Recombinant human stem cell factor stimulates differentiation of mast cells from dispersed human fetal liver cells

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Recombinant Human Stem Cell Factor Stimulates Differentiation of Mast Cells From Dispersed Human Fetal Liver Cells

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We have previously shown the development in vitro of tryptase+ human mast cells from fetal liver cells cocultured with murine 3T3 fibroblasts. In this study, recombinant human stem cell factor (rhuSCF), the ligand for the c-kit proto-oncogene product called Kit, stimulated the growth and differentiation primarily of mast cells from dispersed fetal liver cells, whereas recombinant human interleukin-3 (rhuIL-3) stimulated the differentiation of basophils along with other cell types. Cultures of fetal liver cells were initiated and maintained in the presence of rhuSCF or rhuIL-3 for up to 6 weeks. Metachromatic cells in cytospins were identified as mast cells primarily on the basis of tryptase expression, and as MC~ or MC~ with immunohistochemistry using monoclonal antibodies against tryptase and chymase, whereas basophils were metachromatic, polymorphonuclear, and lacked these proteases. Levels of tryptase and histamine were measured by radioimmunoassay, tryptase and chymase activities by peptide hydrolysis, and cell surface Kit by flow cytometry with the monoclonal antibody YB5.B8. The predominant presence of mast cells occurred only in the cultures supplemented with rhuSCF. The percentage and total number of mast cells increased over time with increasing concentrations of rhuSCF and reached a plateau at 55 ng/mL. At this concentration of rhuSCF, mast cells first appeared by day 7; by day 42, 106% of the starting number of cells were present and 85% of these were tryptase+, 31% being weakly chymase+. These mast cells appeared immuno- by ultrastructural criteria; most cells were mononuclear, but some had nuclei with deeply divided lobes. DNA synthesis in tryptase+ mast cells at days 21 and 28 of culture with rhuSCF was demonstrated by incorporation of bromodeoxyuridine. Calculated levels of histamine (1.2 pg/mast cell) and tryptase (0.9 pg/mast cell) were similar to those determined previously in coculture experiments with murine 3T3 fibroblasts. Chymase activity was undetectable in most cell extracts. On day 0, 4% to 20% of fetal liver cells expressed cell surface Kit. In the presence of rhuSCF, the percentages and total numbers of Kit+ cells and the apparent concentration of Kit per cell increased along with the number of tryptase+ cells. In the presence of rhuIL-3, toluidine blue+, tryptase- cells first and maximally appeared at day 14 (11% ± 2.5%). The percentage of these toluidine blue+ cells then declined to about 6% by days 21 and 35, while the total number of positive cells declined over 10-fold. Kit+ cells in the presence of rhuIL-3 declined from 9% on day 3 to 2% on day 35. Thus, rhuSCF induces the differentiation of Kit+, tryptase+, toluidine blue+ mast cells, whereas rhuIL-3 induces the differentiation of Kit+, tryptase-, toluidine blue- basophils. In conclusion, human SCF appears to be a major growth factor for the differentiation of human mast cells.

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Supported by National Institutes of Health (NIH) Grants No. AI-20487 and AI-27517 to L.B.S. and NIH Grant No. AI-01076 and a charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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0006-4971/92/8012-0004$3.00/0

Blood, Vol 80, No 12 (December 15), 1992: pp 3009-3021

3009
5-fluorouracil, SCF alone stimulates colony formation, and SCF also acts synergistically with GM-CSF, IL-3, IL-6, and IL-1β to stimulate the growth of multilineage colonies. Recombinant rat SCF (rraSCF) acts synergistically with other growth factors on early murine hematopoietic progenitor cells that express the stem cell antigen (SCA+) and lack terminal lineage markers (Lin-). These findings suggest that SCF acts directly on early hematopoietic progenitor cells. In rodents, SCF has also been shown to be an MGF, as demonstrated by its ability to stimulate proliferation and maturation of IL-3-dependent mast cell lines. Furthermore, rraSCF results in the appearance of large numbers of mast cells at local cutaneous injection sites in normal and in mast cell-deficient SI/SI4 mice and increases numbers of both connective tissue and mucosal mast cells in a normal pattern of distribution when administered systemically to rats. Of further interest, both transmembrane and soluble forms of SCF are active on mast cells and, in combination with appropriate cytokines, on other hematopoietic cells, but survival of primordial germ cells and SCF-dependent cell adhesion is observed only with the transmembrane form.

In contrast to the situation in rodents, recombinant human SCF (rhuSCF) alone does not stimulate colony formation in agar cultures of normal human BM and BM from baboons. Analogous to rodents, synergistic effects on the number and size of colonies obtained with multipotential, erythroid, and granulocyte-macrophage progenitors from human BM are observed when either human or murine rSCF is added to rhuGM-CSF, rhuIL-3, and recombinant human erythropoietin (rhuEpo), respectively. SCF does not synergize with recombinant human macrophage-CSF (rhuM-CSF), and exhibits only a modest effect with recombinant human granulocyte-CSF (rhuG-CSF). The effects of rhuSCF were found to be most potent on a highly immature population of CD34+ cells that lack antigens associated with myeloid or lymphoid lineages, and do not contain a high proportion of colony-forming cells. In vivo administration of rhuSCF to baboons by continuous intravenous infusion resulted in increased numbers of erythrocytes, neutrophils, monocytes, lymphocytes, eosinophils, and basophils in peripheral blood, as well as an increase in marrow cellularity and in the absolute number of colony-forming units-granulocyte-macrophage (CFU-GM) and burst-forming units-erythroid (BFU-E). It appears that rhuSCF expands early multipotential, myeloid, and erythroid progenitor cells that can then proliferate and differentiate in response to the lineage-specific CSFs.

In studies by Kirshenbaum et al., using human BM cells, rhuSCF alone exhibited minimal capacity to stimulate production of mast cells and basophils, whereas rhuIL-3 was shown to stimulate the development of basophils and much lesser numbers of mast cells, and rhuSCF added to rhuIL-3 enhanced the effects seen with IL-3 alone without a selective effect on mast cells. Also, other studies have shown previously that stimulation of human fetal liver cells, BM, or cord blood cells with rhuIL-3 induces the transient appearance of basophils. Despite the focus of these prior studies, it is important to emphasize that IL-3 has pleiotropic effects and also stimulates growth and differentiation of other cell types, eg, eosinophils.

In humans, two types of mast cells have been identified based on protease composition. The MCTC type of mast cell contains the proteases tryptase, chymase, and carboxypeptidase and a cathepsin G-like proteinase; the MC(T) type of mast cell contains only tryptase, and basophils do not have appreciable quantities of any of these proteases. Human mast cells predominantly of the MC(T) type and MC(T) type, respectively, have been grown in vitro from progenitor cells present in cord blood and in fetal liver during coculture with murine 3T3 fibroblasts. Because SCF is expressed by murine 3T3 fibroblast cell lines, in which it serves to promote the adhesion of rodent mast cells, it may serve as a growth factor and adhesion molecule for human mast cells. Indeed, rraSCF and rmoSCF act on various rodent and human hematopoietic progenitors about equally well, although rhuSCF is nearly 800-fold less active than rat SCF on a mouse mast cell line, MC-9. In this study, we show that the addition of rhuSCF alone stimulates the proliferation and differentiation of Kit+ human mast cells in cultures of dispersed fetal liver cells, whereas rhu-IL-3 preferentially stimulates the development of Kit+ basophils.

MATERIALS AND METHODS

Materials. Histopaque, RPMI 1640, L-glutamine, nonessential amino acids, fetal calf serum (FCS), penicillin, streptomycin, amphotericin B, peroxidase-conjugated streptavidin, 30% hydroxyethylcarbazole, naphthol AS-MX phosphate, and fast blue RR were obtained from Sigma Chemical Co (St Louis, MO). Murine monoclonal antitryptase antibody (B7), murine monoclonal antitryptase antibody (G3), and goat polyclonal antitryptase antibodies were prepared in house as described previously. B7 was conjugated to biotin-N-hydroxysuccinimide and G3 to alkaline phosphatase. Bromodeoxyuridine (BrdU) and peroxidase-conjugated anti-BrdU antibody were obtained from Becton Dickinson Microbiology Systems (Cockeysville, MD). rhuSCF and rhuIL-3 were obtained from Angen (Thousand Oaks, CA). The monoclonal antibody (MoAb) YB8/B9 against human Kit was used as described previously.

 Cultures of dispersed fetal liver cells. Human fetal livers, 15 to 20 weeks of gestational age, were obtained at therapeutic abortions. The experimental protocol was reviewed and approved by the human studies committee at the Medical College of Virginia. Each liver was minced finely with scissors and the resulting cell suspension filtered over a #80 mesh stainless steel sieve. Approximately 2 to 3 × 10^6 cells were recovered per gram of tissue. Cell viability always exceeded 95%. Dispersed cells were resuspended at 2 to 3 × 10^6 cells/mL in complete RPMI (RPMI 1640 supplemented with 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 10 mmol/L HEPES, 2-mercaptoethanol [50 μmol/L], penicillin [100 U/mL], streptomycin [50 μg/mL], amphotericin B [250 ng/mL], and 10% FCS) and subjected to density gradient sedimentation over an equal volume of histopaque. Low-density cells recovered at the interface were resuspended at 2 × 10^6 cells/mL in complete RPMI and plated in 24-well or 96-well tissue culture plates (Costar, Cambridge, MA). To each well was added equal volumes of the cell suspension and of complete RPMI containing various concentrations of cytokine. Cells were cultured in a Nuair incubator (Nuair Inc, Plymouth, MN) at 37°C and in 5% CO₂. Half of the culture medium was replaced weekly with complete
RPMS with or without rhuSCF. At the time of collection, adherent cells were digested from the plates, in one series of experiments by treatment with 0.25% (wt/vol) trypsin, 0.1 mol/L EDTA for 5 minutes at 37°C and in another series of experiments by vigorous pipetting; in each case, adherent cells were combined with nonadherent cells. Cytocentrifuge preparations were obtained at the initiation of cultures and at various time points thereafter, air dried, and stored at room temperature for 1 to 2 weeks until subjected to double immunohistochemistry for the determination of mast cell numbers and mast cell phenotype. At the initial time point, 1 x 10^6 cells were centrifuged and the pellet stored at -70°C; at other time points, the remaining cells were centrifuged and the cell pellet, as well as the culture supernatant, were stored at -70°C for future determination of mediator content.

**Immunohistochemistry.** Cytocentrifuge preparations were subjected to double immunohistochemical labeling for determination of mast cell phenotype, as described previously,42 using sequential staining with biotinylated murine monoclonal antichymase antibody (B7-B, 2.5 μg/mL) and murine monoclonal antitryptase antibody conjugated to alkaline phosphatase (G3-AP, 6 μg/mL). With this technique, chymase+ , trypase+ granules are visualized in brown, and chymase−, trypase+ granules are visualized in blue. Cells in which all cytoplasmic granules stained brown and cells in which a mixture of blue and brown cytoplasmic granules appeared were both identified as MCtc cells. Cells containing blue cytoplasmic granules only were identified as MCt cells. Percentages of total cells comprising mast cells were determined under phase-contrast microscopy to identify unstained cells on the cytoplasts. Alternatively, cytocentrifuge preparations were stained with 0.5% toluidine blue in 0.5 N HCl for 2 hours at room temperature to detect cells with metachromatically staining granules.

**Incorporation of BrdU and 3H-thymidine.** Cell growth was determined by [3H]-thymidine (0.2 μCi/culture; Du Pont, Pont, Boston, MA) uptake during the last 20 hours of the culture period for cells, by counting of viable cells with the use of a Buerker hemocytometer (American Optical, Buffalo, NY) and trypsin blue exclusion, and by incorporation of BrdU. Cultures of fetal liver cells stimulated with various concentrations of rhusCF were pulsed with 10 μmol/L BrdU for 4 hours under standard culture conditions at days 21 and 28. Cells were then recovered and subjected to cytocentrifugation, fixation in 70% ethanol for 15 minutes, and incubation in methanol with 0.6% H2O2 for 30 minutes to inhibit endogenous peroxidase. Indirect immunohistochemistry was performed with a goat polyclonal IgG antitryptase antibody (26 μg/mL overnight at 4°C) and alkaline phosphatase-conjugated swine antigoat IgG antibody (1:50, 1 hour at room temperature) as described previously.49 The reaction product was visualized with fast blue RR. Incorporated BrdU was detected immunohistochemically, as described previously.

To denature DNA and expose incorporated BrdU, slides stained for BrdU were incubated in 0.07 N NaOH for 5 minutes and then neutralized with 0.1 mol/L borate buffer, pH 8.5. Morphologic detail was compromised by this procedure. Indirect immunoperoxidase was then performed using an overnight incubation with murine monoclonal anti-BrdU antibody (7 μg/mL at 4°C and peroxidase-conjugated goat antigoat IgG antibody (1:50) for 1 hour at room temperature. The reaction was visualized with 3-amino-9-ethylcarbazole and 0.01% H2O2 as a reddish brown reaction product. Cells undergoing DNA synthesis that had incorporated BrdU were recognized by the presence of a brown reaction product in the nucleus, and mast cells were identified by the presence of blue, trypase+ cytoplasmic granules.

**Mediator assays.** Frozen cell pellets were thawed and resuspended at 3 x 10^6 cells/mL in 0.01 mol/L 2-[N-Morpholinol] ethanesulfonic acid (MES) buffer, pH 6.0, 2 mol/L NaCl. Cell extracts were prepared by sonication of the cell pellets at 4°C with a Model W-225R sonicator and microtip (Heat Systems-Ultrasonics, Poughkeepsie, NY) at power 2, 50% pulse cycle, 10 pulses times two. Supernatants containing solubilized material were obtained by centrifugation of the sonicate at 12,000 rpm for 45 minutes at 4°C in a microcentrifuge.

**Electron microscopy.** Fetal liver cells were cultured in the presence of rhusCF (55 ng/mL) for 42 days and processed for electron microscopy as described previously.46 Cells were fixed in 2.5% glutaraldehyde, 3% paraformaldehyde, 0.1 mol/L cacodylate buffer, pH 7.2, for 45 minutes at 4°C, washed in 0.1 mol/L cacodylate buffer with 5% sucrose, post-fixed in 1% osmium tetroxide in cacodylate buffer at 4°C for 1 hour. The specimen was then dehydrated in graded ethanol, transferred through propylene oxide, and embedded in araldite. Thin sections, 60 to 100 nm, were cut on a Sorval MT-5000 ultramicrotome (Dupont, Newtown, CT). Immunogold labeling with antitryptase and antichymase antibodies, followed by sequential staining with uranyl acetate and lead citrate, was performed and evaluated with a JEM 1200EX electron microscope (JEOL, Akishima, Japan) as previously described.

**Flow cytometry.** Analyses of Kit expression was performed by flow cytometry using MoAb YB5.B8 against the Kit protein.41,44 Cells to be labeled (0.2 to 0.5 x 10^6 cells) were incubated for 1 hour in RPMI-10% human serum to block Fc receptor binding of MoAb and were then washed three times in PBS-1% BSA-0.1% sodium azide (washing buffer) at 4°C. YB5.B8 (ascites diluted 1:200) was incubated with the cells at 4°C for 30 minutes. Cells were washed three times with washing buffer and then incubated with a fluorescein isothiocyanate (FITC)-labeled F(ab′2) fragment of rabbit antirabbit IgG (Dako Corp, Carpinteria, CA) at 4°C for 30 minutes. Cells were washed three times as above and resuspended in PBS-1% paraformaldehyde and stored at 4°C until analyzed in a FACScan (Becton Dickinson, San Jose, CA), typically within 4 hours. For each sample, 5000 to 10,000 cells were analyzed. The percentage of cells staining with YB5.B8 was determined in the population of cells with higher relative forward light scatter (see Fig 8), but expressed as a percentage of the whole cell population. Because of noise and higher autofluorescence in the population with low forward light scatter, we were unable to calculate positive cells in that population. Thus, our calculations may underestimate the true percentage of positive cells at early time points, but not at late time points, when most cells exhibit high forward light scatter. The net percentage of positive cells was calculated by subtracting the percentage of positive cells with an isotype-matched negative-control MoAb (1% to 5%) from the percentage of positive cells with YB5.B8.

**Statistical analysis.** An analysis of variance (ANOVA) was used. Results are expressed as mean ± SEM, except where indicated otherwise.
specimen were cultured with rhuSCF at 13.75 ng/mL, 27.5 ng/mL, 55 ng/mL, and 110 ng/mL, or in control medium for 21 days. Half of the culture medium was replaced weekly with fresh medium containing the corresponding concentration of rhuSCF. Each experiment was performed in duplicate. As shown in Fig 1, tryptase+ cells developed in cultures supplemented with rhuSCF; the percentage of mast cells in the culture increased with increasing doses of rhuSCF, reaching a plateau of 42% mast cells at 55 ng rhuSCF/mL. No chymase+ cells were seen. The percentage of cells staining metachromatically with toluidine blue also followed a similar dose-response curve. By contrast, in the absence of rhuSCF, tryptase+ and/or toluidine blue+ cells comprised less than 1% of the total cells remaining in culture after 21 days. Also, the numbers of viable cells recovered after 21 days of culture increased in a dose-response fashion with rhuSCF; compared with the numbers of cells initially plated, only 20% were recovered in the absence of rhuSCF, whereas 44%, 101%, 147%, and 212% were recovered at rhuSCF concentrations of 12.75 ng/mL, 27.5 ng/mL, 55 ng/mL, and 110 ng/mL, respectively.

**Time course of mast cell development from dispersed fetal liver cells cultured with rhuSCF.** Dispersed fetal liver cells (1 × 10⁶ cells/mL) were cultured in the presence or absence of rhuSCF at 55 ng/mL for up to 6 weeks. Half of the culture medium was replaced at weekly intervals as above. Cultures were analyzed at weekly intervals for the total numbers of viable, tryptase+, and metachromatic cells; for mast cell phenotype; and for mediator content. Figure 2 shows the results of time course experiments. As shown earlier, the total number of cells surviving in the absence of rhuSCF decreased over time, such that only 6% of the cells initially plated were recovered by day 42. No tryptase+ cells were seen in these cultures. In contrast, in the presence of rhuSCF at 55 ng/mL, cell proliferation occurred, as evidenced by an increase in the total number of cells recovered at various periods during the culture as compared with the number of fetal liver cells initially plated. Tryptase+ cells were first detected after 1 week of culture; the percentage as well as the absolute number of tryptase+ cells increased gradually over time. Between 80% and 90% of the total cells by 4 to 6 weeks of culture were mast cells that stained strongly with antitryptase antibody (Fig 3A and B). Although most tryptase+ cells were mononuclear, multinucleated or multilobed cells were occasionally noted. Mast cells in cyt centrifuge preparations examined at earlier time points exhibited weaker staining and tryptase+ particulate material was sometimes detected outside of cells. Metachromasia after staining a representative mast cell preparation is shown in Fig 3C. The majority of mast cells developing in cultures supplemented with rhuSCF were of the MC₅ phenotype. No chymase staining was seen in the mast cells present at days 7 and 14 of culture. At later time points, the mean percentage ± SD of mast cells showing faint staining for chymase was 16 ± 5% at day 21 (n = 6), 33 ± 42% at day 28 (n = 4), 36 ± 40 at day 35 (n = 2), and 31 ± 27 at day 42 (n = 3) of culture. Figure 3D shows a field demonstrating the presence of both MC₅ and MC₆ cells.

To directly compare the effects of rhuSCF and rhuIL-3 on mast cell and basophil development from fetal liver cells, dispersed fetal liver cells were divided and placed into culture with rhuSCF (50 ng/mL), rhuIL-3 (1 ng/mL), or media alone for up to 5 weeks. Cells in culture were dispersed by mechanical agitation and counted. Cytospins were analyzed for metachromasia with toluidine blue and for tryptase by immunohistochemistry, and cells in suspension were analyzed for Kit by flow cytometry. Compared with total cell numbers at day 0, cell numbers in cultures with rhuSCF and rhuIL-3 were relatively unchanged for the first 2 to 3 weeks and then declined, whereas those with...
media alone steadily declined to about 10% by day 35 (Fig 4A). The explanation for the decline in rhuSCF-stimulated cells in these experiments compared with those in Fig 2 is not known, but could reflect better dispersion of adherent cells at later time points by trypsinization, slightly higher rhuSCF concentrations used in the prior experiments, or experimental variability among different fetal livers. In cultures stimulated with rhuIL-3, less than 0.5% of cells stained positively for tryptase in all cases except one, which at day 35 contained 7% tryptase+ cells (Fig 4B). However, the percentage of toluidine blue+ cells increased to 11% ± 2.5% by day 14 (Fig 4C), but then declined to about 6% ± 2% by day 21 and remained around 6% on day 35. These cells were polymorphonuclear. Thus, these toluidine blue+, tryptase− cells, most likely, are basophils. The correlation between tryptase+ and toluidine blue+ cells in rhuSCF- and rhuIL-3-stimulated cultures is shown in Fig 4D. The regression coefficient of linearity with rhuSCF was 0.99. Values for cells from rhuIL-3-stimulated cultures examined on days 14, 21, or 35 did not fall within the 95% confidence intervals.

As expected, the levels of histamine and tryptase in extracts from cells cultured with rhuSCF increased over time, corresponding with increased numbers of mast cells.
Fig 4. Comparison of rhuSCF with rhuL-3 on growth and development of mast cells and basophils from fetal liver cells. Fetal liver cells were cultured with (●) rhuSCF (50 ng/mL), (○) rhuL-3 (1 ng/mL), or (△) media alone. For all data points, n = 5 or 6, except for day 35 with media alone, where n = 3, and day 35 with rhuL-3, where n = 4. Adherent cells were dispersed by mechanical agitation and combined with nonadherent cells. The total numbers of cells (A), and the percentages of cells staining positive for tryptase (B) and toluidine blue (C) are shown. In (○), the correlation between tryptase+ and toluidine blue+ cells is illustrated. In (D), the correlation between tryptase+ and toluidine blue+ cells is illustrated.

(Fig 5). Levels of immunoreactive histamine and tryptase per mast cell also increased with time such that mast cells recovered from 1-week-old cultures contained a mean ± SD of 0.3 ± 0.14 pg of histamine per mast cell and 0.2 ± 0.06 pg of tryptase per mast cell, whereas mast cells recovered from 5- to 6-week-old cultures contained a mean of 1.22 ± 0.6 pg of histamine per mast cell and 0.92 ± 0.1 pg of tryptase per mast cell. Levels of enzymatically active tryptase (mU/10^6 total cells) also increased with increasing numbers of mast cells from 0.05 ± 0.03 at day 7 of culture to 3.4 ± 0.9 at day 35 of culture. These levels corresponded to microunits of enzymatically active tryptase per mast cell of 6.5 × 10^{-4} ± 5 × 10^{-4} at day 7 of culture to 4 × 10^{-3} ± 1 × 10^{-3} at day 35 of culture. In contrast, chymase activity was detectable only in one of the cultures of fetal liver cells; at day 42, a level of 0.2 × 10^{-3} μU/mast cell was measured, corresponding to 0.014 pg/mast cell based on the specific activity of purified skin-derived chymase (data not included). In other samples, the level of chymase was estimated to be less than 0.01 pg/mast cell.

Immunoreactive levels of histamine and tryptase were measured in the culture supernatants recovered at various time points during the culture period. As seen in Fig 5, levels of both mediators increased with time and with
increasing numbers of mast cells. The percentages of the total mediator content (medium + cell extract) found in the media at 5 and 6 weeks were comparable, and averaged 29% ± 9% for histamine and 14% ± 12% for tryptase.

**Fig 5.** Levels of tryptase and histamine in cell extracts and in the media of fetal liver cells cultured in the presence of 55 ng rhuSCF/mL for up to 42 days. (■) Percentages of tryptase+ cells. Data are expressed as mean ± SEM (n ≥ 3) or range (n = 2), where for day 0 and day 21 extracts n = 4; for day 7, day 35, and day 42 n = 2; for day 14 and day 28 n = 3; and for media n = 2 for day 7, day 21, day 35, and day 42; and n = 3 for day 14 and day 28. n, the number of different experiments. Adherent cells were collected by trypsinization and combined with adherent cells. (□) Histamine; (□) tryptase; (-) cell extract; (—) media.

**Cell growth.** The effect of rhuSCF and of rhuIL-3 on growth of fetal liver cells in liquid culture was assessed at 1, 3, and 6 days of cultures. Each cytokine was added at 0.1, 1, 10, or 100 ng/mL. Both rhuIL-3 and rhuSCF stimulated the cells to incorporate [³H]dThd in a dose-dependent pattern (Fig 6). Due to the variation in absolute cpm incorporated between different preparations of fetal liver cells, the results are expressed as a percentage of the maximal response to facilitate analysis of data from all experiments together. rhuSCF at 1 ng/mL significantly stimulated the incorporation of [³H]dThd at days 1, 3, and 6 compared with media alone, whereas cultures with rhuIL-3 at 0.1 to 10 ng/mL were significantly stimulated at days 3 and 6. The cytokine concentration at which maximal [³H]dThd incorporation was observed was 10 to 100 ng/mL for rhuSCF and 1 to 10 ng/mL for rhuIL-3. Neither in the presence of rhuIL-3 nor in the presence of rhuSCF was the cell number significantly changed at the day 1 and day 3 time points compared with day 0. However, by day 6, a statistically significant (P < .05) increase in cell numbers in cultures stimulated with each cytokine over media controls was observed, approximately 2.5-fold with rhuSCF at 10 ng/mL and 100 ng/mL and twofold with rhuIL-3 at 1 ng/mL, 10 ng/mL, and 100 ng/mL (Fig 6). This is consistent with the higher rates of DNA synthesis observed with these cytokine concentrations (Fig 6, upper panel). However, these numbers were not significantly higher than cell numbers at day 0. Also, this analysis could not distinguish the growth of progenitors of mast cells, basophils, or other cell types from one another.

To assess whether DNA synthesis is ongoing in mast cells, dispersed fetal liver cells were cultured in the pres-
ence of 55 ng rhuSCF/mL, and incorporation of BrdU into the nucleus of proliferating cells was assessed on days 21 and 28. Mast cells that are tryptase+ and BrdU+ or BrdU− are shown in Fig 3E. Tryptase+ cells comprised 57% and 65% of the total cells at days 21 and 28, respectively. As compared with the initial number of cells plated, respective cell numbers increased 1.3-fold and 0.9-fold in this experiment. Also, incubation with BrdU for 4 hours did not have an adverse effect on cell viability. Of the total cells at day 21, 39% incorporated BrdU, 57% were tryptase+, and 31% were both BrdU+ and tryptase+. Of the total cells at day 28, 46% were BrdU+, 65% were tryptase+, and 46% were positive for both markers.

Electron microscopy. Cell pellets from 6-week-old cultures of fetal liver cells supplemented with rhuSCF (55 ng/mL) were processed for electron microscopy. Mast cells were identified by immunogold labeling with monoclonal antitryptase antibody (5 nm gold particles) and rabbit polyclonal antichymase antibodies (15 nm gold particles). Mast cells also could be identified by their numerous cytoplasmic granules and plasma membranes with varying degrees of surface folds. Mast cell nuclei, though usually rounded, were sometimes folded. Granules exhibited a variety of architectural features. In some cases, as in the cell shown in Fig 7A and C, granules were compact and electron dense, whereas in others, as in Fig 7B and D, granules were larger and less electron dense. Most mast cells contained a few granules with scrolls along with lamellae, but gratings and lattices were not observed. All granules were labeled with 5 nm gold particles, representing the presence of tryptase. Chymase labeling was more variable and always less intense, as shown in Fig 7C and D. In this particular preparation, 47% of the mast cells were weakly stained with B7-biotin by light microscopy, but chymotryptic activity was not detectable.

Expression of c-kit. To monitor cell surface expression of Kit, binding of YB5.B8 to fetal liver cells was assessed by flow cytometry. Figure 8 shows examples of the flow cytometric analysis of Kit expression on fetal liver cells grown in the presence of rhuSCF (50 ng/mL) for 3 and for 35 days. In the left panels, side light scatter (cell density) is plotted against forward light scatter (cell size); in the right panels, fluorescence intensity after staining with YB5.B8 is plotted against relative cell number. Cells analyzed on day 35, compared with those analyzed on day 3, were larger, more dense, and contained a greater proportion of Kit+ cells. As shown in the right panels, those cells with high forward light scatter exhibited a higher intensity of fluorescence, suggesting a greater amount of Kit, on day 35 than on day 3.

The development of Kit+ cells over time in the presence of rhuSCF, IL-3, or media alone is depicted in Fig 9. A mean of 10% ± 3% (range, 2% to 20%) of the low-density fetal liver cells expressed Kit before culture. The variation in percentages was unrelated to the age of the fetus. Stimulation of fetal liver cells with rhuSCF (50 ng/mL) increased the percentage of cells expressing Kit receptor over time. At day 14, the percentage was significantly higher compared with rhuIL-3–stimulated or control cultures. The percentages of Kit+ cells showed a strong linear correlation with the percentages of tryptase+ cells (P < .05). In rhuIL-3–stimulated cultures, the percentage of Kit+ cells declined slightly to 9% ± 1.5% by day 3 and to 2% ± 0.4% by day 35 (Fig 9), values that were not significantly different from control cultures.

DISCUSSION

This study shows that rhuSCF, the ligand for Kit, promotes the development and growth of human mast cells from precursor cells found in fetal liver. This effect did not require the addition of other exogenous cytokines, although a potential effect of endogenous cytokines produced by fetal liver cells cannot be ruled out. After 4 to 6 weeks of suspension culture in the presence of rhuSCF at 50 or 55 ng/mL, a mean of about 80% of the cells in culture had tryptase+, metachromatic cytoplasmic granules, identifying them as mast cells. Mature human mast cells were previously reported to be Kit+. Approximately 10% of the low-density fraction of fetal liver cells and essentially all of the tryptase+ cells that develop in the presence of rhuSCF express Kit as analyzed by flow cytometry with YB5.B8 anti-Kit antibody.

Surface markers coexpressed with Kit on human BM cells included CD34+/CD33−, CD34+/CD33+, and CD34−/CD33−.51 and on human fetal liver cells included CD11a, CD44, CD55, and VLA-4.52 However, in neither study were surface markers correlated with a particular cell lineage. Stimulation of fetal liver cells with rhuSCF increased the percentage and total number of cells expressing Kit (Fig 8). rhuSCF also increased the size and density of the cells and the apparent density of Kit on the cell surface (Fig 9). After 35 days of culture, up to 80% (mean, 52%) of the cells expressed Kit, corresponding to the percentage of tryptase+ mast cells. Because only about 1 × 105 fetal liver cells express Kit initially, it is likely that this population was selectively expanded by rhuSCF, although conversion of Kit− to Kit+ cells has not been excluded. The demonstration in the current study that 71% of the tryptase+ cells developing by day 28 in the presence of 55 ng rhuSCF/mL incorporated BrdU supports the concept that selective proliferation of mast cells occurs under the present culture conditions. Whether the initial Kit+ population was composed of committed mast cell progenitors, of multipotential progenitor cells, or of both cell types is unknown. The finding of Kit on human mast cells developed in culture is in agreement with the observation that mature mast cells express Kit.50,52 Thus, human mast cells might respond to SCF throughout their development.

In contrast to cells cultured with rhuSCF, rhuIL-3–stimulated cultures yielded few mast cells at any time point (≤2%, ≤3 × 105). Instead, cells classified as basophils on the basis of metachromatic, tryptase− granules, a Kit− cell surface, and a multilobed nucleus were detected. From 105 fetal liver cells, maximal numbers and percentages of basophils were detected at 14 days of culture, when 1.2 × 105 basophils (11% purity) were found with rhuIL-3 (1 ng/mL). In terms of the relative proportions of mast cells and basophils observed with rhuIL-3, the present results...
HUMAN MAST CELL DIFFERENTIATION BY SCF

are similar to those reported previously with human BM cells. Other cell types whose differentiation or survival is known to be affected by IL-3, such as eosinophils, were not examined in the current study. Exposure of different types of murine cell lines to CSFs, including IL-3, reportedly downregulates c-kit-derived messenger RNA (mRNA) expression. In the current study, the percentage of cells expressing Kit declined in the presence of rhuIL-3 or of media alone to a similar extent. The effect of combinations of rhuIL-3 and rhuSCF on the growth and development of mast cells and basophils was not explored in this study. However, preliminary results suggest that development of mast cells from fetal liver cells in the combined presence of rhuSCF (50 ng/mL) and rhuIL-3 (1 ng/mL) was not substantially altered. Although the effects of rhuSCF and rhuIL-3 on fetal liver cells in the current study seemed to be direct, other factors produced by cells stimulated with rhuSCF or rhuIL-3 may be involved.

The biologic role of SCF in mast cell development is well established in the rodent system. Thus, mice with mutations at the W or Sl locus resulting in deficient Kit or Kit-ligand (SCF), respectively, are mast cell deficient. When murine mast cells derived from the peritoneal cavity (connective tissue mast cells) and from IL-3–dependent BM cells grown in vitro (immature connective tissue mast cells) were plated in methylcellulose, colony formation occurred best when
IL-3, IL-4, and rraSCF were used together. By themselves, rraSCF and IL-4 were not effective inducers of mast cell colonies. IL-3, by itself, did support colony formation with IL-3-dependent BM-derived mast cells, but not with peritoneal-derived mast cells. In liquid culture, rraSCF increased proliferation of mouse peritoneal-derived and mouse IL-3-dependent BM-derived mast cells and induced maturation of the IL-3-dependent BM-derived mast cells as evidenced by a 28-fold increase in histamine levels per cell and the cellular production of heparin rather than chondroitin sulfate proteoglycan. Long-term culture of lineage-depleted mouse BM cells with rraSCF by itself resulted predominantly in the growth of mast cells. A single administration of rraSCF intravenously to rats caused transient neutrophilia (2 to 12 hours) and lymphocytosis (0.5 to 2 hours) and myeloid and erythroid hyperplasia of the marrow at 6 hours. Daily injections of rraSCF for 2 weeks caused a striking mast cell hyperplasia in the marrow and other tissues and the appearance of mast cells in the bloodstream. Careful analysis of mast cell type using protease markers of mucosal and connective tissue mast cells in rats showed that systemic administration of rraSCF results in the growth of both mucosal and connective tissue mast cells. Thus, SCF plays a major role in mast cell development in rodents. For most hematopoietic progenitor cells, SCF appears to act preferentially on multilineage or immature lineage-committed cells to render them more responsive to the effects of various CSFs. For mast cells, at least in liquid culture, the effects of SCF do not require additional exogenous growth factors and occur at all stages of mast cell development.

Previous studies with human or primate material have not reported the selective development of mast cells in vitro when SCF was used alone, in contrast to a study with mouse BM. These results with human and primate cells may be explained by the fact that special stains to identify mast cells on the basis of mast cell protease content were not used, that culture in methylcellulose or other semisolid media may not be optimal for mast cell development, and that longer periods of culture may be necessary for mast cells to appear. Finally, the precursor cells used in the above studies were derived mostly from human BM, whereas human fetal liver cells were used as a source of progenitor cells for the present study.

Development of mature mast cells was observed by coculture of mononuclear cells of cord blood and fetal liver with murine 3T3 fibroblasts. The content of histamine and tryptase in mast cells developing from fetal liver cells cultured in the presence of rhuSCF was similar to that
reported in mast cells developed from fetal liver cells cocultured with murine 3T3 fibroblasts. Spontaneous release of histamine in the culture medium was also similar in both studies, reaching 30% to 40% of the total cellular histamine content. Tryptase appeared in the culture media in the present study at 3 and 4 weeks of culture, whereas tryptase levels were essentially undetectable in the culture supernatants of fetal liver cells cocultured with murine 3T3 fibroblasts for 1 month. The mechanism explaining the apparent difference in tryptase secretion between mast cells developing in coculture with fibroblasts and those developing with rhuSCF is not obvious. The appearance of both tryptase and histamine in the media may occur due to secretion stimulated by the conditions of culture or to mast cell turnover. In this respect, rraSCF administered subcutaneously to mice results in Kit-dependent activation of mast cells in the dermis; in humans, SCF primes mast cells and basophils for activation by various secretagogues. As shown in Fig. 7, the ultrastructural morphology of mast cells at day 42 often shows swollen granules of low electron density, consistent with activation. Thus, SCF may play a role in the functional response of mast cells as well as in their growth and differentiation.

Whether SCF is the only growth factor produced by murine 3T3 fibroblasts that is involved in the development of mast cells and whether soluble SCF functions differently in this capacity than the transmembrane form of SCF expressed on cell surfaces remain to be demonstrated. In this respect, it is interesting to note that the morphologic appearance of mast cells developed with soluble rhuSCF is less developed than those grown in coculture with fibroblasts and that chymase+ cells, when they develop, show weak chymase staining when rhuSCF is used alone, and strong chymase staining under coculture conditions. That the weak staining for chymase accurately reflects cellular levels was supported by our inability to detect chymase enzymatic activity in all but one of the cell extracts, suggesting a level of less than 0.01 pg/mast cell in most cultures. This compares with 4.5 pg chymase/mast cell from adult skin and approximately 0.6 pg/mast cell from newborn foreskin and is consistent with our experience that immunohistochemistry on cytospin preparations is more sensitive than measurements of protease activity in extracts of dispersed mast cells. Also, grating and lattice structures, characteristic of mast cells of the MC\(_{TC}\) type both in normal tissues and in cocultures of cord blood cells with fibroblasts, were not observed with rhuSCF-dependent fetal liver-derived mast cells. Instead, granules of varying degrees of lucency, containing lamellae, scrolls, and particulate and amorphous material were observed. Thus, one may postulate that cell-cell contact involving Kit+ cells and cells expressing the transmembrane form of SCF on their surface is required for full expression of the chymase gene, or that factors in addition to SCF may be required.

Whether the effect of rhuSCF on fetal liver cells is altered in the presence of other cytokines remains to be determined. If such were the case, one would expect similar results to those seen with human BM, in which SCF enhances the effects of various CSFs. On the other hand, mast cell development from hematopoietic progenitor cells cultured in the presence of SCF alone may represent a "default pathway," occurring optimally in a permissive environment, such as skin, lung, and bowel, in which SCF may be the dominant cytokine. A corollary to this hypothesis is that other cytokines may create a nonpermissive environment for mast cell development, such as in BM. This hypothesis will require further experimentation.

Fetal liver cells appear to be enriched in progenitors of mast cells based on the abundance of mast cells that develop from this source. Approximately 10% of fetal liver cells are Kit+, as compared with 1% to 2% Kit+ cells in cord blood (T. Ishizaka, personal communication, 1992), 4% ± 2% in human BM, and 1% to 4% in unfractionated fetal liver cells recovered after perfusion with an MoAb against Kit. This cell population may account for progenitors of mast cells as well as for other cell types that require additional growth factors for growth and differentiation. Thus, fetal liver cells are particularly well-suited to studies of the development of human mast cells in vitro in the presence of rhuSCF.

ACKNOWLEDGMENT

We thank Scott Howard for expert technical assistance and Gloria Smith for excellent secretarial assistance.

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