Establishment of a Human Acute Promyelocytic Leukemia-Ascites Model in SCID Mice


Acute promyelocytic leukemia (APL, FAB-M3) is characterized by the specific chromosome translocation (15;17) and fusion protein PML-RARα as well as its unique response to the differentiation inducer, all-trans retinoic acid (ATRA).\(^{1,3}\) Although clinical treatment with ATRA results in a high rate of complete remission (CR), we are still at the beginning of understanding the pathogenesis of APL and the mechanisms underlying ATRA-induced differentiation. In addition, most patients treated with ATRA alone after CR will develop early clinical relapse\(^{6}\) or require intensive chemotherapy to prevent relapse. To this end, an appropriate and stable animal model of APL is important to study these issues and to measure the in vivo differentiation effect of drugs including ATRA. The availability of NB4 cells (a human APL cell line with t(15;17) and PML-RARα protein) prompted us to explore the possibility of establishing an in vivo model for human APL using these cells.

Severe combined immunodeficiency (SCID) mice lacking functional B and T lymphocytes provide a model for studying the biology of human normal or malignant tissue transplants that do not grow in nude mice, and for evaluating the efficacy of drugs on these tissues.\(^{5,7}\) It has been shown that normal myeloid or leukemic cells can grow in SCID mice. In recent years, cells from some human malignant hemopathies, such as acute lymphocytic leukemia, acute granulocytic leukemia (FAB-M1, -M2, -M4, and -M5), and multiple myeloma have been successfully engrafted in SCID mice, opening new prospects for the study of basic mechanisms and treatment of human hematologic diseases.\(^{6,11}\) In this report, we show our results from using NB4 cells to establish a human APL-ascites SCID mouse model, the characterization of engrafted leukemic cells, and the efficacy of chemotherapy drugs as well as ATRA in experimental therapy.

MATERIALS AND METHODS

Mice

Male and female SCID mice, 4 to 8 weeks of age, were bred and maintained under a specific pathogen-free condition in the Shanghai Institute of Materia and Medica, Chinese Academy of Sciences.

Cell Line

NB4 cells maintained in vitro were used to create the SCID mouse APL-ascites model.\(^{15}\) Before intraperitoneal inoculation (IP) into mice, NB4 cells in exponential growth were suspended in RPMI 1640 at a concentration of \(1 \times 10^6\) mL\(^{-1}\).

NB4 Engraftment and Serial Passage

For the first transplantation, two SCID mice were inoculated with \(1 \times 10^6\) NB4 cells. One month after inoculation, ascites appeared in the peritoneal cavity of the animals. Ascitic cells, \(1 \times 10^6\), (hereafter, A-NB4) were administered IP into each mouse for passage, and for each passage two to five mice were used.

Drug

ATRA (molecular weight [MW] 300.42) was obtained from Shanghai Huang He Pharmaceutical Laboratory and suspended in 0.5% carboxymethyl cellulose (CMC). Adriamycin (ADM) was obtained from Farmitalia Carlo Erba SPA, Italy.
Characterization of A-NB4 Cells and Organ Infiltration After Nine Passages

Morphology. For morphologic evaluation, ascitic cell slides were prepared with a cytopsin (Shandon, Cheshire, UK) (10^4-10^5 cells/slide) and stained with Wright's stain. Pathologic examinations were also carried out in order to estimate the possible infiltration of A-NB4 cells in different organs.

Clonogenic assay. NB4 cells (2.5 x 10^5) and A-NB4 cells (2.5 x 10^5) from three SCID mice were plated in 24-well petri dishes with 0.5 mL 25% McCoy 5A Medium, containing 25% blast promoting activity (BPA) and 0.3% agarose. After incubation in 5% CO2 at 37°C for 10 days, colonies (>=30 cells) were counted.17

Cyto genetics. Chromosome studies were performed on NB4 cells maintained in vitro and A-NB4 cells from SCID mice using standard R banding techniques. Karyotype analysis was carried out according to ISCN criteria.14

RT/PCR analysis of PML-RARa fusion mRNA. RT/PCR assay for PML-RARa was carried out with both parental NB4 and A-NB4 cells, as well as different organs of SCID mice carrying A-NB4 ascites according to the method established in our laboratory.15

Immunologic staining for PML in pathology sections of different organs. Immunologic staining of pathology sections of different organs was performed using antihuman PML antibody as previously described.16

Differentiation assays. NBT reduction and expression of cell surface differentiation antigens CD11b and CD33 were performed with previously described methods16 to measure differentiation induction.

Experimental therapy. The effect of ATRA and ADM on A-NB4 cells was studied in SCID mice during the ninth to twelfth passage. For ATRA treatment, SCID mice were inoculated with 1 x 10^6 A-NB4 cells and treated by intragastric tube at a daily dose of 25 or 50 mg/kg six times per week, or by IP injection at a dose of 5 or 10 mg/kg every other day (three times a week). The total treatment schedule was 4 weeks. Larger doses or more frequent use of ATRA were shown to be toxic to the animals (data not shown). ADM was administered IP every other day (three times a week) for 3 weeks, with a daily dose of 1 mg/kg.

RESULTS

Serial Passage of A-NB4 Cells in SCID Mice

One month after inoculation with 1 x 10^6 NB4 cells, the abdomen contained moderate ascites with numerous A-NB4 cells. The ascites containing A-NB4 cells could be engrafted through serial IP inoculations into SCID mice to produce new ascites for further passage. The A-NB4 cells grew exponentially from day 6 after transplantation and reached a quasi-stationary state by day 15 (Fig 1). The percentage of cells in S-phase during exponential growth (day 12: 46.1% ± 2.9%) was higher than that of stationary growth (day 19: 38.2% ± 1.6%). We have successfully performed 18 passages in SCID mice in 67 mice. The interval between passages is 3 weeks with a 100% success rate of tumor induction in each passage. The survival time of the animal depended on the number of A-NB4 cells inoculated, 21.6 ± 2.3 days with 1 x 10^6 cells and 28.2 ± 1.2 days with 1 x 10^5 cells. Further decreasing the inoculated cells to 1 x 10^4 resulted in prolongation of the survival time to 36 days in three of four SCID mice and failure of tumor induction in the remaining one mouse. Only one of four SCID mice developed tumor when 1 x 10^4 A-NB4 cells were inoculated, while no tumor induction was found in SCID mice inoculated with 1 x 10^6 cells. The number of in vivo tumorigenic cells was thus estimated as one per 0.4 to 1 x 10^4 A-NB4 cells.

At necropsy the SCID mice bearing APL-ascites had enlarged spleens weighing 120 to 210 mg (three- to fivefold of those of the control). Many small tumor nodules were scattered on the liver surface, mesentery, and peritoneum. Microscopically, the tumors in the peritoneal cavity were hypercellular with scanty connective tissue. Tumor cells were pleomorphic, and varied in size with a high nuclear-cytoplasm ratio and obvious nucleoli. The cytoplasm was basophilic. Mitotic tumor cells were easily found. The tumor cells aggregated in a sheet-like pattern, and spread superficially on the surface of the liver, spleen, and kidney, but no deep infiltration was seen. The enlarged spleen showed reactive cell proliferation. No obvious A-NB4 infiltration was observed in major organs including heart, liver, kidney, lung, and brain, as well as peripheral blood and bone marrow of SCID mice bearing APL-ascites. These observations were confirmed by immunofluorescence staining using anti-PML antibody specifically recognizing A-NB4 cells. Interestingly, "nested" RT/PCR using central portions of the above-mentioned organs showed the presence of the long type PML-RARa transcript (data not shown)17 despite careful washing, including the last wash buffer from each organ, which was negative for PML-RARa transcript, eliminating the possibility of contamination. Therefore, there was some microinfiltration in organs with A-NB4 cells as measured by RT/PCR.

Biologic Properties of A-NB4 Cells

Cells from ascites (A-NB4 cells) were similar in morphology (large size, high nuclear/cytoplasm ratio, fine chromatin

structure with visible nucleoli, basophilic cytoplasm) to NB4 cells maintained in vitro, except for the presence of a large number of vacuoles in the former. Phenotypic analysis using monoclonal antibodies against CD11b and CD33 showed that the percentage of CD11b positive A-NB4 cells (46.5% ± 10.3%) was higher than that of the NB4 cells (16.0% ± 6.0%), while the percentage of the CD33 positive A-NB4 cells (78.6% ± 13.5%) was lower than that of NB4 cells (94.3% ± 4.5%) maintained in vitro. Clonogenic cells in A-NB4 (6.3 ± 1.2 × 10⁴ cells) was significantly lower than that of NB4 maintained in vitro (15.4 ± 0.9/10⁴ cells). Cytogenetically, A-NB4 cells have the same karyotype as the original NB4 cells, which is hypotetraploid with the typical chromosome translocation t(15;17) of APL (Fig 2A and B). The long type PML-RARα transcripts, normally maintained in the NB4 cell line, was also present in A-NB4 cells (Fig 3).

Differentiation induction by ATRA was studied in A-NB4 cells. In vitro ATRA treatment of A-NB4 cells induced a significant differentiation as measured by morphologic and phenotypic parameters (data not shown). NBT reduction was increased by 9.6- to 27-fold (Fig 4A). These results were comparable with parental NB4 cells. However, the response of A-NB4 cells to in vivo ATRA treatment was relatively weak. There was no significant difference in CD11b and CD33 expression between the ATRA-treated group and the control group. The NBT reduction performed before and after two IP administrations of ATRA in the same mice showed an increment of 2.8- to 4.4-fold (Fig 4B). We also examined the differentiation status of A-NB4 cells on day 25 after transplantation in animals receiving ATRA experimental therapy IP protocol (see Materials and Methods). Most cells displayed a maturation to the myelocyte stage, but no segmented granulocytes could be observed. The NBT reduction was mildly increased (2.0- to 4.2-fold) compared with the control (Fig 4C).
Fig 3. RT/PCR of PML-RARα fusion mRNA of A-NE4 cells obtained from the APL-ascites model (mouse 1, 2, and 3) compared with in vitro cultured NB4 cells. Control RNA was from non-APL human cells. The size marker is pGEM 7Zf/HaeIII. Note that PCR products of the same size corresponding to the long type fusion transcript are seen in both ascitic A-NE4 cells and parental NB4 cells.

**Therapeutic Efficacy of ATRA and ADM in APL-Ascites Model in SCID Mice**

When SCID mice bearing A-NB4-ascites were treated with two doses of ATRA (5 or 10 mg/kg each time), the mean survival of these two groups was 28.1 days and 29.1 days, significantly higher than that of the control (21.6 days, \( P < .001 \)). No apparent effect was seen in the groups treated with intragastric gavage of ATRA (data not shown). On the other hand, ADM resulted in a better survival rate (44.6 ± 5.5 days) \( P < .001 \) compared with the control group; \( P < .01 \) compared with the ATRA IP-treated group; Fig 5).

**DISCUSSION**

The NB4 cell line was derived from peripheral blood cells of a patient with APL during the second relapse of the disease, it has been maintained in vitro for 5 years and undergoes differentiation induction by ATRA. Heterotransplantation of these cells into nude mice was unsuccessful (Zhang and Zhu, unpublished data), necessitating the use of SCID mice as described in this work. Our study indicated that although the ascitic cells (A-NB4) share the genotypic features with the parent NB4 cell line, there were some phenotypic modifications in terms of the morphology (vacuolation), immunophenotype (altered CD11b and CD33 expression), and clonogenicity (decreased in vitro clonogenic fraction). These modifications could be caused by a selection or modulation process during the in vivo expansion of NB4 cells and perhaps also occur in the patient with APL.
The survival of SCID mice with APL-ascites significantly increased as a result of ADM treatment and provides a pre-clinical model for the screening of chemotherapy drugs. Our data thus far shows that in vivo treatment with ATRA prolongs the survival time of A-NB4 SCID mice but does not eradicate the tumor. Indeed, the differentiation of A-NB4 cells with ATRA treatment in vivo did not appear to be profound since NBT reduction was only increased 2- to 4-fold (Fig 4, B and C). A-NB4 cells matured only to the myelocyte stage when treated with ATRA. This relatively weak differentiation is in line with the limited improvement of survival of the animals. This modest in vivo therapeutic effect may relate to the heavy burden of tumor cells present at the time of treatment or a low bioavailability of ATRA in SCID mice. A-NB4 cells were as differentiation responsive to in vitro ATRA treatment as cultured NB4 cells when measured by NBT reduction (see Fig 4A), suggesting that IP administration of ATRA may not be readily bioavailable. However, the NB4-SCID mice did respond to treatment with ATRA and may represent the first animal model demonstrating the therapeutic efficacy of differentiation induction. It is possible that a more profound effect could be obtained with a different route of administration or the use of more potent retinoids or retinoids used in combination with non-retinoid differentiation inducers, as recently described.18-20 ADM, as in patients with APL, significantly prolonged the survival of A-NB4 SCID mice. This in vivo model allows for developing combination cytotoxic-differentiation therapy, which is predicted in vitro to significantly enhance differentiation and apoptotic cell death in leukemic cells.21-23 Moreover, this animal model could also be used to address other basic or practical questions, such as the transforming ability of the PML-RARα fusion gene24-27 by blocking the expression of PML-RARα in A-NB4 cells, or the study of the mechanism of relapse after ATRA treatment.

An authentic leukemia in SCID mice would be a more ideal model than IP inoculation of A-NB4 cells in ascites. Such a model is important for studying the interaction between APL cell proliferation, infiltration, and normal hematopoiesis, as well as normal bone marrow stroma. These data would help in understanding the pathogenesis and treatment of human APL. Unfortunately, we have failed to induce leukemia in SCID mice by direct intravenous injection of cultured NB4 cells. Hematopoietic growth factors including stem cell factor and a fusion protein of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) together with irradiation allowed human AML cells to grow in vivo in hematopoietic tissues.4 Whether these additional treatments will allow NB4 cells or fresh human APL cells to proliferate in the bone marrow as well as peripheral blood in recipient SCID mice is worth further investigation.

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