Leukemic Blast Cell Colony Formation in Semisolid Culture With Erythropoietin: A Case Report of Acute Poorly Differentiated Erythroid Leukemia

By Masao Tomonaga, Itsuro Jinnai, Masuko Tagawa, Tatsuhiko Amenomori, Kenji Nishino, Eichi Yao, Hiroaki Nonaka, Kazutaka Kuriyama, Yoshiharu Yoshida, Tatsuki Matsuo, Michito Ichimaru, and Tadashi Suematsu

The bone marrow of a patient with acute undifferentiated leukemia developed unique colonies after a 14-day culture in erythropoietin (EPO)-containing methylcellulose. The colonies consisted of 20 to 200 nonhemoglobinized large blast cells. Cytogenetic analysis of single colonies revealed hypotetraploid karyotypes with several marker chromosomes that were identical to those found in directly sampled bone marrow. The concurrently formed erythroid bursts showed only normal karyotypes. No leukemic colony formation was observed in other culture systems with either colony-stimulating activity (CSA) or phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM). The leukemic colonies exhibited a complete EPO-dose dependency similar to that of the patient's normal BFU-E. Although cytochemical and immunologic marker studies of the bone marrow cells failed to clarify the cell lineage of the leukemic cells with extraordinarily large cell size, ultrastructural study revealed erythroid differentiation such as siderosome formation and ferritin particles in the rhophyeocytosis invaginations. These findings indicate that the patient had poorly differentiated erythroid leukemia and that some of the clonogenic cells might respond to EPO in vitro. Corresponding to this biological feature, the leukemic cells were markedly decreased in number in response to repeated RBC transfusions, and partial remission was obtained. These observations suggest that erythroid leukemia distinct from erythroleukemia (M6) with a myeloblastic component, can develop as a minor entity of human acute leukemia.

MATERIALS AND METHODS

Case Report

The patient, a 50-year-old man, was admitted to a local hospital on September 10, 1983, because of a heel bone fracture. No abnormality was found in his peripheral blood, but pancytopenia developed rapidly during the next 2 months. He was transferred to our department on November 21, 1983. On admission, he was anemic and appeared acutely ill. No lymphadenopathy or splenomegaly was found. The liver was palpated 3 cm below the right costal margin. The hemoglobin (Hb) level was 5.8 g/dL. The WBC count was 3.6 x 10^9/L with 52% mature neutrophils, 40% lymphocytes, 2% eosinophils, and 6% monocytes. The platelet count was 41 x 10^9/L. Initial bone marrow aspirate was highly cellular, with 6.8% myeloblasts, 1.0% promyelocytes, 5.9% promyelocytes, 6.8% myelocytes, 7.4% metamyelocytes, 32.8% mature neutrophils, 1.6% pronormoblasts, and 2.8% normoblasts. Marked hypogranularity and cytoplasmic pyknosis were noted. The patient was given 3 U of packed RBC for the next 3 days and recovered.

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Fig 1. May-Grunwald Giemsa (MG) stain of the initial bone marrow (November 28, 1983) shows an increase (31.6%) of primitive blast cells with uniformly large cell sizes, a finely reticulated chromatin pattern, and several vacuoles. A few of them resemble normal pronormoblasts, exhibiting eccentric nucleus and deeply basophilic cytoplasm with perinuclear halo (original magnification ×400; current magnification ×264.). These abnormal blasts were negative for myeloperoxidase, specific and non-specific esterases, and hemoglobin stains. The bone marrow has well-preserved normal granulocytic elements, but those of the erythroid series are markedly reduced in number.

Cytological Analysis

Cytotoxic stains used were myeloperoxidase (MPO), dual-esterase stain for naphthol-ASD chloroacetate esterase and α-naphthyl butyrate esterase, acid phosphatase, β-glucuronidase, periodic acid-Schiff (PAS), and Hb. Ultrastructural study of bone marrow cells was performed by routine double staining with uranyl acetate and lead citrate and cytochemical staining for MPO.

For immunological marker analysis, several monoclonal antibodies such as J5 (common ALL antigen, CALLA, Coulter Immunology, Hialeah, FL); Leu 1 and HLA-DR (Becton Dickinson, Mountain View, CA), and OKM 1 (Ortho Pharmaceuticals, Raritan, NJ) were used. Terminal deoxynucleotidyl transferase (TdT) was detected by indirect immunofluorescence using anti-TdT antibody (Bethesda Research Labs, Gaithersburg, MD).

Colony Formation

CFU-E and BFU-E Assay. Bone marrow buffy coat cells were collected after centrifugation at 1,200 rpm for 10 minutes. Cells (2 x 10⁶) were plated in 1 mL of α-medium containing a final concentration of 0.88% methylcellulose, 30% fetal calf serum (FCS), 1% bovine serum albumin (BSA) and 1 U/mL of EPO (step III, Connaught, Canada) and cultured in a 35-mm Falcon plastic dish. For testing EPO-dose response by L-CFU, BFU-E, and CFU-E, EPO was added to final concentrations of 0, 0.1, 0.25, 0.5, and 1.0 U/mL, respectively. CFU-E and BFU-E were scored at day 7 and day 14, respectively.

CFU-GM Assay. Bone marrow light-density mononuclear cells (10⁷) were cultured in a 35-mm Falcon plastic dish containing 1 mL of α-medium with 0.88% methylcellulose, 20% FCS, 10% giant cell tumor-conditioned medium (GTC-CM) (GIBCO, Grand Island, NY). Cell aggregates consisting of >20 cells were scored as colonies on day 8.

L-CFU assay. T cell-depleted bone marrow mononuclear cells (2 x 10⁶) were plated in a 35-mm Falcon plastic dish containing 0.88% methylcellulose and 10% PHA-LCM. After 7-day incubation, cell aggregates consisting of >20 cells were scored as colonies. This method allowed the detection of L-CFU colonies in 32 (82.5%) of 40 AML cases.

Cytogenetic Analysis

Bone marrow cells were processed directly for chromosomal preparation, and cytogenetic analysis was performed using standard methods and/or the G-banding method.

Chromosome analyses of individual colonies were performed by our own method described elsewhere. In brief, individual colonies were aspirated and transferred into a droplet (10 μL) of prewarmed 0.075 mol/L of KCl solution placed on a poly-L-Lysine-coated slide. After thorough mixing, the slide remained for 35 minutes at 37°C to allow the cells to attach. The slide was fixed, first with 30% fixative (3:1 methanol-acetic acid diluted with 0.075 mol/L of KCl), second with 20% ethanol diluted with 0.075 mol/L of KCl, and third with 100% fixative. The slide was then flame-dried and stained with Giemsa and/or Q-banded. This method yielded analyzable metaphases even from small colonies containing <40 cells. All laboratory examinations and in vitro assays were performed with informed consent from the patient.

RESULTS

Cytological Features of the Leukemic Cells

The undifferentiated, extraordinarily large leukemic cells were MPO⁺, both types of esterase⁺, acid phosphatase⁺ (86%), β-glucuronidase⁺, PAS⁺, and Hb⁺. They were also ultrastructurally MPO⁺. Immunological marker study disclosed CALLA⁺, Leu 1⁺, TdT⁺, OKM 1⁺, and HLA-DR⁺ (12%). Transmission electron microscopic examination revealed distinct siderosome formation in the cytoplasm of <5% of the leukemic cells and fairly abundant ribophycoplastic invaginations and vesicles containing ferritin particles in half of the leukemic cells (Fig 2A, B, C).

Cytogenetic Findings in Bone Marrow Cells

Results of the serial cytogenetic studies are shown in Table 1. Abnormal hypotetraploid karyotypes were found on initial examination. Most of them had several identical marker chromosomes (Fig 2A). Repeated examinations revealed a considerable karyotypic instability (data not shown). Normal karyotypes were also present, but as a minor population. During the first partial remission, a clone with hypodiploid karyotypes was also observed. The relationship of the hypodiploid clone to the hypotetraploid clone could not be clearly explained. The hypotetraploid clone may represent the giant leukemic blast cells because they are known to have an excess amount of chromosomal materials.

Leukemic Blast Cell Colony Formation in EPO-Containing Culture

Erythroid colony assay of the bone marrow performed at the time of partial remission (January 6, 1984) disclosed bizarre colonies (Fig 3) consisting of 20 to 200 large nonhemoglobinized cells as well as well-hemoglobinized erythroid burst formation (Table 2). These two types of colonies reached maximum number on day 14 and did not form a single mixed colony. The cells from the bizarre colonies were uniformly large blast cells on MG-stained cytopsin-smears.
and resembled the leukemic cells in the bone marrow. They were negative for Hb, dual-esterase, and MPO stains. Cytogenetic analysis from individual day 14 colonies including normal-appearing erythroid bursts obtained from the relapsing bone marrow (February 20, 1984) was performed (Table 3). Karyotypes from the bizarre colonies revealed hypotetraploidy with several identical marker chromosomes, indicating that they belonged to a single abnormal clone (Fig 4B). Furthermore, these karyotypes carried marker chromosomes identical to those observed in the hypotetraploid karyotypes of the directly sampled bone marrow (Fig 4A). In contrast, karyotypes from erythroid bursts were all normal, showing 46, XY.

No leukemic colony formation was observed after cultur-
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Table 2. Serial Assays of Colony-Forming Cells

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<tr>
<td>CFU-GM</td>
<td>90 ± 6</td>
<td>15 ± 2</td>
<td>1 ± 1</td>
<td>448 ± 14</td>
<td>56 ± 8</td>
</tr>
<tr>
<td>CFU-E</td>
<td>—</td>
<td>120 ± 20</td>
<td>—</td>
<td>121 ± 37</td>
<td>59 ± 18</td>
</tr>
<tr>
<td>BFU-E</td>
<td>—</td>
<td>54 ± 10</td>
<td>16 ± 1</td>
<td>80 ± 12</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>L-CFU in EPO culture*</td>
<td>—</td>
<td>10 ± 3</td>
<td>15 ± 3</td>
<td>0</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>L-CFU in PHA-LCM culture†</td>
<td>—</td>
<td>—</td>
<td>0</td>
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EPO, erythropoietin; PHA-LCM, phytohemagglutinin-stimulated leukocyte-conditioned medium. L-CFU, leukemic colony-forming unit. Each colony number shows the mean ± SD of triplicate cultures.

Normal value for each colony-forming unit: CFU-GM 117 to 210 (mean 160) per 1 x 10^6 light-density bone marrow cells; CFU-E 145 to 230 (mean 182) per 2 x 10^6 bone marrow buffy coat cells; BFU-E 72 to 122 (mean 94) per 2 x 10^6 bone marrow buffy coat cells.

*Counted on day 14 of culture.
†Counted on day 7 of culture.

specimen obtained on April 24, 1984. The response curve of L-CFU was similar to those of CFU-E and BFU-E but appeared to be slightly higher at EPO concentration of 0.1 U/mL. A few leukemic colonies with <30 cells developed spontaneously without EPO.

DISCUSSION

The extraordinarily large leukemic blast cells of this patient lacked evidence of differentiation toward specific cell lineage on conventional cytotoxic and cell surface marker analyses. Ultrastructural investigation demonstrated, however, at least in some of the leukemic cells, distinct siderosome formation in the cytoplasm and ferritin particles in the rhopoeicotic invaginations on the cell surface membrane. These findings provide evidence of erythroid differentiation24,25 proceeding in the leukemic cell population. Recently, Reiffers and co-workers reported two similar cases of acute undifferentiated leukemia.26 Clinical and hematologic features common to these three cases are (a) relatively advanced age (>50 years), (b) poor response to conventional chemotherapy, (c) low WBC count with minimal or absent leukemic blasts in the peripheral blood, (d) extraordinarily large leukemic cells, (e) high ploidy and instability of karyotype, (f) ultrastructural evidence of erythroid differentiation and (g) lack of simultaneous proliferation of peroxidase-positive myeloid precursor cells. These characteristics suggest the possibility that this type of acute leukemia is a clinical entity distinct from erythroleukemia (M6) with a myeloblastic component,27 and may be a poorly differentiated type of acute erythremia.28-31

It is not yet clear whether the immunologic markers of erythroid differentiation such as glycoporphin A and spectrin can be demonstrated on these poorly differentiated leukemic cells. Detection of such markers using monoclonal or heterologous antibodies may reveal an erythroid nature of undifferentiated leukemia.32-34 These markers were also demonstrated, however, on AML blasts35 and K562 cells with simultaneous expression of myeloid antigen.36 Therefore, combined ultrastructural and immunological analyses of future cases of undifferentiated leukemia must be conducted to clarify the kind of markers that provide the earliest and consistent evidence of erythroid differentiation in leukemic cells.

The bizarre nonhemoglobinized colonies that developed concurrently with the patient’s normal erythroid bursts in EPO-containing culture apparently derived from the leukemic clone, as evidenced by the presence in single colonies of a common karyotypic abnormality identical to that of the leukemic clone in the bone marrow. The L-CFUs from our patient did not develop colonies in other culture systems with GCT-CM or PHA-LCM, indicating that the L-CFUs responded to EPO itself in the culture medium. There remains a minor possibility of response to another activity, however, such as burst-promoting activity (BPA) contaminating the partially purified EPO material. We found no report of leukemic blast cell colony formation in BFU-E assay or leukemic colonies expressing erythroid markers in other culture systems. Clonal culture studies in acute erythroleukemia (M6) are also few.37,38 Suda and co-workers examined colony formation in the bone marrow cells from four patients with erythroleukemia, using a plasma clot system containing EPO and Minden’s PHA-LCM system.37 They observed defective erythroid colony formation in all cases. They did not note blast cell colony formation in EPO-containing system, although the PHA-LCM system yielded blast cell colonies that evidenced granulocytic differentiation. Thus, their findings in erythroleukemia are distinct from our present observation.

The L-CFUs from our patient showed an almost complete dependency when EPO was added to the culture. The

Table 3. Cytogenetic Analysis from Single Colonies Growing in Erythroid Culture*

<table>
<thead>
<tr>
<th>Colony</th>
<th>No. of Colonies Aspirated</th>
<th>No. of Colonies with an Analyzable Metaphase†</th>
<th>No. (%) of Colonies With a Karyotype of 46, XY</th>
<th>Hypodiploidy</th>
<th>Hypotetraploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFU-E ‡</td>
<td>24</td>
<td>11</td>
<td>11 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-CFU ‡</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6 (100%)</td>
</tr>
</tbody>
</table>

*Culture for the bone marrow cells of February 20, 1984, when the leukemia began to relapse from partial remission.
†All metaphases were photographed and karyotyped.
‡Aspiration of each colony was performed on day 14 of culture. L-CFU: leukemic colony-forming unit.
Fig 5. Erythropoietin (EPO) dose–response curves. The dose–response curve of leukemic colony forming unit (●) is similar to those of normal BFU-Es (●) and CFU-Es (●), but the L-CFUs show a slightly higher sensitivity to EPO at a low concentration of 0.1 U/mL. A few small leukemic colonies developed without EPO. The colony numbers at EPO 1.0 U/mL were regarded as 100%.

Fig 4. (A) Representative G-banded karyotype of the metaphases obtained from directly sampled bone marrow (November 28, 1983) shows hypotetraploidy and several marker chromosomes. Most of the metaphases carry marker chromosomes, indicating that they belong to a single abnormal clone. (B) Q-banded karyotype of the metaphase obtained from a leukemic colony shows hypotetraploidy and several marker chromosomes. Markers 2, 3, 4, 7, and 9 are common between the metaphases, and markers 2, 4, and 9 are identical to markers 2, 4, and 6, respectively, of the karyotype of the direct bone marrow sample, providing evidence that the blast cell colonies derive from the leukemic clone.
ACKNOWLEDGMENT

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(HTB9-CM). Nothing is known at present about the kind of activity that in the various conditioned media stimulates L-CFUs and supports the cell lineage-specific differentiation of L-CFUs. Hoang and co-workers recently separated leukemic blast growth factor (LBGF) from BPA and possibly from GM-CSF by Sephadex G100 fractionation of HTB9-CM. Our observation of EPO dependent leukemic colony formation suggests that factor(s) requirement of L-CFU for


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