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Potential application of dominant negative retinoic acid receptor genes for ex vivo expansion of hematopoietic stem cells

Research Article

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Summary

It is difficult to expand hematopoietic stem cells ex vivo by stimulation with hematopoietic cytokines, since any cytokine thus far tested induces differentiation as well as growth. It is therefore important to consider how to inhibit differentiation of hematopoietic stem cells during ex vivo culture. For this purpose, we have constructed retroviral vectors expressing dominant-negative retinoic acid receptor-α (RARα) genes. The immature hematopoietic cell lines 32D and FDCPmix, transduced with the dominant-negative RARα-expressing vectors, remained blastic or promyelocytic and continued to grow without differentiation even under the differentiation-inducing conditions. This block of differentiation could be overcome by treatment with all-trans retinoic acid, suggesting that the transduced cells still retained their differentiating ability. This leads to the possible application of dominant-negative RARα genes for the ex vivo expansion of hematopoietic stem cells in combination with hematopoietic cytokines. For clinical applications, however, dominant-negative RARα genes should be removed from host cells after ex vivo expansion. We have, therefore, incorporated two loxP sites on either side of the RAR gene in each vector so that removal of the integrated differentiation-blocking RARα genes from the transduced cells could be achieved using the Cre recombinase/loxP system (reversible integration of a gene of interest). We are investigating efficient ways to introduce the Cre recombinase into host cells.

I. Introduction

The possibility of ex vivo hematopoietic stem cell expansion is of interest for both gene therapy and transplantation applications (Dunbar and Young, 1996). Several recent papers describe the so-called “expansion” of human progenitor and stem cells with various combinations of hematopoietic cytokines. Most studies report increases in total cell number, colony-forming units (CFUs), or total $CD34^+$ cells, probably inadequate surrogates for true stem cells. Clinical studies using ex vivo expanded cell populations have not proven maintenance or expansion of either short- or long-term

repopulating ability (Brugger et al., 1995; Holyoake et al., 1997). Gene marking studies in rhesus monkeys indicate that ex vivo expansion of mobilized peripheral blood cells for 10-14 days in the presence of interleukin (IL)-3, IL-6, stem cell factor (SCF), FLT-3 ligand, and stromal cells resulted in no increase in initial engraftment and diminished long-term engraftment (Tisdale et al., 1998). A recent report of graft failure after hematopoietic cell transplantation of ex vivo expanded cells supports the conclusion that "expansion" conditions may differentiate hematopoietic stem cells and damage engrafting cells and that committed progenitors do not contribute to even short-term engraftment (Holyoake et al., 1997). From these studies, for true ex vivo expansion of hematopoietic stem cells, inhibition of differentiation seems to be required during ex vivo culture.

The retinoic acid receptors (RARs) are members of the steroid/thyroid hormone receptors that function as ligand-inducible transcription factors (Evans, 1988). RARs (predominantly RARα) are involved in regulating hematopoiesis. Retinoic acid (RA) induces the HL-60 human leukemia cell line to differentiate into mature neutrophils, and this process is mediated through RARs (Breitman et al., 1980; Collins et al., 1990). In most cases of acute promyelocytic leukemia, the gene of RARα on chromosome 17 is translocated and fused with the PML gene on chromosome 15 (Alcalay et al., 1990; Borrow et al., 1990; De The et al., 1990), and the leukemic cells from these patients can be induced by RA to differentiate into mature neutrophils both in vitro and in vivo (Huang et al., 1988). In this paper, we show that immature hematopoietic cells transduced with dominant-negative RARα-expressing retroviral vectors were not differentiated even under the differentiation-inducing conditions. For possible future applications, the mutant RARα genes need to be removed from the integrated vector sequences after ex vivo culture. We incorporated loxP sites on the retroviral vectors so that the dominant-negative RARα genes could be removed by treatment with the Cre recombinase (Dale et al., 1991; Bergemann et al., 1995).

II. Results

A. Construction of retroviral vectors

We have constructed three retroviral vectors (**Fig. 1**). All vectors are bicistronic and have the neomycin resistance gene (neo) as the second gene. An internal ribosome entry site (IRES) was used for bicistronic expression (Morgan et al., 1992). The first genes are laid between two loxP sites. MXLLneo is the mock vector only containing the neo gene and was used as the negative control vector. MXL403Lneo and MXLELneo have RAR403 and RARE genes, respectively, between two loxP sites as the first genes. Both RAR403 and RARE genes are dominant-negative mutants of the human RARα gene. RAR403 is a C-terminus-truncated form which encodes a peptide of 403 amino acids (Tsai et al., 1993). RARE has a glycine to glutamic acid change at amino acid 303 (Saitou et al., 1994).

B. Expression of dominant-negative RAR genes in transduced cells

Fig. 2A shows the Northern blotting of FDCPmix cells transduced with the mock vector (MXLLneo) or the dominant-negative RARα-expressing vector (MXL403Lneo). The MXL403Lneo vector expressed two forms of the RAR403 gene transcripts (a full length of the message and a spliced form) in the transduced FDCPmix cells, although the mock vector MXLLneo did not. **Fig. 2B** shows the Western blotting of the 32D cells transduced with the mock vector (MXLLneo) or the dominant-negative RARα-expressing vector (MXL403Lneo or MXLELneo). MXL403Lneo expressed the RAR403 (C-terminus-truncated RAR) protein at the molecular weight of 50 kDa and MXLELneo expressed the RAR protein that has a single amino acid residue substituted at the same molecular weight as the intact human RARα protein (55 kDa).

C. Differentiation block of transduced 32D and FDCPmix cells

32D is a murine IL-3-dependent hematopoietic progenitor cell line. IL-3 stimulates growth of 32D cells but granulocyte colony-stimulating factor (G-CSF) stimulates differentiation of 32D cells into neutrophils. As shown in **Fig. 3A**, growth of 32D cells by treatment with IL-3 was not affected whether they were transduced with any of the vectors, MXLLneo, MXL403Lneo, or MXLELneo. However, when 32D cells were transduced with the dominant-negative RARα-expressing vectors (MXL403Lneo and MXLELneo), the 32D cells still continued to grow by treatment with the differentiation-inducing cytokine G-CSF, although the cells transduced with the mock vector (MXLLneo) were differentiated into neutrophils and died by treatment with G-CSF within three weeks (**Fig. 3B**). The 32D cells transduced with the RARE-expressing vector showed less growth for the first two weeks but they grew well thereafter to the same levels as those cells transduced with the other dominant-negative RARα (RAR403)-expressing vector.

FDCPmix is a murine multipotent hematopoietic cell line that is dependent on IL-3 for growth. IL-3 stimulates growth of FDCPmix cells but granulocyte-macrophage

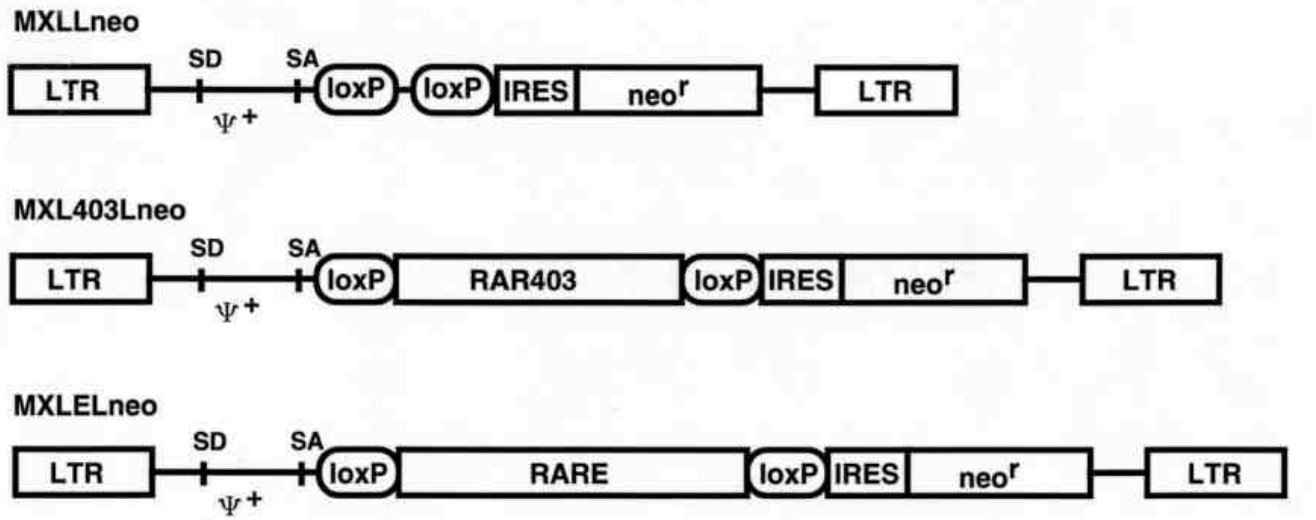
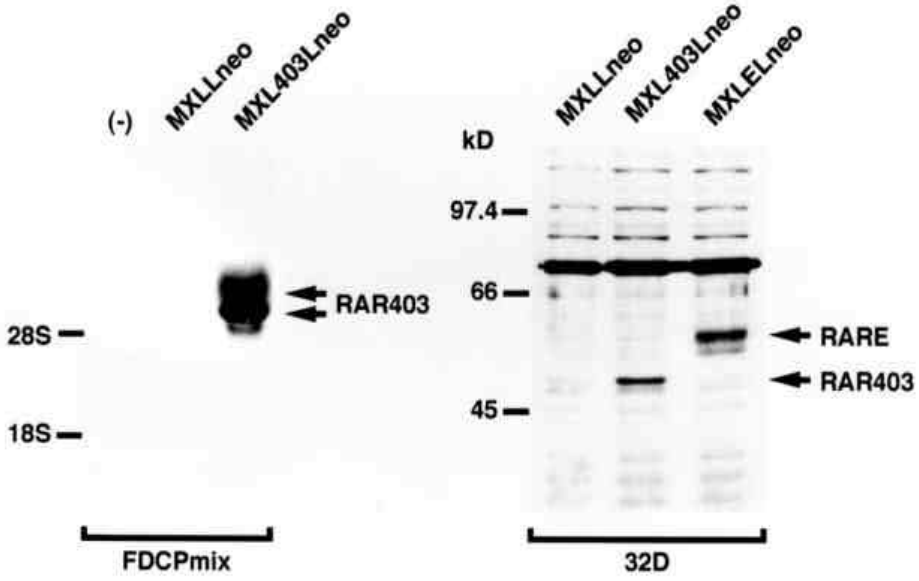


Fig. 1 Schematic structure of three retroviral vectors. All three vectors are bicistronic and have the neomycin resistant gene (neo) as the second gene. An internal ribosome entry site (IRES) was used for bicistronic expression. The first genes are laid between two loxP sites. MXLLneo is the mock vector only containing neo. MXL403Lneo and MXLELneo have the dominant-negative RARα genes, RAR403 and RARE, respectively, between two loxP sites as the first genes.

Fig. 2 Expression of the dominant-negative RARα genes in 32D and FDCPmix cells. (**A, to the left**) Northern blotting of the FDCPmix cells transduced with either MXLLneo or MXL403Lneo vector. Total cellular RNA (20 μg/lane) was applied into an agarose gel, transferred to a membrane, and hybridized with a radiolabeled RARα gene. A full-length and a spliced form of the RAR403 gene were expressed in MXL403Lneo-transduced FDCPmix cells. (**B, to the right**) Immunoblotting of the 32D cells transduced with either MXLLneo, MXL403Lneo, or MXLELneo vector. Total cell lysates (10 μg/lane) were applied into SDS-PAGE, transferred to a membrane, probed with antiserum against the RARα protein. The molecular weight of the RAR403 is 50 kDa and that of RARE is 55 kDa.



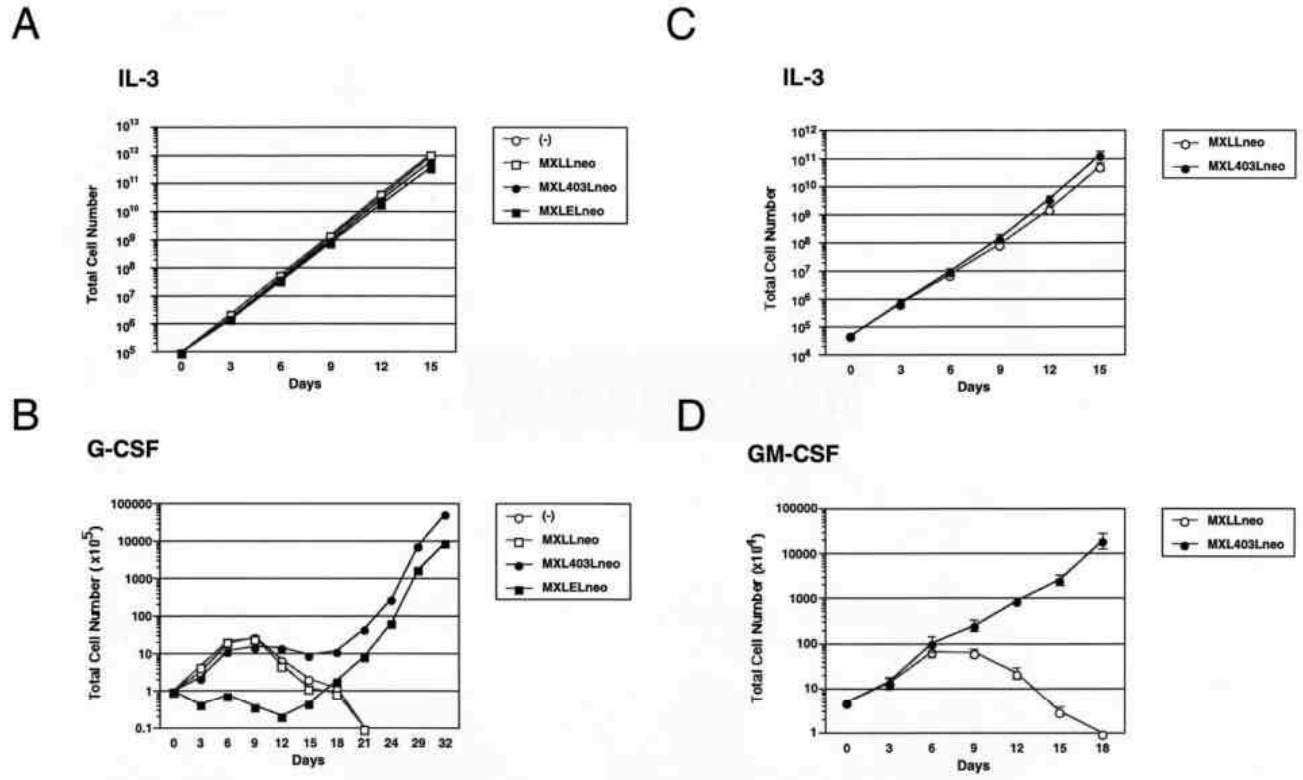


Fig. 3 Growth of the transduced 32D cells after stimulation with the growth-inducing cytokine IL-3 (A) and the differentiation-inducing cytokine G-CSF (B). Growth of the transduced FDCPmix cells after stimulation with the growth-inducing cytokine IL-3 (C) and the differentiation-inducing cytokine GM-CSF (D). Both cells transduced with the dominant-negative RARα-expressing vectors (MXL403Lneo and MXLELneo) were not differentiated but continued to grow under the differentiation-inducing conditions, while cells transduced with the mock vector (MXLLneo) were differentiated into neutrophils and died within 3 weeks under the same conditions.

colony-stimulating factor (GM-CSF) stimulates differentiation of the cells into neutrophils. Similar growth curves were obtained to those of 32D cells. Growth of FDCPmix cells by treatment with IL-3 was not affected whether they were transduced with the mock vector (MXLLneo) or the dominant-negative RARα-expressing vector (MXL403Lneo) as shown in **Fig. 3C**. However, when FDCPmix cells were transduced with the dominant-negative RARα-expressing vector, they continued to grow even in the presence of the differentiation-inducing cytokine GM-CSF, while FDCPmix cells transduced with the mock vector were differentiated into neutrophils and died by day 18 (**Fig. 3D**).

Figs. 4A and **4B** show the morphological changes of 32D and FDCPmix cells, respectively, which were transduced with the mock vector (MXLLneo) or the dominant-negative RARα-expressing vectors (MXL403Lneo and MXLELneo) under the differentiation-inducing conditions. The 32D and FDCPmix cells transduced with the mock vector were differentiated into neutrophils by day 15 and the cells died by day 24 under the differentiation-inducing conditions. However, the 32D and FDCPmix cells transduced with the dominant-negative RARα-expressing vectors remained blastic or promyelocytic cells on day 24 and did not show neutrophilic differentiation even under the differentiation-inducing conditions. There were no differences in morphological changes between the cells transduced with the vectors expressing the two forms of the dominant-negative RARs (RAR403 and RARE).

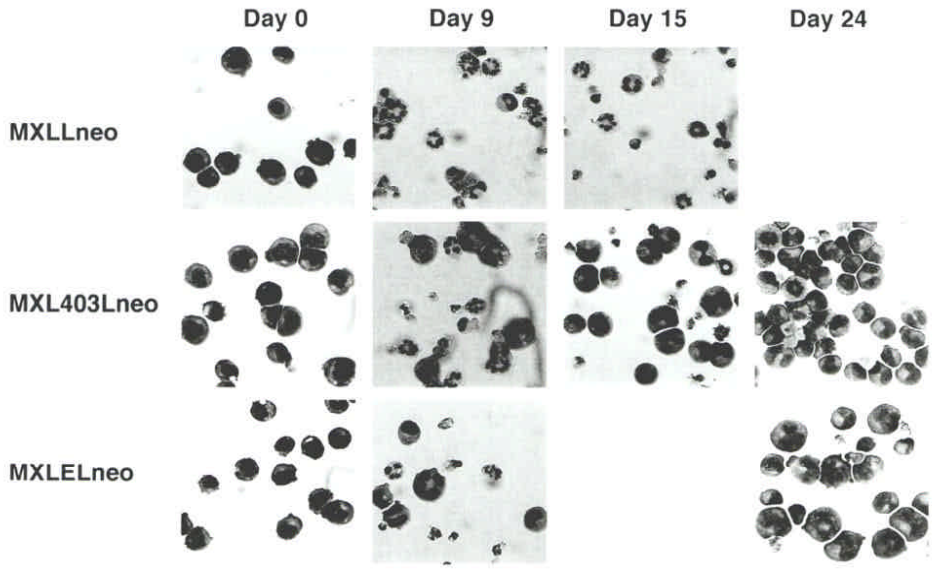


Fig. 4 (**A**) Morphology of transduced 32D cells after stimulation with the differentiation-inducing cytokine G-CSF. (**B**) Morphology of FDCPmix cells after stimulation with the differentiation-inducing cytokine GM-CSF. Both cells transduced with the dominant-negative RARα-expressing vectors (MXL403Lneo and MXLELneo) remained blastic or promyelocytic cells under the differentiation-inducing conditions, while cells transduced with the mock vector (MXLLneo) were differentiated into neutrophils and died by day 24 under the same conditions.

Fig.4A

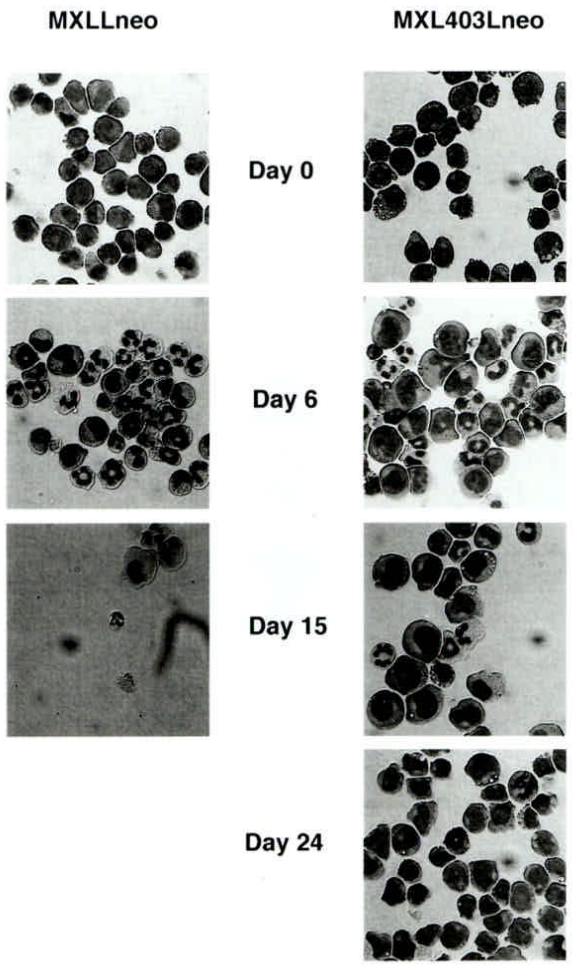


Fig. 4B

D. Effect of ATRA on differentiation block

The block of differentiation of the transduced 32D cells into neutrophils could be overcome by treatment with all-trans retinoic acid (ATRA). **Fig. 5** shows that ATRA-induced neutrophilic differentiation of 32D cells which were transduced with the dominant-negative RARα–expressing vector (MXLELneo). The half-optimal concentration was about 1 μM and the optimal concentration was 10 μM. For comparison, the serum concentration of RA was estimated to be 1-10 nM.

III. Discussion

Several groups have reported that dominant-negative RARs block differentiation of hematopoietic progenitor cells into neutrophils (Tsai et al., 1993; Saitou et al., 1994; Tsai et al., 1994). We have also confirmed that the dominant-negative mutants of the human RARα gene inhibited the differentiation of 32D and FDCPmix cells into neutrophils by using bicistronic retroviral vectors. In these vectors, the mutant RARα genes were laid between two loxP sites so that they could be removed by addition of the Cre recombinase, and a therapeutic gene could be placed as the second gene instead of neo. We have used in this paper two dominant-negative forms of the RARα (RAR403 and RARE) genes. RAR403 is the prototype of the dominant-negative RARα, which is the C-terminus-

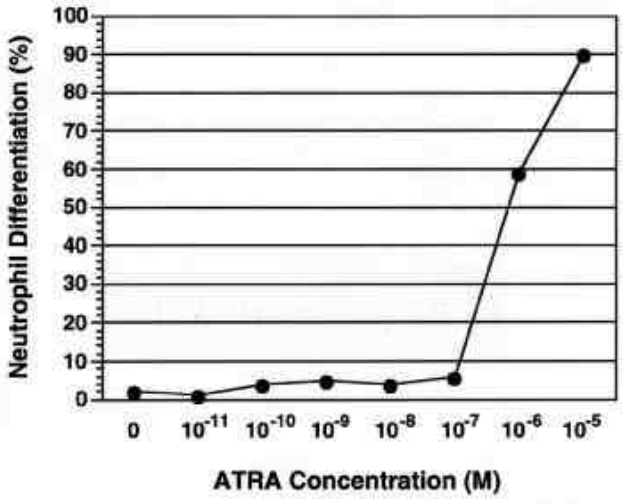


Fig. 5 All-trans retinoic acid (ATRA)-induced neutrophilic differentiation of the transduced 32D cells. 32D cells were transduced with the dominant-negative RARα (RARE)-expressing vector to allow the cells to grow without differentiation even in the presence of the differentiation-inducing cytokine G-CSF. These cells could be differentiated by treatment with ATRA. The horizontal axis shows the concentrations of ATRA and the vertical axis shows the ratio of neutrophils to all cells.

deleted form (Tsai et al., 1993). RARE is another dominant-negative form which has a single amino acid residue substituted (Saitou et al., 1994). Both of them could inhibit the differentiation of 32D and FDCPmix cells into neutrophils. There were no significant differences between the two dominant-negative forms in terms of their ability to block differentiation.

Tsai et al. showed that the expression of RAR403 in normal mouse bone marrow cells leads to a differentiation block in the neutrophil lineage at the promyelocytic stage (Tsai et al., 1993). They also showed that murine lymphohematopoietic progenitors, immortalized by a retroviral vector expressing RAR403, proliferate as an SCF-dependent clonal line that spontaneously generates pre-pro-B lymphocytes and myeloid progenitors. The developmental blocks imposed by the dominant-negative RARα are mapped to the pre-CFU-GM as well as to the neutrophilic promyelocyte stages (Tsai et al., 1994). The block to CFU-GM formation may increase the probability of self-renewal of hematopoietic stem cells.

Therefore, the strategy to use dominant-negative RARs might be applied to ex vivo expansion of hematopoietic stem cells in combination with some cytokines such as SCF, FLT-3 ligand, and thrombopoietin. These cytokines are able to stimulate the proliferation of immature hematopoietic cells but they also stimulate the differentiation of these cells to some degree. Dominant-negative RARs possibly inhibit differentiation but will not inhibit growth of the cells induced by these cytokines. However, this strategy needs to be examined in vivo, since there are no in vitro assays to determine exactly whether or not hematopoietic stem cells can be expanded.

The differentiation ability of cells transduced with dominant-negative RARα-expressing vectors should be restored after the ex vivo culture period for clinical applications. By treatment with ATRA, the differentiation block was overcome, leading to the production of mature hematopoietic cells as shown in **Fig. 5**, suggesting that the transduced cells still retained the ability to differentiate. The vectors in this study were designed so that the mutant RARα gene sequences could be removed from host cells after ex vivo culture. The vectors have loxP sites and the mutant RARα genes can be eliminated by treatment with the Cre recombinase. We are now examining methods to remove the mutant RARα genes from the integrated vector sequences in the transduced cells. For efficient removal of the genes inserted between loxP sites, high transfer efficiency of the Cre recombinase gene would be necessary although its transient expression would suffice.

IV. Materials and Methods

A. Cells and reagents

Ecotropic packaging cell line BOSC23 cells were maintained in Dulbecco's modified essential medium (Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (Filtron, Brooklyn, Australia). 32D cells were maintained in RPMI 1640 medium (Life Technologies) containing 10% fetal calf serum and 50 U/ml murine IL-3. FDCPmix cells were maintained in Fisher's medium (Life Technologies) containing 20% horse serum and 100 U/ml murine IL-3. Human recombinant G-CSF was provided from Chugai Pharmaceutical (Tokyo, Japan). Murine GM-CSF was purchased from Life Technologies. Supernatant from C3H10T1/2 cells transfected with the mouse IL-3 expression plasmid was used as a source of murine IL-3 and it contains 10,000 U/ml murine IL-3. All-trans retinoic acid (ATRA) was purchased from Sigma (St. Louis, MO).

B. Retroviral plasmids

The RAR403 gene (EcoRI-NheI fragment from pCMX403; Tsai et al., 1993) was subcloned into the EcoRI and SmaI site of pBS SK+ (pBS403). The EcoRI-BamHI fragment of pBS403 containing the RAR403 gene was inserted between two loxP sites of plox[2] (provided by Dr. J. D. Marth; Orban et al.,

1992)(pL403L). The HindIII-SmaI fragment containing loxP-RAR403-loxP of pL403L was inserted into the cloning site (HindIII-NotI site) of the retroviral plasmid pMX (provided by Dr. T. Kitamura; Onishi et al., 1996) (pMXL403L). The IRES-neo sequence (XbaI-XhoI fragment of p1.1cIneo; Kodaira et al., 1998) was inserted into the SalI site of pMXL403L (pMXL403Lneo). pMXLLneo was obtained by removal of the RAR403 gene (BamHI-BamHI fragment) from pMXL403Lneo. pMXLELneo was obtained by inserting the RARE gene (EcoRI-BamHI fragment of pCMXRARE; Saitou et al., 1994) into the BamHI site of pMXLLneo. The dominant-negative RARα (RAR403 and RARE) genes originated from the human RARα gene. All restriction enzymes were purchased from Takara Shuzo (Shiga, Japan).

C. Retroviral vectors

BOSC23 was transfected with retroviral plasmids by lipofectamine (Life Technologies) according to the manufacturer's protocol. Two or three days after transfection, supernatants were harvested and filtered. They were used as ecotropic retroviral vectors.

D. Transduction

Six-well plates were coated with retronectin (provided by Takara Shuzo) at the concentration of 96 μg/ml for 2 hr followed by blocking with 2% bovine serum albumin fraction V (Sigma) for 30 min. Cells were suspended in viral supernatants at the density of $2x10^5$/ml and 1 ml of cell suspension was added into each well. After 2-hour incubation at 37°C, another 1 ml of viral supernatants was added into each well. G418 selection (300 mg/ml active for FDCPmix cells and 800 μg/ml active for 32D cells) was started 24 hours after infection.

E. Growth assays and morphological examination

Bulk 32D cells that were transduced and G418-resistant were resuspended in RPMI 1640 medium containing 10% fetal calf serum and 10 ng/ml human G-CSF for the differentiation-inducing conditions, or in RPMI1640 medium containing 10% fetal calf serum and 50 U/ml murine IL-3 for the regular (proliferation-inducing) conditions. The cells were plated in 12-well dishes at the density of 1×10^5 cells per well. Cells were counted and replated at the density of 1×10^5 cells per well every three days. Bulk FDCP cells that were transduced and G418-resistant were suspended in Fisher's medium containing 20% horse serum, 5 ng/ml murine GM-CSF and 1 U/ml murine IL-3 for the differentiation-inducing conditions, or in Fisher's medium containing 20% horse serum and 100 U/ml murine IL-3 for the regular (proliferation-inducing) conditions. The cells were plated in 12-well dishes at the density of 5×10^4 cells per well. Cells were counted and replated at the density of 5×10^4 cells per well every three days. Cells were stained with Wright-Giemsa and were observed under a microscope.

F. ATRA-induced differentiation

32D cells were transduced with the MXLELneo vector. When the cells grew in an exponential manner two or three weeks after transduction, ATRA was added to the culture medium at various concentrations. Forty-eight hours after treatment with ATRA, the cells were examined under a microscopy.

G. Antiserum

The domain A (the N-terminal region) of the human RARα gene was amplified by PCR. The primers were 5'-ATT GGA TCC ATG GCC AGC AAC AGC AGC TCC and 5'-TCA GAA TTC GGC TGG GGA TGG TGT GCT ATA. The PCR product was inserted into pGEX and the plasmid coding to the GST-fusion protein containing the domain A of the RARα was transformed into BL21 strain of *Escherichia coli*. The resulting transformants were induced with isopropyl-1-thio-β-D-galactopyranoside to produce a GST fusion protein. The bacteria were collected by centrifugation and resuspended in buffer containing 20 mM Tris-HCl pH 7.4, 50 mM ethylenediaminetetraacetic acid, 150 mM NaCl, and 1% Triton X-100. Vigorous sonication was performed followed by centrifugation. The GST fusion protein was purified through glutathion beads columns (Pharmacia, Piscataway, NJ). Rabbits were immunized against the GST-fusion protein and antiserum against the RARα protein was prepared.

H. Immunoblotting

Cells were lyzed with buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 100 IU/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Ten μg of cell lysates was loaded into each well of 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were probed with antiserum against the RARα protein using an ECL Western blotting detection kit (Amersham, Piscataway, NJ) according to the manufacturer's protocol.

I. Northern blotting

Total cellular RNA was extracted with an RNA extraction kit Isogen (Nippon Gene, Tokyo, Japan). Twenty μg of RNA was loaded into each well of agarose gels and transferred onto HybondN+ (Amersham). Membranes were hybridized with a radiolabeled human RARα gene (the EcoRI-BamHI fragment of pCMXhRARα; Saitou et al., 1994). Radiolabeling of a probe was performed by using a DNA labeling kit (Amersham).

Acknowledgments

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