Growth of Human Myeloid Leukemias in the Human Marrow Environment of SCID-hu Mice

By Reiko Namikawa, Ryuzo Ueda, and Seishi Kyoizumi

It has been shown previously that multilineage human hematopoiesis is maintained within human fetal bone marrow (BM) fragments implanted into severe combined immunodeficient (SCID) mice. We describe here an application of this animal model, the SCID-hu mouse, to the study of human myeloid leukemias. BM cells from 8 patients with various types of myeloid leukemias were injected directly into human bone grafts in the SCID-hu mouse. Cells from 7 patients grew in the human marrow without spreading to the mouse marrow. Cells from 6 of these patients were successfully transferred in vivo to secondary SCID-hu recipients. The surface phenotype and the cytologic features of the leukemia cells were conserved during passage in vivo. Thus, human myeloid leukemia cells could be reproducibly propagated in the human marrow environment in SCID-hu mice. The differentiation of promyelocytic leukemia cells in the SCID-hu mice was induced by all-trans retinoic acid, suggesting that the biologic features of the leukemia cells were maintained as well. Finally, evidence for a leukemic progenitor cell population in one case of acute myelogenous leukemia was provided with this system. This model may provide a useful tool for studying the biology of human myeloid leukemia as well as for evaluating new therapeutic modalities for myeloid leukemias.

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Both acute and chronic myeloid leukemias are highly lethal hematologic malignancies characterized by abnormal proliferation and differentiation of hematopoietic progenitor cells. They are clonal diseases that are heterogeneous with respect to genetic changes involved in leukemogenic processes, surface phenotypes, and therapeutic response. Despite recent advances in the identification of molecular events involved in myeloid leukemia, little is known about the regulatory mechanisms of proliferation and differentiation of myeloid leukemia cells due to the limitations of experimental systems. Many studies have been performed in vitro with the established myeloid leukemia cell lines that may not be representative of their in vivo counterparts.

Previous attempts have been made to create animal models of human myeloid leukemias using the immunodeficient or immune-deprived mouse. However, growth of acute myelogenous leukemia (AML) cells in nude mice or irradiated mice was self-limited, and only two cases of AML were reported to be serially passaged in vivo. More recently, severe combined immunodeficient (SCID) mice have been shown to be better hosts for a variety of human malignancies, including leukemias. Acute lymphoblastic leukemias of both B-cell and T-cell origin have been shown to grow and to spread in the SCID mouse. It has also been reported that cells from some human myeloid leukemias can proliferate in the SCID mouse bone marrow (BM). A severe limitation to all of these models is the possibility that the human leukemia cells that grow in the mouse are variants different than those that grow in human hematopoietic microenvironments.

The SCID-hu mouse model with human hematopoietic function was developed by implanting human fetal bone fragments into SCID mice. In comparison to other animal models for studying human hematopoiesis, this model is quite unique because both human hematopoietic cells and the human hematopoietic microenvironment are engrafted in the mouse. Multilineage human hematopoietic activity was maintained in the grafts in the absence of exogenous growth factor treatment. The cellular composition in the implanted marrow is comparable with that of normal fetal marrow.

Evidence supporting the idea that the regulatory mechanisms of human hematopoiesis could functionally be maintained in the grafts has been gathered. Exogenously provided human hematopoietic growth factors induced the expansion of the expected cellular compartments. The effects of hematotoxic agents such as ionizing irradiation could be evaluated with this system.

In this report, we describe the SCID-hu model for studying human myeloid leukemia created by introducing patients' BM cells into human bone grafts in the SCID mouse. In contrast to previously published SCID models in which human leukemia cells proliferate in mouse organs, human myeloid leukemia cells were found to grow selectively in the human hematopoietic microenvironment in the SCID-hu mice. It seems likely, therefore, that leukemogenic events that occur in the patients' marrow can be reproduced more precisely in the SCID-hu model than in previous immunodeficient mouse models. A relevant in vivo animal model for the study of the biology of leukemia and for the evaluation of new therapeutic modalities can thus be...
ated. In the present study, the in vivo effect of a differentiating agent, all-trans retinoic acid (ATRA), on acute promyelocytic leukemia cells was examined. Furthermore, we used this animal model to identify a leukemic progenitor cell population in one case of AML.

**MATERIALS AND METHODS**

**Patient samples.** BM samples from myeloid leukemia patients were obtained with informed consent. The cells from AML cases were obtained at initial diagnosis and classified according to French-American-British (FAB) criteria as M1 (3 cases), M2 (1 case), M3 (2 cases), and M4 (1 case). A patient (patient no. 1) with chronic myelogenous leukemia (CML) was diagnosed to be in myeloid blast crisis (CML-BC) phase with a blast cell population of 30% in the BM sample. Mononuclear cells were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density sedimentation and were then cryopreserved in RPMI-1640 (GIBCO, Grand Island, NY) containing 10% dimethylsulfoxide (Merck, Darmstadt, Germany) and 10% fetal bovine serum (FBS). After thawing, cells were washed with RPMI-1640 containing 10% FBS and used for flow cytometric analysis and implantation.

**SCID-hu mice.** Homozygous C.B-17 scid/scid mice (SCID) were bred, treated with antibiotics as described, and used when 6 to 8 weeks old. The collection of fetal tissues and construction of SCID-hu mice with human fetal long bone implants have been previously described. In brief, the femurs and tibias of 19- to 23-gestational-week-old fetuses were cut into fragments (approximately 5 × 5 × 10 mm) and implanted subcutaneously into SCID mice. The human fetal tissues were obtained with informed consent from agencies in compliance with regulations issued by each state and the federal government. Cell suspensions prepared from thymus of individual fetal donors were analyzed for the HLA allotypes.

**Injection of leukemia cells.** After thawing, BM cells of leukemia patients (0.4 to 2.0 ×10⁶ viable cells) were resuspended in 20 μL of RPMI-1640 containing 10% FBS and injected into a microliter syringe (Hamilton Co, Reno, NV) directly into the human fetal bone grafts. In all cases, the bone grafts had been implanted subcutaneously 6 to 8 weeks before the injection of leukemia cells. Combinations of bone and leukemia donors were selected to be disparate for commonly distributed HLA allotypes so that the origin of the cells in human bone implant could later be traced.

In vivo passage of leukemia cells to secondary recipients was performed in a similar fashion. In brief, cell suspensions were prepared from bone injected with leukemia cells as described below. Cells (0.5 to 2.0 ×10⁶) were then injected into bone grafts of other SCID-hu mice with the appropriate HLA allotypes.

**Antibodies.** Mouse monoclonal antibodies (MoAbs) against major histocompatibility complex (MHC) class I antigens were directly conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). These included FITC-W6/32 (monomorphic HLA class I determinant), PE-MA2.1 (HLA-A, B7), PE-BB7.2 (HLA-A2), PE-BB7.1 (HLA-B7, Bw42), and PE-MB40.2 (HLA-B7, B40), FITC-anti-LeuM1 (CD15), PE-anti-LeuM9 (CD33), PE-anti-Leu12 (CD19), FITC-anti-CALLA (CD10), and FITC-anti-HLe1 (CD45) were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA).

**Flow cytometry.** Single-cell suspensions were prepared from human bone grafts by mincing tissues with scissors in cold RPMI-1640 containing 10% FBS. Cells were then treated with ammonium chloride to lyse red blood cells and stained for immunofluorescence as previously described. Cells from mouse peripheral blood and BM were examined as well. Before analysis, propidium iodide was added at a final concentration of 10 μg/mL to selectively gate out dead cells. Multiparameter flow cytometry was performed using the FACScan system (Becton Dickinson Immunocytometry Systems). The percentage of leukemia cells was calculated as the percentage of patient’s HLA allotype-positive cells per total human cells in the individual samples. In each experiment, isotype-matched antibodies were included as negative controls.

In the experiments designed to investigate the leukemic progenitor cell activity, leukemia cells from SCID-hu mice were stained with PE-CD33 and FITC-CD15 and sorted into CD33+CD15- and CD33+CD15+ populations by FACStar (Becton Dickinson Immunocytometry Systems). Cell suspensions were maintained at 4°C during sorting to avoid the loss of CD15 antigen. Cells with intermediate levels of CD15 expression were not collected.

**Histology.** Cytocentrifuge slides were prepared and stained with the Wright-Giemsa stain. Histologic sections of the bone grafts were prepared as described previously.

**Administration of ATRA.** ATRA (Sigma Chemical, St Louis, MO) was suspended in absolute ethanol at an initial stock concentration of 10 mg/mL. A solution of ATRA was prepared freshly at every dosing by adding 23 μL of stock solution into 300 μL of distilled water and administered orally through a gavage needle twice daily (0.45 mg/day). All dilutions were performed in subdued light and the gavage syringe was wrapped with aluminum foil. Treatment was initiated on 18, 22, and 24 weeks after injection of leukemia cells.

**RESULTS**

**Implantation of human myeloid leukemia cells into SCID-hu mice.** Cryopreserved BM cells from 1 case of CML myeloid blast crisis and 7 cases of various types of AML patients were directly injected into human fetal bone fragments of SCID-hu mice. The growth of human leukemia cells injected human BM as well as mouse BM were analyzed by flow cytometry 4 to 56 weeks after injection. The results of primary injection are summarized in Table 1. Each row represents data from an individual graft unless indicated.

The growth of BM cells from a CML patient (patient no. 1) was observed in 4 of 4 animals injected (Table 1). Because patient no. 1 was HLA-B7+, the cells derived from this patient could be defined by HLA-allootypic MoAb, MB40.2. At 14 weeks after injection, approximately 30% of the cells recovered from the human BM graft were W6/32+/MB40.2+ CML cells, whereas 40% were W6/32+/MB40.2+, normal human hematopoietic cells of bone donor origin (Fig 1A, fourth animal in Table 1). Presumably, cells negative for W6/32 and MB40.2 (30%) were infiltrated mouse cells. The cells prepared from the graft 20 weeks after injection were mostly W6/32+/MB40.2+, patient no. 1–derived cells (Fig 1B). These cells contained three distinctive populations defined by combined staining with myeloid markers, CD33 and CD15 (CD33+CD15−, CD33+CD15+, CD33−CD15+) (Fig 1C). Few B-lineage cells could be detected by CD19 and CD10 staining (Fig 1D). Histologic examination of this tissue showed that the marrow space was completely replaced by leukemia tissue consisting of atypical blast cells, granulocytic cells, and megakaryocytes with nuclear abnormalities; no normal hematopoietic foci were left (Fig 2A and B). It was observed that the leukemia...
Table 1. Growth of Human Myeloid Leukemia Cells in the SCID-hu Mouse

<table>
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<tr>
<th>Patient No.</th>
<th>Diagnosis (FAB)</th>
<th>Sample (%)</th>
<th>No. of Cells Injected</th>
<th>Time After Injection (wk)</th>
<th>Human BM</th>
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<td>99</td>
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Abbreviation: ND, not done.
* Percentage of human cells in the individual graft was obtained as the percentage of W6/32+/CD45+ population in total nucleated cells.
† Percentage of leukemia cells was calculated as the percentage of patient’s HLA allotype-positive cells per total human cells in the individual samples. For patient no. 5, who was negative for antibodies against HLA allotypes used in these experiments, the percentage of leukemia cells was calculated based on the myeloid marker analysis and scatter analysis of human cell populations.
‡ Leukemia cells in mouse BM were examined with W6/32 and CD45 antibodies.
§ Cells from 3 or 2 animals were pooled and analyzed.

Tissue grew even outside of the marrow graft. The major cell types observed in Wright-Giemsa-stained cytospin preparations were blast cells and cells of eosinophilic, basophilic, and neutrophilic lineage in myelocytic stage (Fig 2C). Megakaryocytes and mature forms of granulocytes were found infrequently. Phenotypic and histologic examination showed that the growing cells were not normal hematopoietic elements of patient no. 1 nor Epstein-Barr virus–transformed B cells, but leukemic cells instead. The cellular composition of CML cells defined by phenotypic analysis with CD33 and CD15 markers as well as by cytology and histology was very similar in all three animals analyzed at 20 weeks or later. Despite the extensive growth of CML cells in the human marrow, CML cells could not be detected in the mouse marrow of any of the 4 mice examined.

Among 3 cases of M1 AML injected into SCID-hu mice, 2 (patients no. 2 and 3) showed extensive growth in human marrow (Table 1). Although the BM cells from patient no. 4 contained a high percentage of viable leukemic blasts, they failed to engraft in vivo. Six of six bone grafts injected with cells from patient no. 2 were completely replaced with leukemia cells at the time of analysis. Flow cytometric analysis showed that the expression of the myeloid markers CD33 and CD15 on AML cells from patient no. 2 before and after implantation were almost identical, with two major populations of CD33 CD15 and CD33 CD15 cells (Fig 3A and B). These two populations were observed reproducibly in all animals with AML cells from patient no. 2. The origin of the leukemia blasts was confirmed by staining with MoAb, MB40.2, which recognized patient no. 2–derived cells (Fig 3C). In this experiment, SCID-hu mice with two human bone implants in the right and left flank were used. Because AML cells were injected into only one of the grafts, spread of the leukemia cells into the uninjected human bone graft was investigated. Interestingly, cells from the un.injected human bone grafts contained MB40.2+ AML cells at the levels of 30% to 90% of recovered human cell population (Fig 3D), whereas no AML cells were detected in the mouse marrow.

Flow cytometric profiles of the cells from the other M1 case, patient no. 3, growing in the SCID-hu mouse are shown in Fig 4, as are those of M2 AML (patient no. 5) and M4 AML (patient no. 8). Growth of AML cells was observed in 3 of 4 mice receiving patient no. 3 cells, 2 of 4 mice receiving patient no. 5 cells, and 3 of 3 mice receiving patient no. 8 cells (Table 1). The HLA types of these cells were confirmed to be of patient origin, except for those of patient...
SCID-hu MOUSE MODEL FOR MYELOID LEUKEMIA

Fig 1. BM cells from a CML patient grow in the SCID-hu mouse. Cells recovered from human BM 14 weeks (A) or 20 weeks (B, C, and D) after injection of cells from patient no. 1 were analyzed by two-color flow cytometry with the antibodies indicated in the individual panels. Cells derived from patient no. 1 could be detected as W6/32+/MB40.2+ population, which constituted 40% (A) and 95% (B) of total human cells. W6/32+/MB40.2+ cells in the sample obtained 20 weeks postinjection consisted of CD33-CD15-, CD33+CD15-, and CD33+CD15+ populations (C); CD19+ and or CD10+ cells were not evident (D).

Blast cells growing in human marrow in SCID-hu mice expressed the CD33 antigen, proving their myeloid origin (Fig 4A, D, and G). Varying levels of expression of CD15 antigen were maintained uniquely to individual cases after implantation into SCID-hu mice. In the examples shown in Fig 4, patients no. 3 and 5 AML cells almost completely replaced the normal marrow hematopoiesis and no other cell types, such as B-lymphoid cells, were found (Fig 4B and E). In contrast, cells recovered from the human marrow 22 weeks after injection of patient no. 8 AML cells still contained normal components of human hematopoiesis of bone donor origin, including CD10+CD19+ B-lymphoid cells (Fig 4H). None of the mice examined had leukemia cells in their marrow (Fig 4C, F, and I).

Cells from two cases of AML diagnosed as M3 were also implanted (Table 1). After thawing, only 7 × 10^5 viable cells were recovered from BM cells of patient no. 6 and 5 × 10^5 cells were injected into 1 SCID-hu mouse. Analysis performed at 40 weeks showed that only 10% of the cells recovered from the graft were of human origin. However, most of these cells had the characteristics of the injected AML cells, CD33+CD15+ phenotype, scatter profile of blast cells, and the HLA allotype of patient no. 6 (HLA-B7). Cells from another case of M3 (patient no. 7) with the t(15;17) translocation were successfully implanted in 2 of 2 animals injected. The cells growing in the SCID-hu mice maintained the HLA type of patient no. 7 (HLA-A2) detected by MoAbs MA2.1 and BB7.2 (data not shown). The surface phenotype of the blast cells growing in the SCID-hu mice stained with MoAbs CD33 and CD15 was similar to that of the BM cells analyzed before injection (Fig 5A and B). Cytologic features of promyelocytic leukemia cells were also maintained with abundant azurophilic granules in the cytoplasm (Fig 5D and E).

In summary, in all but 1 case (patient no. 4), detectable growth of myeloid leukemia cells was observed reproducibly in the injected human BM. The yield of leukemia cells differed between 10^6 and 10^8, depending on the incubation period and the number of cells injected. In most of the cases, 1 to 10 × 10^7 cells could be recovered. Histologic examination at early time points showed localized growth of leukemic blast cells inside the marrow cavity coexisting with normal hematopoietic cells (data not shown), concordant with results obtained by flow cytometry (as shown, eg, in Fig 1A). As a function of time, leukemia cells replaced normal hematopoiesis inside marrow space and eventually the growing mass extended outside the marrow (Fig 2A). Grafts at
this stage had a greenish color by gross examination, regardless of the subtype in the FAB classification. Bone structures could still be observed and involvement of the stromal elements was found by histology even in the outgrowing areas. Flow cytometry of the cell suspensions prepared from these grafts did not show any evidence of engraftment of leukemia donor-derived normal hematopoiesis, such as the growth of patients’ HLA positive nonmyeloid cells. Cell suspensions freshly prepared from these completely replaced marrows could successfully be transferred into human bone grafts of secondary SCID-hu host by direct injection (Table 1). The surface phenotype and cytologic features of the leukemia cells were stably maintained in the secondary passages (data not shown). Cells from the peripheral blood of leukemia-bearing animals were analyzed by flow cytometry with various combinations of antibodies reacting to myeloid leukemia cells. No leukemia cells could be detected by this method except for 2 animals with the secondary passage of patient no. 3 cells. In these animals, 1% and 4% of total nucleated cells in the peripheral blood were positive for CD33 and the HLA allotype of patient no. 3 (MA2.1 and BB7.2) (data not shown).

Experiments using SCID-hu mice with two human bone implants at distant sites were performed with the secondary passages of patients’ no. 3, 5, 7, and 8 according to the experimental design described above. Spread of AML cells into uninjected human bone grafts was observed in all cases 2 to 5 months after injection of leukemia cells into one of the bone grafts. In contrast, no leukemia cells were detected in mouse marrow of these animals. Thus, human myeloid leukemia cells spread into hematopoietic organ in a species-specific fashion.

Treatment of M3 leukemia cells with ATRA. Three SCID-hu mice bearing M3 leukemia cells from patient no. 7 (passage 2) were treated with ATRA at a dose of 0.45 mg/day administered orally twice a day. This dose was selected based on that used clinically (45 mg/m²/day) assuming a mouse body surface area of 100 cm². Phenotypic and cyto-
logic changes of leukemia cells were examined after 3 to 9 days of treatment. In normal myeloid differentiation pathways, maturation towards the granulocytic lineage is characterized by the acquisition of the CD15 antigen. Because most of the blast cells of patient no. 7 were CD33+CD15- (Fig 5A), the CD15 antigen was chosen as a marker for the differentiation. In vitro experiments with M3 leukemia cells showed that CD15 could be a marker for the differentiation of promyelocytic leukemia cells upon stimulation with RA, suggesting that expression of CD15 antigen would be an earlier indicator than CD16 antigen for detecting the induced differentiation.

In the experiments described here, the samples were confirmed to be completely replaced by HLA-A2+ leukemia cells and the percentages of CD33+CD15- cells and CD33+CD15+ cells were normalized to a total of 100% CD33+ cells (Table 2 and Fig 5). A small piece of the graft (secondary passage) was biopsied on day 3 of treatment and analyzed. No significant changes in the expression of CD15 or in cytology were observed in comparison to the original cells and controls (primary and secondary passage cells without treatment). On day 7 of treatment, the mouse was killed for analysis. A significant portion of leukemia cells (27%) was positive for CD15 at this timepoint (Table 2 and Fig 5C). Similar results were obtained from 2 other SCID-hu mice with secondary passage cells. The population of CD15+ cells increased to 14% and 54% after 7 and 9 days of treatment, respectively. Induction of granulocytic differentiation was confirmed by cytology. After 7 days of treatment with ATRA, more differentiated forms of myeloid cells with lobulated nucleus and neutrophilic granules could be observed among the promyelocytic cells (Fig 5F). Thus, it was shown that promyelocytic leukemia cells growing in the SCID-hu mouse could respond to ATRA and differentiate towards mature neutrophilic cells.

Leukemic progenitor cell population in AML blasts. As described, AML cells from patient no. 2 contained two populations defined by the myeloid markers CD33 and CD15. We compared the leukemic progenitor cell activity in less mature (CD33+CD15-) and more differentiated (CD33+CD15+) populations by transferring varying numbers of sorted cells into secondary SCID-hu mice. Two independent experiments were performed with a total of 45 SCID-hu mice created from 5 bone donors. Leukemia cells from 3 or 2 animals were harvested, pooled, stained with PE-CD33 and FITC-CD15, and then sorted into CD33+CD15- and

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**Fig 3.** Flow cytometric analysis of the AML cells derived from BM of patient no. 2. The surface phenotype of BM cells from patient no. 2 (M1) before implantation (A) and the cells recovered from SCID-hu mouse 16 weeks after implantation (B) were compared by staining with CD33 and CD15 antibodies. Cells recovered from injected (C) or uninjected (D) human bone grafts were analyzed with MB40.2 antibody, which recognized patient no. 2-derived cells. Almost all of the cells (99%) from the injected BM were MB40.2+ (C), whereas approximately 50% of the total human cells recovered from the uninjected human bone graft in the same animal were MB40.2+ AML cells (D). To examine leukemic progenitor cell activity, cells from human BM of 2 SCID-hu mice were pooled, stained, and sorted into CD33+CD15- and CD33+CD15+ populations. Reanalysis of sorted cells showed that the population sorted as CD33+CD15- (E) or CD33+CD15+ (F) maintained 95% and 6% of CD33+CD15- and 4% and 94% of CD33+CD15+ cells, respectively. The level of growth of leukemia cells in secondary hosts was expressed as the percentage of MB40.2+ cells in the total human cells. Human BM 9 weeks after injection of 10⁶ CD33+CD15+ cells contained 45% of patient no. 2-derived (MB40.2+) AML cells (G). Cells recovered from human marrow 10 weeks after injection of 10⁶ CD33+CD15- cells contained both CD33+CD15- and CD33+CD15+ populations (H). This sample was confirmed to be completely replaced by patient no. 2-derived (MB40.2+) AML cells.
CD33+CD15+ populations. It was confirmed that these samples were completely replaced by leukemia donor-derived cells by staining with MB40.2 and BB7.1 antibodies. The purity of sorted cells from two independent sorts was 95% and 95% for the CD33+CD15− population and 92% and 94% for the CD33+CD15+ population, respectively (Fig 3E and F). Growth of leukemia was analyzed by flow cytometry 8 to 11 weeks after injection of 10^6 and 10^5 cells and 9 to 14 weeks with lower doses of cells. The growth of AML cells was indicated as the percentage of HLA-B7+ (MB40.2+, BB7.1+) cells among total human hematopoietic cells detected by combined staining with MoAbs, W6/32 and CD45. For example, the percent of leukemia cells in the sample shown in Fig 3G was 45%.

The results summarized in Table 3 clearly show that the CD33+CD15− population is more efficient in transferring leukemia into the secondary hosts. Five of five animals receiving 10^6 CD33+CD15− cells and 3 of 5 animals receiving 10^5 CD33+CD15− cells had detectable levels of leukemic cell growth, whereas none of 10 animals receiving the same number of CD33+CD15+ cells showed evidence of leukemia growth. It was also observed that the leukemia that developed in the secondary hosts consistently contained both CD33+CD15− and CD33+CD15+ populations, similar to the original leukemia cells, regardless of the injected population (Fig 3H).

**DISCUSSION**

In previous studies, we showed that the biologic features of human hematopoiesis can be maintained in human fetal BM implants in the SCID-hu mice. Therefore, it seemed likely that the implanted human marrow could provide a suitable environment for the growth of leukemia cells. Using the SCID-hu mouse as a leukemia model would be especially advantageous for myeloid leukemias, which are known to be difficult to grow and to maintain as xenotransplants. We reasoned that myeloid leukemia cells injected into human marrow might encounter an environment similar to that in the patients' BM and that the cellular interactions necessary for the growth of leukemia cells would be maintained.

We show here that BM cells from myeloid leukemia patients can indeed grow in human fetal bone implanted in the SCID mouse. Cells from 1 case of CML-BC and 6 of 7 cases of AML were successfully propagated in the human marrow environment. It is notable that, in addition to the common AML subtypes such as M1, M2, and M4, 2 of 2 cases of M3 showed the growth in the SCID-hu mouse. M3 leukemia...
Fig 5. Induction of differentiation of promyelocytic leukemia cells growing in the SCID-hu mice by ATRA treatment. Two-color flow cytometry with myeloid differentiation markers, CD33 and CD15 (A, B, and C), and cytologic examination with Wright-Giemsa-stained cytospin preparations (D, E, and F) were performed on cells of patient no. 7 (M3) before implantation (A and D), on cells from human BM of the SCID-hu mouse with the secondary passage of patient no. 7 cells (B and E), or on the cells from human BM of the SCID-hu mouse with the secondary passage of patient no. 7 cells 7 days after treatment with ATRA (0.45 mg/day) (C and F). The percentages in (B) and (C) were normalized so that total CD33+ cells become 100% (see Table 2). (D, E, and F original magnification × 187.)

cells have not been successfully shown to grow in any other model, likely due to their relatively advanced stage of maturation and lower proliferative activity. The fact that M3 cells grow in the SCID-hu mouse suggests an important supportive role for the human BM environment in the growth of myeloid leukemia cells. The reason(s) why cells from patient no. 4 failed to grow in the SCID-hu mice remain unclear. Although correlations between clinical history and growth in mice has been suggested in the past, the clinical course of patient no. 4 was not any more benign than other cases reported here. A complete remission was not achieved by chemotherapy and the patient died 70 days after diagnosis with bacterial sepsis. It is possible that the condition of bone grafts used for this experiment was not optimum, even though the viability of the injected leukemic blasts was confirmed.

Among the successfully implanted 7 cases, cells from 6 cases could be transferred in vivo. The surface phenotypes and cytologic features of leukemia cells growing in the SCID-hu mice were comparable with those of the original patient BM cells and were stably maintained in secondary passages. Little variability was observed in the expression of myeloid differentiation antigens on leukemia cells among individual animals. This observation is extremely important from a practical point of view because it enabled us to test the efficacy of new therapeutic modalities, such as differentiating agents, biologic modifiers, and cytokines, in a statistically significant manner. It was shown in this report that the differentiation of promyelocytic leukemia cells was induced in vivo by a known differentiation-inducing agent.

Table 2. Effects of ATRA on Promyelocytic Leukemia Cells

<table>
<thead>
<tr>
<th>Passage</th>
<th>Treatment</th>
<th>CD33+CD15</th>
<th>CD33+CD15*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original cells</td>
<td>NA</td>
<td>96%</td>
<td>0%</td>
</tr>
<tr>
<td>1</td>
<td>No</td>
<td>99%</td>
<td>1%</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>99%</td>
<td>1%</td>
</tr>
<tr>
<td>2</td>
<td>ATRA 3 d*</td>
<td>97%</td>
<td>3%</td>
</tr>
<tr>
<td>2</td>
<td>(0.45 mg/d) 7 d*</td>
<td>73%</td>
<td>27%</td>
</tr>
<tr>
<td>2</td>
<td>7 d</td>
<td>86%</td>
<td>14%</td>
</tr>
<tr>
<td>2</td>
<td>9 d</td>
<td>44%</td>
<td>56%</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.

* Samples were derived from the same graft biopsied on days 3 and 7.
ATE IN THE HEMATOPOIETIC ORGANS OF SCID MICE.* WE ALSO OBSERVED THAT CELLS FROM ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) PATIENTS CAN PROLIFERATE IN THE HEMATOPOIETIC ORGANS OF SCID MICE AS W6/32+/MB40.2+ CELLS AMONG TOTAL HUMAN CELLS DEFINED AS W6/32+/CD45+. It has been reported by other investigators that cells from acute lymphoblastic leukemia (ALL) patients can proliferate in the hematopoietic organs of SCID mice. It was reported that AML cells could grow in the SCID mouse. These results, together with our findings, suggest that the subclones that can proliferate without stimuli from human hematopoietic microenvironment or, alternatively, that can proliferate by the stimuli provided by species cross-reactive, mouse molecules preferentially grow out in SCID models, whereas it does not occur in the SCID-hu model.

ATRA,23,24 with stable in vivo passages of M3 leukemia. In previously published studies, the establishment of serially transplantable AML cells was very limited. Even in SCID models, only one case of AML (M5) was reported to be successfully passed.12 The high frequency of establishment of serially transplantable AML in SCID-hu mice again suggests the advantages of having leukemia cells grow in a human, as opposed to a mouse, hematopoietic environment.

It has been reported by other investigators that cells from acute lymphoblastic leukemia (ALL) patients can proliferate in the hematopoietic organs of SCID mice. We also observed that cells from 2 cases of ALL (L1) implanted into SCID-hu mice grew in the injected human marrow and spread as well into the mouse marrow (data not shown). In contrast, the spread of myeloid leukemia cells into mouse hematopoietic organs could not be detected in any of the animals examined, suggesting the dependency of myeloid leukemia cells on the human hematopoietic microenvironment. The observation that human myeloid leukemia cells could spread into the second human bone grafts at a distant site but not to murine marrow in the same mouse would support this possibility.

However, the dependency might be altered in certain conditions because myeloid leukemia cells were shown to grow and spread in SCID mice.11,12 The mechanisms underlying the different growth behavior of myeloid leukemia cells found in the SCID-hu mouse and the SCID mouse are unclear. However, it is possible that species cross-reactive or non-cross-reactive hematopoietic growth factors play important roles. It was reported that AML cells could grow in the SCID mouse marrow when human hematopoietic growth factors (a fusion protein of human interleukin-3 [IL-3] and human granulocyte-macrophage colony-stimulating factor [GM-CSF]) were provided exogenously.30 On the other hand, human myeloid leukemia cell lines dependent on either IL-3 or GM-CSF were not able to grow in the SCID mouse.12 Thus, species-specific cytokines such as IL-3 and GM-CSF clearly play an important role for proliferation of myeloid leukemia cells in the SCID mouse. These results, together with our findings, suggest that the subclones that can proliferate without stimuli from human hematopoietic microenvironment or, alternatively, that can proliferate by the stimuli provided by species cross-reactive, mouse molecules preferentially grow out in SCID models, whereas it does not occur in the SCID-hu model.

In vitro studies have shown that only a small subset of AML cells has the ability to produce leukemic blast cell colonies in semisolid cultures. These clonogenic cells are a distinct subpopulation of leukemic cells and have a less differentiated phenotype when compared with the rest of the population of each patient. It has been suggested that this population of cells acts in vivo as leukemic progenitor cells to maintain the rest of more differentiated leukemic cell populations.3,11-13 However, little is known about the regulatory mechanisms of proliferation and differentiation of myeloid leukemia cells in vivo. In this study, the more differentiated populations in the leukemia cells from patient no. 2 (Fig 3A and B) and patient no. 8 (Fig 4G) were observed to be maintained after implantation into human marrow. The results from the leukemic progenitor cell experiments clearly showed that differentiation of less mature populations occurred in the SCID-hu mice. Furthermore, the differentiated potential of promyelocytic leukemia cells (patient no. 7) was shown to be maintained in the SCID-hu mice as discussed above. These observations suggest that the proliferation and differentiation of leukemic myeloblasts were regulated in the SCID-hu mice as they are in the patient's marrow and, therefore, that this model might provide a unique opportunity for studying the mechanisms of regulation.

In this regard, the CML case (patient no. 1) is of particular interest because differentiation towards multilineage cells was observed. This case was diagnosed as myeloid blast crisis phase with 30% of blast cells in the marrow. After implantation into SCID-hu mice, the cellular composition of CML cells was still maintained. In addition to blast cells (20% to 30%), atypical cells of eosinophilic, basophilic, and neutrophilic myeloid lineages as well as the megakaryocytic lineage were observed. Results from macroscopic, microscopic, and flow cytometric analyses together indicated that these relatively differentiated cell types were derived from the CML clone, although the presence of molecular marker, bcr/abl, in individual cells was not confirmed. Thus, CML blast cells proliferated in the human marrow of SCID-hu mouse, yet maintained their ability to differentiate into multiple cell types, similar to events observed in the patient's marrow.

Finally, we addressed the question of whether leukemic progenitor cell populations could be identified in vivo with this model. Because AML cells from patient no. 2 contained two populations of cells defined by the expression of the myeloid differentiation antigens, CD33 and CD15, the leukemogenic activity of CD33+CD15− and CD33+CD15+ populations was compared in vivo. The results showed clearly that the phenotypically less mature, CD33+CD15−...
population had at least 100-fold higher activity to transfer leukemia than the phenotypically more differentiated CD33+CD15- population. Surprisingly, only 10^6 cells of the CD33+CD15- population were required to transfer leukemia. It is conceivable that CD33+CD15- cells contaminating (<10%) in the sorted CD33+CD15+ population contributed to the tumorigenic activity observed in animals with injections of large numbers of CD33+CD15+ cells. Thus, as suggested by in vitro studies, the leukemic progenitor cell activity was preferentially found in the less differentiated population of leukemic blasts. It was also noticed that leukemia cells growing in the secondary hosts contained both CD33+CD15- and CD33+CD15+ populations, indicating that the CD33+CD15+ population was derived from the CD33+CD15- population. These results suggest that the CD33+CD15+ population in this particular AML case is inclusive of a cell type that has leukemic progenitor cell activity in vivo. This is the first in vivo demonstration of the leukemic progenitor cells with self-renewal capacity and the ability to differentiate to nonproliferative cells.

It would therapeutically be of great importance to understand the regulatory mechanisms of growth and differentiation of these leukemic progenitor cells. There are many other important questions that remain to be understood in the biology of human myeloid leukemias, such as the interactions between leukemia cells and hematopoietic stromal cells and the influence of leukemia cells on normal hematopoiesis. We believe that the leukemia model in the SCID-hu mouse reported here will provide a useful in vivo experimental system to address these questions.

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REFERENCES


Growth of human myeloid leukemias in the human marrow environment of SCID-hu mice

R Namikawa, R Ueda and S Kyoizumi