The FLT3 Ligand Potently and Directly Stimulates the Growth and Expansion of Primitive Murine Bone Marrow Progenitor Cells In Vitro: Synergistic Interactions with Interleukin (IL) 11, IL-12, and Other Hematopoietic Growth Factors

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Summary

The recently cloned murine flt3 ligand (FL) was studied for its ability to stimulate the growth of primitive ($Lin^-Sca\text{-}1^+$) and more committed ($Lin^-Sca\text{-}1^-$) murine bone marrow progenitor cells, alone and in combination with other hematopoietic growth factors (HGFs). Whereas FL was a weak proliferative stimulator alone, it potently synergized with a number of other HGFs, including all four colony-stimulating factor (CSF), interleukin (IL) 6, IL-11, IL-12, and stem cell factor (SCF), to promote the colony formation of $Lin^-Sca\text{-}1^+$, but not $Lin^-Sca\text{-}1^-$ or erythroid progenitor cells. The synergistic activity of FL was concentration dependent, with maximum stimulation occurring at 250 ng/ml, and was observed when cells were plated at a concentration of one cell per culture, suggesting that its effects are directly mediated. 2 wk of treatment with FL in combination with IL-3 or SCF resulted in the production of a high proportion of mature myeloid cells (granulocytes and macrophages), whereas the combination of FL with G-CSF, IL-11, or IL-12 resulted predominantly in the formation of cells with an immature blast cell appearance. Accordingly, FL in combination with G-CSF or IL-11 expanded the number of progenitors more than 40-fold after 2 wk incubation. Thus, FL emerges as a potent synergistic HGF, that in combination with numerous other HGFs, can directly stimulate the proliferation, myeloid differentiation, and expansion of primitive hematopoietic progenitor cells.

Hematopoiesis is tightly regulated, at least in part through the complex and opposing actions of soluble stimulatory and inhibitory hematopoietic growth factors (HGF)[1] (1–3). An important, if not essential phenomenon in early hematopoiesis is synergy among HGFs, in that most primitive progenitors can only be recruited to proliferate in vitro by the combined stimulation of multiple HGFs (1–3). In contrast, committed progenitors can usually be recruited by stimulation with a single cytokine, although synergism between multiple cytokines might result in enhanced proliferation of each progenitor cell (3). A number of HGFs could be grouped as synergistic HGFs since they have little or no in vitro effect on proliferation alone, and act predominantly to enhance the growth promoting activity of other HGFs, such as the CSFs and Epo (1–3). The synergistic HGFs include IL-1, IL-6, IL-11, IL-12, leukemia inhibitory factor (LIF), and stem cell factor (SCF).

The growth-promoting effects of SCF appear to be predominantly on primitive progenitors or stem cells, although more committed progenitors of the myeloid/erythroid and mast cell lineage also respond to SCF (4–8). Furthermore, SCF is a very potent growth stimulator when combined with other cytokines, and no other cloned HGF can synergize with as many cytokines as SCF (7). Thus, potent synergistic interactions on the growth of primitive hematopoietic progenitors has been observed between SCF and the CSFs, rHu-erythropoietin (Epo), IL-1, IL-6, IL-11, and IL-12 (4–14).

The effects of SCF are signaled through the c-kit tyrosine kinase receptor (15, 16) that is closely related to c-fms (17) and the flt3/flk-2 receptor (18, 19). Whereas c-fms appears to be expressed on both primitive and mature myeloid progenitors, flt3/flk-2 is thought to be expressed exclusively on

[1] *Abbreviations used in this paper:* BFU-E, burst-forming unit erythroid; CFC, colony-forming cells; Epo, rHu-erythropoietin; FL, flt3 ligand; HGF, hematopoietic growth factor; HPP, high proliferative potential; LPP, low proliferative potential; rMu, recombinant murine; rr, recombinant rat.

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primitive progenitors (19). The successful cloning of the flt3 ligand (FL) (20–22) has allowed us to investigate the potential role of this cytokine in hematopoiesis. The cloning papers demonstrated that FL synergized with IL-3, IL-6, and SCF to enhance the growth of primitive murine hematopoietic progenitor cells (20, 21), but the potential interaction of FL with other HGFs, as well as its effects on differentiation and expansion of progenitors, were not examined. We here demonstrate the direct and potent ability of FL to synergize with a number of HGFs to induce the growth of primitive murine bone marrow progenitor cells.

Materials and Methods

Hematopoietic Growth Factors. Recombinant murine flt3 ligand was cloned and purified as previously described (20). Purified recombinant murine (rMu)IL-3 was from Peprotech Inc. (Rocky Hill, NJ). Purified rHuG-CSF, rMuGM-CSF and recombinant rat (rr)SCF were generously supplied by Dr. Ian K. McNiece (Amgen Biologicals, Thousand Oaks, CA). rHuCSF-1 was kindly provided by Dr. Michael Geier (Cetus Corp., Emeryville, CA), and rHUIL-6 and rHuIL-11 was a gift from Genetics Institute (Cambridge, MA). rMuIL-12 was a generous gift from Dr. Stan Wolf (Genetics Institute, Cambridge, MA). Epo was purchased from Cilag AG (Schaffhausen, Switzerland). Growth factors were used at predetermined optimal concentrations; 20 ng/ml rMuGM-CSF, 50 ng/ml rHuG-CSF, 20 ng/ml rMuIL-3, 50 ng/ml rHuCSF-1, 100 ng/ml rrSCF, 50 ng/ml rHuIL-6, 50 ng/ml rHuIL-11, 50 ng/ml rMuIL-12, 5 IU/ml rHuEpo, and 250 ng/ml rMuFL.

Isolation of Lin$^-$Sca-1$^+$ and Lin$^-$Sca-1$^-$ Bone Marrow Cells. Lin$^-$ bone marrow cells were isolated from femurs of normal C57BL/6 mice (5–8 wk old), according to a previously described protocol (14, 23). Briefly, low density bone marrow cells were incubated at 4°C for 30 min in a cocktail of predetermined optimal concentrations of antibodies; RA3-6B2 (B220 antigen; PharMingen, San Diego, CA), RB6-8C5 (GR-1 antigen; PharMingen), MAC-1 (Serotec Ltd., Oxfordshire, UK), Lyt-2 (CD8; PharMingen), L3T4 (CD4; PharMingen), and TER-119 (gift from Dr. Tatsuo Kina, Kyoto University, Kyoto, Japan). Sheep anti-rat IgG (Fc)-conjugated immunomagnetic beads (Dynal, Oslo, Norway) were added at a cell to bead ratio of 1:20, and incubated at 4°C for 30 min. Labeled (Lin$^+$) cells were removed by a magnetic particle concentrator (Dynal), and Lin$^-$ cells recovered from the supernatant. Lin$^-$Sca-1$^+$ cells were isolated as described previously (14, 23, 24). Briefly, 4–6 × 10^7 Lin$^-$ cells were resuspended per ml of complete IMDM and incubated for 30 min on ice with either FITC-conjugated rat anti-mouse Sca-1 antibody (clone E13161-7; PharMingen) or an isotype-matched control antibody. The cells were washed twice, and Lin$^-$Sca-1$^-$ and Lin$^-$Sca-1$^+$ cells sorted on a Epics Elite Cell Sorter (Coulter Electronics, Hialeah, FL). The final recovery of Lin$^-$Sca-1$^+$ cells was 0.05–0.1% of the unfractionated bone marrow.

Single Cell Proliferation Assay. Lin$^-$Sca-1$^+$ or Lin$^-$Sca-1$^-$ cells were seeded in microtiter plates (Nunc, Kamstrup, Denmark) at a concentration of 1 cell per well in a volume of 20 μl IMDM (GIBCO BRL, Paisley, UK) supplemented with 20% FCS (BioWhittaker, Inc., Walkersville, MD), penicillin, and L-glutamine (complete IMDM). In some experiments, an Epics Elite Cell Sorter equipped with a single-cell depositor was used to plate single cells. This method assures reproducibly that >99% of the wells contain one cell. Similar results were obtained with both methods. Wells were scored for colony growth (>50 cells) and clusters (10–50 cells) after 12–14 d of incubation at 37°C and 5% CO_2 in air. In some experiments individual colonies were sampled, transferred to slides by a cytospin centrifuge, and examined morphologically after Giemsa staining.

Replating Experiments/Colony Formation in Semisolid Medium. After 14 d of incubation of Lin$^-$Sca-1$^+$ bone marrow cells in complete IMDM supplemented with different growth factor combinations, cells were counted, and 500 cells plated in a two-layer agarose culture as previously described (25). α-MEM (GIBCO BRL, Gaithersburg, MD) supplemented with 20% FCS was used in the colony assays. Cytokines were incorporated into a 1-ml underlayer with Sea plaque agarose (FMC BioProducts, Rockland, ME) at a final concentration of 0.5%, whereas cells were incorporated into a 0.5-ml overlayer with a final sea plaque agarose concentration of 0.3%. After 12–14 d of incubation at 5% O_2, 10% CO_2, and 85% N_2, cultures were scored for colony-forming cells (CFC) with low or high proliferative potential (LPP-CFC and HPP-CFC, respectively) according to established criteria (25, 26). Erythroid colony assays were performed in complete IMDM and 1.2% (final concentration) methylcellulose (Methocel; Fluka Chemie AG, Buchs, Switzerland). Cultures were scored for burst-forming unit erythroid (BFU-E) colonies after 7–8 d of incubation at 37°C, in 5% CO_2, in air. In some experiments, IMDM with 20% FCS was replaced by a serum-free medium (x-vivo 15, BioWhittaker) supplemented with 1% detoxified bovine serum albumin (Stem Cell Technologies Inc., Vancouver, Canada)

Cell Morphology/Immune Phenotyping. Lin$^-$Sca-1$^+$ cells were plated in complete IMDM and incubated for 12–14 d with predetermined optimal concentrations of cytokines at 37°C and 5% CO_2 in air. Cell morphology was determined after Giemsa staining of cytospin preparations. Cells were examined for expression of GR-1 and MAC-1 after 11–12 d of incubation in FL + G-CSF or FL + IL-11. Cells were harvested, washed, and incubated for 30 min on ice with predetermined optimal concentrations of FITC-conjugated anti-GR-1 antibody (PharMingen) and a PE-conjugated anti-Mac-1 antibody (PharMingen), or isotype matched irrelevant control antibodies (PharMingen). Fluorescence intensity was analyzed by a Coulter Epics Elite FACS.

Results

As few as 100 Lin$^-$Sca-1$^+$ cells can radioprotect 50% of lethally irradiated mice, and long-term reconstitute all cell lineages in the blood (23, 24; Veiby, O. P., unpublished data). FL (30–1,000 ng/ml) stimulated few or no colonies of Lin$^-$Sca-1$^+$ bone marrow cells, and no colonies were formed in response to IL-11 (50 ng/ml) alone (Fig. 1). However, in combination with IL-11, FL stimulated in a concentration-dependent manner the colony formation of Lin$^-$Sca-1$^+$ progenitor cells (Fig. 1). A maximum stimulation (67 colonies) was seen at FL 250 ng/ml, and an ED_{50} was observed at ~60 ng/ml of FL. A similar ED_{50} was observed when IL-12 (50 ng/ml) was combined with escalating concentrations of FL, and maximum colony formation (39 colonies) was again seen in response to 250 ng/ml FL (Fig. 1). Since these experiments were performed on Lin$^-$Sca-1$^+$ cells cultured at one cell per well, they suggested that FL in combination with IL-11 or IL-12 in a direct manner can potently stimulate the growth of primitive hematopoietic progenitor cells.

Next, Lin$^-$Sca-1$^+$ progenitors were stimulated by 250 ng/ml of FL and increasing concentrations of IL-11 and IL-12. Maximum colony formation was observed at 5 to 50 ng/ml

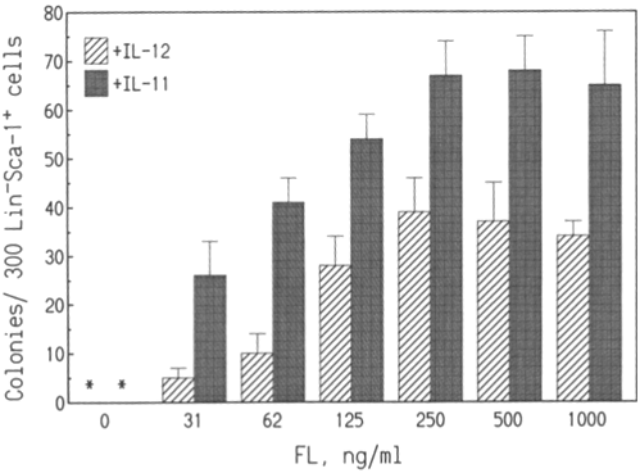

Figure 1. Concentration-response of FL-induced colony formation in combination with IL-11 and IL-12. Lin⁻Sca-1⁺ cells were plated at one cell per well in 20 μl complete IMDM supplemented with rHuIL-11 (50 ng/ml) or rMuIL-12 (50 ng/ml), and exposed to increasing concentrations of rMuFL as indicated. Wells were scored for colony formation after 12–14 d of incubation at 37°C, in 5% CO_2, in air. Results represent the mean (SEM) of four separate experiments. * No colonies were formed in response to IL-11 or IL-12 in the absence of FL.

of IL-11 and 20 ng/ml of IL-12, whereas the ED_{50} was 0.5 ng/ml and 0.2-2 ng/ml for IL-11 and IL-12, respectively (data not shown).

Lin⁻Sca-1⁺ bone marrow cells plated at a concentration of one cell per well (300 wells per group) were next cultured in FL (250 ng/ml) in combination with other cytokines. In these experiments we also examined whether the ability of FL to enhance colony formation of Lin⁻Sca-1⁺ progenitor cells was due to recruitment of additional progenitors, and/or an increase in the size of responding clones from clusters to colonies. These studies demonstrated that the synergistic effects of FL in general were due to an increase in the total number of proliferating clones, in that both the number of clusters and big colonies was enhanced (Table 1). FL alone stimulated only the formation of a low number of clusters, and in agreement with previous reports (20, 21), FL synergized with IL-3 to increase the cloning frequency of Lin⁻Sca-1⁺ progenitor cells (from 23 to 98 clones). Not previously shown, FL also potently enhanced G-CSF–stimulated colony growth. Whereas G-CSF alone stimulated the formation of only five clusters and one colony, the addition of FL resulted in the formation of 14 clusters and 69 colonies (Table 1). Although the combination of FL and CSF-1 was not at all as potent as FL + IL-3 or FL + G-CSF, FL enhanced the number of CSF-1–responsive clones from 7 to 21. Similarly, GM-CSF–induced clonogenic progenitors were increased from 6 to 38 in the presence of FL (Table 1). Interestingly, a synergistic enhancement of FL was also observed on SCF-stimulated colony formation (from 12 to 45 clones). Finally, whereas no colonies and only a low number of clusters were observed in response to IL-6, IL-11, or IL-12 alone, FL increased the number of proliferative clones to 62, 72, and 42, respectively. Thus, FL directly and synergistically enhances the growth

Table 1. *The Effect of FL on Colony Formation of Lin⁻Sca-1⁺ Progenitor Cells*

Cytokines	Clone size*				Total clones/ 300 cells
	1	2	3	4	
Medium	0(0)	0(0)	0(0)	0(0)	0(0)
FL	3(2)	0(0)	0(0)	0(0)	3(2)
IL-3	8(1)	9(1)	3(2)	3(1)	23(3)
FL + IL-3	14(4)	41(6)	26(5)	18(2)	98(8)
G-CSF	5(3)	1(1)	0(0)	0(0)	6(3)
FL + G-CSF	14(3)	50(5)	16(4)	3(1)	82(7)
CSF-1	3(1)	3(1)	1(1)	0(0)	7(2)
FL + CSF-1	6(1)	10(2)	3(1)	2(1)	21(2)
GM-CSF	1(0)	3(1)	2(1)	0(0)	6(2)
FL + GM-CSF	10(3)	13(5)	8(3)	7(3)	38(10)
CSF	3(0)	4(1)	5(2)	0(0)	12(3)
FL + SCF	15(4)	22(5)	8(2)	1(1)	42(9)
IL-6	2(1)	0(0)	0(0)	0(0)	2(1)
FL + IL-6	13(2)	40(7)	6(2)	3(1)	62(9)
IL-11	2(1)	0(0)	0(0)	0(0)	2(1)
FL + IL-11	13(2)	36(5)	16(4)	7(2)	72(12)
IL-12	0(0)	0(0)	0(0)	0(0)	0(0)
FL + IL-12	12(3)	23(4)	6(3)	0(0)	42(7)

Lin⁻Sca-1⁺ bone marrow cells were isolated as described in Materials and Methods, plated at one cell per well in 20 μl complete IMDM, and supplemented with predetermined optimal concentrations of cytokines (Materials and Methods). Wells were scored for cell growth after 12–14-d incubation at 37°C and 5% CO_2 in humidified air.

* Scoring criteria: 1, 10–50 cells; 2, 50 cells to 10% of well covered by cells; 3, Cells covering 10–50% of well; 4, >50% of well covered by cells. The results represent the mean (SEM) of five separate experiments.

of primitive murine hematopoietic progenitor cells in combination with multiple HGFs.

We have recently demonstrated that IL-12, in combination with SCF is a potent synergistic growth factor for Lin⁻Sca-1⁺ bone marrow progenitor cells (14). Here, we compared the ability of FL and SCF to stimulate colony formation in combination with IL-12 (Table 2). Whereas FL (250 ng/ml) in combination with IL-12 (50 ng/ml) stimulated the formation of 37 clusters or colonies, the combined action of SCF (100 ng/ml) and IL-12 induced the outgrowth of 55 clones (Table 2). However, the most dramatic difference between the FL + IL-12 and SCF + IL-12 response was with regard to the size of the clones formed (Table 2). Specifically, whereas FL + IL-12 stimulated the formation of only four colonies covering more than 10% of the well and no colonies covering over 50% of the well, SCF and IL-12 in combination resulted in formation of 17 colonies covering 10–50%, and 15 colonies covering more than 50% of the well. Furthermore, the trifactor combination of SCF + FL + IL-12 stimulated 97

Table 2. *Synergistic Effects of IL-12 on FL and SCF-stimulated Colony Formation of Lin⁻Sca-1⁺ Progenitor Cells*

Cytokines	Clone size				Total clones/ 300 cells
	1	2	3	4	
FL	2(1)	0(0)	0(0)	0(0)	2(1)
SCF	3(1)	4(2)	6(2)	0(0)	13(3)
FL + SCF	17(4)	20(5)	6(2)	0(0)	43(10)
FL + IL-12	14(3)	19(4)	4(2)	0(0)	37(5)
SCF + IL-12	10(2)	14(4)	17(5)	15(4)	55(6)
FL + SCF + IL-12	9(3)	31(6)	29(7)	28(6)	97(11)

Lin⁻Sca-1⁺ bone marrow cells were plated at one cell per well in the presence of predetermined optimal concentrations of cytokines (Materials and Methods) as indicated, and scored for cell growth after 12–14-d incubation at 37°C, in 5% CO_2 in air. Scoring criteria were as in Table 1. Results represent the mean (SEM) of four separate experiments.

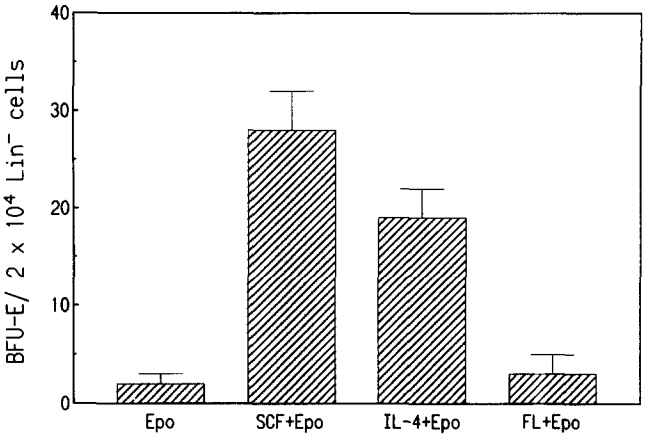

Figure 2. Effects of SCF, IL-4, and FL on erythroid colony formation. Lin⁻ cells were isolated, and plated at 2 × 10^4 cells in semisolid medium as described in the Materials and Methods. Cultures were done in triplicates, and supplemented with predetermined optimal concentrations of cytokines (Materials and Methods) as indicated. BFU-E colony formation was scored after 7–8 d of incubation at 37°C, in 5% CO_2, in air. Results represent the mean (SEM) of three separate experiments.

colonies out of 300 Lin⁻Sca-1⁺ cells, and 28 of these covered more than 50% of the well (Table 2).

To investigate whether more committed hematopoietic progenitor cells were also responsive to FL, Lin⁻Sca-1⁻ cells were cultured in FL (250 ng/ml), in the absence or presence of other HGFs. No colonies were formed in response to FL alone, or in combination with IL-11 or IL-12 (data not shown). In agreement with previous studies (13), IL-3 and G-CSF stimulated colony formation of Lin⁻Sca-1⁻ progenitor cells (25 ± 4 and 5 ± 2 colonies, respectively), but FL did not synergize with IL-3 or G-CSF (28 ± 3 and 7 ± 2 colonies, respectively).

In contrast to IL-4 and SCF, FL did not stimulate BFU-E colony formation of Lin⁻ bone marrow cells in combination with Epo (Fig. 2), suggesting in agreement with previous studies (20, 21), that FL might not stimulate the growth of erythroid progenitor cells. Furthermore, in serum-free cultures FL did not enhance the number of BFU-E colonies formed in response to IL-3 + Epo or IL-3 + SCF + Epo (data not shown).

To determine the morphology of the progeny of Lin⁻Sca-1⁺ cells formed in response to FL, 2,000 Lin⁻Sca-1⁺ cells were cultured in the presence of FL in combination with other cytokines for 2 wk, and cytospin preparations were analyzed morphologically. No viable cells were observed after 14 d of incubation with FL alone. However, after 7–10 d of incubation with FL a population of 100% blast cells was observed, whereas SCF-stimulated cultures in comparison contained a high proportion of granulocytes (data not shown). Whereas IL-3 alone stimulated the formation of 47% granulocytes and 50% macrophages after 12–14 d of incubation, FL + IL-3 stimulated the formation of 80% macrophages and only 16% granulocytes (Table 3). Only 3% undifferentiated blast cells were observed in response to IL-3, both in the absence and presence of FL (Table 3). In contrast, cultures supplemented with FL + G-CSF contained 65% undifferentiated blasts, 6% granulocytes, and 29% macrophages (Table 3). In agreement with other studies (10, 13, 14), SCF alone stimulated the formation of predominantly granulocytes (90%), and a low number of macrophages (4%) and blast cells (6%). Similarly, 73% granulocytes, 9% macrophages, and 18% blasts

Table 3. *Effects of FL on the Differentiation of Lin⁻Sca-1⁺ Bone Marrow Cells*

Cytokines	Cells	Percent granulocytes	Percent macrophages	Percent blasts*
	× 10^4			
IL-3	8(4)	47(7)	50(8)	3(2)
FL + IL-3	36(11)	16(5)	80(9)	3(3)
FL + G-CSF	27(10)	6(2)	29(9)	65(8)
SCF	2(1)	90(4)	4(2)	6(3)
FL + SCF	5(1)	73(5)	9(3)	18(7)
FL + IL-11	26(8)	5(2)	35(8)	60(8)
FL + IL-12	8(3)	7(3)	17(6)	77(9)
SCF + IL-12	59(9)	91(5)	4(3)	5(2)

Two thousand Lin⁻Sca-1⁺ cells were plated in 300 μl complete IMDM supplemented with predetermined optimal concentrations of cytokines (Materials and Methods) as indicated. Total cell numbers were counted, and cytospin preparations were stained with Giemsa after 12–14-d incubation. Morphology was examined under a light microscope. At least 100 cells were examined per group in each experiment. Results are the mean (SEM) of six separate experiments. In the presence of G-CSF, IL-11, IL-12, of FL alone no or almost no viable cells were observed (not shown).

were observed in response to FL + SCF (Table 3). Only 5% of the cells harvested from cultures supplemented with FL + IL-11 were granulocytes, whereas 35% were macrophages, and 60% blast cells. Similarly, 7% of the cells formed in response to FL + IL-12 were granulocytes, 17% macrophages, and 77% undifferentiated blast cells (Table 3). In comparison SCF + IL-12 stimulated almost exclusively the production of granulocytes (91%), and a very low number of blasts (5%).

To further support the finding that Lin$^-$Sca-1$^+$ progenitors produce myeloid progeny in response to FL, we examined the expression of MAC-1 and GR-1, two myeloid cell surface antigens (27, 28). After 11–12 d of incubation in FL + G-CSF, 25% of the cells expressed GR-1 and 60% MAC-1 (Table 4). Similarly 28 and 78% of cells formed in response to FL + IL-11 were positive for GR-1 and MAC-1, respectively (Table 4).

300 Lin$^-$Sca-1$^+$ cells were next cultured individually, and for each treatment a total of at least 50 colonies covering more than 5% of the well were sampled in six experiments to determine the direct effect of FL on the relative production of CFU-G, CFU-M, CFU-GM, and CFU-blast (CFU-B). In response to IL-3 a mean of 19 ± 4 clones were formed. 46 ± 12% of the sampled colonies were CFU-G, 11% CFU-M, 43% CFU-GM, whereas no CFU-B were observed (data not shown). In contrast, only 3 ± 2% of the colonies produced in response to FL + IL-3 (87 ± 9 clones total) were CFU-G, 62 ± 16% were CFU-M, 33 ± 9% CFU-GM, and 2 ± 1% CFU-B. FL + G-CSF (76 ± 8 clones) stimulated predominantly CFU-GM (46 ± 12%), a high number of CFU-B (23 ± 8%), 12 ± 4% CFU-M, and 19 ± 5% CFU-G. FL + IL-11 (75 ± 13 clones) stimulated 2 ± 1% CFU-G, 37 ± 5% CFU-M, 38 ± 7% CFU-GM, and 23 ± 6% CFU-B. In response to FL + IL-12 (38 ± 6 clones) 11 ± 3% of sampled colonies were CFU-G, 56 ± 8% CFU-GM, and 33 ± 8% CFU-B. In comparison, 81 ± 14% of examined colonies induced by SCF + IL-12 (59 ± 8 clones) were CFU-G, 2 ± 1% CFU-M, 16 ± 4%, and 1 ± 1% CFU-B (data not shown).

When Epo in four experiments was included in the cultures of Lin$^-$Sca-1$^+$ cells stimulated with FL + IL-11 or FL + G-CSF, the number of CFU-G, CFU-M, CFU-GM, and CFU-B did not change significantly, and no BFU-E and only a low number of mixed (myeloid plus erythroid) colonies (2 ± 1 and 1 ± 1 in response to FL + IL-11 and FL + G-CSF, respectively) were observed (data not shown).

The fact that FL in combination with some HGFs, such as IL-11 and G-CSF appeared to increase the production of cells with an immature morphology after as much as 2 wk of incubation, led us to examine whether FL might in fact induce an expansion of the number of progenitors after the same period of time. This was done by measuring the number of HPP-CFCs, demonstrated to be among the most primitive hematopoietic progenitors measurable in vitro, as well as LPP-CFC, that represents a population of more committed progenitor cells (25, 26). The input of 2,000 Lin$^-$Sca-1$^+$ cells at the start of the incubation produced 108 HPP-CFC and 234 LPP-CFC colonies in response to a cocktail of CSF-1 + IL-1 + IL-3 + SCF (Table 5). After 14 d of incubation, the FL + IL-11–stimulated culture contained 1.48 × 10^5 cells, which represented a 74-fold expansion of the total number of cells. These cells contained a total of 5032 HPP-CFC (47-fold increase), and 9786 LPP-CFC (42-fold increase). Similarly, a 98-fold increase in cell number was observed when Lin$^-$Sca-1$^+$ cells were stimulated by FL + G-CSF, and this corresponded to a 40-fold and 101-fold increase in HPP-CFC and LPP-CFC, respectively (Table 5). Finally, we examined the ability of FL + IL-3 to expand the number of progenitors, since this combination appeared much less efficient in maintaining a high proportion of immature cells than FL + IL-11 and FL + G-CSF (Table 3). Although this combi-

Table 4. *Expression of MAC-1 and GR-1 on Lin$^-$Sca-1$^+$ Progeny Stimulated by FL*

Cytokines	GR-1–positive cells	MAC-1–positive cells
	%	%
FL + G-CSF	25(7)	60(8)
FL + IL-11	28(6)	78(11)

2,000 Lin$^-$Sca-1$^+$ bone marrow cells were incubated in predetermined optimal concentrations of FL + G-CSF or FL + IL-11 for 11–12 d. Cells were harvested, and 5 × 10^4 cells were incubated with FITC-conjugated GR-1– or PE-conjugated MAC-1 antibodies as described in the Materials and Methods. Results are from three separate experiments and represent the mean (SEM) percentage of GR-1– and MAC-1–positive cells, as compared to cells incubated with irrelevant control antibodies.

Table 5. *Amplification of HPP-CFC and LPP-CFC from Lin$^-$Sca-1$^+$ Progenitor Cells by FL*

Cytokines	Total cells	HPP-CFC	LPP-CFC
	× 10^4		
Input	0.2	108 (43)	234 (75)
FL + IL-11	14.8 (2.5)	5032 (1367)	9786 (2417)
FL + G-CSF	19.6 (4.1)	4312 (952)	23520 (4762)
FL + IL-3	18.5 (3.9)	93 (55)	1480 (370)

2,000 (input) Lin$^-$Sca-1$^+$ cells were plated in complete IMDM in the presence of predetermined optimal concentrations of cytokines as indicated. After 14 d of incubation the total number of nucleated cells was determined for each group, and 500 cells were plated in the double layer colony assay (Materials and Methods) in the presence of predetermined optimal concentrations of CSF-1, IL-1, IL-3, and SCF. Following another 12–14 d of incubation at 37°C, in 5% O_2 and 10% CO_2, in air, cultures were scored for HPP-CFC and LPP-CFC colony growth. In each experiment triplicate cultures were done for each group. Results represent the mean (SEM) of three separate experiments.

nation also potently stimulated the total cell production (93-fold increase), no amplification of the number of HPP-CFC was observed. However, an expansion of LPP-CFC (sixfold) was observed (Table 5).

Discussion

In agreement with recent studies (20, 21), we found FL alone to be a weak proliferative stimulus for $Lin^-Sca\text{-}1^+$ progenitors, and in fact much weaker than SCF alone. However, potent synergistic effects of FL was observed in combination with IL-3, GM-CSF, G-CSF, CSF-1, SCF, IL-6, IL-11, and IL-12. The degree of synergy between FL plus IL-3 and FL plus SCF was more pronounced in the present study, than reported in the original cloning papers (20, 21). Since no dose–response of FL was reported in these studies, it is possible that the original studies were not performed with optimal concentrations of FL. Alternatively, the difference might be due to the fact that different progenitor cell populations were used in the different studies.

Interestingly, the strongest synergy was observed when FL was combined with ligands signaling through members of the hematopoietin receptor family (G-CSF, GM-CSF, IL-3, IL-6, IL-11, and IL-12), whereas weaker synergy was seen when FL was combined with one of the other ligands for tyrosine kinase receptors (CSF-1 and SCF). Maximum stimulation of the proliferation of $Lin^-Sca\text{-}1^+$ progenitors required as much as 125–250 ng/ml of FL. In comparison, optimal effects of SCF are observed at 100 ng/ml. Since we have recently observed a tendency of the murine but not the human FL to aggregate (Lyman S. D., unpublished observations), the optimal concentration of FL might prove to be lower.

The present studies demonstrate that IL-12 also potently enhances FL-stimulated proliferation of $Lin^-Sca\text{-}1^+$ progenitors, although less efficiently than in combination with SCF. In addition, the potent trifactor combination of FL + SCF + IL-12 stimulated one out of three $Lin^-Sca\text{-}1^+$ progenitors to proliferate, many of them forming very large colonies. Thus, the present data further underscores the ability of IL-12 to act as a direct synergistic growth factor on primitive hematopoietic progenitor cells.

In agreement with others (20, 21), we found no effect of FL on Epo-stimulated BFU-E colony formation, at variance with the potent synergy observed between SCF and Epo. Since our studies were performed on restricted progenitor cell populations, further studies are required to rule out the involvement of flt3 signaling on erythroid and other committed progenitor cells. The fact that FL supports the formation of mature myeloid cells suggest that FL might also support the growth of subsets of more mature hematopoietic cells. However, at the moment it appears that SCF affects a broader range of murine hematopoietic progenitors than FL.

The morphological examination of cells formed by $Lin^-Sca\text{-}1^+$ progenitors after as much as 12–14 d in culture, demonstrated that FL in combination with other HGFs could potently stimulate the production of cells with an immature blast appearance. Although immunophenotyping demonstrated that the majority of the cells formed in response to FL + G-CSF and FL + IL-11 expressed myeloid cell surface antigens, the replating studies demonstrated by day 14 a more than 40-fold increase in the number of HPP-CFCs. Thus, FL can in combination with other cytokines induce myeloid differentiation, but also expand the number of primitive (HPP-CFC) and more committed (LPP-CFC) bone marrow progenitors. Thus, FL might prove efficient in a transplantation setting.

A potential important role of FL in early hematopoiesis was also supported by a recent study where antisense oligonucleotides against STK-1 (the human homologue of murine flt3), potently inhibited colony formation of $CD34^+$ human bone marrow cells, in particular in long-term bone marrow cultures (29). However, since no genetic mutations of flt3 or FL affecting hematopoiesis have yet been identified, the physiological role of FL in hematopoiesis remains to be determined.

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