FLT3/FLK2 Ligand Promotes the Growth of Murine Stem Cells and the Expansion of Colony-Forming Cells and Spleen Colony-Forming Units

By Susan Hudak, Brisdell Hunte, Janice Culpepper, Satish Menon, Charles Hannum, LuAnn Thompson-Snipes, and Donna Rennick

The effect of FLT3/FLK2 ligand (FL) on the growth of primitive hematopoietic cells was investigated using Thy1-Sca1+ stem cells. FL was observed to interact with a variety of factors to initiate colony formation by stem cells. When stem cells were stimulated in liquid culture with FL plus interleukin (IL)-3, IL-6, granulocyte colony-stimulating factor (G-CSF), or stem cell factor (SCF), cells capable of forming colonies in secondary methylcellulose cultures (CFU-c) were produced in high numbers. However, only FL plus IL-6 supported an increase in the number of cells capable of forming colonies in the spleens of irradiated mice (CFU-S). Experiments with accessory cell-depleted bone marrow (Lin- BM) showed that FL alone lacks significant colony-stimulating activity for progenitor cells. Nevertheless, FL enhanced the growth of granulocyte-macrophage progenitors (CFU-GM) in cultures containing SCF, G-CSF, IL-6, or IL-11. In these assays, FL increased the number of CFU-GM initiating colony formation (recruitment), as well as the number of cells per colony (synergy). Many of the colonies were macroscopic and contained greater than 2 x 10^5 granulocytes and macrophages. Therefore, FL appears to function as a potent costimulus for primitive cells of high proliferative potential (HPP). FL was also observed to costimulate the expansion of CFU-GM in liquid cultures of Lin- BM. In contrast, FL had no growth-promoting effects on progenitors committed to the erythroid, megakaryocyte, eosinophil, or mast cell lineages.

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MATERIALS AND METHODS

Animals. C57Bl/Ka Thy1.1 mice were bred and maintained at Simonsen Laboratory (Gilroy, CA). CBA/J mice were purchased from Simonsen Laboratory.

Growth factors. Purified recombinant human erythropoietin (epo) [specific activity (spec act), greater than 10^6 U/mg] and mouse stem cell factor (SCF; spec act, 10^5 U/mg) were purchased from R & D Systems (Minneapolis, MN) and Genzyme (Cambridge, MA), respectively. Purified recombinant murine GM-CSF (spec act, 1.3 x 10^6 U/mg) was a gift of Schering Plough Research Institute (Kenilworth, NJ). Purified human G-CSF (spec act, 3 x 10^7 U/mg), murine IL-3 (spec act, 2 x 10^5 U/mg) and murine IL-6 (spec act, 4 x 10^5 U/mg) were provided by Drs G. Zuwalski, A. Miyajima, and S. Menon, respectively (DNAX, Palo Alto, CA). Supernatants of Cos-7 cells transfected with cDNA encoding murine IL-11 were provided by Dr F. Lee (DNAX). One unit of Cos-7 cell-expressed IL-11 was defined as the amount of factor that stimulates half-

From the Departments of Immunology and Molecular Biology, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA.

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Address reprint requests to Donna M. Rennick, PhD, DNAX Research Institute of Molecular and Cellular Biology, 901 California Ave, Palo Alto, CA 94304.

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maximal \(^{3}H\)-thymidine incorporation by a factor-dependent cell line plated at 5 \times 10^5 cells per mL. Purified recombinant murine FL (spec act, 2 \times 10^3 U/mg) was produced at DNAX. Briefly, a mouse FL fragment, encoding amino acid residues 28 to 162, was isolated by polymerase chain reaction (PCR) using the T118 cDNA clone as a template. This fragment was inserted into the expression vector pET3a. Inclusion bodies were isolated from transformed Escherichia coli carrying FL-pET3a and solubilized in Tris buffer, pH 8.5, containing 6 mol/L guanidine HCl and 10 mol/mL l-ithiocreitol (DTT). The solubilized inclusion bodies were renatured by dilution in 50 mmol/L Tris, pH 8.5, containing 2.5 mol/L reduced glutathione, 0.5 mol/L oxidized glutathione, and 0.15 mol/L NaCl. The renatured protein was purified by sequential chromatography on an anion exchange column (POROS-Q; PerSeptive Biosystems, Cambridge, MA) at pH 7.5 and on a cation exchange column (Poros S; PerSeptive Biosystems) at pH 3.0. Protein fractions were assayed for activity using the Ba/F3 cell line expressing murine FLK2/FLT3, as described below. Active fractions were pooled, lyophilized, and stored at 4°C. Pyrogen levels were determined by the Limulus Amebocyte Lysate method (Whittaker Bioproducts, Walkersville, MD) and were found to be approximately 2 EU/mg of protein.

**Antibodies.** Monoclonal antibodies specific for murine IL-6 (MP2-20F3), M-CSF (5A1), and GM-CSF (22E5) were a gift of Dr. J. Abrams (DNAX). Each of these antibodies was used at 15 pg/mL. Antibodies specific for CD4 (GK1.5), CD8 (53.6.7), B220 (RA3-6B2), Mac1 (M1/70), GR1 (RB6-8C5), and erythrocytes (Ter-119). Lineage-positive cells were depleted using magnetic particles coated with goat antitrat antibodies (PerSeptive Diagnostics, Cambridge, MA) in two successive rounds of treatment.

**Purification of Thy^+^Sca+^Lin-^BM**. BM cells were isolated from femurs and tibias of 6- to 8-week-old mice, then overexpressed by the Ba/F3 cell line expressing murine FLK2/FLT3 as described below. Active fractions were pooled, lyophilized, and stored at 4°C. Pyrogen levels were determined by the Limulus Amebocyte Lysate method (Whittaker Bioproducts, Walkersville, MD) and were found to be approximately 2 EU/mg of protein.

**Antibodies.** Monoclonal antibodies specific for murine IL-6 (MP2-20F3), M-CSF (5A1), and GM-CSF (22E5) were a gift of Dr. J. Abrams (DNAX). Each of these antibodies was used at 15 pg/mL. Antibodies specific for CD4 (GK1.5), CD8 (53.6.7), B220 (RA3-6B2), Mac1 (M1/70), GR1 (RB6-8C5), and erythrocytes (Ter-119). Lineage-positive cells were depleted using magnetic particles coated with goat antitrat antibodies (PerSeptive Diagnostics, Cambridge, MA) in two successive rounds of treatment.

**Lineage-depleted bone marrow cells (Lin^-^BM).** BM cells were isolated from femurs and tibias of 6- to 8-week-old mice, then overlaid on Lymphopaque (Accurate Chemicals, Westbury, NY), and centrifuged at 1,000g for 20 minutes. Cells were removed from the interface, washed, and incubated with unmodified rat monoclonal antibodies specific for CD4 (GK1.5), CD8 (53.6.7), B220 (RA3-6B2), Mac1 (M1/70), GR1 (RB6-8C5), and erythrocytes (Ter-119). Lineage-positive cells were depleted using magnetic particles coated with goat antitrat antibodies (PerSeptive Diagnostics, Cambridge, MA) in two successive rounds of treatment.

**Purification of Thy^+^Sca-^1^^Lin-^BM.** The protocol used is a modification of that described previously by Spangrude et al. Briefly, BM cells from C57Bl/Ka Thy.1 mice were prepared by isolation of the interface from Lymphopaque followed by incubation with rat monoclonal antibodies specific for CD4, CD8, B220, Mac1, GR1, and erythrocytes. Lineage-positive cells were removed by two rounds of depletion with antitrat coated magnetic particles. The remaining cells were stained in suspension with phycoerythrin (PE)-goat antitrat antibodies (Biomed, Foster City, CA), fluorescein isothiocyanate (FITC)-conjugated anti-Thy 1.1 (19XES), biotinylated anti-Sca-1 (E13 161.7), and Texas Red-conjugated streptavidin (Biomed). Cell separation was performed on a dual laser FACStar<sup>TM</sup> (Becton Dickinson, Milpitas, CA). An initial sort gate was set to select for cells with intermediate forward light scatter and low-to-negative staining with PE/propidium iodide (lineage marker negative, viable cells designated Lin^-^). Secondary sorting criteria were intermediate levels of fluorescein staining (Thy+) and high levels of Texas Red staining (Sca-1^+^).

** Colony-forming assays.** Either 1 \times 10^5 nonadherent BM cells, 5 \times 10^5 Lin^-^BM cells, or 1.5 \times 10^5 sorted Thy^+^Sca^-^1^ cells were seeded in 35-mm culture dishes containing 1 mL modified Iscove's medium (GIBCO, Grand Island, NY), 20% fetal calf serum (FCS; GIBCO), 50 \mu mol/L 2-mercaptoethanol, and 0.8% (wt/vol) methylcellulose. All cultures were supplemented with saturating concentrations of FL, various growth factors, or a combination of these, as indicated in Results. Plates were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂. After 7 or 14 days of culture, the number and size of colonies were analyzed. Cell morphologies were determined after sequentially isolated colonies were applied to glass slides and stained with Wright-Giemsa (Sigma, St. Louis, MO). For megakaryocyte and eosinophil colony formation, agar (0.3% wt/vol) cultures were used. After 7 days of incubation, the agar cultures were fixed in 2.5% glutaraldehyde and stained for acetylcholinesterase (megakaryocytes) or with Luxol blue (eosinophils) and counterstained with hematoxylin. For mast cells, methylcellulose cultures were incubated for 21 days. Sequentially isolated colonies were stained with toluidine blue, and mast cells were identified by their metachromatic granules.

**Liquid culture.** Thy^-^Sca^-^1^ cells (400) or 5 \times 10^4 Lin^-^BM cells were seeded in 1.5-mL microcentrifuge tubes in a total volume of 315 \mu L of modified Iscove's medium, 20% FCS (vol/vol), 50 \mu mol/L 2-mercaptoethanol, and various growth factors. After 7 days in culture, cells were harvested, washed, and counted. Cells were resuspended in medium and cultured in methylcellulose to detect colony-forming cells (CFU-c). A combination of hematopoietic growth factors (SCF + IL-3 + IL-6 + epo) was used in the colony-forming assays to support the development of all cell lineages. The net increase in CFU-c was calculated based on the number of colonies formed by the original Lin^-^BM population and the number of colonies observed in the secondary cultures.

**Spleen colony-forming unit (CFU-s) assay.** The CFU-s assay was performed by injecting various concentrations of cells into lethally irradiated recipients (six mice per group). Spleens were removed 12 days after transplantation and fixed in Tellycsniczky's fixative (70% ethanol:acetic acid:formalin at 20:1:1). The number of macroscopic colonies per spleen was determined, and the mean number of CFU-s detected per six recipients was used to calculate the total number of CFU-s generated per culture.

**Statistical analysis.** Levels of significance for comparisons between samples were determined by Student's t test. When no significance level was given, the results were not statistically different from control values.

**RESULTS**

**Dose response study.** Units of FL activity were established by measuring the survival of the pro-B cell line Ba/F3 transfected with a cDNA clone encoding mouse FLTK2. The dose response of the stable transformants called Baft cells is shown in Fig 1A. This assay has been used to standardize all purified preparations of recombinant FL used in this study. In our previous study, 25 U/mL of native FL was used to costimulate colony formation by Thy^-^Sca^-^1^ stem cells in the presence of IL-3. To determine the amount of recombinant FL required to stimulate optimal growth of Thy^-^Sca^-^1^ cells, varying concentrations of FL were added to stem cell cultures containing 300 U/mL of IL-3 (Fig 1B). Two different preparations of FL stimulated maximum colony numbers when used at 30 U/mL or more as defined by the Baft cell assay. To ensure that all colony assays contained saturating concentrations of FL, 100 U/mL (500 ng/mL) was used in all subsequent experiments.
FL interacts with a selected set of factors to stimulate colony formation by Thy1Sca1+ stem cells. The data presented in Fig 2A confirm that FL alone does not initiate the clonal growth of Thy1Sca1+ cells, whereas it enhances their growth when combined with IL-3 or IL-6. FL was also found to promote colony formation by Thy1Sca1+ cells when combined with SCF, GM-CSF, G-CSF, or IL-11. No colonies were observed when FL was combined with IL-1, IL-10, or M-CSF (data not shown). When the costimulatory actions of FL and SCF were compared, FL always supported lower colony numbers than SCF, regardless of the second factor present. Furthermore, FL did not significantly increase colony numbers in cultures that already contained SCF plus another factor. The one exception was the higher number of colonies observed with FL plus SCF and IL-11.

The data presented in Fig 2B show the number of stem cell colonies that achieved a diameter of greater than 0.5 mm and contained greater than 2 x 10^5 cells. It was found that FL, like SCF, was capable of interacting synergistically with GM-CSF, G-CSF, IL-3, or IL-6 to generate colonies containing large numbers of cells. Interestingly, only small colonies were observed when FL and SCF were combined in the absence of another factor. This result suggests that FL and SCF can synergize with other factors, but not with each other, to support the continuous proliferation of stem cell progeny. However, this did not appear to be the case, as combining FL and SCF with a third factor always resulted in greater numbers of cells per colonies than could be supported by any two-factor combination containing either FL or SCF. Indeed, both FL and SCF were required to generate large colonies in the presence of IL-11.

Cellular composition of stem cell colonies. The colonies costimulated by FL (shown in Fig 2) were sequentially isolated and analyzed for their cellular composition after staining with Wright-Giemsa. All colonies supported by FL plus GM-CSF, G-CSF, SCF, IL-6, or IL-11 contained only granulocytes and macrophages. Most colonies stimulated by FL plus IL-3 were also found to consist of granulocytes and macrophages. FL plus IL-3 did not stimulate a higher proportion of mixed colonies than those stimulated by IL-3 alone (2%). Similarly, FL plus IL-3 and SCF did not increase the incidence of mixed colonies above that supported by IL-3 plus SCF (approximately 12%). Based on these results, we have concluded that FL can enhance cell production. However, the types of cells that arise in these stem cell colonies are determined by the actions of the other factors present.

During our morphologic analyses, large numbers of undifferentiated cells (blasts) were observed in colonies grown for 10 to 14 days in the presence of FL plus IL-3, IL-6, G-CSF, or SCF. In contrast, few blasts were detected in colonies grown in FL plus GM-CSF. These results suggested that FL may interact with some but not all factors to expand a primitive population of cells in the absence of differentiation. Additional studies to investigate the significance of this finding are presented below.

**FL costimulates the expansion of CFU-c and CFU-s in stem cell cultures.** FL was tested for its ability to expand...
clonogenic cells in 7-day liquid cultures of Thy"Sca" cells. We also determined the total number of cells produced in these cultures. It was found that FL plus IL-3, IL-6, or G-CSF supported a small increase in cell number over the input value of 400 and greatly enhanced cell production (Fig 3A). Similarly, enhanced production was observed when stem cells were precultured in SCF plus IL-6 or G-CSF. Only a modest increase in cell numbers was stimulated by FL plus SCF. This outcome was not unexpected based on the small size of the stem cell-derived colonies supported by these two factors in our primary methylcellulose cultures.

None of the individual factors were able to support the expansion of CFU-c in liquid cultures of Thy"Sca" cells (Fig 3B). However, high numbers of CFU-c were generated when FL was combined with other factors. The most dramatic expansion was obtained with FL plus IL-6 (30-fold) and with FL plus G-CSF (greater than 20-fold). The precultured cells were assayed for CFU-c in secondary methylcellulose cultures containing a combination of factors (IL-3, IL-6, SCF, and epi) known to support the growth of many hematopoietic cell lineages. The sizes of the colonies were variable, ranging from a few hundred to thousands of cells. Approximately 30% of the colonies were large and multicentric. Although the majority of the colonies consisted of granulocytes and/or macrophages, there was a small number (3% to 5%) of large, mixed colonies. Despite the inability of FL to directly enhance the outgrowth of mixed colonies in primary cultures, it was capable of costimulating the proliferation of primitive cells from which multipotential progenitors were derived.

Figure 3C shows that day-12 CFU-s were increased five-fold when stem cells were precultured in FL plus IL-6. Interactions between FL and the other factors (IL-3, SCF, or G-CSF) did not result in the expansion of CFU-s. Instead, these factor combinations supported CFU-s numbers equivalent to or below the input number. A significant but less impressive expansion of CFU-s occurred in the presence of SCF plus IL-6 when compared with that obtained with FL plus IL-6.

**FL does not support the growth of CFU-c in Lin- BM cultures.** Experiments with unseparated BM cells showed that FL alone could stimulate only a small number of colonies as compared with GM-CSF (Fig 4). In contrast, FL was unable to stimulate colony formation of Lin- BM cells above background levels. These results suggested that factors produced by accessory cells in unseparated BM cultures may have contributed to the colony formation observed with FL. This was confirmed by showing that the number of colonies induced by FL was diminished in unseparated BM cultures containing anti-CSF antibodies (Fig 4). Therefore, it appears that FL does not possess a strong colony-stimulating activity but can serve as a cofactor.

**FL enhances the growth of granulocyte-macrophage colony-forming units (CFU-GM) of low and high proliferative potential (HPP).** Although FL alone did not support significant colony formation by Lin- BM cells, it markedly increased the number of GM colonies present in cultures containing SCF, G-CSF, or IL-6 (Fig 5). The most striking finding was the ability of FL to interact with G-CSF, IL-6, or IL-11 to generate macroscopic colonies (Fig 5, hatched bars) containing greater than 2 x 10^3 cells. Such colonies comprised more than 30% of all colonies formed in the presence of FL plus IL-6 or IL-11. Only a few macroscopic colonies were observed in cultures stimulated with FL plus SCF. In contrast with these results, FL had no affect on the number or size of the colonies stimulated by M-CSF or GM-CSF (Fig 5). Furthermore, FL did not increase the total number of large and small colonies stimulated by IL-3. However, there were twice as many cells in colonies measuring greater than 0.5 mm in diameter when FL was used as a cofactor with IL-3 (Fig 5).

Colonies were sequentially isolated from each treatment group to verify their cellular composition. A small number of mixed colonies (3%) were present in cultures containing IL-3 but their frequency was not significantly changed by costimulation with FL. The colonies from all other groups consisted entirely of neutrophilic granulocytes and macrophages. This was also true of the macroscopic colonies, although some differences between the treatment groups were
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Colonies per 1×10^5 cells

<table>
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<th>Factor</th>
<th>FL + anti-CSF antibodies</th>
<th>FL + isotype control</th>
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<tr>
<td>GM-CSF</td>
<td>Unseparated BM</td>
<td>Lin^- BM</td>
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Fig 4. FL does not support colony formation in accessory cell-depleted bone marrow cultures. Unseparated (1×10^5 cells per plate) and Lin^- BM cells (5×10^3 cells per plate) were cultured with FL (100 U/mL) or GM-CSF (200 U/mL). Some cultures were also supplemented with a mixture of neutralizing antibodies specific for GM-CSF (15 μg/mL), M-CSF (15 μg/mL), G-CSF (15 μg/mL), and IL-6 (15 μg/mL) or with isotype control antibodies (60 pg/mL) as indicated. Data are reported as mean values ± SD of triplicate plates from three independent experiments (n = 9). *P < .05 compared with groups not treated with antibodies or groups treated with isotype control antibodies.

Fig 5. FL enhances colony formation by CFU-GM. Methylcellulose cultures containing 5×10^3 Lin^- BM cells were supplemented with FL and other growth factors as indicated. The concentration of each factor used is the same as that indicated in the Fig 2 legend. Data are reported as mean values ± SD of triplicate cultures from three independent experiments (n = 9). The hatched portions of the bars indicate the number of GM colonies measuring greater than 0.5 mm in diameter and containing more than 2×10^4 cells. Average numbers of cells per colony were determined after large colonies (greater than 0.5 mm) were pooled from one plate per treatment group. *P < .05 compared with groups not supplemented with FL.

Colonies per 5x10^3 cells

<table>
<thead>
<tr>
<th>Factor</th>
<th>Cells/colony measuring &gt;0.5mm</th>
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<tbody>
<tr>
<td>SCF</td>
<td>25950</td>
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<tr>
<td>G-CSF</td>
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<td>IL-6</td>
<td>27150</td>
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<td>IL-3</td>
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<td>M-CSF</td>
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noted. The macroscopic colonies supported by FL plus IL-11 contained predominately macrophages, whereas those supported by FL plus SCF, IL-6, or G-CSF contained predominately granulocytes. Furthermore, the macroscopic colonies generated in the presence of FL contained large numbers of undifferentiated cells, suggesting that primitive cells were expanded in the absence of differentiation. Figure 6 shows the appearance of cells grown in IL-6 as compared with those grown in IL-6 plus FL.

Effects of FL on the expansion of CFU-GM in liquid cultures of Lin^- BM cells. Lin^- BM cells were cultured in liquid medium containing FL in the presence or absence of other factors. After 7 days, the precultured cells were assessed for CFU-c activity in secondary methylcellulose cultures. When factors were present individually in the precultures, only FL was found to expand CFU-c numbers (17-fold) over the input value of 118 (Fig 7). An even greater expansion of CFU-c (greater than 40-fold) was observed when FL was combined with SCF, IL-6, or G-CSF (Fig 7). The colonies formed after plating of the precultured cells were relatively small (containing 100 to 400 cells) and were comprised of granulocytes and/or macrophages.

FL does not promote the growth of erythroid burst-forming units (BFU-e), mast cell colony-forming units (CFU-mast), eosinophil colony-forming units (CFU-eo), or megakaryocyte colony-forming units (CFU-meg). We have investigated the possibility that FL combined with appropriate lineage-specific growth factors may enhance colony formation by different types of progenitor cells. Our results show that FL lacks erythroid-promoting activities in epo-dependent BFU-e assays (Fig 8). A number of factors appear to regulate megakaryocytopoiesis (ie, IL-3, IL-6, IL-10, and IL-11). We observed that FL alone did not support the growth of megakaryocyte progenitors or augment the generation of megakaryocyte colonies in the presence of IL-3 (Fig 8).

We tested the ability of FL to promote the growth of eosinophil progenitors. In these studies, FL had no detectable activity when combined with IL-3, GM-CSF, or IL-5 (Fig 8). In earlier studies with Lin^- BM cells, FL appeared to
DISCUSSION

Our initial studies showed that FL is incapable of supporting colony formation by Thy<sup>+</sup>Sca<sup>1</sup> stem cells. The failure of FL to induce the clonal growth of these primitive cells was not surprising due to their requirement for signaling by multiple factors. Significant colony formation was observed when FL was combined with either IL-3 or IL-6. Herein, it is shown that FL also promotes stem cell growth when combined with SCF, G-CSF, GM-CSF, or IL-11. In contrast, FL was ineffective when combined with IL-1, IL-10, or M-CSF. These results cannot be attributed to the absence of any stem cell-stimulating activities by these latter factors and may simply indicate that not all factor interactions lead to enhanced responses.

Because FL and SCF signal through different but related tyrosine kinase receptors, we compared their actions in stem cell assays. In the presence of other factors, FL was found to be less effective than SCF in recruiting Thy<sup>+</sup>Sca<sup>1</sup> cells to form colonies. Furthermore, the recruiting activity of FL was redundant with that of SCF, as FL usually did not cause additional colony formation when SCF was present. In these same cultures, however, FL and SCF exhibited synergistic actions with respect to the total number of maturing cells that could be derived from a single stem cell. Therefore, combining both FL and SCF with a third factor (ie, IL-3, IL-11, G-CSF, or GM-CSF) invariably supported the generation of larger colonies. The mechanism responsible for this...
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One important goal of our studies was to determine whether the actions of FL on Thy<sup>+</sup>Sca1<sup>+</sup> stem cells resulted in the generation of primitive cells that retained clonogenic properties. After Thy<sup>+</sup>Sca1<sup>+</sup> cells were precultured for 7 days in FL plus IL-3, IL-6, G-CSF, or SCF, the numbers of CFU-c recovered were greatly increased over the input number. Although all of our factor combinations supported CFU-c production, only FL plus IL-6 stimulated the expansion of day-12 CFU-s. We also found that SCF plus IL-6 supported an increase in both CFU-c and CFU-s numbers, albeit to a lesser extent than FL plus IL-6. The ability of SCF to interact selectively with IL-6 to expand these two clonogenic populations in stem cell cultures has been reported by others. The latter results were also in contrast with findings in the human system, as strong synergies between FL and IL-3 or GM-CSF were responsible for the enhanced growth observed with human CD34<sup>+</sup> cells.

Studies are in progress to determine whether FL can enhance the clonal growth of various types of lineage-committed progenitor cells. See Materials and Methods for details about specific assays and the identification of cells comprising colonies generated in these cultures. IL-4 and IL-10 were each added at 100 U/mL. All other factors were present at the same concentrations as indicated in the Fig 2 legend. Data are reported as the mean values ± SD of triplicate cultures from three independent experiments (n = 9).

Fig 7. CFU-c recovered from liquid cultures of Lin<sup>-</sup> BM cells. Five thousand Lin<sup>-</sup> BM cells were cultured for 7 days in FL in the presence and absence of other factors. The total numbers of CFU-c per culture are shown. Data are presented as the mean ± SEM of triplicate cultures from two independent experiments (n = 8). *P < .05 as compared with the input number obtained with Lin<sup>-</sup> BM cells not cultured before CFU-c assay.

Fig 8. FL has no effect on colony formation by BFU-e, CFU-meg, CFU-eo, or CFU-mast. The ability of FL to either support or enhance the clonal growth of various types of lineage-committed progenitor cells was evaluated. See Materials and Methods for details about specific assays and the identification of cells comprising colonies generated in these cultures. IL-4 and IL-10 were each added at 100 U/mL. All other factors were present at the same concentrations as indicated in the Fig 2 legend. Data are reported as the mean values ± SD of triplicate cultures from three independent experiments (n = 9).

cultures. We have also combined FL with a variety of factors known to induce colony formation and found that FL possesses a strong potentiating effect on GM progenitors. No effect on the growth of other types of progenitor cells was detected. Similar results have been obtained with human cells. In our murine assays, FL dramatically augmented the number and size of GM colonies if combined with G-CSF, IL-6, or IL-11. Little or no potentiation was observed when FL was combined with IL-3, GM-CSF, or M-CSF. These latter results were also in contrast with findings in the human system, as strong synergies between FL and IL-3 or GM-CSF were responsible for the enhanced growth observed with human CD34<sup>+</sup> cells. It is possible that the discrepancies observed between human and murine assays may reflect fundamental differences between the species. Distinguishing between these possibilities will require further investigation.

In Lin<sup>-</sup> BM cultures costimulated with FL, some of the type of synergy has yet to be defined. It has been argued that increased cell production occurs when the cofactors involved (in this case, FL and SCF) provide different but complimentary signals or stimulate different progeny based on the differential expression of cytokine receptors.

One important goal of our studies was to determine whether the actions of FL on Thy<sup>+</sup>Sca1<sup>+</sup> stem cells resulted in the generation of primitive cells that retained clonogenic properties. After Thy<sup>+</sup>Sca1<sup>+</sup> cells were precultured for 7 days in FL in the presence of in vivo reconstitution of lymphoid and myeloid lineages and capable of protecting mice from lethal irradiation. In our murine assays, FL dramatically augmented the number and size of GM colonies if combined with G-CSF, IL-6, or IL-11. Little or no potentiation was observed when FL was combined with IL-3, GM-CSF, or M-CSF. These latter results were also in contrast with findings in the human system, as strong synergies between FL and IL-3 or GM-CSF were responsible for the enhanced growth observed with human CD34<sup>+</sup> cells. It is possible that the discrepancies observed between human and murine assays may reflect fundamental differences between the species. Distinguishing between these possibilities will require further investigation.

In Lin<sup>-</sup> BM cultures costimulated with FL, some of the
GM colonies were enormous, containing greater than 2 $\times$ 10^6 cells. Such colonies are known to be formed by a subset of progenitor cells of HPP. The growth requirements of CFU-HPP are complex, as stimulation by two or more factors is needed to initiate their growth and to produce optimal numbers of maturing cells.2-15 CFU-HPP have been shown to be the precursors of CFU-c of lower proliferative potential.36-38 Our results indicate that FL in conjunction with G-CSF, IL-6, or IL-11 supports the growth of CFU-HPP. These results agree with those obtained with human CD34+ cells, where the growth of CFU-HPP was elicited by FL plus GM-CSF or IL-3. The colonies formed by CFU-HPP in our murine cell cultures contained large numbers of blasts as well as maturing granulocytes and macrophages. Based on this observation, we tested the possibility that substantial numbers of clonogenic cells had been generated. In the absence of other factors, we found that FL alone supported a 17-fold expansion of CFU-c in suspension cultures of Lin- BM cells. It is conceivable that the actions of FL were dependent on cosignals provided by interactions between Lin- BM cells, or that mature accessory cells were generated rapidly when some of the Lin- BM cells attached to plastic. In liquid cultures, unlike semisolid cultures, these events are unavoidable. Regardless of whether FL alone is sufficient to support the proliferation of pre-CFU, the production of CFU-c was clearly augmented if FL were combined with SCF, G-CSF, or IL-6.

The growth characteristics of the CFU-c derived from Thy"Scal+ or from Lin- BM precultures were slightly different, although the same factor combinations were used for their generation. Specifically, the CFU-c from Thy"Scal+ precultures formed large, multicentric colonies containing mixtures of granulocytes and macrophages. A few colonies (3% to 5%) contained additional cell types and blasts, suggesting they were formed by multipotential CFU-c. In contrast, the CFU-c from Lin- BM precultures formed smaller colonies and contained only mature granulocytes and/or macrophages. Apparently, the CFU-c generated in Lin- BM cultures were late GM-committed progenitors with relatively low proliferative potential. Therefore, it is likely that they were derived from an early population equivalent to GM-committed CPU-HPP. Because considerable expansion of CFU-c occurred in both the Thy"Scal+ and Lin- BM precultures, FL seems to be very effective in regulating the sequential development of early and late CFU-c from ancestral cells.

Stromal cells are known to play an essential role in supporting hematopoiesis in the bone marrow microenvironment. The local production of growth factors by stroma is believed to provide most of the signals required for normal stem cell and progenitor cell development. The isolation of FL from a bone marrow stromal cell line1 suggests that this factor may contribute to steady-state hematopoiesis. Therefore, we have studied the activities of FL in the presence of factors that are derived mostly, if not exclusively, from stromal cells. Presently, the role that FL plays in the de novo generation of pluripotential stem cells is unknown. However, we have shown that FL interacts with certain stromal-derived factors to initiate stem cell proliferation, resulting in the production and expansion of primitive descendents that are believed to comprise reserve progenitor cell pools (ie, CFU-s and CFU-c). Furthermore, interactions between FL and specific stromal factors appear to favor the generation of CFU-GM and to enhance the subsequent proliferation of CFU-GM. Based on the results of these and previous studies,1 FL appears to optimize not only the growth of early hematopoietic populations but to skew bone marrow-dependent myelopoiesis toward the preferential production of granulocytes and monocytes.

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REFERENCES


HEMATOPOETIC EFFECTS OF FLT3/FLK2 LIGAND


34. Muench MO, Schneider JG, Moore MAS: Interactions among colony-stimulating factors, IL-1β, IL-6, and kit-ligand in the regulation of primitive murine hematopoietic cells. Exp Hematol 20:339, 1992


FLT3/FLK2 ligand promotes the growth of murine stem cells and the expansion of colony-forming cells and spleen colony-forming units

S Hudak, B Hunte, J Culpepper, S Menon, C Hannum, L Thompson-Snipes and D Rennick