Growth of Clonogenic Myeloblastic Leukemic Cells in the Presence of Human Recombinant Erythropoietin in Addition to Various Human Recombinant Hematopoietic Growth Factors

By Yoshinobu Asano, Seiichi Okamura, Tsunefumi Shibuya, Mine Harada, and Yoshiyuki Niho

The effects of human recombinant erythropoietin (rEpo) in the presence of other stimulators on the growth of clonogenic leukemic blast cells from ten Japanese patients with acute myeloblastic leukemia were studied with an in vitro leukemic blast colony assay in methylcellulose culture. With the addition of rEpo alone, no leukemic blast colony formation was stimulated in any of the cases examined. However, when rEpo and phytohemagglutinin lymphocyte-conditioned medium (PHA-LCM) were added to the culture simultaneously, in contrast to results with PHA-LCM alone, the number of leukemic blast colonies formed was significantly increased in two of the ten cases (P < .01). These two cases were classified as M1 according to the French-American-British (FAB) classification. This enhancing effect of rEpo was observed with human recombinant granulocyte/macrophage colony-stimulating factor (rGM-CSF) or human recombinant interleukin-3 (rIL-3) but was not observed with human recombinant granulocyte CF (rG-CSF).

MATERIALS AND METHODS

AML blast cells. Leukemic blast cells from ten Japanese patients with AML were studied. Leukemic types were diagnosed according to the French-American-British (FAB) classification of acute leukemia (Table 1). Mononuclear cells from the peripheral blood were obtained by density-gradient centrifugation, and T lymphocytes were removed by E rosetting. The remaining cells were used for leukemic blast colony assay, which was a modification of the technique of Buick et al., as previously described, and immunofluorescence assay.

Semisolid culture procedures. Blast cells were plated in plastic 96-microwell plates in alpha medium (GIBCO, Grand Island, NY) containing 0.88% methylcellulose and 10% fetal calf serum (FCS). Each well, containing 2 x 10^4 cells in 0.1 mL medium with appropriate stimulators, was incubated for six days in a moist atmosphere of 5% CO2 in air at 37°C, and compact colonies were counted using an inverted microscope. The colonies broke up and became diffuse after prolonged incubation. Individual colonies were picked out, and the cells were placed directly on slides. After being air-dried and fixed in absolute methanol, they were stained with Giemsa solution. The cells within the colonies were similar in morphology to the patients' leukemic blast cells.

Phytohemagglutinin–leukocyte-conditioned medium (PHA-LCM). Mononuclear cells from the peripheral blood of healthy human volunteers were obtained by density-gradient centrifugation. After being washed with phosphate-buffered saline (PBS), the cells were resuspended in RPMI 1640 medium (GIBCO) supplemented with 1% PHA (HA15, Wellcome Diagnostics, Dartford, England) and 10% FCS. Ten-milliliter aliquots of cell suspension at 2 x 10^6 cells/mL were each seeded into a tissue culture dish 9 cm in diameter and incubated in a moist atmosphere of 5% CO2 in air at 37°C. Culture supernatants were harvested on day 3 and stored until use (PHA-LCM). PHA-LCM was added at an optimum concentration of 10% to the semisolid culture when indicated.

Hematopoietic factors. The human rEpo (101 U/mg protein) was a gift from Snow Brand Milk Products (Tokyo). It was purified from the supernatant of a mammalian cell line, BHK-21, and transfected with a plasmid containing a cloned human genomic Epo gene.

Human rGM-CSF was provided by Dr K. Arai (DNAX Research Institute, Palo Alto, CA). Activities of CSFs were measured using the method of Nicola et al.

Human rG-CSF (2.5 to 10 x 10^4 U/mL) was a gift from Chugai Pharmaceutical (Tokyo). It was purified from the supernatant of Chinese hamster ovary cells containing a plasmid expression vector that incorporates the human G-CSF gene.

Human rIL-3 was purchased from Genzyme (Boston). It was produced in yeast containing a plasmid expression vector that incorporates the human IL-3 gene and contained 10^6 U/mg protein according to the manufacturer.

Sufficient blast colony formation was observed following addition of 1,000 U/mL GM-CSF, 10 ng/mL G-CSF, or 500 U/mL IL-3. Our experiments were performed with these concentrations.

Immunofluorescence assay. Antibody reactivity with the leukemic blast cells was determined by indirect immunofluorescence. Aliquots of 2 x 10^6 blast cells were incubated for 30 minutes at 4°C with 10 µL antilymphoblast A (GPA) monoclonal antibody (Cosmo Bio, Tokyo). After being washed, the cells were further incubated with fluorescein-conjugated goat anti-mouse IgG (Becton Dickin-
RESULTS

We examined the effects of rEpo on the growth of clonogenic leukemic blast cells from ten AML patients with an in vitro leukemic blast colony assay. GPA on the surface of blast cells from nine patients was also examined. In no case were blast cells significantly stained with anti-GPA antibody.

The numbers of blast colonies formed with the addition of each stimulator are shown in Table 2. With the addition of 1 U/mL rEpo alone, no leukemic blast colonies were formed in any case except in cases 4 and 6, in which spontaneous colonies were formed without addition of stimulator and colony growth was not further stimulated by the addition of rEpo. On the other hand, with addition of 10% PHA-LCM in all cases examined, many colonies were observed, although marked variation in responsiveness of clonogenic cells to PHA-LCM was also observed. Spontaneous colony growth was further stimulated by addition of PHA-LCM in cases 4 and 6.

Table 2. Effect of rEpo Addition With PHA-LCM on Leukemic Blast Colony Formation

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis (FAB)*</th>
<th>Leukocytes (×10⁶/L)</th>
<th>Blasts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>M</td>
<td>M1</td>
<td>43,800 ± 90</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>F</td>
<td>M1</td>
<td>12,900 ± 90</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>F</td>
<td>M2</td>
<td>9,400 ± 67</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>M</td>
<td>M4</td>
<td>125,500 ± 97</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>M</td>
<td>M4</td>
<td>31,500 ± 83</td>
<td>83</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>F</td>
<td>M2</td>
<td>24,100 ± 94</td>
<td>94</td>
</tr>
<tr>
<td>7</td>
<td>78</td>
<td>M</td>
<td>M2</td>
<td>20,800 ± 85</td>
<td>85</td>
</tr>
<tr>
<td>8</td>
<td>49</td>
<td>M</td>
<td>M2</td>
<td>16,000 ± 83</td>
<td>83</td>
</tr>
<tr>
<td>9</td>
<td>61</td>
<td>F</td>
<td>M3</td>
<td>6,800 ± 59</td>
<td>59</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>F</td>
<td>M5</td>
<td>130,000 ± 98</td>
<td>98</td>
</tr>
</tbody>
</table>

*FAB classification was determined as previously described.²

Numbers of leukemic colonies are shown as means ± SD/10⁶ cells as determined from triplicate plates.

Fig 1. Dose–response relationships between concentration of added rEpo with 10% PHA-LCM and enhanced effects on blast colony formation. Values are normalized to the stimulatory effect added. The most remarkable enhancing effect was observed between 1 and 2 U/mL rEpo; therefore, all further experiments in this study were performed with 1 U/mL rEpo added.

Next, we examined the types of recombinant hematopoietic growth factors that were able to produce the phenomenon described above. The numbers of blast colonies formed with rEpo added along with other recombinant hematopoietic growth factors are shown in Table 3. A substantial increase in the number of colonies was produced by addition of rEpo with rGM-CSF in five cases (cases 1 through 5) and with rIL-3 in three cases (cases 1, 3, and 4), whereas the stimulatory effect of rG-CSF was not enhanced by addition of rEpo in any case.
Table 3. Effect of rEpo Addition With Human Recombinant Hematopoietic Growth Factors on Leukemic Blast Colony Formation

<table>
<thead>
<tr>
<th>Case No.</th>
<th>rGM-CSF (1,000 U/mL)</th>
<th>With rEpo (1 U/mL)</th>
<th>rIL-3 (500 U/mL)</th>
<th>With rEpo (1 U/mL)</th>
<th>rG-CSF (100 ng/mL)</th>
<th>With rEpo (1 U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>600 ± 39</td>
<td>767 ± 47*</td>
<td>655 ± 15</td>
<td>755 ± 39†</td>
<td>280 ± 51</td>
<td>322 ± 65</td>
</tr>
<tr>
<td>2</td>
<td>992 ± 98</td>
<td>1,482 ± 49§</td>
<td>972 ± 57</td>
<td>988 ± 50</td>
<td>512 ± 56</td>
<td>547 ± 81</td>
</tr>
<tr>
<td>3</td>
<td>508 ± 56</td>
<td>650 ± 36‡</td>
<td>1,032 ± 49</td>
<td>1,342 ± 93*</td>
<td>32 ± 6</td>
<td>43 ± 12</td>
</tr>
<tr>
<td>4</td>
<td>299 ± 15</td>
<td>339 ± 9†</td>
<td>327 ± 12</td>
<td>369 ± 16‡</td>
<td>102 ± 11</td>
<td>109 ± 10</td>
</tr>
<tr>
<td>5</td>
<td>45 ± 11</td>
<td>77 ± 6†</td>
<td>122 ± 12</td>
<td>135 ± 29</td>
<td>3 ± 2</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>6</td>
<td>682 ± 27</td>
<td>650 ± 37</td>
<td>662 ± 50</td>
<td>643 ± 63</td>
<td>362 ± 39</td>
<td>355 ± 29</td>
</tr>
<tr>
<td>7</td>
<td>30 ± 4</td>
<td>33 ± 2</td>
<td>52 ± 6</td>
<td>45 ± 8</td>
<td>33 ± 5</td>
<td>38 ± 8</td>
</tr>
<tr>
<td>8</td>
<td>272 ± 22</td>
<td>317 ± 24</td>
<td>398 ± 29</td>
<td>452 ± 41</td>
<td>622 ± 39</td>
<td>672 ± 29</td>
</tr>
<tr>
<td>9</td>
<td>147 ± 17</td>
<td>138 ± 10</td>
<td>204 ± 14</td>
<td>197 ± 9</td>
<td>38 ± 9</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>10</td>
<td>115 ± 8</td>
<td>128 ± 6</td>
<td>92 ± 8</td>
<td>102 ± 6</td>
<td>27 ± 6</td>
<td>32 ± 5</td>
</tr>
</tbody>
</table>

Numbers of leukemic colonies are shown as means ± SD/10⁶ cells as determined from triplicate plates.

*Significantly different by unpaired t test.
†P < .02.
‡P < .05.
§P < .01.

Dose–response curves of leukemic blast colonies formed by the addition of rGM-CSF with and without rEpo in two cases are shown in Fig 2. In both cases, the stimulatory effects on colony formation reached a plateau level at a rGM-CSF concentration of 100 U/mL, irrespective of the presence of rEpo. In addition, the number of leukemic blast colonies stimulated by rEpo plus rGM-CSF was greater than that stimulated by rGM-CSF alone at each concentration of rGM-CSF, and the slopes of the dose–response curves did not differ very much, i.e., the doses of rGM-CSF for half-maximum stimulation were almost the same.

**DISCUSSION**

Leukemic blast CSA has been demonstrated in PHA-LCM1 or human bladder carcinoma cell line 5637-conditioned medium.18 With the recent development of genetic engineering, human recombinant hematopoietic growth factors have become available for the study of CSA. Human GM-CSF,2,3 G-CSF,4,5 and IL-367 are capable of stimulating CSA. Recently, rEpo was shown to stimulate proliferation of clonogenic erythroid leukemic blast cells from human erythroblastic leukemia patients,19 and some murine erythroleukemia cell lines have membrane receptors for Epo.20,21 Because the effects of rEpo on the growth of clonogenic myeloblastic leukemic cells have not been well documented, however, we examined the effects of rEpo on growth of clonogenic myeloblastic leukemic blast cells to determine whether any synergistic effects occurred with other recombinant hematopoietic growth factors.

We observed that rEpo alone was unable to stimulate myeloblastic leukemic blast colony growth in any of the cases examined. However, when rEpo and PHA-LCM were added to cultures simultaneously, the number of leukemic blast colonies formed was significantly increased in two of ten cases in comparison with PHA-LCM alone. Because PHA-LCM contains several kinds of CSFs,22 we examined which factors contributed to these rEpo-enhancing effects, using human recombinant CSFs; an enhancing effect of rEpo was observed with either rGM-CSF or rIL-3 added, but not with rG-CSF added.

Two possibilities for the mechanism of this rEpo-enhancing effect were considered. The first possibility was that rEpo directly or indirectly increased the sensitivity of clonogenic leukemic blast cells to rGM-CSF or rIL-3. The second was the presence of some clonogenic cell populations requiring rEpo and either rGM-CSF or rIL-3 simultaneously for growth. To clarify this, we examined the dose–response...
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curves of leukemic blast colonies formed by addition of rGM-CSF with and without rEpo, as shown in Fig 2. Colony formation was dependent on the GM-CSF, and the number of colonies formed with rEpo was consistently greater at every examined concentration of GM-CSF. The slopes of the dose–response curves did not differ greatly, and the doses of GM-CSF for half-maximum stimulation were nearly the same. From these findings, we deduced that a population exists requiring both GM-CSF and Epo for blast colony formation in addition to another population that can form colonies responding to GM-CSF alone. A similar phenomenon involving synergism between GM-CSF and G-CSF has been reported in some AML cases, and this was also attributed to the presence of clonogenic cell populations requiring both GM-CSF and G-CSF for growth.

In normal hematopoiesis, Epo acts as a growth factor for erythropoiesis. However, experiments in a mouse model showed that Epo is not required for erythroid burst or, probably, for multipotency colony formation, during the first few days of culture in the presence of IL-3, even though Epo is required for the final stages of differentiation. Our present results showed that rEpo alone was unable to stimulate the growth of clonogenic myeloblastic leukemic cells, suggesting that these cells have almost no receptors for Epo before culture. One possible interpretation is that the expression of Epo receptors occurred during the course of culture.

Recently, many instances of leukemic cells bearing mixed-lineage surface markers have been reported. The mechanism of mixed leukemia has been proposed to be aberrant gene expression in leukemic cells (lineage infidelity) or leukemic change occurring in multipotential progenitors (lineage promiscuity). In the same way, we offer two hypotheses as to why rEpo affected the growth of clonogenic leukemic blast cells in the presence of rGM-CSF or rIL-3: the first hypothesis is aberrant gene expression of the Epo receptor on leukemic blast cells in the presence of rGM-CSF or rIL-3, and the second alternative is leukemic change occurring in multipotential progenitors that have the potential to differentiate into either erythroid or myeloid cells. We ruled out the possibility of leukemia-containing erythroid conditioned by human bladder carcinoma cell line 5637. Int J Cell Cloning 5:504, 1987

REFERENCES


Growth of clonogenic myeloblastic leukemic cells in the presence of human recombinant erythropoietin in addition to various human recombinant hematopoietic growth factors

Y Asano, S Okamura, T Shibuya, M Harada and Y Niho