B cells, monocytes, activated T cells |
| CD3 (Gen Trak Inc)                           | – –           | T-cell receptor            |
| CD45 (Gen Trak Inc)                          | ± ±           | Leukocyte common antigen   |
| CD14 (Gen Trak Inc)                          | – –           | Monocytes, macrophages, granulocytes |
| CD16 (Gen Trak Inc)                          | – –           | NK cells, granulocytes     |
| CD19 (Gen Trak Inc)                          | – –           | B cells                   |
| CD 42b (Armac Inc, Westbrooke, ME)           | – –           | Platelet gpII              |
| CD 56 (Gen Trak Inc)                         | – –           | NK cells                  |
| CD W64 (Harlen Inc, Indianapolis, IN)        | – –           | Monocytes                 |
| c-kit (Dr A. Ulrich or Armac Inc)            | + +           | CFCs, mast cells           |
| c-fms/CSF-1 receptor (Oncogene Science Inc, Manhassey, NY) | – –       | Monocytes                 |

Abbreviation: NK, natural killer.
priming (Amersham International, Amersham, UK) to a specific activity of 4 to 8 × 10⁷ 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 Frischhaft. DNA (10 mg) was digested with *Pst I* and *HindIII* (New England Biolabs, 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.

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 10⁶/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-Giemsa–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 dispersed 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 α 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 ± 13.6 v 39.1 ± 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.14,15

**Table 2. Summary of Long-term Mast Cell Cultures Obtained to Date**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Time in Culture</th>
<th>CFUs Detected Until Day</th>
<th>Doubling Time</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>5 mo</td>
<td>ND</td>
<td>—</td>
<td>Cryopreserved</td>
</tr>
<tr>
<td>37</td>
<td>3 mo</td>
<td>55</td>
<td>—</td>
<td>Cryopreserved at day 87</td>
</tr>
<tr>
<td>38</td>
<td>7-8 mo</td>
<td>70</td>
<td>6-12 d</td>
<td>Still growing</td>
</tr>
<tr>
<td>41</td>
<td>5-6 mo</td>
<td>70</td>
<td>4-5 d</td>
<td>Still growing</td>
</tr>
<tr>
<td>45</td>
<td>2 mo</td>
<td>ND</td>
<td>—</td>
<td>Still growing</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
Two c-kit RNA species were detected in the long-term cultures of cord blood cells (Fig 4A). Yarden et al reported 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 ultrastructural 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 10⁴ 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, Mitsui 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 Mitsui 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 refedding 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.43,44 Furthermore, rat mast cells produce NGF in an autocrine fashion (R. Montalcini, personal communication) and human mast cells produce IL-4.45 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.

ACKNOWLEDGMENT

We thank Y. Jiang, N. Hamel, and H. Ralph for their technical assistance; B. Alhadeff for the cytogenetics; J. Pack for manuscript preparation; Drs. L. Sozza, J. Egrie, and K. Zsebo (Amgen, Thousand Oaks, CA) and S. Clark (Genetics Institute, Cambridge, MA) for providing us with pure recombinant growth factors; the nursing staff of the Labor and Delivery Unit of the New York Hospital for the collection of cord blood samples; and Dr. P. Besmer (Sloan-Kettering Institute, New York, NY) for his helpful discussions.

REFERENCES


15. Valtiere M, Tweardy DJ, Caracciolo D, Johnson K, Mavilio F,


19. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, Hsu SY, Birkett NC, Okino KH, Murdock DC, Jacobsen FW, Langley KE, Smith KA, Takeishi T, Cattanach BM, Galli SI, Suggs SV: Stem cell factor is encoded at the Spr locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell 63:213, 1990


Long-term generation of human mast cells in serum-free cultures of CD34+ cord blood cells stimulated with stem cell factor and interleukin-3

B Durand, G Migliaccio, NS Yee, K Eddleman, T Huima-Byron, AR Migliaccio and JW Adamson