Establishment of a retinoic acid-resistant human acute promyelocytic leukaemia (APL) model in human granulocyte–macrophage colony-stimulating factor (hGM-CSF) transgenic severe combined immunodeficiency (SCID) mice

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Summary To understand the mechanisms and identify novel approaches to overcoming retinoic acid (RA) resistance in acute promyelocytic leukaemia (APL), we established the first human RA-resistant APL model in severe combined immunodeficiency (SCID) mice. UF-1 cells, an RA-resistant APL cell line established in our laboratory, were transplanted into human granulocyte–macrophage colony-stimulating factor (GM-CSF)-producing SCID (hGMtg SCID) mice and inoculated cells formed subcutaneous tumours in all hGMtg SCID mice, but not in the non-transgenic control SCID mice. Single-cell suspensions (UF-1/hGMtg SCID cells) were similar in morphological, immunological, cytogenetic and molecular genetic features to parental UF-1 cells. All-trans RA did not change the morphological features of cells or their expression of CD11b. RA did not alter the growth curve of cells as determined by MTT assay, suggesting that UF-1/hGMtg SCID cells are resistant to RA. These results demonstrate that this is the first RA-resistant APL animal model that may be useful for investigating the biology of this myeloid leukaemia in vivo, as well as for evaluating novel therapeutic approaches including patients with RA-resistant APL.

Keywords: acute promyelocytic leukaemia; severe combined immunodeficiency mice; human granulocyte–macrophage colony-stimulating factor; retinoic acid; drug resistance

All-trans retinoic acid (RA) can induce terminal differentiation of the leukaemic cells, resulting in complete remission in most patients with acute promyelocytic leukaemia (APL) (Huang et al. 1988; Kanamaru et al. 1996; Warrell et al. 1991). However, the majority of patients became RA resistant with continuous treatment with RA (Warrell et al. 1993). Mechanisms and strategies to overcome RA resistance in APL are still unclear, and it is a serious clinical problem for differentiation-inducing therapy. Recently, we established a novel APL cell line (UF-1) with RA-resistant features that will be a useful model for studies on the block of differentiation of the leukaemic cells (Kizaki et al. 1996a). However, studies based on the analysis of cell lines in vitro may not reflect in vivo conditions. Thus, a suitable in vivo model for human APL is critical for investigating mechanisms of RA resistance and to develop novel therapeutic drugs for patients.

Severe combined immunodeficient (SCID) mice have been used as a model for studying the biology of human disease. Unlike lymphoid leukaemic cells, human myeloid leukaemic cells have been difficult to propagate in SCID mice (Uckun et al. 1996). It has been shown that cytokines, and fetal bone and thymus, increase the reconstitution capacity of the human haematopoietic system in SCID mice (Lapidot et al. 1994). To address these problems, we have established human granulocyte–macrophage colony-stimulating factor (GM-CSF)-producing transgenic SCID (hGMtg SCID) mice (Miyakawa et al. 1996). In this report, we demonstrate and characterize the first human RA-resistant APL model using UF-1 cells and hGMtg SCID mice.

MATERIALS AND METHODS

Cells and chemicals

The RA-resistant APL cell line (UF-1) was established in our laboratory (Kizaki et al. 1996a). RA-sensitive HL-60 and NB4 cells (the latter a gift from Dr M Lanotte, hôpital St. Louis, Paris, France) (Lanotte et al. 1991) were maintained in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) containing 15% fetal bovine serum (FBS: Hyclone Laboratories. Logan, UT, USA), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin in a humidified atmosphere with 5% carbon dioxide. All-trans RA was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in 100% ethanol to a stock concentration of 1 mM, stored at -20°C and protected from light.

Transgenic (Tg) SCID mice producing human GM-CSF

Human GM-CSF transgenic (hGMtg) SCID mice used in this study were newly produced as described previously (Miyakawa et al. 1996). Briefly, equal numbers of pCDSRthGM-CSF and
pCDSRothIL-3 plasmids (provided by Dr Y Takebe, National Institute of Infectious Diseases, Tokyo, Japan) (Takebe et al. 1988) linearized with ApaLI (Takara Shuzo, Tokyo, Japan) were mixed and microinjected into prenuclear stage embryos obtained by crossing BDF1 females with C.B-17-scid or C57B6/J (B6J)-scid males. A Tg mouse producing hGM-CSF in the sera born of two founder mice, which carried both pCDSRothGM-CSF and pCDSRothIL-3 in their genomes, was crossed with B6J-scid to obtain hGM-CSF producing scid/scid offspring. The mice were maintained by back-crossing with B6J-scid under specific pathogen-free conditions in our laboratory. Serum levels of human GM-CSF were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) in triplicate according to the manufacturer’s instruction.

**Inoculation of UF-1 cells into hGMtG SCID mice**

UF-1 cells (1 x 10⁷ cells) were inoculated either intraperitoneally (i.p.) or subcutaneously (s.c.) into hGMtG SCID and control B6J SCID mice. Mice were pretreated with 3 Gy of total-body irradiation, a sublethal dose that may enhance acceptance of xenografts (Miyakawa et al. 1996). Leukaemic cell growth was assessed by daily measurements of the dimension of subcutaneous nodules.

When the animals showed severe wasting, they were not observed further and the day of sacrifice was recorded to estimate lifespan according to the UKCCCR guidelines (Workman et al. 1988). Surviving mice were sacrificed at the end of the experiment and sectioned for microscopic examination of the tumour.

**Analysis of leukaemic cells and tissue infiltration**

**Morphology and histopathology**

A leukaemic tumour nodule was removed from SCID mice, cut into small pieces with a scalpel and then gently ground in a nylon cell strainer within a tissue culture dish containing RPMI-1640 medium. Single-cell suspensions (UF-1/GMtG SCID cells) were collected and morphology was evaluated from cytospin slide preparations with Giemsa stain. To estimate the infiltration of the leukaemic cells in different organs, tissue sections from mice were fixed in 10% formalin and paraffin embedded, and then stained with haematoxylin and eosin.

**Cytogenetic studies**

Chromosomes of UF-1/GMtG SCID cells were analysed by standard Giemsa banding techniques as described previously (Kamada et al. 1981).
Table 1 Engraftment of UF-1 cells in hGMTg SCID mice

| Mice          | Sex | Age (weeks) | Serum levels of hGM-CSF (pg ml⁻¹) | Route of inoculation | Engraftment (days after inoculation) | Findings                      |
|---------------|-----|-------------|----------------------------------|----------------------|-------------------------------------|-------------------------------|
| hGMTg SCID    |     |             |                                  |                      |                                     |                               |
| 1             | M   | 13          | 6440                             | s.c.                 | + (27)                              | Subcutaneous tumour           |
| 2             | M   | 13          | >10 000                          | i.p.                 | + (52)                              | Subcutaneous tumour. ascites. |
| 3             | F   | 10.5        | 6640                             | s.c.                 | + (20)                              | Subcutaneous tumour           |
| 4             | F   | 10.5        | 6760                             | i.p.                 | + (59)                              | Subcutaneous tumour. abscess  |
| B6J SCID      |     |             |                                  |                      |                                     |                               |
| 1             | M   | 10.5        | –                                | s.c.                 | –                                   | Subcutaneous tumour           |
| 2             | M   | 10.5        | –                                | i.p.                 | –                                   |                               |
| 3             | F   | 10.5        | –                                | s.c.                 | + (83)                              |                               |
| 4             | F   | 10.5        | –                                | i.p.                 | –                                   | (tumour/total body weight 1.6×) |

Twenty-four hours after 3 Gy of TBI, SCID mice were injected s.c. or i.p. with 1 × 10⁶ UF-1 cells.

Surface marker analysis

Cell-surface antigens were detected by immunofluorescence staining with monoclonal antibodies including CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD13, CD14, CD19, CD20, HLA-DR, CD33, CD34, CD38 and CD41 (Becton Dickinson, Mountain View, CA, USA). The cells were analysed by flow cytometry (FACScan, Becton Dickinson) and the data represent the mean of triplicate experiments.

RT-PCR assay for PML/RARα fusion transcript

RT-PCR assay for PML/RARα was carried out with both parental UF-1 and UF-1/GMTg SCID cells, as well as control HL-60 cells as previously described (Kizaki et al. 1996a).

FISH analysis

To confirm the cytogenetic findings, fluorescence in situ hybridization (FISH) was performed on slides from the same culture as cytogenetics with specific DNA probes for RARα and PML (Hiorns et al. 1994).

Assays for cellular proliferation and differentiation

Parental UF-1 and UF-1/GMTg SCID cells, as well as NB4 cells, were cultured with all-trans RA (10⁻⁶–10⁻⁸ M) for 4 days. Cellular proliferation was measured using a non-radioactive cell proliferation assay system (MTT assay; Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer’s protocol. For analysis of cellular differentiation, cells were examined by morphometry using Giemsa staining and expression of cell-surface CD11b. Cells were incubated for 60 min with human AB serum (Sigma) to block Fc receptors and then stained with phycoerythrin (PE)-conjugated mouse anti-human CD11b antibody (Becton Dickinson) (Kizaki et al. 1996b).

RESULTS

Inoculation of UF-1 cells into hGMTg SCID mice

RA-resistant human APL cells (UF-1) (1 × 10⁶) were injected either intraperitoneally (i.p.) or subcutaneously (s.c.) into four hGMTg SCID mice and four control B6J SCID mice. Endogenous serum human GM-CSF levels were detected in all hGMTg SCID mice (6640–10 000 pg ml⁻¹) but not in any non-transgenic B6J SCID mice (Table 1). UF-1 cells formed tumours at the injected site as subcutaneous tumours in 4 out of 4 hGMTg SCID mice between days 20 and 59 (Table 1). Subcutaneous tumour, intra-abdominal tumour and ascites were detected in only one mouse with i.p. injection (Table 1). All of the tumours were composed of leukaemic cells; however, no obvious infiltration was observed in the major organs in both hGMTg SCID and control B6J mice (Figure 1A and B, and data not shown). Only 1 out of 4 control mice developed a small subcutaneous tumour: this was found at autopsy (Table 1).

Morphology of UF-1/GMTg SCID cells

Single-cell suspensions from tumours were collected and cultured in RPMI-1640 medium. Cells proliferated without any haematoopoietic growth factors and were designated as UF-1/GMTg SCID cells. UF-1/GMTg SCID cells were similar in morphology to parental UF-1 cells. UF-1/GMTg SCID cells and parental UF-1

Table 2 Reactivity of parental UF-1 cells and UF-1/GMTg SCID cells with monoclonal antibodies

| Monoclonal antibodies (% of positive cells) |
|-------------------------------------------|
| CD3 | CD4 | CD5 | CD7 | CD8 | CD10 | CD11b | CD13 | CD14 | CD19 | CD20 | HLA-DR | CD33 | CD34 | CD38 | CD41 |
|-----|-----|-----|-----|-----|------|-------|------|------|------|------|--------|------|------|------|------|
| Parental UF-1  |     |     |     |     | 5    | 38    | 22   | <1   | 1    | 1    | 91     | <1   | 65   | 1    |
| UF-1/GMTg SCID | 1   | 85  | <1  | 1   | 35   | <1   | 1    | 1    | 8    | 1    | 44     | 1    |

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cells showed large and often lobulated nuclei with a few nucleoli, and contained large azurophilic granules that were compatible with hypergranulocytic promyelocytes (Figure 1C and D).

**Surface marker analysis**

Phenotypic analysis using various monoclonal antibodies in parental UF-1 and UF-1/GMTg SCID cells is summarized in Table 2. Leukaemic cells displayed the same phenotype as parental cells. Both types of cells were positive for CD7, CD13 and CD38. Interestingly, parental UF-1 cells were 91% positive for CD33, whereas UF-1/GMTg SCID cells were only 8% positive (Table 2).

**Cytogenetic and FISH studies**

Cytogenetic analysis of G-trypsin-banded karyotypes was performed on 20 metaphases. Both UF-1/GMTg SCID cells and parental UF-1 cells showed t(15;17) (q22;q11–12) and additional abnormalities of add(1)(q44), add(6)(q12) and add(7)(q36) (Figure 2). To confirm the cytogenetic studies, we performed FISH analysis using metaphase chromosomes from UF-1/GMTg SCID cells. PML/RARα fusion signals were detected in all samples (Figure 3).

**RT-PCR analysis of PML/RAR-α fusion transcripts**

We also examined the expression of PML/RAR-α chimeric transcript in UF-1/GMTg SCID cells by using RT-PCR. The PML/RAR-α transcript was detected in both parental UF-1 and UF-1/GMTg SCID cells, but not in HL-60 cells (negative control). These results were confirmed by subsequent Southern blotting of the PCR products (Figure 4).

**Effects of all-trans RA on proliferation and differentiation of NB4, parental UF-1 and UF-1/GMTg SCID cells**

RA-sensitive NB4 cells, parental UF-1 and UF-1/GMTg SCID cells were incubated with all-trans RA (10⁻¹⁰ to 10⁻⁸ M) for 4 days. All-trans RA inhibited cellular proliferation of NB4 cells in a dose-dependent manner (Figure 5A). By contrast, the absorbance of MTT was changed more gradually in parental UF-1 and UF-1/GMTg SCID cells, suggesting that all-trans RA did not affect cell growth at 10⁻¹⁰ to 10⁻⁸ M RA. However, cell proliferation decreased by 30% and 40%, respectively, after parental UF-1 and UF-1/GMTg SCID cells were exposed to higher concentrations of all-trans RA (10⁻⁶ M) (Figure 5A).

Induction of differentiation of these cell lines into mature granulocytes by all-trans RA was assessed by morphology and expression of CD11b using FACS analysis (Figure 5B). NB4 cells were differentiated towards mature granulocytes by RA, whereas all-trans RA did not cause morphological differentiation of parental UF-1 and UF-1/GMTg SCID cells towards either mature granulocytes or monocytes (data not shown). CD11b expression in NB4 cells was increased by RA in a dose-dependent manner. In contrast, all-trans RA did not alter CD11b expression in UF-1/GMTg SCID cells except at 10⁻⁶ M. These results were similar to those obtained in parental UF-1 cells, suggesting that both parental UF-1 and UF-1/GMTg SCID cells were resistant to induction of cellular differentiation by all-trans RA.

**DISCUSSION**

APL is characterized by the t(15;17) translocation, which fuses the PML gene on chromosome 15 to the RAR-α gene on chromosome 17, and this PML/RAR-α fusion transcript may be involved in the leukaemogenesis of APL (Warrell et al. 1993). All-trans RA is now being used in the treatment of APL as differentiation-inducing therapy (Huang et al. 1988; Warrell et al. 1991; Kanamuru et al. 1995). Although a high proportion of patients with APL achieve complete remission with all-trans RA, most patients will develop early clinical relapse and eventual resistance to retinoids (Warrell et al. 1993). To date, most approaches have not been successful in overcoming RA resistance in patients; thus, a suitable animal model of APL is important.

SCID mice provide a model system to study the biology of human leukaemias and explore the feasibility and efficacy of novel therapeutic approaches to leukaemia. Many studies have
been done to establish a SCID model of human leukaemias, including acute myelocytic leukaemia (AML). However, unexplained graft failures have been observed, and inoculation of myeloid leukaemic cells into SCID mice has generally been less successful than lymphoid leukaemic cell inoculation (Lord et al. 1991; Sawyers et al. 1992; Namikawa et al. 1993). Therefore, several investigations have attempted to improve engraftment of human myeloid leukaemic cells in SCID mice using various haematopoietic growth factors and human fetal tissues (Terpstra et al. 1995). Recently, Lapidot et al. (1994) have reported that primary leukaemic cells injected into sublethally irradiated SCID mice that were treated with cytokines (PIXY321 and SCF) resulted in highly reproducible engraftment in mice. To resolve these problems, we have established transgenic mice producing human GM-CSF with homozygous scid gene (hGMTg SCID mice) (Miyakawa et al. 1993). We have already shown that functional hGM-CSF αβ receptor-transfected Ba/F3 cells formed tumours and invaded organs in this hGMTg SCID model, suggesting that these mice would be a useful model for human myeloid leukaemias. In the present study, we created a human RA-resistant APL SCID mouse model using this system.

The growth pattern of subcutaneously inoculated human primary myeloid leukaemic cells in SCID mice has correlated with clinical outcomes (Yan et al. 1996). APL cells have strikingly low proliferation potential in vitro; therefore, leukaemic cells from patients with APL are more difficult to transplant into SCID mice than other types of myeloid leukaemia (Cesano et al. 1992; Yan et al. 1996). We have previously established and characterized a novel APL cell line (UF-1) with RA-resistant features (Kizaki et al. 1996a). This cell line had an enhanced proliferation in the pres-
has been only one APL mouse model using RA-sensitive NB4 cells and SCID mice (Zhang et al. 1996a). NB4 cells (1 × 10^6 cells) were transplanted into the peritoneum of SCID mice and then appeared as NB4 ascites cells (A-NB4), which differentiated into mature granulocytes by all-trans RA (Zhang et al. 1996a). In contrast to A-NB4 cells, all-trans RA did not change the morphological features and CD11b expression or growth rate of UF-1/GMTg SCID cells, indicating that these cells are resistant to RA. Thus, these mice are the first RA-resistant APL animal model.

UF-1/GMTg SCID cells were positive for CD7, CD13 and CD38 and negative for CD34. These results are similar to parental UF-1 cells (Kizaki et al. 1996a). Interestingly, CD33 and CD14 were expressed in parental UF-1 cells but not in UF-1/GMTg SCID cells. The reason for this finding is unclear. Because CD14 is preferentially expressed in monocyte-like cells, a certain change in cell phenotype might be occurring. In addition, perhaps the CD33-negative leukaemic cells had a growth advantage in vivo during establishment and development of this APL mouse model. It has been reported that leukaemic cell proliferation and high levels of blast colony-forming units (AML-CFU) were observed in CD34-positive cells, and CD34-negative cells were poorly engrafted into SCID mice (Lapidod et al. 1994), suggesting that expression of CD34, but not CD33, is important for engraftment. Consistent with this report, we could not reproducibly inoculate UF-1 cells into control B6J SCID mice. In marked contrast, UF-1 cells were successfully engrafted in human GM-CSF, producing hGMTg SCID mice. We also have successfully transplanted a variety of leukaemic cell lines, including NB4 (promyelocytic leukaemia; Lanotte et al. 1991), UT-7 (megakaryocytic leukaemia; Komatsu et al. 1991) and TF-1 (erythroid leukaemia; Kitamura et al. 1989), into these transgenic SCID mice (data not shown). In particular, UT-7 and TF-1 cells are leukaemic cell lines that require GM-CSF and IL-3 for growth and survival. These results suggest that this mouse system is more adaptive to myeloid leukaemic cells and may be a useful in vivo model of human myeloid leukaemia. The leukaemic cells spreading to the haematopoietic tissues, including bone marrow and peripheral blood, would be an ideal model for human leukaemia. Therefore, further studies and additional treatment of hGMTg SCID mice will be needed to develop an ideal animal model.

In summary, using hGMTg SCID mice, we established a human RA-resistant APL mouse model. In addition to intra-abdominal and ascites manifestations of APL, we successfully transplanted UF-1 cells into hGMTg SCID mice as subcutaneous tumours. Recent clinical and in vitro studies in China have shown that arsenic trioxide is an effective and safe drug in the treatment of APL patients refractory to all-trans RA (Chen et al. 1996). However, no animal studies on arsenic trioxide exist to determine the lethal dose and detailed pharmacokinetics in vivo. Therefore, this RA-resistant APL model will be useful for investigating the development of novel therapeutic strategies including arsenic trioxide and the mechanisms of RA resistance in myeloid leukaemia.

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