In recent years multiple cytokines have been cloned that can stimulate hematopoietic progenitor cell growth in vitro as well as in vivo.\(^1\) \(^3\) The colony-stimulating factors (CSFs), which include granulocyte-CSF (G-CSF), granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (M-CSF or CSF-1), and interleukin-3 (IL-3 or multi-CSF), have been considered unique in their ability to stimulate the clonal growth of myeloid bone marrow (BM) progenitor cells in vitro, in the absence of other cytokines.\(^1\) \(^3\) However, it has become clear that the optimal in vitro growth of more primitive progenitors can be achieved only through synergistic interactions of multiple cytokines.\(^1\) \(^3\) Thus, a number of cytokines have been identified, which have little or no ability to directly stimulate hematopoietic progenitor cell growth alone, but in combination with CSFs, erythropoietin (Epo), or stem cell factor (SCF); also called mast cell growth factor, kit ligand, and steel factor, they can potentially enhance the growth of primitive progenitors.\(^1\) \(^3\)

SCF is considered to be a key regulator of hematopoiesis, based on the hematopoietic defects observed in Steel or W/W mutant mice.\(^4\) As a single growth factor, SCF has a weak stimulatory activity on hematopoietic progenitor cell growth in vitro. However, its unsurpassed ability to synergize with the CSFs, Epo, multiple interleukins, and other cytokines results in efficient recruitment of progenitors with high-proliferative capacities. The hematopoietic growth promoting effects of SCF are most pronounced on primitive progenitors or stem cells.\(^5\) \(^16\) However, more mature progenitors committed to the myeloid, erythroid, and mast cell lineages do also respond to SCF.\(^6\) \(^16\)

The effects of SCF are signaled through the c-kit tyrosine kinase receptor,\(^17\) \(^18\) which is related to c-fms\(^9\) and fli3/lk-2.\(^20\) \(^22\) c-fms, the receptor for CSF-1 is predominantly expressed on mature myeloid progenitors, whereas c-kit is expressed on primitive, as well as committed, progenitors. In contrast, fli3/lk-2 has been found to be preferentially expressed on primitive hematopoietic progenitor cells, suggesting, perhaps, a unique role in the regulation of early hematopoiesis.\(^20\) Recently, the ligand for fli3 (FL) was cloned, and the cloning papers demonstrated its ability to stimulate the growth of murine and human BM progenitor cells.\(^23\) \(^25\) Thus, FL might prove to be an attractive candidate for use in stimulation of hematopoietic recovery in patients receiving myelosuppressive treatment.

The present studies were undertaken to explore the ability of purified recombinant human FL to stimulate the in vitro growth of hematopoietic progenitor cells in adult human BM in serum-containing as well as serum-deprived cultures. In particular, we addressed the ability of FL to synergize with other cytokines to directly stimulate the growth of primitive, as well as more mature, subsets of CD34\(^+\) progenitor cells. Here we demonstrate that although the most potent stimulatory activities of FL is observed on primitive progenitors, it can also enhance the growth of more committed human CD34\(^+\) BM cells, including erythroid progenitors.

**Materials and Methods**

Hematopoietic growth factors. Recombinant human (rh) FL was cloned and purified as previously described.\(^23\) rhIL-3 and rhGM-CSF were generously provided by Dr Steven Gillis (Immunex Corporation, Seattle, WA). rhSCF and rh-G-CSF were generously supplied by Dr Ian K. McNiece (Amgen Inc, Thousand Oaks, CA), and rhIL-6 was a gift from Genetics Institute (Cambridge, MA). rhCSF-I was kindly supplied by Dr Michael Geier (Cetus Corp, Emeryville, CA). rhEpo was purchased from Cilag AG (Schaaffhausen, Switzerland). Unless otherwise indicated, all hematopoietic growth factors were formed by CD34\(^+\)CD71\(^-\) cells in response to FL + IL-3 + SCF or FL + IL-3 or SCF + IL-3. Similar findings were observed in serum-containing and serum-deprived cultures. Whereas FL did not enhance burst-forming unit-erythroid (BFU-E) colony formation of CD34\(^+\) BM cells in the presence of serum, a low number of BFU-E colonies were formed in response to FL plus erythropoietin (Epo) under serum-deprived conditions. In addition, FL both in serum-containing and serum-deprived cultures stimulated colony formation of more committed myeloid progenitors in CD34\(^+\)CD71\(^-\) BM cells. Thus, FL potentely stimulates the growth of primitive and more committed human BM progenitor cells.

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(HGFs) were used at predetermined optimal concentrations; rhG-CSF 20 ng/mL, rhGM-CSF 50 ng/mL, rhIL-3 20 ng/mL, rhCSF-1 50 ng/mL, rhSCF 50 ng/mL, rhIL-6 50 mg/mL, and rhEpo 5 IU/mL.

**Cell separation.** Human BM cells were obtained by iliac crest aspiration from normal adult volunteers with informed consent and the approval of the Ethics Committee of The Norwegian Radium Hospital. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). Positive selection of CD34+ cells was performed according to a previously described method. Brieﬂy, BM mononuclear cells were rosetted with Dynabeads M-450 directly coated with the CD34 monoclonal antibody (MoAb) 561 for 45 minutes at 4°C on an apparatus that provided tilting and gentle rotation. The bead to total cell ratio was 1:1. Rosetted cells were attracted to a samarium cobalt magnet and nonrosetting cells were removed by pipetting. Rosetted cells were washed four times. Detachment of beads from positively selected cells was performed by incubation with anti-Fab antiserum (DETAChaBEAD, Dynal) at a concentration of 35 mg/mL for 1 hour at room temperature. Isolated cells, free of beads, were washed and counted. The purity of CD34+ cells isolated by this method was reproducibly >90% as determined by flow cytometric analysis.

To enrich and separate primitive and more mature progenitors, we took advantage of the differential expression of the transferrin receptor (CD71) previously described on these cells. CD71, being brightly expressed on the cell surface of erythroid progenitors, is also a marker associated with cell proliferation and activation. Thus, as conﬁrmed by the studies of Lansdorp et al, primitive, noncycling progenitors are enriched, and cycling committed progenitors depleted, in cell populations selected on the basis of low CD71 expression.

To isolate cells with the CD34+CD71+ and CD34+CD71− phenotypes by cell sorting, positively selected CD34+ cells were stained with anti-CD34 (HPCA-2; Becton Dickinson, San Jose, CA) directly conjugated to phycoerythrin (PE), and antiCD71 (Ber-T9; Dakopatts, Copenhagen, Denmark) directly conjugated to fluorescein isothiocyanate (FITC). Isotype-matched FITC- and PE-conjugated irrelevant mouse MoAbs served as controls. Cell sorting was performed on an Epics Elite cell sorter (Coulter Electronics, Hialeah, FL). A sort gate within a dual parameter cytogram of forward light scatter against 90° side scatter was drawn. A second amorphous gate was drawn on two-color cytograms, and sort equations were set to positively sort cells satisfying both gates.

**Colony assay.** Test cells were plated in a volume of 1 mL Iscove's modiﬁed Dulbecco's medium (IMDM) (GIBCO, Paisley, UK) containing 20% fetal calf serum (FCS; Sera-lab, Sussex, England) 1.0% methylcellulose (Methol 4000 mPa.s, Fluka AG, Buchs, Switzerland), 5 × 10−7 M 2-mercaptoethanol, 300 mg/mL glucose, 66 mg/L penicillin, 100 mg/mL streptomycin, and recombinant human growth factors as indicated. Serum-deprived cultures were set up in X-Vivo 15 medium (BioWhittaker, Inc, Walkersville, MD) containing 1% detoxiﬁed bovine serum albumin (Stem Cell Technologies, Vancouver, Canada), 1% methylcellulose, glutamine, streptomycin, and penicillin. After 2 weeks of incubation at 37°C and 5% CO2 in air, colonies (≥40 cells) were counted using an inverted microscope. Colonies derived from erythroid burst-forming units (BFU-E) were assessed and counted as previously described.

**Single-cell proliferation assay.** Test cells were seeded in 96-well plates (Nunc, Kastrup, Denmark) at a concentration of 1 cell per well (300 wells per group) in 20 μL IMDM containing 20% FCS (Sera-lab), 1% glutamine, penicillin, and streptomycin (complete IMDM), or in X-Vivo 15 (BioWhittaker) containing 1% bovine serum albumin (Stem Cell Technologies), glutamine, streptomycin, and penicillin. Wells were scored for cell growth after 2 weeks of incubation at 37°C and 5% CO2 in air. Similar results were obtained whether cells were plated by dilution at one cell per well or were plated individually by a single cell depositor coupled to an Epics Elite cell sorter, assuring that more than 99% of the wells had one cell.

**Cell morphology.** CD34+ cells were plated in complete IMDM, and incubated for 2 weeks at 37°C and 5% CO2 in air in the presence of predetermined optimal concentrations of the growth factors indicated. Cell morphology was determined following May-Grünwald Giemsa staining of cytospin preparations. Morphological examination of individual colonies was performed on May-Grünwald Giemsa-stained cell preparations made by picking colonies and dispersing the cells on glass slides with a jet air stream.

**Statistical analysis.** All results were expressed, where applicable, as the mean ± standard error of mean (SEM) of data obtained from three or more separate experiments. The statistical signiﬁcance of differences between groups was determined using the paired t-test.

### RESULTS

**FL stimulates IL-3-induced colony formation of CD34+ BM progenitor cells.** To study the effects of FL on colony formation by CD34+ BM progenitor cells, we ﬁrst examined the colony-forming ability of FL plus IL-3 at different concentrations of FL, as well as IL-3. IL-3 (20 ng/mL) alone induced the formation of 16 ± 3 colonies of 2,000 CD34+ cells plated (Fig 1A). The supplementation of FL enhanced IL-3-induced colony formation in a concentration-dependent manner, with 50% of maximum stimulation occurring at a FL concentration just below 3 ng/mL, whereas the maximum stimulation of 277% was observed at 12 ng/mL. Whereas maximum colony numbers were obtained at FL 6 to 12 ng/mL, maximum increase in colony size occurred at 25 to 50 ng/mL (not shown).

FL (50 ng/mL) as a single growth factor promoted the formation of 4 ± 1 colonies of 1,000 CD34+ cells plated (Fig 1B). IL-3 enhanced FL-induced colony formation in a concentration-dependent manner, with 50% of maximum stimulation occurring at a IL-3 concentration of 0.1-1 ng/mL, and a maximum stimulation of 575% was observed at 10 ng/mL.

**Effects of FL on colony formation of CD34+ BM cells in combination with other HGFs.** To investigate the ability of FL to modulate colony formation of CD34+ BM progenitors, the growth promoting effects of multiple HGFs were tested in the absence or presence of FL. As single growth factors, FL, SCF, as well as CSF-1, all being ligands for tyrosine kinase receptors, had weak colony-promoting activities (Fig 2). However, a potent synergy was observed combining FL with SCF or CSF-1, increasing colony numbers 14-fold (P < .05) and 43-fold (P < .05), respectively. Similarly, a synergistic growth response was observed combining FL with G-CSF, GM-CSF, or IL-3, augmenting colony formation 1.6-fold (P = .05), 3.4-fold (P < .05), and 3.8-fold (P < .05), respectively. Thus, FL can synergize with multiple HGFs to increase colony formation of CD34+ BM progenitor cells.

**Comparison of the ability of FL and SCF to stimulate the growth of CD34+ progenitor cells.** Because both FL and SCF appear to be potent stimulators of hematopoietic progenitor cell growth, it was of particular interest to compare the in vitro hematopoietic effects of these cytokines. FL and...
SCF did not differ in their ability to synergistically enhance IL-3--induced colony formation, increasing colony numbers 3.8-fold and 3.5-fold, respectively (Fig 3). When IL-3, FL, and SCF were combined, a striking recruitment of additional colony-forming progenitors was observed, resulting in colony numbers nine-fold higher than induced by IL-3 alone, and 2.3-fold \((P < .05)\) and 2.5-fold \((P < .05)\) higher than that obtained in response to IL-3 plus FL and IL-3 plus SCF, respectively (Fig 3). IL-6 did not induce colony formation as a single growth factor, and FL appeared to more potently enhance IL-6--stimulated colony formation than SCF, in that
2.3-fold more colonies appeared in the presence of IL-6 plus FL than IL-6 plus SCF. However, this difference did not reach statistical significance \( P = .1 \). Furthermore, FL potently enhanced colony formation induced by IL-3 plus IL-6, increasing colony numbers 3.1-fold \( P < .05 \). Finally, as previously suggested, the supplementation of FL did not affect colony formation of committed CD34+ erythroid progenitors (BFU-E) stimulated by Epo or Epo plus SCF in serum-containing cultures (Fig 4). Together, these results demonstrate that FL, similar to SCF, can interact with numerous HGFs including the CSFs, to synergistically enhance colony formation of CD34+ BM progenitor cells.

**Direct proliferative effects of FL.** To determine whether the potent stimulation of CD34+ progenitor cell growth in response to FL was directly mediated, we next plated CD34+ BM cells at a concentration of one cell per well. In addition, we enumerated both clusters (10 to 40 cells) and colonies (>40 cells). Interestingly, FL and IL-3 as single growth factors did not differ in their ability to induce clonal proliferation (>10 cells), and the combination of FL plus IL-3 had an additive, but not synergistic effect on the total number of proliferative clones (Table 1). However, whereas FL and IL-3 induced the formation of 8 ± 2 and 10 ± 2 colonies (>40 cells), respectively, a synergistic growth response with regard to colony formation was observed combining FL and IL-3, resulting in the growth of 39 ± 6 colonies. In particular, the growth of large colonies (covering more than 10% of the well) was potently enhanced by the combination, as FL and IL-3 individually only stimulated zero and two such colonies, respectively, whereas they in combination recruited eight large colonies (Table 1). Thus, it appears that the predominant effect of FL on unfractionated CD34+ BM cells when combined with IL-3, is stimulation of the formation of large colonies.

As FCS might contain growth factors possibly interfering with the defined growth factors supplemented, the above experiments were repeated using a serum-deprived culture system. These experiments confirmed the ability of FL as a single factor to induce cluster formation (Table 2). Likewise, a potent synergy with regard to colony formation occurred combining FL and IL-3, in that FL and IL-3 as single factors supported the growth of 1 ± 1 and 3 ± 2 colonies, respectively, whereas 13 ± 4 colonies were formed in the presence of FL plus IL-3 (Table 2). Although the supplementation of FL plus IL-3 induced the proliferation of more clones than the total of clones stimulated by the two factors given individually \((30 \div 21)\), the present results support that increasing the size of proliferative clones is the foremost effect observed when combining FL and IL-3.

**Effect of FL on differentiation of progeny to CD34+ BM cells.** To determine the effects of FL on the differentiation
EFFECTS OF FLT3 LIGAND ON CD34+ PROGENITORS

of myeloid progenitors, CD34+ BM cells were grown in the presence of FL and/or IL-3, and cytospin preparations from three separate experiments were analyzed morphologically. After 2 weeks in the presence of FL (50 ng/mL), 16% ± 1% of the cells could be identified belonging to the granulocytic lineage, whereas 71% ± 7% of the cells were macrophages (Table 3). In contrast, IL-3 (20 ng/mL) induced the formation of 60% ± 8% granulocytic cells, and 34% ± 4% macrophages. Interestingly, in the presence of FL plus IL-3, 23% ± 5% and 71% ± 7% of the cells developed into granulocytes and macrophages, respectively, demonstrating the ability of FL in combination with IL-3 to direct progenitors towards macrophage development. Similarly, also FL plus SCF and FL plus IL-3 plus SCF predominantly stimulated macrophage development (Table 3).

Effects of FL on specific subsets of BM progenitors. To delineate the effects of FL on specific types of BM progenitors, individual colonies were picked after 2 weeks of incubation and examined morphologically. As a single growth factor, FL predominantly promoted the formation of macrophage (M) colonies (Table 4). When FL was added to IL-3 plus Epo, the number of GM and M colonies were 19% and 17%, respectively, as compared with 9% and 3%, respectively, in the presence of IL-3 plus Epo alone. Of note, FL plus IL-3 plus Epo supported the formation of 3.7-fold more mixed erythroid and granulocyte/macrophage (Mix) colonies than IL-3 plus Epo. In contrast to SCF plus Epo, the tri-factor combination (FL plus SCF plus Epo) supported the formation of GM, M, as well as blast (B1) cell colonies. Adding IL-3 to the tri-factor combination resulted in an increased proportion of GM and M colonies (Table 4).

FL did not support colony formation (>40 cells) of CD34+ cells in semisolid serum-deprived media. Under such conditions, 20% of colonies formed in response to IL-3 plus Epo consisted of eosinophil and neutrophil granulocytes, but no GM or M colonies was observed (Table 5). Interestingly, and in contrast to what occurred in the presence of serum, the addition of FL did not significantly stimulate the development of GM or M colonies.

Effects of FL on colony formation of primitive (CD71+) and more mature (CD71+) subsets of CD34+ BM progenitor cells. SCF has been demonstrated to preferentially promote the survival and proliferation of primitive progenitor cells. To study whether FL shares the same pattern of activity, the growth response of CD34+CD71+ cells, a subset enriched in primitive progenitors, as opposed to more mature CD34+CD71+ progenitors was determined. Significant differences were observed between these two progenitor cell populations in terms of differential responses to FL. Specifically, whereas the addition of two growth factors (IL-3 plus FL, or IL-3 plus SCF) promoted the growth of 3.4-fold (P < .05) and 3.8-fold (P < .05) more colonies by CD34+CD71+ than by CD34+CD71- cells, the number of colonies formed in response to the supplementation of three growth factors (IL-3 plus FL plus SCF), did not differ between the two progenitor populations (Fig 5). Accordingly, colony formation of CD34+CD71+ progenitor cells in response to IL-3 plus FL plus SCF did not exceed that of IL-3 plus FL or IL-3 plus SCF (Fig 5). In contrast, the addition of FL stimulated 4.5-fold more colonies of CD34+CD71+ progenitors than IL-3 plus FL or IL-3 plus SCF (Fig 5).

### Table 3. Effects of FL on the Differentiation of CD34+ BM Progenitors

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Blasts</th>
<th>Granulocytes</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>12 ± 1</td>
<td>16 ± 1</td>
<td>73 ± 2</td>
</tr>
<tr>
<td>IL-3</td>
<td>2 ± 1</td>
<td>60 ± 8</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>FL + IL-3</td>
<td>6 ± 2</td>
<td>23 ± 5</td>
<td>71 ± 7</td>
</tr>
<tr>
<td>FL + SCF</td>
<td>5 ± 3</td>
<td>17 ± 8</td>
<td>78 ± 9</td>
</tr>
<tr>
<td>FL + IL-3 + SCF</td>
<td>12 ± 4</td>
<td>23 ± 2</td>
<td>65 ± 5</td>
</tr>
</tbody>
</table>

CD34+ cells were plated in complete IMDM at a density of 5.0 x 10^3 cells/mL and incubated for 14 days at 37°C and 5% CO2 in air in the presence of predetermined optimal concentrations of the growth factors indicated (Materials and Methods). Cell morphology was determined after May-Grünwald Giemsa staining of cytospin preparations. The results represent mean percentages ± SEM of three separate experiments.

### Table 4. Effects of FL on Subsets of BM Progenitors in Serum-Containing Cultures

<table>
<thead>
<tr>
<th>FL</th>
<th>G (%)</th>
<th>Eos (%)</th>
<th>GM (%)</th>
<th>M (%)</th>
<th>Bl (%)</th>
<th>Mix (%)</th>
<th>E (%)</th>
<th>Total No. CFU-C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>IL-3 + Epo</td>
<td>9</td>
<td>12</td>
<td>9</td>
<td>3</td>
<td>65</td>
<td>36</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>FL + IL-3 + Epo</td>
<td>3</td>
<td>8</td>
<td>19</td>
<td>17</td>
<td>11</td>
<td>42</td>
<td>54</td>
<td>9 ± 9</td>
</tr>
<tr>
<td>SCF + Epo</td>
<td>3</td>
<td>97</td>
<td>50</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL + SCF + IL-3 + Epo</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>9</td>
<td>78</td>
<td>59</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

A total of 1 x 10^3 CD34+ cells were plated in 1 mL serum-containing methylcellulose, and incubated for 14 days at 37°C and 5% CO2 in air in the presence of predetermined optimal concentrations of the growth factors indicated (Materials and Methods). Cell morphology was determined after May-Grünwald Giemsa staining of individual colonies dispersed on glass slides. The results represent mean percentages of three separate experiments. Colonies were scored as representative of CFU-G and CFU-Eos (>90% neutrophil and >80% eosinophil granulocytes, respectively), CFU-GM (>10% granulocytes, >10% macrophages), CFU-M (>90% macrophages), CFU-BI (>80% blast cells), CFU-Mix (presence of erythroid and granulocyte/macrophage elements), or BFU-E (all erythroid cells).

### Table 5. Effects of FL on Subsets of BM Progenitors in Serum-Deprived Culture

<table>
<thead>
<tr>
<th>FL</th>
<th>G (%)</th>
<th>Eos (%)</th>
<th>GM (%)</th>
<th>M (%)</th>
<th>Bl (%)</th>
<th>Mix (%)</th>
<th>E (%)</th>
<th>Total No. CFU-C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>15</td>
<td>7</td>
<td>5</td>
<td>72</td>
<td>21</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>FL + IL-3 + Epo</td>
<td>6</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>72</td>
<td>39</td>
<td>6 ± 6</td>
</tr>
</tbody>
</table>

A total of 1 x 10^3 CD34+ cells were plated in 1 mL serum-deprived methylcellulose media (Materials and Methods), and incubated for 14 days at 37°C and 5% CO2 in air in the presence of predetermined optimal concentrations of the growth factors indicated (Materials and Methods). Cell morphology was determined after May-Grünwald Giemsa staining of individual colonies dispersed on glass slides. The results represent mean percentages of three separate experiments. Scoring criteria: see note to Table 4.
In serum-deprived semisolid media, FL, SCF, and Epo did not promote colony formation as single growth factors. Interestingly, whereas FL did not affect erythroid colony formation in serum-containing cultures (Fig 4), it promoted the formation of 5 ± 2 and 9 ± 3 BFU-E colonies of CD34+ cells and CD34+CD71+ cells, respectively, when combined with Epo in the absence of serum (Fig 6). These colonies, like erythroid colonies stimulated with other growth factor combinations in serum-deprived cultures, did not display the typical red color of hemoglobinized erythroid cells, but based on their growth pattern, they appeared erythroid, and this was confirmed by May-Grünwald Giemsa-staining and morphologic examination of individually picked colonies. In contrast to what was observed in the presence of serum, these results suggest that FL can synergistically interact with Epo inducing erythroid progenitor cells to grow. Furthermore, similar to what occurred in serum-containing cultures, the addition of two growth factors (IL-3 plus FL, or IL-3 plus SCF) promoted the growth of 9.6-fold and 5.8-fold more colonies by CD34+CD71+ than by CD34+CD71- cells, whereas the number of colonies formed in response to the supplementation of three growth factors (IL-3 plus FL, or IL-3 plus SCF), did not differ significantly (P = .2) between the two progenitor populations (Fig 6). Finally, colony formation of CD34+CD71+ progenitor cells in response to IL-3 plus FL plus SCF was 2.2-fold and 2.3-fold more than that of IL-3 plus FL and IL-3 plus SCF, respectively (Fig 6). In comparison, the addition of FL stimulated 14.9-fold and 9.3-fold more colonies of CD34+CD71+ progenitors than IL-3 plus FL or IL-3 plus SCF, respectively (Fig 6). Collectively, the results of serum-containing, as well as serum-deprived, cultures suggest that FL preferentially recruits primitive progenitors when acting in combination with IL-3 plus SCF.

**DISCUSSION**

The selective expression of flt3/flk-2 on primitive murine hematopoietic progenitor cells suggested that FL might preferentially stimulate the growth of primitive hematopoietic progenitor cells. In agreement with this notion, the cloning papers, as well as other recent studies, demonstrated that the stimulatory effects of FL is predominantly observed on the most primitive murine hematopoietic progenitor cells. The ability of FL to stimulate the growth of primitive human hematopoietic progenitor cells in fetal liver has been established. In addition, FL stimulates the growth of human CD34+ progenitors in human adult BM as would be expected from the expression of flt3 on CD34+ cells. However, clearly the CD34+ cell population includes most, if not all progenitors in the BM, and accordingly it is a highly heterogeneous mixture of primitive and more mature progenitors allowing limited conclusions to be drawn about differential effects on mature and primitive progenitor cells.

The present study demonstrates that FL can stimulate the growth of CD34+ progenitors in the absence of other exogenously added cytokines both in serum-containing and serum-
deprived cultures. This effect appears to be directly mediated, as the growth-promoting activity was observed at the single cell level. The clones formed in response to FL alone were mostly small, representing predominantly clusters (>10 cells) rather than colonies (>40 cells). Interestingly, optimal recruitment of CD34+ progenitor cell growth was observed at 6 to 12 ng/mL of human FL, which is considerably lower than the optimal concentration of murine FL for enhancement of murine progenitor cell growth (250 ng/mL). As demonstrated by others, FL had, unlike SCF, no effect on the growth of CD34+ erythroid progenitor cells in serum-containing cultures. However, in the absence of serum, a synergistic growth response with regard to erythroid colony formation was observed combining FL and Epo, demonstrating that FL, although much less potently than SCF, can act as a synergistic growth enhancing factor of erythroid progenitor cells. The differential effect of FL in the absence and presence of serum might be due to components present in serum specifically capable of inhibiting the stimulatory effects of FL on erythroid progenitor cell growth. In that regard, we have recently demonstrated that endogenous transforming growth factor (TGF)-β in serum-containing cultures potently inhibits growth of murine erythroid progenitor cells, as well as FL-stimulated growth of primitive murine progenitors (Jacobsen SEW, manuscript submitted).

In serum-containing cultures, the majority of nonerythroid colonies induced by FL plus IL-3 plus Epo, as well as those formed by adding SCF to this growth factor combination were GM or M colonies. Similarly, whereas stimulation with IL-3 alone induced mostly granulocyte production (60%), predominantly macrophages (71%) were formed in bulk liquid cultures stimulated by FL plus IL-3. In contrast, morphologic examination of individual colonies grown in serum-deprived media supplemented with IL-3 plus Epo, or FL plus IL-3 plus Epo showed few or no GM and M colonies, suggesting that serum-derived components might interfere with the differentiation program of progenitors stimulated by combinations of FL, IL-3, and Epo, promoting the growth of progenitors capable of macrophage differentiation.

FL synergistically and potently enhanced colony formation of CD34+ BM progenitors in combination with SCF, GM-CSF, IL-3, and CSF-1, whereas colonies formed in response to G-CSF were less dramatically affected. When the total number of CD34+ clones (clusters plus colonies) were scored in single cell experiments, the combination of IL-3 and FL resulted in an additive rather than synergistic stimulation. This could be explained by IL-3 and FL recruiting overlapping populations of CD34+ progenitors when acting separately, whereas the combined action of the two cytokines recruits additional, presumably more primitive progenitors with higher proliferative potentials. Alternatively, FL and IL-3 might recruit distinct and nonoverlapping populations of CD34+ progenitors, whereas the two cytokines combined increase the proliferative capacity of these progenitors without recruiting additional progenitors.

The addition of FL to SCF plus IL-3-stimulated cultures of unfractionated CD34+ BM cells, increased colony formation 2.5-fold. The comparative studies on primitive (CD71+) and more committed (CD71−) subsets of CD34+ BM progenitors under both serum-containing and serum-deprived conditions suggested that these additional recruited progenitors were predominantly located in the primitive CD34+CD71− subpopulation. Specifically, FL and IL-3 stimulated little or no growth of CD34+CD71+ progenitors when acting individually, but the combination demonstrated synergistic activity. More importantly, whereas FL in the presence of serum did not enhance colony formation of CD34+CD71+ progenitors stimulated by IL-3 plus SCF, it did increase colony formation of the more primitive CD34+CD71− progenitor cells more than fourfold in combination with IL-3 plus SCF. However, these experiments did also establish that FL can stimulate the growth of more committed (CD34+CD71−) progenitors in human BM as well, alone and in combination with other cytokines. Thus, it appears that the activity of FL is not limited to the most primitive progenitors in human BM, as might have been predicted from the restricted expression of flt3 on primitive murine BM progenitors, and from recent studies on murine progenitors.

The addition of FL to SCF plus IL-3-stimulated cultures of hematopoietic cells potently inhibited growth of murine erythroid progenitor cells,38 as well as FL-stimulated growth of primitive murine progenitors (Jacobsen SEW, manuscript submitted).

In conclusion, the human FL is a potent and direct growth stimulator of human CD34+ BM progenitor cells in combination with multiple cytokines. The activities of FL appear to be partially overlapping, but distinct from those of SCF. Although its most potent activity as a synergistic factor is observed on primitive CD34+ progenitors, it can also stimulate the growth of more committed progenitor cells. Based on its in vitro activity, FL might prove clinically useful for stimulation of hematopoietic recovery in myelosuppressed patients, and in vivo expansion of hematopoietic progenitor cells.

ACKNOWLEDGMENT

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The FLT3 ligand is a direct and potent stimulator of the growth of primitive and committed human CD34+ bone marrow progenitor cells in vitro

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