RAPID COMMUNICATION

Fluorouracil Selectively Spares Acute Myeloid Leukemia Cells With Long-Term Growth Abilities in Immunodeficient Mice and in Culture

By Wim Terpstra, Rob E. Ploemacher, Arie Prins, Kirsten van Lom, Karin Pouwels, Albertus W. Wognum, Gerard Wagemaker, Bob Löwenberg, and Jenne J. Wielenga

A subset of leukemic cells is assumed to maintain long-term growth of acute myeloid leukemia (AML) in vivo. Characterization of these AML progenitor cells may further define growth properties of human leukemia. In vitro incubations with 5-fluorouracil (5-FU) have been used for enrichment of normal primitive hematopoietic stem cells. By analogy to normal hematopoiesis, it was hypothesized that primitive leukemic stem cells might be kinetically more inactive than colony-forming cells (colony-forming units-AML [CFU-AML]). To examine this hypothesis, conditions were established for incubation with 5-FU that eliminated all CFU-AML. These conditions selected a 5-FU-resistant AML fraction that was evaluated for its capacity for long-term growth by transplantation into mice with severe combined immunodeficiency (SCID) and long-term culture in the quantitative cobblestone area-forming cell (CAFC) assay. Transplantation of the 5-FU-resistant fraction of four cases of AML into SCID mice resulted in growth of AML. Whereas no CFU-AML survived, 31% to 82% of primitive (week-6) CAFC were recovered from the 5-FU-treated cells. Hematopoietic cells proliferating in the CAFC assay were shown to be leukemic by cytologic, cytogenetic, or molecular analysis. The reduction of AML growth as determined by outgrowth of AML in SCID mice was in the same order of magnitude as the primitive (week-6) CAFC reduction. This indicates that both assays measure closely related cell populations and that the CAFC assay can be used to study long-term growth of AML. These results show a hierarchy of AML cells that includes 5-FU-resistant progenitors. These cells are characterized as primitive (week-6) CAFC and as leukemia-initiating cells in SCID mice.

IT IS CURRENTLY ASSUMED that a subset of leukemic cells maintains long-term growth of acute myeloid leukemia (AML) in vivo. Investigation of this subset may be useful for understanding the outgrowth and relapse of AML. This would require the isolation and functional characterization of the subpopulation of leukemic cells with the capacity to maintain AML.1

Isolation of precursors from normal bone marrow (BM) has required the combination of functional assays and purification strategies. One particular purification strategy is based on incubation with 5-fluorouracil (5-FU). 5-FU is toxic to cells in S-phase and this antimitabolite interferes with mRNA splicing in metabolically active cells.2 5-FU resistance is a property of murine hematopoietic cells with long-term in vivo repopulating abilities (LTRA) and long-term abilities in vitro, indicating that these cells are mainly in a kinetically quiescent state (G0-phase).3,4 Early human hematopoietic progenitors showed similar resistance to 5-FU.5,6 Prolonged incubation of unseparated human hematopoietic cells with 5-FU selects for quiescent pluriotent cells with an immature phenotype.7,8

For identification of AML progenitors, clonogenic assays in semisolid media have been used. This resulted in identification of the AML colony-forming unit (CFU-AML). Thymidine suicide studies showed that a high proportion of CFU-AML are synthesizing DNA.9 It is likely that CFU-AML may be eliminated by concentrations of 5-FU that allow for the survival of quiescent subsets of primitive leukemic cells, as is the case in normal hematopoiesis.

We sought to identify AML cells resistant to 5-FU. In analogy to normal BM, such AML cells may constitute a primitive subset of AML that is enriched for AML cells with long-term growth abilities. We selected conditions for incubation with 5-FU that eliminated all CFU-AML. The capacity of the 5-FU-resistant AML fraction to initiate leukemia in vivo was investigated by transplantation into mice with a severe combined immunodeficiency (SCID). In addition, the long-term in vitro abilities were investigated in the cobblestone area-forming cell assay (CAFC assay), a limiting-dilution type long-term culture system for hematopoietic progenitor cells.7,8,10,11

MATERIALS AND METHODS

AML cells. Peripheral blood (PB) samples were obtained after receiving informed consent from four patients presenting with AML diagnosed according to the criteria of the French-American-British Committee (FAB).12 AML cases were selected for their abilities to proliferate in SCID mice and the presence of a leukemia-specific marker. AML no. 1 had a normal karyogram. The leukemic growth characteristics in the CAFC assay and SCID mice of AML no. 1 have been described before.13 AML case no. 2 showed 47XX, +8, del(7)(q22-q36) as the cytogenetic markers. AML no. 3 had normal cytogenetics, but carried a point mutation in the granulocyte colony-stimulating factor receptor (G-CSF-R) gene.14 AML no. 4 had a translocation (9:22) (Table 1). Nucleated cells were isolated as auffy coat. The cells were cryopreserved in 10% dimethylsulfoxide, 20% heat-inactivated fetal calf serum (FCS), and Iscove's modified Dulbecco's medium (IMDM; GIBCO, Breda, The Netherlands). After thawing by stepwise dilution, the viability as assessed by trypan blue staining ranged from 72% to 92%.

Incubation of AML cells with 5-FU. 5-FU (Sigma, St Louis, MO) was dissolved in phosphate-buffered saline (PBS). AML cells

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(2 × 10^9/L) were incubated in 175 cm² polystyrene tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) in IMDM (GIBCO) and 10% FCS with 5-FU at 0, 15, 25, 40, and 80 µg/mL for 24 hours at 37°C in an atmosphere of humidified 5% CO₂. Equivalent proportions of the flasks based on input values were used in the various assays (CFU-AML, CAFC, and SCID mouse transplantations), without corrections for cell loss or viability to assess true variations in the recovery of specific subsets of cells.

Serum-free colony culture assay. Cells (the equivalent of 4 × 10⁶ input cells) were plated in 35-mm dishes (Becton Dickinson, San Jose, CA) in IMDM (GIBCO) containing 0.9% methylcellulose, 13% bovine serum albumin (BSA; Sigma), insulin (0.025 U/mL), linoleic acid (2.8 µg/mL), cholesterol (7.8 µg/mL), sodium selenite (0.18 ng/mL), P-mercaptoethanol (3.5 µL/mL), and human transferrin (0.62 mg/mL). Assays were performed in triplicate in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; Behringwerke AG, Marburg, Germany; 200 ng/mL) and interleukin-3 (IL-3; Sandoz, Basel, Switzerland; 5 ng/mL). Colonies were scored after 14 days of incubation at 37°C in a humidified atmosphere.

### Table 1. Characteristics of the Analyzed Cases of AML

<table>
<thead>
<tr>
<th>AML Case</th>
<th>FAB Classification</th>
<th>Leukemic Marker*</th>
<th>IL-3–Dependent in SCID Mice†</th>
<th>Immunophenotype (% of positive cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD34</td>
<td>CD38</td>
</tr>
<tr>
<td>1</td>
<td>M2</td>
<td>None</td>
<td>No</td>
<td>24%</td>
</tr>
<tr>
<td>2</td>
<td>M1</td>
<td>+.del(17q22-36)</td>
<td>No</td>
<td>30%</td>
</tr>
<tr>
<td>3</td>
<td>M1</td>
<td>R-G-CSF gene mutation</td>
<td>Yes</td>
<td>25%</td>
</tr>
<tr>
<td>4</td>
<td>M4</td>
<td>t(9;22)</td>
<td>Yes</td>
<td>40%</td>
</tr>
</tbody>
</table>

Abbreviations: FAB, French-American-British; R-G-CSF, G-CSF receptor.

* A leukemia-specific characteristic of the AML described.
† IL-3–dependent indicates that, for extensive AML proliferation in vivo, SCID mice have to be injected with human IL-3 (60 µg intraperitoneally, 5 days a week).
‡ +, >90% of the AML cells staining with a specific antibody as determined by flow cytometry and compared to a IgG 1-FITC or IgG 1PE control; −, <10% of the AML cells staining with a specific antibody as determined by flow cytometry and compared with a IgG 1-FITC or IgG 1PE control.

### Table 2. Growth Characteristics of 5-FU Exposed and Control AML Cell Populations in SCID Mice and in Vitro

<table>
<thead>
<tr>
<th>AML Case</th>
<th>AML Cell Population*</th>
<th>% AML Mouse BM ± SD† (no. of mice)</th>
<th>CFU-AML (10⁶)</th>
<th>Week 2 CAFC (10³ ± SEM)</th>
<th>Week 6 CAFC (10³ ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2461</td>
<td>10 ± 4</td>
<td>52 ± 6</td>
<td>39 ± 28</td>
</tr>
<tr>
<td>1</td>
<td>Untreated</td>
<td>91 ± 5 (5)</td>
<td>0</td>
<td>1.7 ± 0.5</td>
<td>32 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>Untreated</td>
<td>19 ± 18 (5)</td>
<td>4</td>
<td>1.9 ± 0.6</td>
<td>11 ± 3.4</td>
</tr>
<tr>
<td>3</td>
<td>Untreated</td>
<td>63 ± 17 (4)</td>
<td>71</td>
<td>6.2 ± 1</td>
<td>5.2 ± 1.46</td>
</tr>
<tr>
<td>4</td>
<td>Untreated</td>
<td>10 ± 13 (3)</td>
<td>271</td>
<td>38 ± 0.4</td>
<td>3.8 ± 0.6</td>
</tr>
</tbody>
</table>

 Abbreviations: BM, bone marrow; SD, standard deviation; SEM, standard error of the mean; CFU-AML, colony-forming unit AML; CAFC, cobblestone area-forming cell.

* AML cells were incubated for 24 hours in IMDM and 10% FCS, with or without 40 µg/mL of 5-FU.
† The percentage of human cells proliferating in the SCID mouse bone marrow was determined by flow cytometry.
‡ The percentage of 5-FU-resistant CFU-AML, week 2 CAFC, and primitive week 6 CAFC were derived from the quotient of the values of the 5-FU–exposed fraction and untreated AML cells.
§ Value below the detection limit of the assay.

**SCID mice and transplantation of AML.** Female specific pathogen-free CB17 scid/scid mice (5 to 8 weeks of age) were purchased (Harlan CPB, Austerlitz, The Netherlands) and housed under specific pathogen-free conditions in a laminar air flow unit. The mice were supplied with sterile food and acidified drinking water with 100 mg/L ciprofloxacin (Bayer AG, Leverkusen, Germany). The plasma Ig level of these mice was determined with an enzyme-linked immunosorbent assay (ELISA) using a sheep antimouse antibody reacting with mouse IgG and IgM (Boehringer Mannheim Biochemica, Penzberg, Germany), and animals with plasma Ig levels greater than 40 µg/mL were excluded. To enhance outgrowth of human hematopoietic cells in vivo, macrophage depletion was performed with the use of 0.2 mL of C1,MDP liposomes intravenously on the day before transplantation, as described.19,21 Total body irradiation (TBI) at 3.5 Gy (dose rate, 0.95 Gy/min) was delivered by a ³²P source (GammaCell, Atomic Energy of Canada, Ottawa, Ontario, Canada) adapted for the irradiation of mice. The graft size was the equivalent of 10 × 10⁶ AML input cells for AML no. 1, the equivalent of 20 × 10⁶ AML input cells for AML no. 3, and the equivalent of 30 × 10⁶ AML input cells for AML no. 4. The transplants, which were suspended in 300 µL Hank’s Balanced Salt Solution (HBSS;
GIBCO) and 0.1% BSA (Sigma), were injected intravenously (IV) into a lateral tail vein. The first two cases of AML had been shown to proliferate in SCID mice without support of human hematopoietic growth factors, and AML no. 3 and 4 were IL-3-dependent. The latter SCID mice received 60 μg of human IL-3 (Gist Brocades, Delft, The Netherlands) in 200 μl HBSS and 1% BSA (Sigma) intraperitoneally, 5 days a week as described.22

Tissue collections. SCID mice were killed by CO₂ inhalation followed by cervical dislocation in accordance with institutional animal research regulations. Cell suspensions were prepared from the BM of both femora and used for flow cytometry and the preparation of cytospin slides. Cytospin slides were stained with May-Grünwald-Giemsa and morphology was microscopically evaluated.

Flow cytometry. The initial leukemias and BM samples from mice that had been transplanted with leukemic grafts were stained or double-stained with the following mouse monoclonal antibodies: CD34-fluorescein isothiocyanate (FITC), CD34-phycoerythrin (PE), CD38-PE, CD34-FITC/CD38-PE, CD34-FITC/HLA-DR-PE, CD34-FITC/Cd34-PE, and finally CD45-FITC/CD33-PE. Mouse IgG₁-FITC and mouse IgG₁-PE-conjugated antibodies and samples from nontransplanted SCID mice were used as controls. The antibodies were obtained from Becton Dickinson. Fluorescence was measured using a FACSCAN flow cytometer and Lysys II software (Becton Dickinson Immunocytometry Systems). Erythrocytes and dead cells were excluded from analysis by gating on forward and orthogonal light scatter. To determine the percentage of human cells in the mouse, BM cells staining with at least two different antibodies for human hematopoietic cells were counted as human cells.23

Detection of G-CSF-R gene mutation. AML no. 3 is characterized by the presence of a point mutation in the G-CSF-R gene resulting in an aberrant G-CSF-R isofrom unique to this case of AML.17 Polymerase chain reaction (PCR) of the G-CSF-R gene was performed as described with the forward primer FW161 5':ACCTTTGTGTTCCACCAAGT-3' (in intron 16) and GR RV1 3':GGTATCGCTGACTTATCATG-5' (nucleotide 2745-2769) followed by digestion with Kpn I, which selectively degrades the PCR product of the mutated G-CSF-R gene.

CAFC assay. The CAFC assay was performed as described.5 Briefly, confluent stromal layers of FM2BD-1 cells in 96-well plates were overlaid with AML cells in a limiting dilution method. The cells were cultured in IMDM supplemented with 20% of a selected horse serum (HS) batch and hydrocortisone (isoene 21-hemisuccinate (10⁻⁸ mol/L final concentration). IL-3 (12.5 ng/mL) and G-CSF (20 ng/mL) were added weekly to the cultures. Input values were the equivalent of 50,000 nucleated cells (NC) per well. Twelve dilutions twofold apart were used for each sample, with 15 replicate wells per dilution. The percentages of wells showing at least one phase-dark hematopoietic clone of at least five cells (cobblestone area [CA]) beneath the stromal layer were determined at 2-week intervals, and CAFC frequencies were calculated using Poisson statistics as described.13 In normal hematopoiesis, stroma-dependent clones that are observed late in culture are indicators of primitive long-term repopulating stem cells in vivo, whereas early appearing clones are tentative indicators of transiently repopulating progenitors.7,8 At intervals, the content of some wells was harvested for additional investigation.

Results

Elimination of CFU-AML by exposure to 5-FU. To determine a concentration of 5-FU eliminating all CFU-AML, a dose titration ranging from 0 to 80 μg/mL of 5-FU was performed. Twenty-four hours of incubation at a concentration of 25 μg/mL of 5-FU reduced the number of AML-CFU to less than 2%, as compared with the colony numbers in the absence of 5-FU in all cases of AML tested (data not shown). A higher concentration of 40 μg/mL of 5-FU eliminated all CFU-AML (Table 2). AML cell viability varied from 50% to 80% in 5-FU-containing cultures, which compared with 65% to 80% viability for control incubations without 5-FU. This indicated that most cells sensitive to 5-FU had not been killed within 24 hours. In subsequent experiments (SCID mouse transplantations and CAFC assay), AML cells were incubated at 40 μg/mL of 5-FU to examine the 5-FU-resistant fraction.

Outgrowth of AML in SCID mice. The 5-FU-resistant fractions of AML cases no. 1 thru 4 were transplanted into SCID mice in individual experiments. Groups of 3 to 5 SCID mice were killed at 35 to 47 days after transplantation. Whereas the 5-FU-resistant fractions of AML did not contain measurable numbers of AML-CFU, in all instances these fractions had the capacity to initiate leukemia in SCID mice (Table 2). The levels of AML infiltration by the 5-FU-resistant fraction in the marrow of SCID recipients were less than those of control transplantations. By comparison of these values of infiltration and those in a cell dose titration of two cases of AML, the 5-FU fraction appeared to be equivalent to

<table>
<thead>
<tr>
<th>Cell Dose Titration of AML Cells Transplanted Into SCID Mice</th>
<th>% AML Cells Mouse BM</th>
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</thead>
<tbody>
<tr>
<td>Cell Dose (×10⁶ per mouse)</td>
<td>AML 1</td>
</tr>
<tr>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>3.3</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>2*</td>
</tr>
<tr>
<td>0.3</td>
<td>1*</td>
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</table>

AML cell proliferation in the bone marrow of SCID mice transplanted with graded doses of AML cells in one experiment. Each percentage represents one SCID mouse evaluated on day 35.

* Evaluated between days 44 and 81.
Fig 1. (A) Identification of trisomy 8 in cells proliferating in the BM of SCID mice that had been transplanted with AML no. 2. Cells with three signals represent leukemic cells. (B) Identification of trisomy 8 in cells proliferating in the CAFC assay. FISH with a probe specific for chromosome 8 on a cell proliferating in the CAFC assay of AML no. 2. The nucleus with three signals represents a leukemic cell in the CAFC assay.

an approximate threefold reduction of the unseparated graft (Table 3).

*Human AML cells proliferate in the SCID mouse BM.* Cells recovered from the SCID mouse BM were characterized as AML cells. Flow cytometry was performed with the same panel of monoclonal antibodies as was used for the initial immunophenotyping of the graft. It was shown that the immunophenotypes of cells recovered from the SCID mouse BM were identical to the AML immunophenotypes of the respective grafts, with the following exceptions: C1)34+/CD38- cells from AML case no. 1 had converted to a CD34-/CD38+ phenotype, as described earlier,14 and CD34+ cells from case no. 3 had converted to a CD34+ phenotype. Cytospin slides prepared from the BM of mice transplanted with AML no. 1 thru 4 showed evident blast cells. The morphologic identification of leukemic blasts was relatively easy due to the considerably larger size of AML blasts as compared with normal mouse BM cells. The percentages of AML blasts counted on the cytospin slides were similar to those determined by flow cytometry (data not shown). FISH performed on BM cells proliferating in SCID mice transplanted with AML no. 2 confirmed the trisomy 8 in the BM of all 3 mice examined. More than 90% of the cells staining with an anticentromere probe for chromosome 8 (human cells) showed 3 fluorescent dots (Fig 1).

*CAFC assay.* In parallel to the SCID mouse transplantations, long-term growth of the 5-FU-resistant fractions was investigated in the quantitative long-term BM culture system, ie, the CAFC assay. Frequency analysis of hematopoietic precursor cells in vitro showed in the number of week-2 CAFC in the 5-FU-exposed fraction a reduction towards 4% to 17% of the unexposed AML cells among the four AML specimens examined. In contrast, more primitive (week-6) CAFC were reduced to 31% to 82% only.

After 6 to 10 weeks of culture, cells were recovered from a series of wells used for the CAFC assay and cytospin preparations were made. Morphologic examination of the hematopoietic cells harvested from the CAFC assay of AML no. 1 showed 92% blast cells among the hematopoietic cells proliferating in the CAFC assay; more mature myeloid cells
were nearly absent. In a CAFC assay of normal peripheral blood stem cells, always less than 15% myeloblasts were counted at week 6, and all morphologic maturation stages of the neutrophil series were represented. FISH analysis of case no. 2 showed the trisomy 8 in 59% of the 52 evaluable cells staining with the anticentromere probe (Fig 1). Morphologically, 75% of all hematopoietic cells in the CAFC assay were myeloblasts. In AML no. 3 cells harvested from the CAFC assay, the presence of the specific mutant G-CSF-R gene was shown using PCR (Fig 2). Morphologic examination of the cells proliferating in the CAFC assay showed 83% myeloblasts. AML no. 4 cells were harvested from the CAFC assay; however, contamination with yeast cells interfered with further investigation.

DISCUSSION

We identified a subset of AML cells devoid of CFU-AML and resistant to 5-FU. This subset initiates AML in SCID mice and contains 31% to 82% of the AML cells with the ability to initiate long-term growth in the CAFC assay (Table 2). The 5-FU-sensitive fraction of normal hematopoietic cells produces a high number of colony-forming units (CFU-C) and week 2 CAFC in culture, whereas the 5-FU-resistant subpopulation contains the great majority of primitive (week-6) CAFCs and primitive normal hematopoietic cells.\textsuperscript{6,9,11} The cell population that we have identified here is enriched for immature leukemic precursors and shares these characteristics with its normal analogue. This cell population may be identical to or closely resemble the leukemic stem cell population. These observations suggest a hierarchy of AML cells similar to that in normal hematopoiesis: a hierarchy from kinetically quiescent immature progenitors identified as primitive (week-6) CAFC and the cells initiating leukemia in SCID mice towards kinetically active mature progenitors such as week-2 CAFC and CFU-AML.

The reduction of AML growth as determined by outgrowth of AML in SCID mice and by the number of week-6 leukemic CAFC was in the same order of magnitude. The hematopoietic cells proliferating in the CAFC assay could be shown to be leukemic by cytologic, cytogenetic, or molecular analysis, although the presence of some normal progenitors could not be fully excluded. These data indicate that both assays measure closely related cell populations and that the CAFC assay can be used for study of long-term growth of AML. The issue of whether the differences observed between the percentage of 5-FU-resistant week-6 CAFC and 5-FU-resistant leukemia-initiating cells in SCID mice are significant requires further investigation.

CFU-AML phenotypes display considerable heterogeneity among patients.\textsuperscript{26,27} Nevertheless, a generally more immature immunophenotype is expressed by CFU-AML as compared with the unseparated AML cell population, and differentiation of CFU-AML in vitro has been observed.\textsuperscript{26,29} CFU-AML recovered from methylcellulose cultures can be replated and produce secondary colonies.\textsuperscript{1} The capacities for (limited) differentiation and self-renewal are consistent with the role of the CFU-AML as a progenitor cell. The data presented here show that the CFU-AML is a relatively mature progenitor and is functionally distinguishable from most cells initiating long-term growth of AML. However, a definitive determination of any long-term potential of CFU-AML would require isolation of this subset.

Another approach towards the isolation of primitive leukemic progenitor cells is based on the immunophenotypical similarities between normal and leukemic progenitors. A fraction of normal BM enriched for repopulating stem cells has been identified as CD34\textsuperscript{+}, CD33\textsuperscript{-}, CD38\textsuperscript{-}, HLA-DR\textsuperscript{-}, Thy-1\textsubscript{low}, negative for specific lineage antigens, and low in rhodamine-123 uptake.\textsuperscript{31,36} In AML, a CD34\textsuperscript{+}/CD38\textsuperscript{-} fraction is present in approximately one third of the cases.\textsuperscript{37}
Cells initiating outgrowth of AML in immunocompromised mice were recovered exclusively from the CD34+/CD38- fraction in at least three cases of AML. These data may not apply to each and every case of AML, because we identified one case with identical abilities to initiate leukemia in SCID mice of both the CD34+ and the CD34- fractions. The heterogeneity of immunophenotype among cases of AML and the apparent lack of functional significance of expression of CD34 in AML may interfere with this approach to identify leukemic progenitors by immunophenotype.

Our data indicate that selection of AML subsets based on functional characteristics such as cytokinetic activity might offer a more generally applicable approach to the investigation of leukemic progenitors.

ACKNOWLEDGMENT

We thank E. van Bodegom for taking excellent care of the mice and T. Visser for technical assistance.

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