Leukemia Inhibitory Factor Differentiation-Inhibiting Activity/Human Interleukin for DA Cells Augments Proliferation of Human Hematopoietic Stem Cells

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Leukemia inhibitory factor (LIF)/differentiation-inhibiting activity (DIA)/human interleukin for DA cells (HILDA) is a cytokine with biologic activities involving a variety of different types of target cells. Here we have tested LIF/DIA for possible effects on the growth and differentiation of normal human hematopoietic cells in culture. As a single agent, LIF/DIA had no effect on colony formation by CD34-positive human bone marrow cells. However, LIF/DIA was as effective as either interleukin-6 (IL-6) or granulocyte colony-stimulating factor (G-CSF) in the enhancement of IL-3-dependent colony formation of very primitive blast colony-forming cells. Studies using neutralizing antibodies against IL-6 or G-CSF demonstrated that this was not due to induction in culture of either of the other known synergistic factors for blast cell colony formation. A 1-day delay in the time course of appearance of blast cell colonies grown in the presence of LIF/DIA relative to those grown in the presence of IL-6 suggests that the different synergistic factors may operate through different mechanisms, although we cannot rule out that high doses of LIF/DIA might yield accelerated blast cell colony formation. Our findings provide evidence that LIF/DIA may play an important role, along with IL-6 and G-CSF, in the regulation of early hematopoietic stem cells.

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MATERIALS AND METHODS

Hematopoietic growth factors and antibodies. Two types of LIF/DIA material were used in the experiments. Conditioned medium (CM) containing recombinant human LIF/DIA was produced after transfection of COS cells with the cDNA clone pCl0-6R in the expression vector pX. Recombinant human GM-CSF (GM-CSF) and macrophage-CSF (M-CSF) were supplied by the Genetics Institute Process Development Laboratory. The IL-3 preparation had a specific activity of 2 x 10⁷ U/mg. Purified recombinant human GM-CSF had a specific activity of 8 x 10⁶ U/mg protein. Recombinant human IL-4, IL-5, IL-6, and M-CSF were isolated from COS cells that had been transfected with cDNA encoding the glycoproteins. Purified human urinary erythropoietin was provided by Dr Takaji Miyake, University of California (Santa Barbara, CA). Anti-IL-6 was a polyclonal IgG rabbit antibody, and anti-G-CSF was an IgG monoclonal antibody provided by Dr Lawrence Souza, AmGen (Thousand Oaks, CA). The neutralizing activities of these antibodies have been described previously.

Cell preparation. Bone marrow cells were obtained from healthy, adult volunteers who gave informed consent after they had been advised of the risks. These studies were approved by the Institutional Review Board for Human Research, Medical University of South Carolina and the Veteran's Administration Medical Center. Mononuclear cells were isolated using Ficoll-Paque (Pharmacia, Inc, Piscataway, NJ) and the nonadherent fraction was obtained after overnight adherence to plastic dishes. Samples were further enriched by positive, indirect immune adherence using anti-CD34 antibody (Human Progenitor Cell Antibody; Becton-Dickinson, Mountain View, CA) as described previously.

Clonal cell culture. CD34-positive marrow cells were cultured in 35 mm Lux suspension culture dishes (Miles Laboratories, Inc, Naperville, IL) using a modification of the technique described by Iscove et al. Briefly, 1 mL of culture contained 2,000 CD34-positive colony-stimulating factor (G-CSF). Thus, LIF/DIA appears to be the third synergistic factor to be documented for augmentation of IL-3-dependent proliferation of early hematopoietic progenitors.
bone marrow cells, α-medium (Flow Laboratories, Inc, Rockville, MD), 1.2% of 1,500 centipoise methylcellulose (Fisher Scientific Co, Norcross, GA), 30% fetal calf serum (Flow Laboratories, Inc), 1% deionized bovine serum albumin (Sigma Chemical Co, St Louis, MO), 5 x 10⁻³ mol/L mercaptoethanol, 2 U/mL of purified human urinary erythropoietin, and 1:100 dilution of LIF/DIA CM, Mock CM, or 100 U/mL IL-3. Dishes were incubated for 14 days at 37°C in a humidified atmosphere of 5% CO₂ in air. The types and sizes of the colonies were estimated in situ as described previously.¹¹

Blast cell colony formation. We analyzed blast cell colony formation using a slight modification of the assay that we recently developed.¹² Briefly, 1 × 10⁴ CD34-positive bone marrow cells were plated in methylcellulose culture containing 2% fetal calf serum, 1% crystallized, deionized bovine serum albumin, 5 × 10⁻³ mol/L mercaptoethanol, 600 µg/mL fully-iron saturated human transferrin (98% pure, Sigma Chemical Co), 10 µg/mL soybean lecithin (Sigma Chemical Co), 6 µg/mL cholesterol (Sigma Chemical Co), and 10⁻³ mol/L sodium selenite (Sigma Chemical Co). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 5% O₂. On day 14 of incubation, 100 U of IL-3 or 100 U with or without synergistic factors were layered over the culture. All blast cell colonies were replated as previously described¹² to confirm their ability to form secondary colonies. Only blast cell colonies with secondary replating capabilities were reported.

RESULTS

Colony formation by CD34⁺ human marrow cells. We first tested the effects of COS cell-CM containing LIF/DIA on colony formation from CD34-positive human marrow cells. A 1:100 dilution of LIF/DIA COS cell-CM was added on day 0 of culture containing 2,000 CD34-positive human marrow cells, and colonies were counted on day 14 on an inverted microscope. The results are presented in Table 1. Colony formation supported by LIF/DIA was similar to that supported by the same dilution of Mock CM. In contrast, IL-3 supported a larger number of colonies, including several erythrocyte-containing mixed colonies. These results indicated that, as a single agent, LIF/DIA does not support proliferation of hematopoietic progenitors. Metcalf has shown similar results in cultures of murine bone marrow cells.¹³

Effects of LIF/DIA on blast cell colony formation. When LIF/DIA COS cell CM was added to culture on day 14 of incubation, it alone did not support blast cell colony formation. However, LIF/DIA revealed a significant enhancing effect on IL-3-dependent blast cell colony formation. Results of two separate experiments are presented in Fig 1. In both experiments, the degree of enhancement of IL-3-dependent blast cell colony formation by LIF/DIA was similar to the enhancement by IL-6 that we reported previously.¹⁴ In addition, there was no difference in the types of secondary colonies between the two groups. A combination of LIF/DIA and IL-6 did not enhance blast cell colony formation over that by IL-6 or LIF/DIA alone, indicating that the targets of these factors overlap significantly. However, the time courses of blast cell colony development supported by LIF/DIA and IL-3 appeared to be one day later than the blast cell colony development supported by IL-6 and IL-3. Purified LIF/DIA also enhanced IL-3-dependent blast cell colony formation. As shown in Table 2, its activity was indistinguishable from that of COS cell-CM containing LIF-DIA.

Blast cell colony enhancement in the presence of antibodies. Earlier we reported that IL-6 and G-CSF can enhance IL-3-dependent proliferation of early hematopoietic progenitors and that part of the synergistic mechanism is to shorten the Go period of the dormant stem cells.¹⁵,¹⁶ In order to test whether or not the synterstic effect of LIF/DIA on human blast cell colony formation is direct or mediated indirectly by IL-6 and/or G-CSF, we studied blast cell colony formation in the presence of neutralizing antibodies against these two factors. We have shown previously that these antibodies can block the synergistic effects of IL-6 and G-CSF on human blast cell colony formation.³ Twenty-five micrograms of anti-IL-6 neutralized that synergistic activity of 20 U of IL-6. Similarly, 25 µg of anti-G-CSF neutralized 100 U of G-CSF. We plated 1 × 10⁴ CD34-positive marrow cells per dish in the presence of 2% fetal calf serum and serum-free culture supplements, added growth factors, and antibodies on day 14 of incubation and observed blast cell colony development. The cumulative incidences of blast cell colonies presented in Fig 2 clearly documented that the synergistic effect of LIF/DIA is not mediated by IL-6 or G-CSF. Again, the time course of blast cell colony development supported by the combination of IL-3 and LIF/DIA was delayed by 1 day relative to the development supported by the combination of IL-6 and IL-3.

Examination of other lymphohematopoietic factors for synergistic activity. Since LIF/DIA is the third factor to be shown to augment IL-3-dependent human blast cell colony formation in culture, we wished to test the other known human lymphohematopoietic factors for possible synergistic activities. We have documented that IL-1α and IL-1β do not augment IL-3-dependent proliferation of human hematopoietic progenitors.¹⁴ Therefore, we tested IL-2 (100 U/mL), IL-4 (100 ng/mL), IL-5 (1:1,000), erythropoietin (Ep) (5 U/mL), M-CSF (1:1,000), and GM-CSF (100 U/mL). None of these factors augmented IL-3-dependent blast cell colony formation except GM-CSF. The numbers of blast cell colonies for experimental versus control groups of each factor were: IL-2, 17/13; IL-4, 12/16; IL-5, 18/15; Ep, 13/14; and M-CSF, 15/15. In the presence of GM-CSF and IL-3, there were six more blast cell colonies than in cultures containing IL-3 alone. However, while all the blast cell colonies revealed secondary colony formation, six of the blast cell colonies produced only secondary colonies restricted to the GM lineages. We reported earlier that
human GM-CSF supports formation of a small number of blast cell colonies that are committed to GM lineages. Therefore, we interpreted these results as indicative of additive effects of GM-CSF.

**DISCUSSION**

We presented evidence earlier that IL-6 and G-CSF enhance IL-3-dependent formation of human\textsuperscript{14} and murine\textsuperscript{15,16} blast cell colonies, and part of the synergistic effects of these factors is to shorten the G\textsubscript{0} period of dormant stem cells.\textsuperscript{15,16} In this article, we have demonstrated that a newly identified factor, LIF/DIA, also augments human blast cell colony formation that is supported by IL-3. The maximal cumulative levels of blast cell colonies supported by the combination of IL-3 and LIF/DIA were similar to those supported by the combination of IL-3 and IL-6. Synergistic effects of LIF/DIA could be demonstrated even in the presence of neutralizing antibodies prepared against IL-6 and G-CSF, indicating that the LIF/DIA effects on early progenitors are not mediated by these factors. The time course of blast cell colony development in the presence of IL-3 and LIF/DIA was identical to the control experiments.

**Table 2. Enhancement of Blast Cell Colony Formation by Purified LIF/DIA and LIF/DIA CM**

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Incidence of Blast Cell Colonies\dagger</th>
<th>Range of Replating (%)</th>
<th>Types of Secondary Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>8</td>
<td>8-53</td>
<td>GM, E, Eo/Baso, E-Mix</td>
</tr>
<tr>
<td>IL-3 + pure LIF/DIA*</td>
<td>14</td>
<td>8-50</td>
<td>GM, E, Eo/Baso, E-Mix</td>
</tr>
<tr>
<td>IL-3 + LIF/DIA CM</td>
<td>15</td>
<td>13-47</td>
<td>Gm, E, Eo/Baso, E-Mix</td>
</tr>
</tbody>
</table>

Abbreviations for colonies are listed in the legend for Table 1.  
*Purified LIF/DIA was used at a final concentration of 20 ng/mL.  
\dagger Number of blast cell colonies detected in six dishes.
EFFECTS OF LIF/DNA ON HUMAN STEM CELLS

Fig 2. The ability of LIF/DIA to enhance IL-3-dependent blast cell colony formation in the presence of neutralizing antibodies prepared against IL-6 and G-CSF. Antibodies were added to culture on day 14 of incubation together with IL-3 with or without LIF/DIA. The concentrations of anti-IL-6 (25 pg) and anti-G-CSF (25 pg) have been shown to neutralize 20 U of IL-6 and 100 U of G-CSF, respectively. The result presented in this figure clearly demonstrated that the synergistic effect of LIF/DIA is not mediated by IL-6 or G-CSF.

in which only IL-3 was added, and was 1 day later than that of the colony formation supported by a combination of IL-3 and IL-6. We are not certain of the significance of this difference in the time course, although it was notable in all three experiments (Figs 1 and 2). The human blast cell colony assay is dependent on the delayed addition of IL-3 on day 14 of incubation, and therefore, the blast cell colonies identified in this system may be restricted to only a later-appearing subpopulation of blast cell colonies. Therefore, the 1-day difference in the time course of blast cell colony development may suggest differences in the mechanisms of these synergistic factors. It is possible, however, that the late effect of LIF/DIA is due to suboptimal conditions of this protein. Due to a limited supply of purified LIF/DIA, we were unable to carry out dose-response studies with LIF/DIA.

LIF/DIA is the third factor to be identified to augment IL-3-dependent proliferation of early human hematopoietic progenitors. It is of interest that all synergistic factors, IL-6, G-CSF and LIF/DIA, were originally identified as differentiation-inducing factors for murine M1 cells. Similar to our earlier studies on murine synergistic factors,16 no other known human lymphohematopoietic factors possessed the ability to augment IL-3-dependent formation of human blast cell colonies. LIF/DIA is active on early embryo stem cells, where it is required for stem cell renewal and the maintenance of pluripotency. Two interesting issues arise when this is considered with the effects we report here on hematopoietic stem cells. First, is LIF/DIA active in other stem cell systems; for example, the skin and gut? Second, to what extent are the same factors and mechanisms used to control both hematopoietic and embryonic stem cell proliferation and differentiation?

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