Recent studies have shown that a high proportion of patients with acute promyelocytic leukemia (APL) achieve complete remission after treatment with all-trans retinoic acid (RA). Nevertheless, despite an initial good response, most patients that received continuous treatment with all-trans RA relapse and develop RA-resistant disease. The 9-cis RA is a high-affinity ligand for retinoid X receptors (RXRs) and also binds efficiently to retinoic acid receptors (RARs); all-trans RA is a ligand for RARs. Both alone are able to induce differentiation of wild-type HL-60 cells. We found that neither all-trans RA nor 9-cis RA (<2 x 10^{-8} mol/L) induced differentiation of RA-resistant HL-60 cells into either mature granulocytes or monocytes. However, morphologic differentiation of the RA-resistant HL-60 cells was induced by 10^{-8} mol/L all-trans RA combined with various concentrations (10^{-12} to 10^{-4} mol/L) of 9-cis RA. Electron microscopic examination also confirmed that the combination of both retinoids induced RA-resistant HL-60 cells to differentiate to mature granulocytes. Functional analysis of differentiation (NBT reduction activity) confirmed the necessity of both analogs to induce differentiation. Also, expression of myeloid-specific differentiation antigens (CD11b and CD14) as well as migration inhibitory factor-related protein (MRP)-8/14 mRNAs were upregulated only in the presence of both retinoids in a dose-dependent manner. In these conditions 3H-thymidine incorporation was inhibited and numbers of viable cells were decreased, suggesting that all-trans RA with 9-cis RA may inhibit cell growth and induce differentiation of RA-resistant HL-60 cells into mature granulocytes. These studies suggest that 9-cis RA in combination with all-trans RA is an effective inducer of RA-resistant HL-60 cells and may have implications for both the biology of retinoids and clinical treatment of RA-resistant acute myelogenous leukemia, including APL patients.

Therefore, in this study, to investigate whether the novel RA compound, 9-cis RA, could overcome RA resistant-leukemic cells, we studied the effects of this novel RA on cell growth, differentiation, and expression of RAR-α and RXR-α mRNA in RA-resistant HL-60 cells in vitro. We now report that 9-cis RA in combination with all-trans RA is an effective inducer of differentiation of RA-resistant HL-60 cells.

**MATERIALS AND METHODS**

Cells. RA-resistant HL-60 cells (generous gift of Dr. R.E. Gallagher, NY) were maintained in serum-free culture medium (Cosmedium 001; Cosmo Bio Co, Tokyo, Japan) with 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO₂ to eliminate the influence of endogenous RA in fetal bovine serum. Serum albumin can bind RA; however, Cosmedium 001 is free of albumin. Thus, this medium should be free of endogenous RA. All-trans RA (10^{-10} mol/L) (Sigma Chemical Co, St Louis, Missouri) and 9-cis RA (10^{-8} to 10^{-4} mol/L) were added to the culture medium.

The biologic effects of RA are mediated by two distinct families of transcription factors: RA receptors (RARs) and retinoid X receptors (RXRs). All-trans RA binds to RARs with high affinity and alters gene expression as a consequence of this direct ligand interaction; however, it has poor binding activity to RXRs. Recently, the ligand of RXRs has been identified as 9-cis RA, which is a stereoisomer of RA and activates both RARs and RXRs. Both heterodimers ofRAR/RXR and RXR homodimers are ligand inducible trans-regulators that modulate the transcription of target genes by interacting with cis-acting specific DNA sequences known as RA response elements (RAREs).

We have previously compared the abilities of 9-cis RA and all-trans RA to induce differentiation and inhibit proliferation of AML cell lines and fresh leukemic cells from patients and have found an enhanced potency of 9-cis RA.

The problem of RA resistance in APL patients extends well beyond this particular disease and may pose a serious problem for differentiation-inducing therapy in general.

**From the Division of Hematology, Keio University School of Medicine; Chugai Pharmaceutical Co, Ltd, Tokyo; the Division of Hematology, Jichi Medical School, Tochigi, Japan; and the Division of Hematology-Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA.**

*Submitted December 22, 1993; accepted March 11, 1994.*

*Supported by grants from the Ministry of Education, Science and Culture in Japan, the New Energy and Industrial Technology Development Organization, the National Institutes of Health Grant No. CA33936, Members of Real Estate Industry Division, and Parker Hughes Fund.*

Address reprint requests to Masahiro Kizaki, MD, Division of Hematology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.

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MO) was always added to the culture medium for the maintenance of RA-resistant HL-60 cells. At least 4 days before each experiment, the cells were washed with phosphate-buffered saline (PBS), then various concentrations of retinoids were added to the medium.

**Chemicals.** All-trans RA was purchased from Sigma, and 9-cis RA was a generous gift of Dr H. Klaus (F. Hoffmann-La Roche, Basel, Switzerland). They were dissolved in 100% ethanol to a stock concentration of 1 mmol/L; stored at −20°C and protected from light. In each experiment, controls were run using the same concentration of ethanol as present in the experimental plates and this concentration of dilutant had no effect on proliferation of cells.

**Assays for cellular proliferation and survival.** The cellular proliferation was measured by incorporation of 3H-thymidine into cellular DNA. RA-resistant HL-60 cells (2 × 10^5) were incubated with

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**Fig 1.** Effects of 9-cis RA and all-trans RA on cell survival and proliferation of RA-resistant HL-60 cells in liquid culture. (A) Effects of retinoids on cell survival of RA-resistant HL-60 cells in liquid culture. RA-resistant HL-60 cells (1 × 10^5/mL) were cultured in the presence of various concentrations of retinoids for 4 days, and viable cells were counted by trypan blue dye exclusion. Results are the mean ± SD of percent of viable cells compared with the number in control dishes without retinoids. Results are from three experiments. (B) Effects of retinoids on proliferation of RA-resistant HL-60 cells. RA-resistant HL-60 cells (2 × 10^4/mL) were incubated with various concentrations of retinoids for 4 days with 1 μCi of 3H-thymidine. Results are expressed as the mean percentage of control dishes that contained no retinoids (mean ± SD from quadruplicate experiments).

**Fig 2.** Comparison of the differentiation-inducing activities of 9-cis RA and all-trans RA. (A) NBT reduction activity. Cells were cultured with either all-trans RA, 9-cis RA, or both for 4 days and differentiation was determined by NBT reduction. Results are expressed as the mean percentage of control dishes that contained no retinoids (mean ± SD from three experiments). (B) Expression of CD11b antigens in RA-resistant HL-60 cells. Cells were treated for 4 days with retinoids (all-trans RA and/or 9-cis RA), and then analyzed by FACS (Materials and Methods section and Fig 3). Data represent the mean of triplicate experiments, the SD was within 10% of the mean. Representative data were shown in Fig 3.
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Assays for cellular differentiation. RA-resistant HL-60 cells (2 x 10^6/mL) were suspended with test materials at appropriate concentrations. After incubation for 4 days, cells were harvested and examined for induction of differentiation by morphology using both light and electron microscopy as described^25 and by nitroblue tetrazolium (NBT).^26 For NBT, cell suspension (2 x 10^6/mL) was mixed with an equal volume of solution containing 1.25 mg/mL NBT (Sigma), 17 mg/mL bovine albumin, and 1 mg/mL 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma) for 30 minutes at 37°C. After incubation, the medium was discarded and the formazan deposits were dissolved by adding 100 μL of dimethyl sulfoxide (DMSO; Sigma) and measured at 580 nm by spectrophotometer. For analysis of cell-surface antigens, a two-color direct immunofluorescence staining technique was used. Cells were incubated for 60 minutes with human AB serum (Sigma) to block Fc receptors and then stained by using fluorescein isothiocyanate (FITC)-conjugated mouse antihuman CD11b and phycocerythrin (PE)-conjugated mouse antihuman CD14 (Becton Dickinson, Mountain View, CA). Control studies were performed with a nonbinding control mouse IgG, isotype antibody (Becton Dickinson). Analysis of fluorescence was performed on a FACS-45 flow cytometer (Becton Dickinson).

Analysis of apoptosis. Apoptosis was determined by morphologic changes and detection of fragmentation of the genomic DNA in RA-resistant HL-60 cells after exposure of various concentrations of retinoids. Genomic DNA was isolated as previously described with some modifications.^27 Briefly, 1 x 10^6 cells were washed in Ca^2+-Mg^2+-free PBS and resuspended in 20 μg of lysase buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA containing 0.5% laurylsarkosinate and 1.25 mg/mL protease K (Sigma) and incubated for 1 hour at 50°C. Ten microliters of RNase A (10 mg/mL) (Boehringer Mannheim, Indianapolis, IN) was added and incubation was performed at 50°C for 60 minutes followed by the addition of 10 μL of 10 mmol/L EDTA, pH 8.0, and 8 μL of 6X loading buffer (0.25% bromophenol blue, 9.25% xylene cyanol, 15% Ficoll 400). DNA was analyzed for fragmentation by electrophoresis in 1.5% agarose gel (GIBCO BRL, Gaithersburg, MD) in Tris-acetate-EDTA buffer stained with ethidium bromide.

DNA isolation and Northern blotting. Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method.^28 Total RNA (20 μg/lane) was electrophoresed on formaldehyde-agarose gels (BRL), and transferred to nitrocellulose membranes (Hybond N+; Amersham Japan, Tokyo). Briefly, the labeled probes were hybridized for 16 to 24 hours at 42°C in 50% formamide, 2X SSC (1X SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0), 5X Denhardt's, 0.1% sodium dodecyl sulfate (SDS), and 0.5% NaHC03 (Sigma). Filters were washed to a stringency of 0.1X SSC at 65°C and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). Autoradiograms were exposed for 24 hours to 3 days. Densitometry was performed for signal intensity on autoradiograms of Northern blots using a digital densitometer (Advantec DM-303; Tokyo, Japan).

DNA probes. A human RAR-α cDNA (Kpn I-EcoRI; 1.9 kb) was purified from plasmid pSG1^23 and a human RXR-α cDNA probe (EcoRI-EcoRI; 1.9 kb) was purified from pSKX3-1.16 β-Actin probe was the EcoRI-BamHI fragment (0.7 kb) from plasmid pHβB-3.31 Migration inhibitory factor-related protein (MRP)-8 and MRP-14 cDNA probes were constructed by polymerase chain reaction (PCR) of first-strand cDNA from 1,25(OH)2D3-treated HL-60 cells, and details were described previously.11 Briefly, the oligoribonucleotide primers used in this study were derived from the published cDNA sequence for MRP-8 and MRP-14^32 and the 24 nucleotides coding the N-terminal and C-terminal amino acids were chosen as primers for both cDNAs,31 with six additional nucleotides for HindIII site to facilitate subcloning. The cDNA fragments, 296 bp for MRP-8 and 387 bp for MRP-14, were subcloned into HindIII site of pGEMzf (+) (Promega, Madison, WI). All probes were labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham) by random priming kits (Takara Shuzo Co, LTD, Tokyo, Japan).
RESULTS

Effects of retinoids on proliferation and cell survival of RA-resistant HL-60 cells in liquid culture. RA-resistant HL-60 cells were cultured for 4 days in serum-free medium containing various concentrations of retinoids (Fig 1). Neither all-trans RA nor 9-cis RA (<2 × 10⁻⁶ mol/L) changed either the numbers of viable cells (Fig 1A) or ³H-thymidine incorporation (Fig 1B). Cell growth and proliferation decreased by only 10% to 20% after the cells were exposed to higher concentration of retinoids (10⁻⁶ mol/L of either all-trans RA or 9-cis RA) for 4 days (Fig 1). 9-cis RA at 10⁻⁶ mol/L was slightly more potent than all-trans RA in inhibiting proliferation of the cells. However, the combination of 10⁻⁶ mol/L all-trans RA and with various concentrations of 9-cis RA (10⁻¹² to 10⁻⁶ mol/L) markedly decreased the numbers of viable cells and inhibited ³H-thymidine incorporation in a dose-dependent manner (Fig 1). Therefore, this combination of all-trans RA and 9-cis RA was surprisingly potent in inhibiting the proliferation of RA-resistant HL-60 cells.

Effects of retinoids on differentiation of RA-resistant HL-60 cells. Induction of differentiation of RA-resistant HL-60 cells into mature granulocytes by retinoids was assessed by NBT reduction activity (Fig 2A), and expression of CD11b and CD14 antigens (Fig 2B and Fig 3). Also we examined morphologic changes of the cells by light (Fig 4) and electron (Fig 5) microscopy. Each of the RA compounds individually did not change percent cell with NBT reduction activity (Fig 2A) or expression of CD11b antigen by fluorescence-activated cell sorter (FACS) analysis compared with control cells (Fig 2B and Fig 3). Exposure of cells to either 10⁻⁶ mol/L all-trans RA or 9-cis RA for 4 days resulted in a slight increase of NBT activity and expression of CD11b antigen. In marked contrast, the combination of all-trans RA (10⁻⁶ mol/L) plus various concentrations of 9-cis RA (10⁻¹² to 10⁻⁶ mol/L) increased the NBT reduction activity at least sixfold compared with either retinoid alone in the RA-resistant cells (Fig 2A). Expression of CD11b antigen in RA-resistant HL-60 cells was increased by 10⁻⁶ mol/L all-trans RA combined with 9-cis RA (10⁻¹² to 10⁻⁶ mol/L) in a dose-dependent manner (Figs 2B and 3).

Morphologically, exposure of RA-resistant HL-60 cells to both 10⁻⁶ mol/L all-trans RA and 9-cis RA for 4 days resulted in differentiation toward mature granulocytes (Fig 4D). However, neither all-trans RA nor 9-cis RA alone induced differentiation of RA-resistant cells into either mature granulocytes or monocytes (Fig 4, A through C). The electron microscopic picture confirmed that the cells cultured with both retinoids differentiated to mature granulocytes (Fig 5). The nuclei displayed considerable segmentation and increased definition of heterochromatin areas, and the cytoplasm contained abundant glycogen particles (Fig 5B).

Taken together, these results suggested that neither all-trans RA nor 9-cis RA alone induced differentiation of RA-resistant HL-60 cells into mature granulocytes; however, together they inhibited leukemic cell growth and induced differentiation of these cells into mature granulocytes.

Modulation of migration inhibitory factor-related protein (MRP)-8/14 mRNA expression by retinoids in RA-resistant HL-60 cells. We generated a monoclonal antibody (MoAb) (clone 5C12) directed against a differentiation-specific antigen. This MoAb immunoprecipitated a complex of MRP-8 and MRP-14, and the expression of MRPs and these mRNAs were found to be regulated during the differentiation
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Fig 5. Electron microscopic pictures of RA-resistant HL-60 cells cultured either in the presence or absence of both retinoids (10^{-6} mol/L) for 4 days. (A) Control RA-resistant HL-60 cells. The nuclear chromatin was primarily euchromatic, and prominent nucleoli were stained. Peroxidase-positive primary granules were detected in the cytoplasm. (B) RA-resistant HL-60 cells differentiated by all-trans RA (10^{-8} mol/L) and 9-cis RA (10^{-8} mol/L) for 4 days. The cells displayed evidence of nuclear and cytoplasmic differentiation characteristics of mature granulocytes. Considerable nuclear segmentation and increased definition of heterochromatin areas were observed. A great many glycogen particles were present in the cytoplasm. (C) RA-resistant HL-60 cells treated with both retinoids for 4 days. The cell showed cytoplasmic and nuclear vesicularization that was compatible with advanced stage of apoptosis.

Effects of retinoids on RAR-α and RXR-α mRNA expression in RA-resistant HL-60 cells. The expression of RAR-α and RXR-α mRNA levels in RA-resistant HL-60 cells cultured in the presence of various concentrations of retinoids was determined by Northern blot analysis (Fig 8). Steady-state mRNA levels of RAR-α and RXR-α in RA-resistant HL-60 cells were not affected by either all-trans RA or 9-cis RA. In addition, levels of RAR-α and RXR-α mRNA did not change by induction of terminal differentiation of RA-resistant HL-60 cells by the combination of both retinoids (Fig 8).
DISCUSSION

Recent studies have shown that a high proportion of patients with APL achieve complete remission after treatment with all-trans RA. However, their remissions are of brief duration and leukemic relapses occur despite continuous treatment with all-trans RA. In this study, we showed for the first time that 9-cis RA combined with all-trans RA is an effective inducer of RA-resistant HL-60 cells. The induction of differentiation of these RA-resistant HL-60 cells did

A. Morphology

B. DNA fragmentation

Fig 6. Modulation of expression of MRP-8/14 mRNA by all-trans RA and/or 9-cis RA in RA-resistant HL-60 cells. Cells were treated with various concentrations of all-trans RA and/or 9-cis RA for 4 days. Total RNA was extracted and analyzed by Northern blot technique and hybridized with [32P]-labeled MRP-8/14 cDNA probes, as described in Materials and Methods (20 µg per lane). Hybridization with [32P]-labeled β-actin probe was performed as a control for the amount of RNA in each lane (lower panel).

Fig 7. Cell death by apoptosis in all-trans RA and 9-cis RA-treated RA-resistant HL-60 cells. (A) After 4 days of culture with both retinoids (10^{-8} mol/L), morphologic evidence of apoptosis occurred in the form of chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies. (B) Agarose gel electrophoresis of genomic DNA at 4 days from control and retinoid-treated RA-resistant HL-60 cells. Lane 1, wild-type RA-resistant HL-60 cells; lane 2, RA-resistant HL-60 cells cultured for 4 days with all-trans RA (10^{-9} mol/L); lane 3, RA-resistant HL-60 cells cultured for 4 days with 9-cis RA (10^{-8} mol/L); RA-resistant HL-60 cells cultured for 4 days in the presence of all-trans RA (10^{-8} mol/L) with 9-cis RA (10^{-9} mol/L) (lane 4) or with 9-cis RA (10^{-8} mol/L) (lane 5), respectively.
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Fig 8. Expression of RAR-α and RXR-α mRNA in RA-resistant HL-60 cells cultured with retinoids. Cells were exposed to various concentrations of all-trans RA and/or 9-cis RA for 4 days. RA-resistant HL-60 cells were cultured in medium alone (lane 1) or medium containing all-trans RA at 10⁻⁴ mol/L (lane 2) and 9-cis RA at 10⁻⁴ mol/L (lane 3). Also, RA-resistant HL-60 cells were cultured with all-trans RA (10⁻⁴ mol/L) plus various concentrations of 9-cis RA (10⁻¹² mol/L, 10⁻¹⁰ mol/L, 10⁻⁸ mol/L, and 10⁻⁶ mol/L) (lanes 4 through 7, respectively). Total RNA was extracted, run on gel (20 µg per lane), and analyzed by Northern blot for RAR-α, RXR-α, and β-actin transcripts. Analyses were performed as described in Materials and Methods.

not alter levels of either RAR-α or RXR-α mRNA. These cells died at least in part by apoptosis after exposure to all-trans RA and 9-cis RA. These cells showed the morphologic features of apoptosis including nuclear chromatin condensation, nuclear fragmentation, cytoplasmic fragmentation, the presence of apoptotic bodies, and endonucleolytic degradation of DNA at nucleosomal intervals. The results suggested that after inducing an irreversible commitment to terminal differentiation, both retinoids may initiate programmed cell death in the maturing cells. The external signals that lead to apoptosis are probably as varied as those that lead to cell differentiation. Inhibition of proliferation in RA-resistant HL-60 cells by the combination of both retinoids may be mediated by two mechanisms; terminal differentiation and apoptosis of the cells.

Several possible mechanisms have been proposed to explain in vitro resistance to retinoids. One obvious explanation is generation of new mutations in retinoid receptors. Several mutant RA-resistant HL-60 subclones have been isolated, and mutation of the ligand-binding domain of RAR-α gene has been identified in one of these cell lines. Other groups have reported the isolation of an RA-resistant APL cell line, NB4-RA' cells. This cell line carries an APL-specific 15;17 translocation resulting in a PML/RAR-α chimeric gene. Recently, a study has shown that this mutant cell line showed the usual PML/RAR-α bands in both Northern and Southern blot assays, but expressed no detectable PML/RAR-α protein, suggesting that the resistance to all-trans RA in this cell was associated with loss of intact PML/RAR-α protein.

In our experiments, we used one clone of RA-resistant HL-60 cells; we have reported that those cells contained a markedly decreased content of ganglioside, especially neolacto series of ganglioside. These cells did not show any increase in the content of gangliosides when cultivated with RA. Also, we showed that these resistant HL-60 cells were found to be differentiated into mature granulocytes when the cells were cultured in the presence of neolacto series ganglioside. These resistant cells have also been reported to have a decrease in the amplification of the oncogene c-myc compared with wild-type HL-60 cells. However, these cells have not been examined for their levels of RAR-α and RXR-α protein by Western blotting, for their expression of other RA receptors and for the enzymes converting for all-trans RA to 9-cis RA on this cell line. Further studies will be required to clarify the molecular mechanism of RA resistance in this cell line.

Another potential mechanism for clinical RA resistance is the pharmacologic alteration in the metabolism of all-trans RA. Continuous treatment with all-trans RA in APL is associated with progressive reduction of plasma concentrations of RA. Possible mechanisms for accelerated clearance are induction of cytochrome P450 enzymes and the increased expression of cellular RA-binding proteins (CRABPs), resulting in lowering plasma levels of active retinoid. Therefore, these patients may only be achieving a concentration of RA that stimulates proliferation without inducing differentiation of leukemic cells. Another possible approach to overcome resistance is to use inhibitors of cytochrome P450 oxidative enzymes, such as ketoconazole and liarozole. The other approach is to develop a suitable formulation of RA to increase plasma levels of RA in those patients that are rapidly metabolizing the compound. Liposomally encapsulated all-trans RA is one of the candidates. However, these and other approaches have failed to overcome RA resistance.

Another possible approach to overcome resistance is to use an alternative retinoid, such as 9-cis RA, which binds to both RARs and RXRs, RXRs interact with several
other steroid receptors, including vitamin D$_3$ receptor (VDR) and thyroid hormone receptor (TR).$^{47-52}$ Studies have shown that RARs required heterodimerization with RXRs for effective DNA binding and function.$^{47-52}$ The RXRs could form either RXR/RXR homodimers or RAR/RXR heterodimers in the presence of 9-cis RA.$^{53}$ We have previously shown that 9-cis RA was more potent than all-trans RA in inhibiting proliferation and inducing differentiation of both myeloid leukemia cell lines and fresh APL cells.$^{21,22}$ Therefore, 9-cis RA is a very potent activator of either RXR/RXR or RAR/RXR dimers. Nevertheless, 9-cis RA was unable to induce the differentiation of RA-resistant HL-60 cells. The combination of both all-trans RA and 9-cis RA may represent a possible approach to therapy for patients with RA resistance in myeloid leukemia and deserves further study. The genes to overcome the block in differentiation in RA-resistant HL-60 cells may depend on transactivation of a gene having an RAR/RXR, but not an RAR/RXR recognition sequence.

Why RA-resistant HL-60 cells were induced to differentiate by 9-cis RA and all-trans RA, but not by either 9-cis RA or all-trans RA alone, is unclear. Because 9-cis RA can bind and activate both RAR/RXR and RAR/RXR dimers and all-trans RA can bind and activate RAR/RXR dimers,$^{53}$ one would predict that the two together should not have a qualitatively different range of biologic action. Potentially, the two ligands together bind receptors that attach to novel retinoic acid responsive elements (RAREs) that might activate a new set of differentiation related genes. Alternatively, the combination of all-trans RA and 9-cis RA may make the existing transactivation pathway much more efficient. Definition of the molecular defect of the RA-resistant cells used in this study will help clarify how the combination of the two ligands are mediating their action.

Retinoid resistance is a serious problem of differentiation-inducing therapy for patients with APL and other leukemias. The data presented in this report strongly suggest that 9-cis RA combined with all-trans RA could overcome RA resistance in myeloid leukemias including APL. Future studies should examine the differentiation-inducing capabilities of the combination of these two ligands on fresh leukemic cells of different phenotypes and on leukemic cells from APL patients with all-trans RA-resistant leukemia.

ACKNOWLEDGMENT

We thank Y. Enomoto (Division of Pathology, Keio University) for his excellent technical assistance of electron microscopy. We also thank Satomi Someya and Kim Burgin for their excellent secretarial help.

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Novel retinoic acid, 9-cis retinoic acid, in combination with all-trans retinoic acid is an effective inducer of differentiation of retinoic acid-resistant HL-60 cells

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