Direct Synergistic Effects of Leukemia Inhibitory Factor on Hematopoietic Progenitor Cell Growth: Comparison With Other Hematopoietins That Use the gp130 Receptor Subunit

By Jonathan R. Keller, John M. Gooya, and Francis W. Ruscetti

Because leukemia inhibitory factor (LIF) has little or no effect on murine hematopoietic progenitor cell growth yet enhances hematopoiesis in vivo, we sought to determine whether the effects of LIF were directly or indirectly mediated, or a combination of both. Although LIF alone or in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) has no effect on colony formation of unfractonated bone marrow cells (BMClC), it enhances M-CSF-induced colony formation. In comparison, LIF synergizes with IL-3, GM-CSF, M-CSF, and Steel Factor (SLF) to promote the colony formation of partially purified lineage-negative (Lin-7) BM progenitors without altering their differentiation. These effects were directly mediated since identical results were observed in single-cell assays. Comparing the effect of LIF with other members of this subclass of hematopoietins (IL-6, oncostatin M [OSM], and ciliary neurotrophic factor [CNTF]), we found that while LIF and IL-6 equally synergize with M-CSF and SLF to promote the colony formation of Lin- BMCs, OSM, and CNTF have no effect. In agreement with OSM's ability to directly bind gp130, preincubation of BMCs with OSM inhibits progenitor cell growth stimulated by the combination of LIF or IL-6 plus SLF. LIF can also directly enhance the growth of further purified more primitive Lin- o-kit progenitor cells in the presence of IL-3, GM-CSF, or SLF. Thus, LIF can directly synergize with growth factors to promote the proliferation of purified hematopoietic progenitors, suggesting that the direct effects of LIF on hematopoietic cell growth can, in part, explain the observed hematopoietic effects in vivo.

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L E U K E M I A inhibitory factor (LIF) is a polyfunctional regulator of cell growth and has been shown to have a broad spectrum of effects on a variety of cell types. Specifically, LIF has been shown to (1) induce the differentiation and reduce the clonogenicity of the mouse myeloid leukemic cell line, M13; (2) maintain the pluripotent phenotype of embryonic stem cells; and (3) induce the production of acute-phase proteins. In addition, LIF was shown to induce the proliferation of the factor-dependent mouse myeloid cell line DA-1a as well as potentiate interleukin-3 (IL-3)–induced proliferation of murine myc-transformed erythroleukemia cell lines.

Although LIF alone or in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to have no effect on normal murine bone marrow (BM) progenitor cell colony formation in vitro, it was subsequently shown to affect megakaryocyte proliferation and maturation in vitro and in vivo. Interestingly, LIF-deficient mice have normal levels of circulating red blood cells, white blood cells, and platelets, but have dramatically decreased numbers of colony-forming unit-spleen cell activity in BM and spleen. Nonetheless, BM cells (BMC) from LIF-deficient mice can reconstitute the hematopoietic system of wild-type lethally irradiated recipients. Taken together, although hematopoietic cell function is not lost in LIF-deficient mice, it is not clear whether LIF acts directly on hematopoietic progenitor stem cells or whether LIF acts indirectly on accessory cells in the microenvironment or a combination of both effects. Therefore, the experiments presented here were designed to determine (1) whether LIF can directly effect the growth of hematopoietic stem/progenitor cell populations, and (2) to compare its effects on BMC growth with other members of this subclass of hematopoietin growth factors including IL-6, oncostatin M (OSM), and ciliary neurotrophic factor (CNTF).

MATERIALS AND METHODS

Mice. Female BALB/c mice 8 to 12 weeks of age were obtained from the Animal Production Area at the Frederick Cancer Research and Development Center (Frederick, MD). Animal care was provided in accordance with the procedures outlined in the “Guide For Care and Use of Laboratory Animals” (publication no. 86-23, Bethesda, MD, National Institutes of Health, 1985). Mice were maintained in a pathogen-free environment.

Cytokines. Recombinant murine IL-3, human OSM, human LIF, rat CNTF, and murine IL-6 were purchased from Peprotech, Inc (Rocky Hill, NJ). Recombinant murine GM-CSF was a gift of Drs Ian McNiece and Thomas C. Boone (Amgen Corp, Thousand Oaks, CA). Recombinant human M-CSF was a gift from Dr Michael Geier (Cetus Corp, Emeryville, CA). Purified murine steel factor (SLF) was a gift of S. Gillis (Immunex Corp, Seattle, WA).

BM cells. Normal murine BMC were obtained by aspirating the femurs of 8-week-old BALB/c mice. Light-density BMC (LDBMC) were separated by centrifugation on lymphocyte separation medium (Organon Teknika Corp, Durham, NC). Cells were washed twice in Iscove's modified Dulbecco's medium (IMDM), and resuspended in IMDM supplemented with 10% fetal calf serum (Inovar, Gaithersburg, MD), 15 mg/L gentamicin, and 3 mg/mL glutamine (complete IMDM).

Purification of lineage-negative (Lin-) BM progenitors. Lin- BMC were purified according to a previously described protocol.

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Briefly, LDBMC were resuspended in complete IMDM and incubated at 4°C for 30 minutes with a cocktail of antibodies, RA3-6B2 and R6B-8C5 (gifts of R. Coffman, DNAX Corp, Palo Alto, CA); MAC-1 (purchased from Boehringer-Mannheim, Indianapolis, IN); Lyt-2, and Lyt4 (purchased from Becton Dickinson Co, Mountain View, CA). Cells were washed twice and resuspended in complete IMDM. Magnetic beads (Dynal, Great Neck, NY) were added at a ratio of 40:1 (beads/cell), and the mixture was incubated for 30 minutes at 4°C. Labeled lineage-positive cells were removed by a magnetic particle concentrator (Dynal) and Lin− cells were recovered from the supernatant.

Fluorescence-activated cell separation of Lin− c-kit+ progenitors. Lin− cells were directly labeled with the phycocerythrin conjugated monoclonal antibody that recognizes c-kit or isotope-matched control purchased from Pharmingen (San Diego, CA). Cells (1 × 10⁶) were incubated for 30 minutes at 4°C with the recommended concentration of antibodies, washed twice in complete IMDM, and resuspended in the same medium to approximately 2 to 5 × 10⁶ Lin− murine BMC cells/mL and separated by fluorescence activated cell separation (Becton Dickinson Co).

Soft agar colony formation. To measure colony formation of murine progenitor cells in vitro, 7.5 × 10⁴ unfractionated BMC or 1 × 10⁵ Lin− cells in 1 mL of complete IMDM and 0.3% sea plaque agarose were plated in 35-mm Lux petri dishes (Miles Scientific, Naperville, IL.), incubated at 37°C in 5% CO₂ for 7 to 10 days, and scored for colony growth (>50 cells). In addition, colonies were stained with 2-iodophenyl-3,4-nitrophenyl-s-phenyltetrazolium chloride (INT; Sigma, St Louis, MO) for photomicroscopy. Briefly, 100 µL of 1% INT in PBS was added to day 7 to 10 soft agar colonies and incubated for 24 hours at 37°C, 5% CO₂ to visualize viable colonies.

Single-cell proliferation assay. The Lin− cells or the Lin− c-kit+ cells were seeded in Terasaki plates, 60 wells per plate, (Nunc, Kamstrup, Denmark) at a concentration of one cell in 20 µL of IMDM supplemented with 10% fetal calf serum and containing purified growth factors as indicated in the text. In the initial experiments, those wells containing greater than two cells were eliminated, whereas those wells with single cells were scored for cell growth (>10 cells) after 7 to 10 days at 37°C, 5% CO₂. The frequency is expressed as the number of single cells that showed growth in 300 single cells examined.

RESULTS

Effect of LIF on hematopoietic growth factor–induced colony formation of unfractionated and Lin-purified BMC. To determine whether LIF could affect the growth of hema-
DIRECT SYNERGISTIC EFFECTS OF LIF

Topoietic progenitor cells in vitro, initial experiments compared the effect of LIF plus or minus GM-CSF, IL-3, or M-CSF on the growth of unseparated (light density BM, LDBM) and progenitor enriched (Lin⁻) BMC in colony assays. As previously shown, a saturating concentration of LIF (200 ng/mL) alone had no effect on colony formation (data not shown) and had no effect on GM-CSF–induced colony formation of unFractionated BMC over a broad dose range of GM-CSF (Fig 1A). Similarly, LIF had no effect on IL-3–induced unFractionated BMC colony formation (Fig 1B). In contrast, LIF enhanced M-CSF–induced colony formation of unFractionated BMC over a dose range of 200 ng/mL to 1 ng/mL of M-CSF (Fig 1C).

Because hematopoietic progenitor cells constitute a small fraction of unseparated BM, we next compared the effects of LIF on the growth of progenitor-enriched Lin⁻ BMC which represents 1% to 5% of the starting marrow population. Unlike the unFractionated BMC population, LIF enhanced the number and size of both GM-CSF– and IL-3–induced Lin⁻ colony formation at concentrations of IL-3 and GM-CSF ranging from 50 ng/mL to .05 ng/mL (Fig 2A and B, and Fig 3). In addition, similar to the results with the unFractionated BM population, LIF enhanced the number and size of M-CSF–induced colony formation of Lin⁻ cells over a dose of M-CSF ranging from 200 ng/mL to 2 ng/mL (Fig 2C, and Fig 3). Finally, although SLF alone promotes the growth of small hematopoietic colonies and clusters (containing <50 cells) with increasing doses of SLF, LIF increases the number and size of SLF–induced colonies over the dose range examined (Fig 2D).

To determine whether LIF had an effect on growth factor–mediated differentiation of hematopoietic progenitor cells, the morphology of 7- to 10-day soft agar colonies were determined by Wright Jenners staining and microscopy. While LIF enhanced IL-3–, GM-CSF–, M-CSF–, and SLF–induced colony formation, it did not significantly alter the morphology (differentiation) of the colonies induced by the growth factors alone (Table 1). Thus, LIF synergizes with GM-CSF, IL-3, M-CSF, and SLF to promote the growth of Lin⁻ BMC in soft agar without altering their differentiation potential.

Fig 3. Photomicrograph of the effect of LIF on growth factor–induced soft agar colonies. Purified Lin⁻ BMCs were plated in soft agar colony assays as described in Materials and Methods and supplemented with GM-CSF (20 ng/mL), IL-3 (30 ng/mL), M-CSF (200 ng/mL) in the presence or absence of LIF (200 ng/mL). Day 7 colonies were stained with 1% 2-iodophenol-3, 4-nitrophenyl-s-phényltetrazolium chloride (INT; Sigma) for photomicroscopy as described in Materials and Methods.
tures supplemented with SLF alone induced small clusters of cells. We have previously shown that both LIF and OSM signal through the LIF receptor/gp130 receptor complex to equally effect embryonic proliferation of factor-dependent BMCs, as was observed by trypan blue exclusion upon the addition of OSM at the concentrations tested (data not shown). Thus, although OSM alone or in combination with other growth factors has no effect on Lin− cell growth, OSM can compete for the synergistic effect of LIF or IL-6 on Lin− colony formation in combination with SLF.

Direct effects of LIF on hematopoietic growth factor–induced proliferation of Lin− negative BMC. To determine whether the effects of LIF on hematopoietic cell growth were direct or indirect, Lin− BMC were seeded into terasaki plates at one cell per well in 20 μL in the presence or absence of IL-3, GM-CSF, M-CSF, or SLF. Similar to the results obtained in soft agar assays, LIF alone had no effect on the growth of isolated Lin− BMC; however, LIF increased the number of isolated Lin− cells that proliferate to form colonies in response to IL-3, GM-CSF, M-CSF, and SLF (Table 2). Furthermore, although LIF had little or no effect on the number of SLF plus IL-3 responsive single cells (1 in 9 cells respond), wells containing greater than 500 cells increased

Table 1. Morphology of LIF-Stimulated Hematopoietic Colonies

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>G (%)</th>
<th>M (%)</th>
<th>GM (%)</th>
<th>Mix (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SLF + LIF</td>
<td>61</td>
<td>7</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>IL-3</td>
<td>75</td>
<td>3</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>IL-3 + LIF</td>
<td>80</td>
<td>8</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>M-CSF</td>
<td>0</td>
<td>93</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>M-CSF + LIF</td>
<td>0</td>
<td>98</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>11</td>
<td>21</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td>GM-CSF + LIF</td>
<td>10</td>
<td>4</td>
<td>88</td>
<td>0</td>
</tr>
</tbody>
</table>

Lin− cells were purified and plated in colony assays as described in Materials and Methods. The culture medium was supplemented with IL-3 (30 ng/mL), GM-CSF (20 ng/mL), SLF (100 ng/mL), CSF-1 (200 ng/mL), and LIF (200 ng/mL). After 7 to 10 days, the agar plug was dried onto glass slides, fixed, and stained using Diff-Quik (Baxter Scientific, McGaw Park, IL). For each group, 100 colonies were examined: granulocyte (G), macrophage (M), granulocyte/macrophage (GM), and mixture (Mix, colonies containing G, M, blastlike, mast cells). Cultures supplemented with SLF alone induced small clusters of cells not scored by this assay (not determined, ND).

Effect of other hematopoietin receptor family members that use the signal transducing subunit gp130 on SLF− and M-CSF− induced Lin− BMC colony formation. It has previously been shown that both LIF and OSM signal through the LIF receptor/gp130 receptor complex to equally effect embryonic cell proliferation, or the differentiation of the myelomonocytic cell line M1. Therefore, because LIF enhanced the growth of Lin− cells in the presence of hematopoietin growth factors, we compared the effect of other hematopoietin growth factors that signal through the gp130 receptor subunit on M-CSF− and SLF−induced Lin− progenitor cell growth including OSM, CNTF, and IL-6. As shown in Fig 2 and reproduced here using a broader dose response range, LIF synergizes with both M-CSF or SLF to promote colony formation of Lin− BMC (Fig 4A and B). In addition, as previously demonstrated, IL-6 also synergizes with M-CSF and SLF to promote colony formation (Fig 4A and B). In contrast, neither OSM nor CNTF had any effect alone or in combination with M-CSF or SLF on the growth of Lin− BMC colony formation. Although OSM had no effect on the growth of BM cells, it promoted the proliferation of factor-dependent DA-1a cells in a dose-dependent manner (data not shown).

Because OSM showed no effect on the growth or proliferation of Lin− cells yet has been shown to bind to gp130 with low affinity, we examined whether OSM could compete for the synergistic effects of LIF on Lin− progenitor colony formation. Therefore, cells were preincubated for 45 minutes with decreasing concentrations of OSM (starting at 20 μg/mL) before plating in soft agar assays in LIF plus SLF or LIF plus IL-6. Preincubation with OSM inhibits Lin− colony formation induced by either LIF plus SLF or LIF plus IL-6 in a dose-dependent manner (Fig 5). Furthermore, preincubation of Lin− cells with OSM had no effect on IL-3 plus SLF-induced growth of Lin− BMC which do not use gp130 to promote growth (Fig 5). In addition, no toxicity of Lin− colonies was observed by trypan blue exclusion upon the addition of OSM at the concentrations tested (data not shown).
from 4 in 300 single cells to 15 in 300 cells in the presence of LIF. Thus, LIF directly synergizes with IL-3, GM-CSF, M-CSF, and SLF to promote the growth of isolated Lin- cells, and LIF can enhance the clone size of progenitors stimulated by the combination of SLF plus IL-3.

It has previously been shown that Lin- c-kit-positive (c-kit) cells are enriched for pluripotent hematopoietic stem cells that are capable of long-term reconstitution in lethally irradiated mouse recipients. Therefore, to determine whether LIF could directly affect the growth of primitive hematopoietic progenitors, Lin- progenitors were further purified by sorting c-kit cells (both dull and bright) by flow cytometry (25% to 35% of the Lin- cells) and then were plated into single cell assays in the presence or absence of growth factors. Similar to the results obtained with Lin- cells, LIF directly increased the number and size of isolated Lin- c-kit cells that form colonies in response to IL-3 or SLF (Table 3). Furthermore, LIF had no significant effect on the number or size of colonies stimulated by the combination of SLF plus IL-3 (Table 3).

DISCUSSION

A variety of studies have shown that LIF can effect the growth of hematopoietic progenitor cells; however, these studies could not determine whether LIF was directly interacting with hematopoietic progenitor cells, whether LIF effects were indirectly mediated through other cell types, or whether the effects were a combination of both. For example, administration of LIF in vivo enhances extramedullary hematopoiesis and increases platelet production in mice, LIF-deficient mice have reduced numbers of colony-forming unit-spleen numbers, and LIF can enhance retroviral mediated gene transfer in conditions where there are mixtures of stromal and hematopoietic cells.

Table 2. Direct Effects of LIF on GM-CSF-, IL-3-, M-CSF-, and SLF-Mediated Proliferation of Lin- Progenitor Cells

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>No. of Responders/300 Cells</th>
<th>Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300 Cells</td>
<td>&gt;200 Cells</td>
</tr>
<tr>
<td>Medium</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LIF</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>13 ± 1</td>
<td></td>
</tr>
<tr>
<td>IL-3 + LIF</td>
<td>27 ± 1*</td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>13 ± 2</td>
<td></td>
</tr>
<tr>
<td>GM-CSF + LIF</td>
<td>17 ± 1*</td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>18 ± 1</td>
<td></td>
</tr>
<tr>
<td>M-CSF + LIF</td>
<td>30 ± 3*</td>
<td></td>
</tr>
<tr>
<td>SLF</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>SLF + LIF</td>
<td>11 ± 1*</td>
<td></td>
</tr>
<tr>
<td>SLF + IL-3</td>
<td>38 ± 2</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>SLF + IL-3 + LIF</td>
<td>34 ± 3</td>
<td>15 ± 1*</td>
</tr>
</tbody>
</table>

Lin- cells were obtained and plated into single cell assays as described in Materials and Methods. Cultures were supplemented with growth factors as described in Table 1. Terasaki plates were scored for growth (>10 cells/well) 7 to 10 days later as described in Materials and Methods. These results are representative of four separate experiments.

* Statistical significance by paired Student's t-test with a P value < .05 when comparing growth factor-induced colony formation in the presence of absence of LIF.

Table 3. Direct Effect of LIF on Growth Factor-Induced Proliferation of Linage-Negative c-kit-Positive Progenitors

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>No. of Responders/300 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Colonies</td>
</tr>
<tr>
<td>Medium</td>
<td>+LIF</td>
</tr>
<tr>
<td>LIF</td>
<td>9±4</td>
</tr>
<tr>
<td>IL-3</td>
<td>12±3</td>
</tr>
<tr>
<td>SLF/IL-3</td>
<td>68±6</td>
</tr>
</tbody>
</table>

Lin- cells were further purified by flow cytometry using directly conjugated c-kit monoclonal antibodies as described in Materials and Methods. Cells were plated and scored in single-cell assays as described in Materials and Methods. Cultures were supplemented with growth factors as described in Table 1. The results are representative of three separate experiments.

* Statistical significance by paired Student's t-test with a P value < .05 when comparing growth factor-induced single cell proliferation in the presence or absence of LIF.
The initial studies to examine the effects of LIF on hematopoietic progenitor cells in vitro examined unfractionated mouse BM and showed that LIF alone or in combination with GM-CSF had no effect on progenitor cell growth, suggesting that the effects of LIF on hematopoiesis summarized above were indirect. In comparison, studies with human hematopoietic progenitors have shown that LIF can enhance or show no effects on growth factor-induced colony formation in vitro. 27,28 In agreement with the initial results on murine BMCs, we found that LIF has no effects on GM-CSF-induced or IL-3-induced colony formation of unfractionated BMC; however, in contrast, we found that it potently enhances M-CSF-induced colony formation. In this regard, autoradiographic analysis of hematopoietic cells from a variety of sources using 125I-LIF showed that LIF receptors were highly expressed on monocyte/macrophage lineage cells,29 and thus may account for the synergistic effects of LIF on macrophage colony formation in combination with M-CSF. Interestingly, comparing the effects of LIF on more purified Lin cells, we found that LIF also synergizes with IL-3, GM-CSF, M-CSF, and SLF to increase the number and size of colonies in soft agar and in single-cell assays (as well as increases the size of colonies stimulated by the combination of IL-3 plus SLF). These effects were probably not detected in the initial studies because they examined unfractionated BMC rather than Lin cells which represent roughly 1% to 5% of the marrow.

It has been previously shown that both LIF and OSM can equally effect ES cell growth and the differentiation of the myeloid cell line M1 which has been, in part, explained by receptor/ligand interactions. In particular, LIF but not OSM binds with low affinity to a unique LIF receptor, whereas OSM but not LIF binds with low affinity to gp130, and that gp130 and LIF-R form the high affinity compatible receptor complex for both OSM and LIF. However, we found that in contrast to LIF, OSM has no effect alone or in combination with other cytokines on normal hematopoietic cell growth in vitro. It is interesting to note that human OSM like LIF can induce the proliferation of murine DA-1a cells in a dose-dependent manner in vitro (data not shown). Furthermore, a 50- to 100-fold excess of OSM could inhibit LIF plus SLF or IL-6 plus SLF induced colony formation of Lin BMCs suggesting that human OSM can bind and effectively compete for the murine signal transducing gp130 receptor. In this regard, we would like to compare murine OSM with human OSM, because it is possible that while human OSM can bind and compete for the murine gp130 receptor, it may show a reduced affinity for a heterodimeric murine receptor complex. Taken together, the data indicate that while LIF receptor and gp130 are present on the colony forming cells, a receptor component or signal transducing component required for OSM-induced proliferation is absent, or OSM has yet an undefined effect other than a proliferative one on BMC. In this regard, OSM has been shown to bind to a separate receptor complex that requires gp130 but does not bind LIF. Although it is not possible to perform binding studies on pure populations of hematopoietic stem/progenitor cells, future studies using radiolabeled ligands looking at low- and high-affinity binding sites on unfractionated and partially purified Lin+ murine BMCs may help to answer this question.

We also show here that LIF can directly synergize with IL-3 or SLF to enhance the growth of Lin+ c-kit+ cells that are enriched for cells that have long-term reconstituting activity in lethally irradiated mice. Thus, the effects of LIF on hematopoietic cell growth are direct and are maximally observed using purified hematopoietic progenitors. It is interesting to note that LIF receptors were detected on an uncharacterized subpopulation of lymphocyte-like cells in normal BM cell populations by autoradiography but not on the majority of lymphocytes. These LIF receptor positive cells could have been progenitor cells because it is difficult to distinguish between these cell types by morphology alone. Thus, our results indicate that the enhancement of hematopoiesis in mice administered exogenous LIF, and the reduced numbers of progenitors detected in the colony-forming unit-spleen (CFU-s) assay in LIF-deficient mice are at least, in part, directly mediated by LIF.

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