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Characterization of Two Novel Retinoic Acid-resistant Cell Lines Derived from HL-60 Cells Following Long-term Culture with all-*trans*-Retinoic Acid

Junichirou Mori, Satoru Suzuki,[1] Masahiro Hara, Atsuko Kaneko, Koh Yamashita, Mieko Kumagai, Takahiro Sakuma, Tomoko Kakizawa, Masanori Yamazaki, Teiji Takeda, Takahide Miyamoto, Kazuo Ichikawa and Kiyoshi Hashizume

Department of Aging Medicine and Geriatrics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621

Either all-*trans*-retinoic acid (RA) or vitamin D3 (VD) induces differentiation of the myeloid leukemia cell line HL-60. RA is available for the treatment of acute promyeloleukemia, although the development of resistance to the agent is a serious problem for differentiation-inducing therapy. To approach the mechanisms of resistance to RA, we developed two novel cell lines, HL-60-R2 and R9, which were subcloned after exposure to increasing concentrations of RA. The growth rate of HL-60-R2 cells was significantly increased by RA treatment, whereas the growth rate of HL-60-R9 was not affected. RA induces apoptosis in the parental HL-60 cells. The number of apoptotic cells, however, was not increased and nitroblue tetrazolium (NBT) reduction was not altered by 1 μM RA in either of the cloned cell lines. Treatment with VD induced monocytic differentiation and increased the expression of CD11b in HL-60 and HL-60-R9 cells, but not in HL-60-R2 cells. Flow cytometric and G-banding analysis demonstrated that R2 cells were near-triploid. The sequencing analysis revealed a deletion of three nucleotides in the sequence of the *RAR*α gene in HL-60-R9 cells, resulting in deletion of codon 286. No mutation was found in HL-60-R2 cells. Taken together, these data indicate that the resistance to RA is caused by the mutation in RARα of HL-60-R9, but by other factor(s), which also affect the VD-response pathways, in HL-60-R2. The abnormal response to VD may be associated with the abnormal ploidy of the R2 cells.

Key words: Retinoic acid — Resistance — Vitamin D — Mutation — HL-60

The basic machinery for cell growth appears to be present in essentially all mammalian cells at all times, but activation and inactivation of the cell division cycle, apoptosis and differentiation are regulated by many different signals that originate from both intracellular and extracellular factors.[1)] Numerous agents which induce differentiation have been reported.[2)] all-*trans*-Retinoic acid (RA), 3,5,3′-triiodo-L-thyronine (T3), and vitamin D3 (VD) are ligands for steroid receptor superfamily members, which have crucial roles in the development of cells, including morphogenesis and apoptosis.[3)]

RA induces cells to mature along the granulocyte pathway, while the monocyte-macrophage lineage is developed by VD.[4)] RA co-operates with VD to promote differentiation of HL-60 cells along the monocyte pathway of maturation.[5)] We previously reported that thyroid hormone alone does not affect the differentiation of HL-60 cells, but potentiates RA-induced apoptosis during differentiation of the cells.[6)] Taken together, it is suggested that the action of RA interacts with that of T3 or VD along the pathways of maturation of HL-60 cells.

Although the RA-induced apoptosis may be due to factors initiated through the two classes of retinoid receptors (RARs and RXRs), the precise molecular mechanisms are still under investigation.[7,8)] The specific translocation of acute promyeloleukemia t(15:17) has been shown to result in a fusion protein between the *RAR*α gene and a gene called *PML*, suggesting that abnormal proliferation and apoptosis arise through the abnormal function of the receptors for RA.[9)] Clinically, RA is used to treat acute promyeloleukemia, but the development of resistance can make it difficult to achieve complete remission.[10)]

To approach the molecular mechanisms of RA-mediated apoptosis, differentiation and proliferation, we initially focused on the development of retinoic acid resistance by HL-60 cells. We established RA-resistant cells and characterized the new phenotypes of these cells by cell counting, nitroblue terazolium (NBT) reduction assay, estimation of the expression of CD11b as a marker of differentiation, cell cycle analysis, cytogenetic studies, and molecular sequencing of RARα. The response to VD was also examined in the RA-resistant cells.

MATERIALS AND METHODS

Chemicals 1,25-Dihydroxyvitamin D_3 was purchased from Solvay Duphar B.V., Weesp, The Netherlands. all-*trans*-Retinoic acid and Actinomycin D were obtained from Sigma Chemical Co., St. Louis, MO. The agents

[1] To whom correspondence should be addressed.
E-mail: soutaro@hsp.md.shinshu-u.ac.jp

were dissolved in 100% ethanol. The final concentration of ethanol was adjusted to 0.1% in all dishes. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma Chemical Co.

Establishment of RA-resistant cells HL-60 cells were obtained from RIKEN Cell Bank (Tsukuba). We cultured the cells in RPMI medium with 10% fetal calf serum for 2 weeks. The parental cultures were propagated in dishes, with passaging and feeding twice weekly to maintain log phase growth. During the stepwise development of resistance, only cells which did not adhere to the flask walls were passaged. Resistance to RA was initiated in this series by exposure of HL-60 cells to 100 p*M* RA and passaging, every 4–5 days, cells which did not adhere to the dish walls. At approximately 2-month intervals the concentration of RA was increased stepwise. The time at each concentration of RA was considered sufficient when the cells were able to proliferate in the presence of 1 μM RA without adherence to the dish walls and at rates approximating to the doubling time of the parental cells in RA-free medium. Six cell lines were subcloned by using the limiting dilution method in the presence of 1 μM RA. Prior to starting the experiments, we grew sufficient cells and stocked them frozen in liquid nitrogen. All of the experiments were performed within 2 months (20 passages) after thawing the frozen stock at 37°C.

Cell counts Prior to incubation with the indicated ligands, cells were distributed equally into 12-well dishes at 1×10^4 cells/ml. The number of cells was counted after staining with trypan blue. Cells were scored as apoptotic if there was evidence of nuclear pycnosis and fragmentation, cytoplasmic condensation, and basophilia, as previously described.[6] Morphological assessment of apoptosis was performed in a blinded fashion, without revealing the initial treatment assignment. Differential counts (at least 200 cells/sample) were performed on cytospin preparations.

NBT reduction assays NBT reduction assays were performed as previously described, with minor modifications.[6] Briefly, the cells were grown in the medium with the indicated concentrations of agents for 5 days, and 2×10^5 cells were aliquoted into 0.2 ml of RPMI medium with 0.8 ml of 0.125% NBT and 200 n*M* 12-O-tetradecanoylphorbol-13-acetate. The cells were incubated at 37°C for 30 min, centrifuged at 1100*g* for 7 min at room temperature, and resuspended in 200 μl of phosphate-buffered saline. Cytospins from aliquots of the samples were stained with Safranin-O (for 5 min). The percentage of NBT-positive cells in each preparation was determined.

Flow cytometric analysis Cell cycle analyses were performed as described on samples permeabilized with 70% ethanol, treated with 2 mg/ml RNase A, and stained with 50 μg/ml of propidium iodide (PI).[11] Cells were analyzed by fluorescence-activated cell sorting (FACS) flow cytometry (Becton Dickinson, San Jose, CA) using the reagents and procedures recommended by the manufacturer. Proportions of cells in G1, S, and G2/M phases of the cell cycle were analyzed using Mod-Fit software (Verity Software House Inc., Maine). The expression of CD11b was estimated by flow cytometry after incubation with phycoerythrin (PE)-labeled anti-human CD11b antibodies (Phamingen, San Diego, CA) for 30 min at 4°C.

Cytogenetic analysis HL-60 and resistant cells in the logarithmic growth phase were incubated with colchicine (Colcemid, Gibco BRL, Gaithersburg, MD) at a final concentration of 0.016 μg/ml of medium for 2 h. The cells were treated with 0.05 *M* KCl for 20 min at room temperature and fixed with methanol:acetic acid (3:1). Preparations were stained by using a modification of the trypsin-Giemsa banding method, and G-banded metaphases were photographed and analyzed.[12]

DNA sequencing DNA was extracted from HL-60 and resistant cells by using a DNA extractor kit (Wako, Osaka) according to the manufacturer's protocol. Oligonucleotide primers for exons 7, 8, and 9 of the *RAR*α gene for polymerase chain reaction (PCR) amplification were synthesized as described by Morosetti *et al*.[13] Total RNA was extracted by using a Mini kit (Quiagen, Valencia, CA). The sequence of the ligand-binding domain of retinoid X receptor (RXR) was carried out by direct sequencing after amplification of the region between nucleotides 673 and 1492, by using the reverse transcriptase (RT)-PCR method. The RT reaction was carried out using 1 μg of total RNA, 100 pmol of random hexamer, 10 U of RNase inhibitor, 200 U of MMLV-RT, and deoxynucleotides (dNTPs, final concentration 0.5 mmol/liter each) in a total volume of 20 μl under the conditions recommended by the suppliers. After cDNA synthesis, 40 cycles of PCR were sequentially carried out. The primers used in this study were as follows:

5′-primer, 5′-ATGAAGCGGGAAGCCGTGCAGGAG-3′;
3′-primer, 5′-GGTGGGCACAAAGGATGGGCCCGC-3′.

Data collection and analysis were performed on an automated DNA sequencer (model 310, Applied Biosystems, Foster City, CA).

Statistical analysis All results are expressed as mean± SD of the indicated determinations. Statistical significance was determined by an analysis of variance followed by the Bonferroni multiple comparison test; asterisks denote significant differences from the results for cells treated with 0.1% ethanol as a control ($P<0.05$).

RESULTS

Characterization of cell growth (Fig. 1) We cloned six cell lines from the parental HL-60 cells. Five-day treatment with 1 μM RA decreased the growth rates in four clones out of six (Fig. 1A). In either HL-60 R2 or R9, five-day treatment with 1 μM RA did not significantly

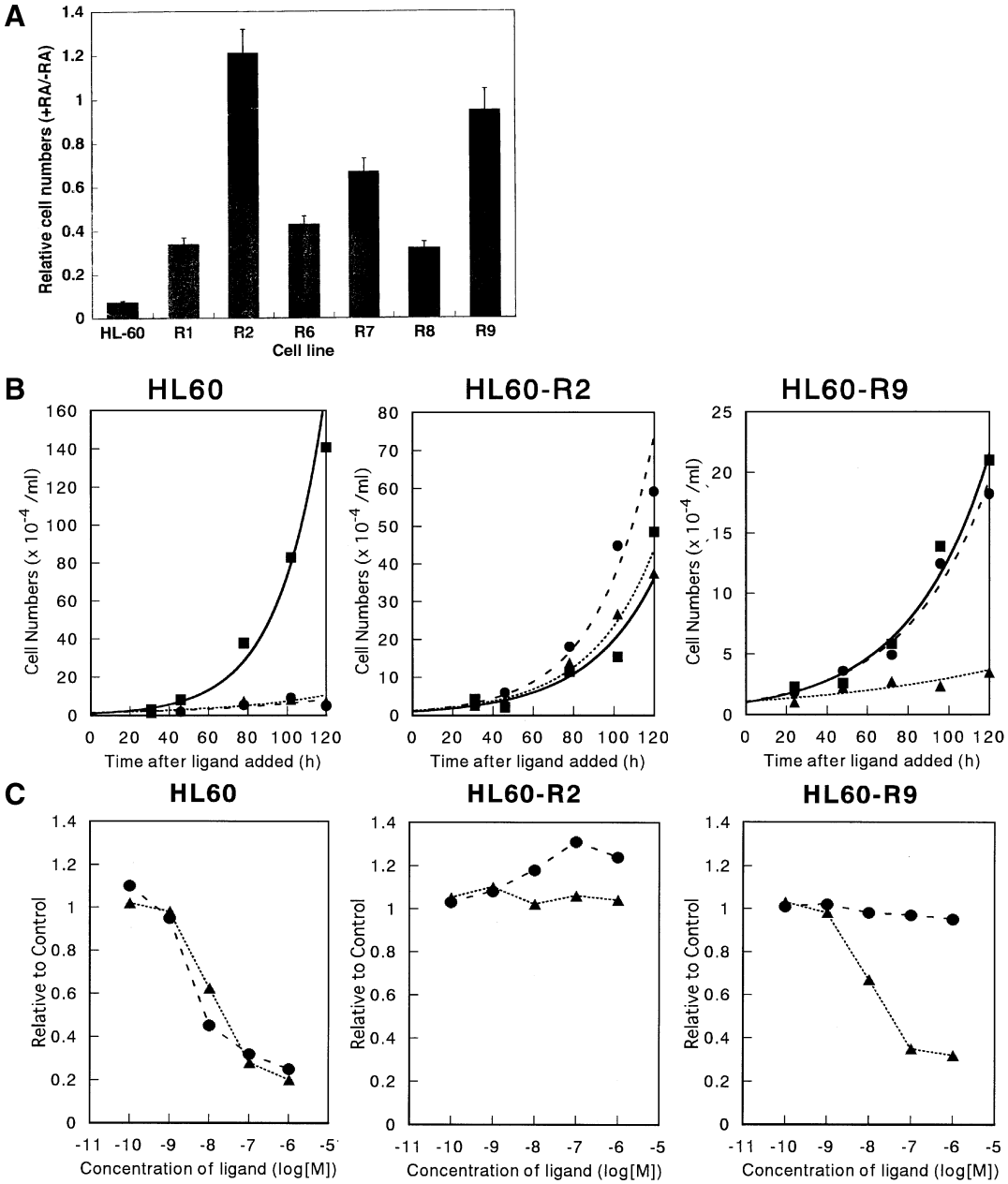

Fig. 1. (A) Effect of 5-day incubation with 1 μM RA on cell numbers of each cell line. Data are presented as the ratio of the cell number recovered after incubation with 1 μM RA relative to that after incubation with 0.1% ethanol for 5 days. The values are the mean±SD of three independent experiments. HL-60, the parental cells; R, resistant cells cloned in this study. (B) Growth curves for HL-60, R2 and R9 cells treated with or without 1 μM RA or VD. RA or VD was added at 0 h, and cell numbers were determined every 24 h. The counting of cell numbers was performed in a blind fashion, without knowledge of the initial treatment assignment. All results were expressed as the mean of five independent experiments, each performed in triplicate. Representative exponential curves calculated from the data are presented. Closed squares, circles, and triangles represent the numbers of HL-60, R2 or R9 cells recovered after treatment with 0.1% ethanol, 1 μM RA, and 1 μM VD, respectively, for the indicated hours. Bold, broken, and dotted lines denote the exponential curves calculated from the counts of the cells recovered after treatment with 0.1% ethanol, 1 μM RA, or 1 μM VD, respectively. Significant positive correlations were observed in all cell lines studied (r>0.9). (C) The effects of various concentrations of RA and VD were evaluated in HL-60, R2 and R9 cells. After 5 days of incubation with the indicated concentrations of compounds, the number of cells was counted. All data were corrected for the number of cells treated with 0.1% ethanol as the control. Closed circles and triangles represent the numbers of cells treated with RA and VD, respectively. The values are the means of three separate experiments. The ligand concentrations are given on the abscissa, and the relative number of cells on the ordinate.

decrease the growth rate (Fig. 1B). Doubling times of R2 and R9 were calculated by counting the cell numbers after staining with trypan blue every 24 h. There was no difference between the growth of cells treated with and without 0.1% alcohol for 5 days as a control (data not shown). The doubling time of either HL-60-R2 or R9 is longer than that of the parental HL-60 cells in the absence of the ligands studied (Table I). RA did not significantly alter the doubling time of HL-60-R9 cells, and the growth rate of HL-60-R2 significantly increased (rather than decreased) in the presence of 1 μM RA. Treatment with VD decreased the growth of the parental HL-60 cells and HL-60-R9, whereas no effect was observed in HL-60-R2 cells. In the parental HL-60 cells, treatment with either RA or VD suppressed the cell number in a dose-dependent manner (Fig. 1C). A dose-dependent increment was observed when we added RA at concentrations between 100 pM and 100 nM, but treatment with VD did not affect the number of R2 cells. In R9 cells, VD suppressed the cell number in a dose-dependent manner, but RA did not.

Microscopical findings Exponentially growing cultures of HL-60 had frequent mitotic figures and morphological features characteristic of a blastic leukemic cell line (Fig. 2). RA-induced differentiation of HL-60 cells was associated with the appearance of apoptotic cells (arrowhead). Apoptotic HL-60 cells could be recognized by the appearance of nuclear condensation and fragmentation, and a marked decrease in cellular volume. Microscopically, more than 90% of the parental cells possessed segmented nuclei after incubation with 1 μM RA for 5 days, indicating that most of the treated cells had differentiated (data

Table I. Effect of RA or VD on Doubling Time

Cell line	Doubling time (h)		
	0.1% ethanol	1 μM RA	1 μM VD
HL-60	16.2±1.8	40.3±3.7	36.2±3.1
HL-60-R2	23.2±2.5	19.1±1.8*	22.2±2.5
HL-60-R9	27.4±2.6	28.5±2.3	69.3±7.1

Doubling times were calculated by using the "curve fit program" in Delta graph software (Polaroid, Tokyo). All results are the mean±SD of five independent experiments, each performed in triplicate. Asterisk denotes a statistically significant difference between the doubling times of HL-60-R2 cells treated with 0.1% ethanol and 1 μM RA (P=0.0144).

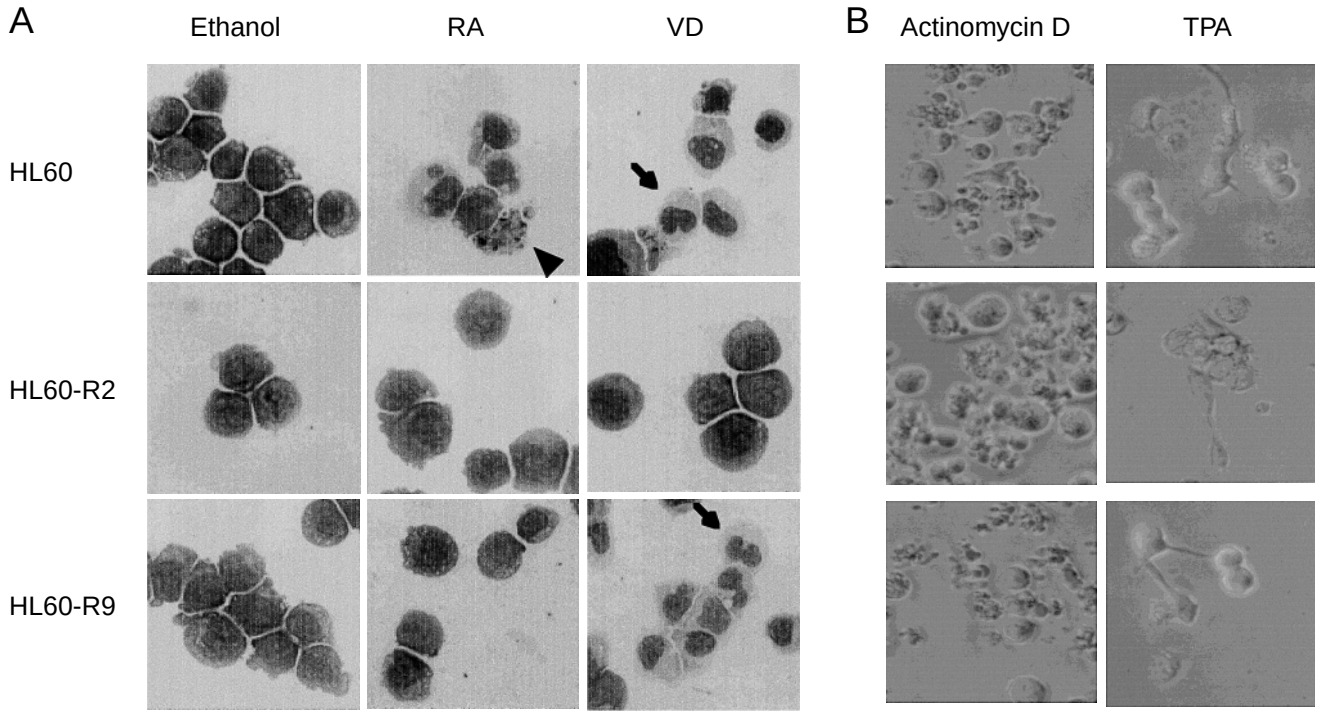

Fig. 2. (A) Effects of RA or VD on the morphology of the cell lines studied. Five days after incubation with 0.1% ethanol or 1 μM of the indicated ligands, 0.2 ml of cells was taken to make cytospin preparations, which were fixed in 100% methanol, and stained with May-Giemsa solution. The arrowhead shows a typical apoptotic cell with fragmented nuclei. The arrows show typical monocytes. Magnification ×400. (B) Effects of Actinomycin D and TPA on the morphology of the cell lines. The cells studied were incubated with 1 μg/ml Actinomycin D for 6 h or 10 nM TPA for 24 h. Phase contrast microscopic findings are shown. Magnification ×400.

not shown). VD induced the differentiation of monocyte-macrophage lineage in HL-60 and R9 cells (arrows). The ligands we studied did not affect the HL-60-R2 cells morphologically. In R9 cells, treatment with RA had no effect. When the two sublines were exposed to 1 μg/ml Actinomycin D, the induction of cell death was observed within 6 h, as in the parental cells (Fig. 2B). Morphologically, 10 n*M* TPA increased the number of attached cells in the parental, R2 and R9 cell lines. As shown in Fig. 3A, the number of apoptotic cells was not increased by treatment with RA in either HL-60-R2 or R9. VD did not affect the induction of apoptosis in any of the cell lines studied.

NBT reduction assays and CD11b expression There was little evidence of spontaneous differentiation, as judged from the low frequency of parental HL-60 cells competent to reduce the NBT dye. Morphologic differenti-

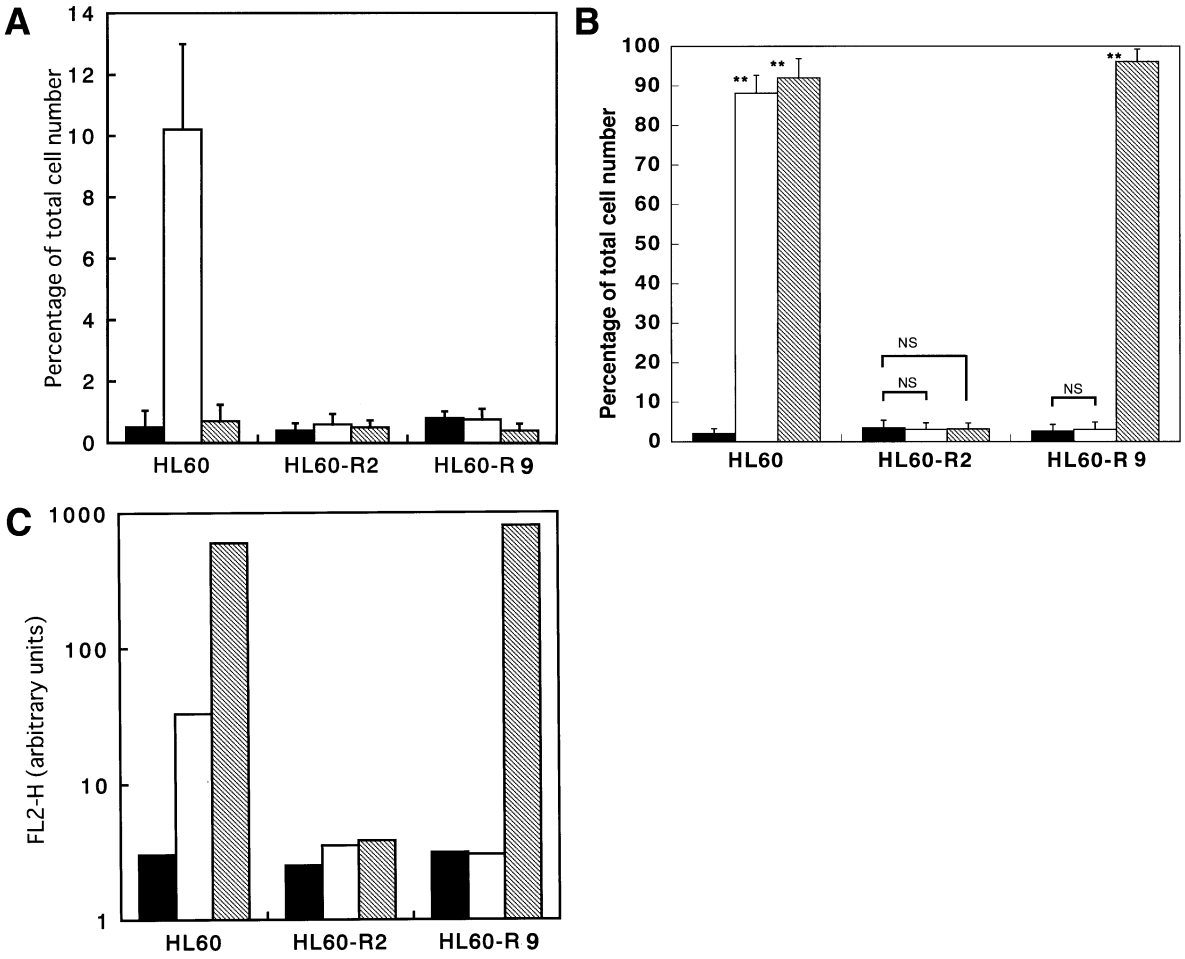

Fig. 3. (A) Effect of RA or VD on the induction of apoptosis in HL-60, R2, or R9 cells. Morphological assessment of apoptosis was performed in a blinded fashion, without knowledge of the initial treatment assignment. Differential counts (at least 200 cells/sample) were performed on the cytospin preparations in Fig. 2. Results are expressed as percent of total counts of the cells. All results are the mean±SD of three independent experiments, each performed in duplicate. (B) Effect of RA or VD on the activity of NBT reduction. Five days after incubation with 0.1% ethanol, or 1 μM of the indicated ligands, cells were recovered. After incubation with NBT and TPA, cells were resuspended in PBS to make cytospin preparations, which were stained with Safranin-O. Black- or blue-staining cells were counted as positive. The percentage of NBT-positive cells in each preparation was determined. All results are the mean±SD of three independent experiments, each performed in duplicate. Asterisks denote statistically significant differences from the results for the cells treated with 0.1% ethanol as the control (P<0.01). NS represents no significant difference. (C) Effect of RA or VD on the expression of CD11b on the surface of HL-60, R2, or R9 cells. Five days after incubation with 0.1% ethanol or 1 μM of the indicated ligands, cells were recovered. The expression of CD11b was estimated by flow cytometry after incubation with phycoerythrin (PE)-labeled anti-human CD11b antibodies for 30 min at 4°C. Twenty thousand cells were counted, and the mean fluorescence intensities were calculated. The results are the mean of two independent experiments.

ation was paralleled by an equivalent increase in the number of NBT^+-HL-60 cells (Fig. 3B). None of the ligands studied induced NBT reduction by HL-60-R2 cells. RA treatment did not increase the number of NBT^+-R9 cells. VD induced NBT^+ cells significantly in HL-60 and R9 cell lines. Expression of the CD11b is up-regulated during differentiation to either granulocytes or monocytes.[14] As shown in Fig. 3C, the treatment with RA did not induce the expression of CD11b in either HL-60-R2 or R9 cells. The expression of CD11b in HL-60-R9 was augmented by the treatment with VD, whereas the expression was not altered in HL-60-R2 cells.

Cell cycle analyses The proportions of cells in the different phases of the cell cycle were determined by flow cytometry. RA increased the fraction of G0-G1 phases and decreased that of S phase, suggesting that RA induced G1 arrest in HL-60 cells (Fig. 4). Neither RA nor VD affected the cell cycle of HL-60-R2. VD induced G1 arrest in HL-60 and R9, but not in HL-60-R2. The mean G1 DNA content in HL-60-R2 was approximately 1.6 times higher than the G1 DNA content of the parental HL-60 cells.

Cytogenetic analysis Ten metaphases were photographed and karyotyped. Representative karyotypes of HL-60 and HL-60-R2 are shown in Fig. 5. The modal chromosome number was consistently near triploid in HL-60-R2 cells. The general findings for R9 cells were essentially the same as those of the parental cells (data not shown). The structural abnormalities mostly involved chromosomes 9, 10, 14, and 16 in HL-60, R2, and R9 cells. t(15;17) was not observed in the parental HL-60 cells or resistant cells that we studied.

Sequence analyses of RARα and RXRα Since the actions of RA are initiated through RARs and RXRs, we sequenced the ligand-binding domains of RARα and RXRα in R2 cells, and of RARα in R9 cells. No mutation was found in R2 cells. There were deletions between nucleotides 856 and 858 in the sequence of RARα in R9 cells (Fig. 6), resulting in loss of codon 286.

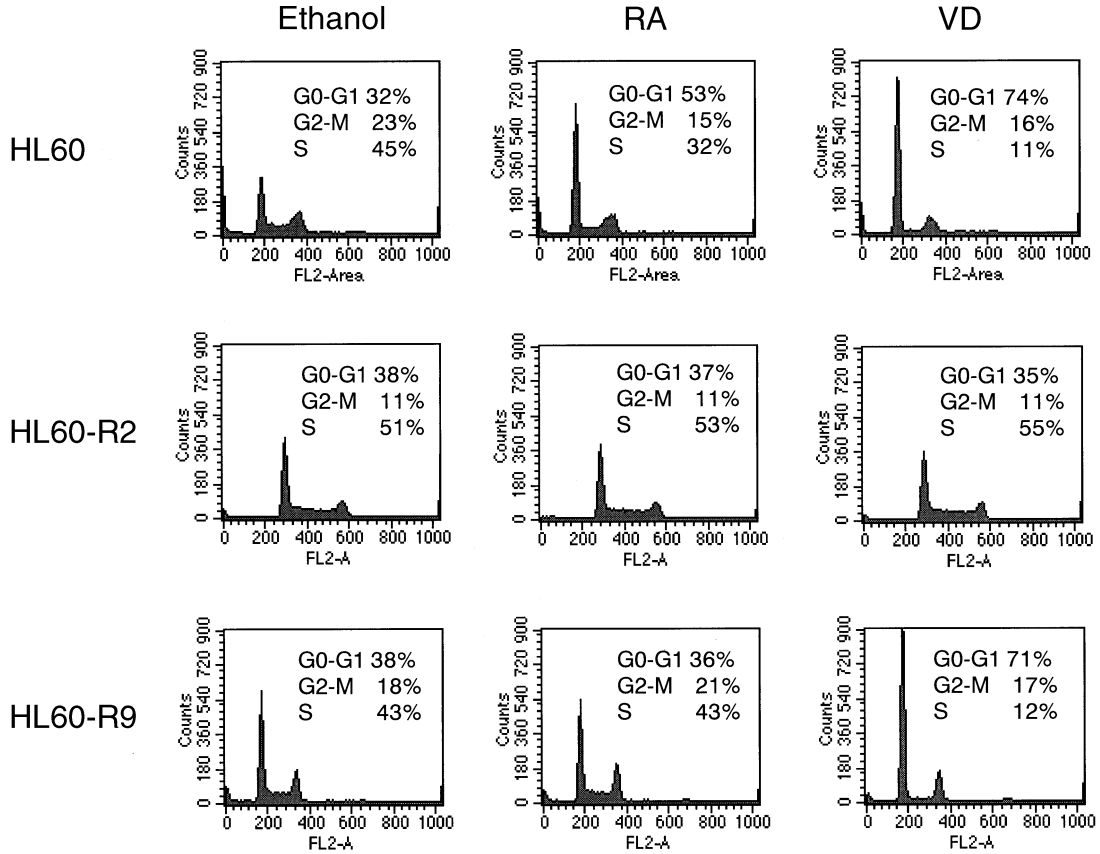

Fig. 4. Flow cytometric profiles of DNA content of nuclei from HL-60, R2, and R9 cells exposed to the indicated concentrations of the ligands. The cells were grown for 5 days in the presence of 0.1% ethanol or 1 μM of the indicated ligands and analyzed by FACS. The abscissa indicates the integrated fluorescence intensity and the ordinate the particle number. Approximately 20,000 particles are represented in each histogram. The percentages of cell cycle phases were calculated from the normal diploid cell population.

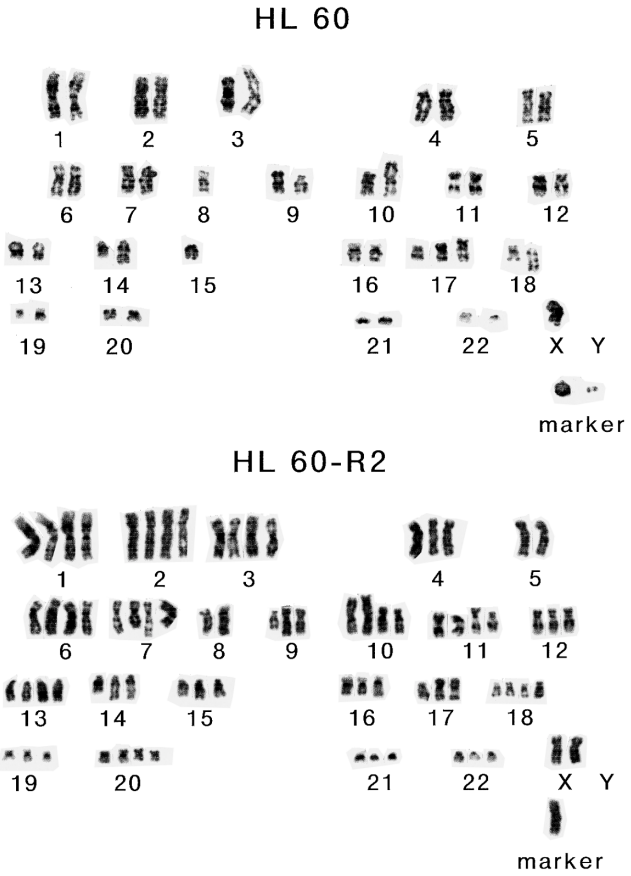

Fig. 5. Cytogenetic analyses of HL-60 and R2 cells after exposure to Colcemid. The upper panel shows a diploid HL-60 cell. The composite karyotype formula was 45-46, X, −X, +3, add(3)(p12-p13), −5, −8, add(9)(p11), +10, der(10)t(10;13)(p11;q12), −14, add(14)(q32), del(14)(q2?q31), add(16)(q2?2), add(16), add(16)(q2?2), −17, add(17)(p11), add(18)(q23), −20, +1-4mar. A near-triploid R2 cell with approximately 77 chromosomes is shown in the lower panel. The composite karyotype formula was 74-78<3n>, XX, −X, +1, +2, +3, −5, +6, +7, −8, add(9)(p11), +10, der(10)t(10;13)(p11;q12), der(10), del(11)(p11), +12, +13, add(14)(q32), add(14), del(14)(q2?q31), +15, add(16)(q2?2)×2, der(16)del(16)(p1?)add(16)(q2?2), add(17)(p11)×2, +18, +19, +20, +mar.

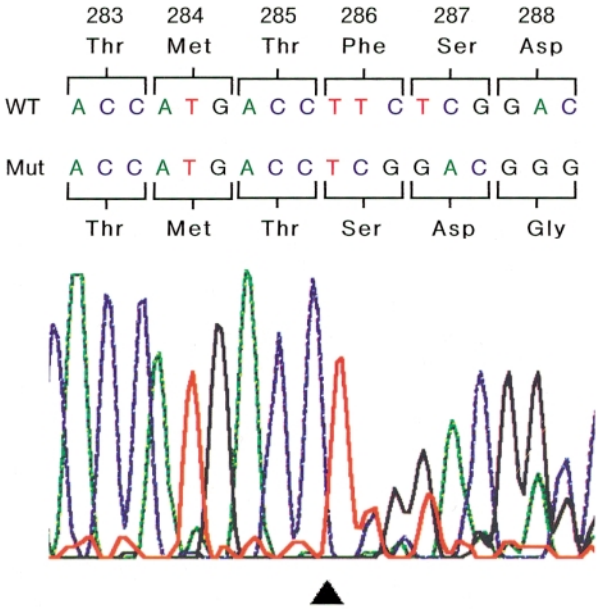

Fig. 6. Automated direct sequence analysis of exon 7 of the *RARα* gene in HL-60-R9. The arrowhead after codon 285 indicates the deletion of three nucleotides in the mutant allele. The nucleotide sequences and corresponding codons of the wild-type and mutant alleles are shown above.

DISCUSSION

Clinically, resistance to RA poses a problem for differentiation-inducing therapy, and the mechanisms of resistance are not fully understood. One of the possible mechanisms is genetic alterations.[15] Robertson *et al.* found the same mutations in the diploid sequences of *RARα* genes in HL-60R cells, which are resistant to RA.[16] However, Morosetti *et al.* found no mutation in the sequence of RARα in a series of tumor cell lines, implying that mutation in RARα is not a common cause of resistance to RA.[13] Moreover, Kizaki *et al.* reported that there was no mutation in the *RARα* gene of UF-1 cells derived from a patient clinically resistant to RA.[17] They suggested that the RA-resistance might be caused by the induction of cytochrome p450 enzymes and P-glycoprotein.[18] Thus, there may be various mechanisms of development of resistance to RA.

We have sub-cloned 6 cell lines after exposure to 1 *μM* RA. In four cell lines out of the six, the growth rates were decreased by 1 *μM* RA although the suppressive potencies of RA in these 4 cloned cell lines were weaker than that in the parental HL-60, implying that these cell lines are partially resistant to RA. The growth rate of HL-60-R2 increased rather than decreased after adding the ligand, and that of R9 was not altered, indicating these cell lines are completely resistant to 1 *μM* RA. Morphological assessment after exposure to Actinomycin D or TPA suggests that the two cell lines were not intrinsically apoptosis- and differentiation-incompetent.

Three major specific characters were found in HL-60-R2 cells. The first is that the growth rate was significantly increased by incubation with RA for 5 days. Since the cell cycle and the induction of apoptosis were not affected by RA, the mechanism of the acceleration of the cell growth in HL-60-R2 cells is unclear. But we speculate that the

time of cell replication may be altered without changing the proportions of cells in different phases of the cell cycle. The second is that HL-60-R2 possesses resistance not only to RA, but also to VD. Similar results were observed in NB4 promyelocytic leukemia cells, as previously reported by Gianni *et al*.[19] This abnormality indicates that the pathways of RA-induced growth arrest and apoptosis are partially common to that of VD-induced growth inhibition. Since the actions of RA and VD are initiated through the heterodimer with their own nuclear receptors and the RXRs, one of the common factors is the RXRs. We found no mutation in the sequence of the ligand-binding domain, including the domain required for heterodimer formation in RXRα. It is possible that mutations were present in some other domain of the RXRα we sequenced, or in other isoforms of RXRs, or that there are abnormalities in co-mediator(s) which interact with RARs and VDRs, as suggested by Mengus *et al*.[20]

The third character is that R2 cells have abnormal ploidy. Little is known about the molecular mechanisms of the control of ploidy in mammalian cells. The tumor suppressor p53 may be required for the maintenance of diploidy, because loss of inactivation of p53 can be associated with tetraploidy or aneuploidy.[21] Cross *et al*. demonstrated a possible role of murine p53 in a spindle checkpoint.[22] HL-60 cells are a p53-defective human cell line.[23] VD-resistant HL-60 cells developed by a method similar to ours were tetraploid.[24] Moreover, cytogenetic analysis of RA-resistant cells, which are also resistant to VD, showed the presence of a hypotriploid karyotype.[19] Thus, loss of p53 may potentially cause abnormal ploidy in HL-60 cells, and the abnormal ploidy may be associated with the mechanisms of vitamin D resistance.

Acute promyelocytic leukemia (APL) is characterized in about 80% of the cases by a cytogenetic abnormality, i.e., a reciprocal translocation of chromosomes 15 and 17, t(15;17).[25] Cytogenetic study showed that R2 and R9 cells have derivative chromosomes 10 resulting from the segregation of t(10;13)(p11;q12), like the parental cells, but t(15;17) was not found in the parental HL-60 cells or resistant cells, suggesting the presence of other mechanisms for development of APL.

Since HL-60-R9 cells possess resistance to RA, we sequenced the RARα and found mutations in the ligand binding domain. Deletion of codon 286, phenylalanine, in one allele of RARα results in a dominant-negative effect on the RA response genes.

Robertson *et al*. first described a mutation in codon 411 of RARα in RA-resistant HL-60 cells.[16] The same C-to-T transition was reported from another laboratory, suggesting that the mutation exists in a small subpopulation of the parental HL-60 or that this is a mutational hot spot.[26] Dore and Momparier reported another mutation in the ligand-binding domain of RARα in RA-resistant HL-60 cells.[27] To our knowledge, R9 cell line is the third clone which possesses mutant RARα among RA-resistant HL-60 cells.

In this study, two RA-resistant cell lines were developed from HL-60 cells by increasing the concentration of RA. The resistance to RA was caused by factor(s) which also affect the VD-response pathways, and the abnormal response to VD may be associated with the abnormal ploidy of one of the resistant cell lines. These cell lines should be useful to approach the molecular mechanisms of action of RA and VD, and the development of resistance.

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