9-cis-Retinoic Acid: Effects on Normal and Leukemic Hematopoiesis In Vitro

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Retinoic acid exhibits effects on the proliferation and differentiation of many hematopoietic cells. Cellular responsiveness to retinoic acid (RA) is conferred through two distinct classes of nuclear receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs). The RARs bind to both 9-cis- and all-trans-RAs, but 9-cis-RA alone directly binds and activates RXR. This suggested that 9-cis-RA could have expanded hematopoietic activities as compared with all-trans-RA. We compared the abilities of 9-cis- and all-trans-RAs to induce differentiation and inhibit proliferation of three acute myelogenous leukemia (AML) cell lines and fresh leukemic cells from 28 patients and found that: (1) 9-cis-RA in general was more potent than all-trans-RA in suppressing the clonal growth of two AML cell lines and 17 AML samples from patients, including four from individuals with acute promyelocytic leukemia (APL). Eleven leukemic samples, including three from patients with chronic myelogenous or chronic myelomonocytic leukemia, were relatively refractory to both retinoids. (2) The range of activities of both retinoids was similar except that the clonal growth of samples from three AML patients were inhibited by 9-cis-RA, but not by all-trans-RA. (3) Both retinoids inhibited the clonal proliferation of leukemia cells without necessarily inducing their differentiation; in fact, the only fresh AML cells that were able to undergo differentiation were from patients with APL and one individual with M2 AML. (4) Both retinoids enhanced myeloid and erythroid clonal growth from normal individuals, and 9-cis-RA showed slightly more stimulation of the myeloid clonal growth than did all-trans-RA. Our study suggests that 9-cis-RA is worthy of further study for the treatment of selected individuals with AML.

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efficiently than all-trans-RA. The 9-cis-RA also binds and activates RARs with a potency that is similar to that of all-trans-RA. Therefore, 9-cis-RA potentially could have a unique range and potency of action on hematopoietic cells. To date, no study has examined the biological activity of 9-cis-RA. In this investigation, we compare the range of action of 9-cis-RA and all-trans-RA on normal and leukemic hematopoiesis.

MATERIALS AND METHODS

Cells. Three human AML cell lines (HL-60, KCL-22, and K562) were studied. HL-60 was derived from a patient with M2 stage of AML; these cells are predominantly at the promyelocytic stage of development. KCL-22 was derived from a patient with undifferentiated chronic myelogenous leukemia (CML) in blast crisis. K562 was established from a patient in CML blast crisis, and has an early erythroid phenotype. All cells were maintained in T flasks with a modified minimum essential medium (Flow Laboratories, Inc, McLean, VA), 10% fetal bovine serum (FBS; J.R. Scientific, Inc, Woodland, CA), penicillin, and streptomycin. Experiments were performed on cells that were in their logarithmic growth phase.

Leukemic cells were obtained from either the peripheral blood or bone marrow of 28 patients: two acute undifferentiated leukemias (AUL), 23 AML, 1 lymphoid blast crisis of CML, 1 chronic phase of CML, and 1 chronic myelomonocytic leukemia (CML-M0/M). AML cases were classified according to the French-American-British (FAB) classification. All M3 leukemia had either a t(15;17) identified by chromosomal analysis and/or a PML-RAR fusion gene, identified by molecular analysis. Leukemic cells from CML patients were harvested from the Philadelphia (Ph) chromosome. Cells were isolated by Ficoll-Hypaque centrifugation, and the mononuclear cells were harvested and washed twice in alpha-medium.

Retinoids. A reported oxidation procedure was modified for the synthesis of 9-cis-RA. A mixture of 9-cis-retinal (Aldrich, Milwaukee, WI) (0.102 g, 0.359 mmol), sodium cyanide (0.184 g, 3.75 mmol), manganese dioxide (1.39 g, 16.0 mmol), and acetic acid (0.06 mL, 0.971 mmol) in methanol (50 mL) was stirred at room temperature under argon in the dark for 18 hours. The mixture was filtered through Celite, the solvent was removed at reduced pressure, and the residue was taken up in ether (50 mL) and washed with water (2 × 20 mL) and brine (10 mL). After drying (MgSO4), the solvent was removed under reduced pressure to give an orange oil (0.092 g), which on flash chromatography using 1% ethyl acetate/99% hexane as eluant afforded methyl 9-cis-retinoate as a yellow oil (0.078 g, 69%), UV (EtOH) λmax 349 nm (ε 18,232). A suspension of the methyl ester (0.070 g) was stirred at 70°C for 60 minutes with human AB serum (Sigma) to block Fc receptors of 300 cells. For analysis of cell surface antigens, cells were incubated for 5 hours under argon in the dark, during which time the material dissolved. After cooling to room temperature, the yellow solution was acidified with glacial acetic acid and the product was extracted with ether (3 × 15 mL). The combined ether extracts were washed with water (2 × 10 mL) and brine (1 × 10 mL), dried (MgSO4), and concentrated at reduced pressure to give the crude acid as a yellow powder (0.070 g). This material was recrystallized from methanol to give 9-cis-RA as a yellow crystalline solid (0.036 g, 80%), melting point 184°C to 187°C; HPLC (C18, 300 MeCN/250 MeOH/150 lPrOH/225 H2O/10 HOAc, 260 nm, 2 mL/min) Rf 19.0 minutes (100%), UV (95% EtOH 1% MeOH) λmax 349 nm (ε 37,254), and was also characterized using IR, 1H NMR, and elemental analysis.

All-trans-RA (Sigma Chemical Co, St Louis, MO) and 9-cis-RA were stored at −20°C. They were dissolved in 100% ethanol at 1 mmol/L and diluted with phosphate-buffered saline (PBS) before use. All experiments were performed in subdued light, and tubes containing retinoids were covered with aluminum foil. Controls were run using the same concentration of ethanol as present in the experimental plates and this concentration of diluent had no effect.

Assay of proliferation and cell survival in liquid culture. HL-60 cells (2 × 10^5/mL) were incubated with either all-trans-RA or 9-cis-RA in α-medium and 15% FBS for 1 week at 37°C in a humidified atmosphere of 5.5% CO2 in air. Viability was assayed by trypan blue dye exclusion.

Clonogenic assay in soft gel culture. Cells (5 × 10^5) from the AML lines were plated in a 35-mm petri dish (Becton Dickinson and Co, Lincoln Park, NJ) with α-medium, 15% FBS, 0.3% agar (Flow Laboratories), and retinoid solution. After incubation for 7 days at 37°C in a humidified atmosphere containing 5.5% CO2, colonies (>40 cells) were counted under a microscope. Leukemic cells (3 to 4 × 10^5) freshly obtained from patients were plated in α-medium, 20% FBS, and 0.3% agar with retinoid solution and 100 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Amgen, Thousand Oaks, CA). The culture dishes were incubated for 14 days, and colonies were counted. For assay of colony-forming units-granulocyte-macrophage (CFU-GM) and burst-forming units-erythroid (BFU-E), human bone marrow was aspirated into heparinized syringes from normal volunteers who had provided informed consent. Freshly aspirated marrow cells were layered over a Ficoll-Hypaque gradient and the light-density, mononuclear cells were used. These cells (2 × 10^5) were plated in 0.9% methylcellulose with 30% FBS, 10% bovine serum albumin (Sigma), 2 U/mL recombinant human erythropoietin (Amgen), 1 × 10^8 mol/L monothioglycerol (Sigma), and retinoid solution. After incubation for 14 days, erythroid colonies were scored. For CFU-GM culture, a total of 2 × 10^5 cells/mL were suspended in α-medium containing 20% FBS, 0.9% methylcellulose, and 100 ng/mL recombinant GM-CSF. The cells were plated in 1 mL portions in 35-mm petri dishes with retinoid solution. The culture plates were incubated for 11 days and colonies were counted.

Assays of induction of differentiation. Leukemic cell lines (2 × 10^5/mL) were incubated with retinoid in α-medium and 15% FBS for 1 week at 37°C in a humidified atmosphere of 5.5% CO2 in air. Fresh leukemic cells (1 × 10^5/mL) from patients were also cultured with retinoid in α-medium and 20% FBS. After incubation for 1 week, cells were harvested and examined for their morphology and ability to reduce nitroblue tetrazolium (NBT). For morphologic examination, cytopsin slides were stained with Giemsa and assessed with a light microscope. The NBT reduction was assayed as previously reported. The percentage of cells containing intracellular blue-black formazan deposits was determined by examination of a minimum of 300 cells. For analysis of cell surface antigens, cells were incubated for 60 minutes with human AB serum (Sigma) to block Fc receptors and then stained by direct immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated mouse antihuman CD11b (Becton Dickinson, Mountain View, CA). Control studies were performed with a nonbinding control mouse IgG1 isotype antibody (Becton Dickinson). Analysis of fluorescence was performed on a FACS flow cytometer (Becton Dickinson).

Statistics. Results of the cell lines and hematopoietic cells from normal individuals represent the mean ± SD greater than or equal to three experiments, each with greater than or equal to triplicate dishes. Levels of significance between samples were determined using the Student's t-distribution. Results of fresh leukemic cells usually represent the mean of greater than or equal to triplicate dishes.

RESULTS

Effects of 9-cis-RA and all-trans-RA on proliferation and cell survival of HL-60 cells in liquid culture. Alterations in proliferation of HL-60 cells were apparent by 3 days of culture with both 9-cis-RA and all-trans-RA (Fig 1). For example,
growth ceased by day 4 of culture when cells were cultured with either 9-cis-RA or all-trans-RA at 10^{-6} mol/L. These cells no longer proliferated even when resuspended in new medium without added retinoid (data not shown). The 9-cis-RA and all-trans-RA were similarly effective in decreasing growth of HL-60.

Myeloid leukemia lines: Effects of 9-cis-RA and all-trans-RA on clonal growth and differentiation. The clonal growth of the human myeloid leukemia cell lines HL-60 and KCL-22 were markedly suppressed by both 9-cis-RA and all-trans-RA in a dose-dependent manner (Fig 2A and B). 9-cis-RA at 10^{-9} to 10^{-7} mol/L was significantly (P < .005) more potent than all-trans-RA in inhibiting clonal growth of HL-60 cells (Fig 2A). The effective dose that inhibited 50% colony formation (ED_{50}) of HL-60 was 2.9 nmol/L for 9-cis-RA and 40.0 nmol/L for all-trans-RA. No difference was observed between the ability of 9-cis-RA and all-trans-RA to inhibit clonal growth of KCL-22 cells (Fig 2B). The clonal growth of the human myeloid leukemia cell line K562 was not inhibited by either 9-cis-RA or all-trans-RA at concentrations up to 10^{-6} mol/L (data not shown).

Both 9-cis-RA and all-trans-RA induced differentiation of HL-60, as measured by NBT reduction (Fig 3A), expression of CD11b (Fig 3B), and morphology (data not shown). 9-cis-RA (10^{-9}, 10^{-8}, and 10^{-6} mol/L) was more potent than all-trans-RA in reducing NBT (P < .01) and in inducing the expression of the myeloid differentiation antigen, CD11b, in HL-60 cells (Fig 3B). The ability of both retinoids to induce differentiation of HL-60 roughly paralleled their abilities to inhibit clonal growth of these cells. Neither 9-cis-RA nor all-trans-RA induced differentiation of either KCL-22 or K562 cells (data not shown).

Fresh leukemic cells: Effects of 9-cis-RA and all-trans-RA on their clonal growth and differentiation. We examined fresh leukemic cells from 2 patients with AUL, 23 patients with AML (M1, 6; M2, 8; M3, 4; M4, 1; M5, 3; M6, 1), 2 patients with CML, and 1 patient with CMMoL (Table 1). Leukemic cells from each patient were studied for clonal
growth in the presence of increasing amounts of 9-cis-RA and all-trans-RA (5 concentrations, $10^{-9}$ to $10^{-10}$ mol/L). The mean colony number of triplicate cultures for each concentration was compared with the colony number of control dishes not containing retinoids. Data for nearly all patients formed a linear curve when plotted on arithmetic graph paper. From each patient’s graph, the ED$_{50}$ for both retinoids was determined. For all retinoid-responsive samples, 9-cis-RA was more potent than all-trans-RA in the inhibition of clonal growth (Table 1). For 6 individuals (patients no. 3, 9, 10, 11, 12, and 20), the ED$_{90}$ of 9-cis-RA was at least 10-fold less than that of all-trans-RA (Table 1). Of a total of 25 patients with AUL and AML, 9-cis-RA and all-trans-RA achieved greater than 50% inhibition of clonal growth in 17 and 14 patients, respectively. The M1 and M2 samples had a diversity of sensitivities, with some being very sensitive and others being resistant to these retinoids. All M3 samples were very sensitive to the effects of the retinoids (Table 1 and Fig 4); inhibition of growth occurred between $0.3$ and $31 \times 10^{-9}$ mol/L. At very low concentrations ($10^{-11}$ to $10^{-10}$ mol/L), two patients had a paradoxical enhancement of clonal growth by all-trans-RA (Fig 4A and B). All M4, M5, M6, CML, and CMMoL samples were relatively insensitive to the growth-inhibiting effects of both retinoids.

Differentiation induced by both retinoids was examined in 10 individuals (patients no. 3, 6, 9, 12, 16, 17, 18, 21, 22, and 28). Only M3 (APL) (patients no. 17 and 18) and M2 (patient no. 12) cells were able to undergo differentiation as measured by NBT reduction (Fig 5A and B) and morphology (granulocytic differentiation, data not shown); both retinoids had similar activity (Figs 5A and B).

**Normal human myeloid and erythroid progenitor cells: Effects of 9-cis-RA and all-trans-RA on clonal growth.** 9-cis-RA was statistically ($P < .05$) more potent than all-trans-RA in the stimulation of CFU-GM at $10^{-9}$ to $10^{-7}$ mol/L, with maximal stimulation occurring at $10^{-8}$ mol/L 9-cis-RA and $10^{-7}$ mol/L all-trans-RA (Fig 6A). Both retinoids significantly ($P < .005$) increased the number of BFU-E (Fig 6B), with maximal stimulation occurring at $10^{-7}$ mol/L. No significant differences occurred in the abilities of both retinoids to enhance clonal growth of BFU-E.

**Table 1. Concentration of 9-cis-RA and all-trans-RA That Inhibited 50% Clonal Growth of Leukemic Cells Freshly Obtained From Patients**

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<th>Patient No.</th>
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<th>all-trans-RA</th>
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Abbreviation: >, 50% inhibition of growth not reached at $10^{-6}$ mol/L retinoid.

* Chronic phase.
† Lymphoid blast crisis.
9-cis-RETINOIC ACID AND HEMATOPOIESIS

DISCUSSION

Interest has developed in the use of retinoids for leukemia. Retinoids were initially found to be capable of inducing differentiation of HL-60 cells as well as inhibiting the clonal proliferation of several myeloid leukemia lines. Further in vitro studies of leukemic cells from 21 patients with either AML or CML found that differentiation occurred only in cells obtained from two patients with either AML or CML. A subsequent study confirmed the differentiation activity of RA on cells from APL patients. More recently, samples from 35 APL patients were cultured in the presence of all-trans-RA (mol/L) for 3 to 5 days. Cells from all but 1 patient demonstrated an increase in differentiation (NBT reduction) after exposure to RA. A comparison was made between the differentiating activity of 13-cis-RA and all-trans-RA on APL cells from 8 patients. Maximal granulocytic differentiation was achieved by mol/L all-trans-RA, whereas a 10-fold higher concentration of 13-cis-RA was required to obtain similar results. All-trans-RA at very low concentrations was able to inhibit the clonal growth of cells from patients with a variety of leukemic subtypes.

A number of case studies suggested that retinoids had antileukemic effects in APL patients, as well as some patients with myelodysplastic syndrome. More recently, three clinical studies have shown that administration of all-trans-RA is able to induce remission in the majority of patients with APL with minimal morbidity. In vitro culture results correlated closely with clinical response to this agent, and may be useful in predicting which patients will benefit from retinoid therapy. Also, in vitro data and limited clinical information suggest that all-trans-RA is superior to 13-cis-RA in the treatment of APL. Despite high remission rates, the majority of patients appear to relapse shortly after a complete response is attained. Relapsing patients are generally not responsive to reinduction with all-trans-RA. An additional difficulty with therapy using all-trans-RA is that it appears to work for only a select fraction of patients, i.e., those with APL.

Although both 9-cis-RA and all-trans-RA can directly bind the RAR subfamily with similar affinity, 9-cis-RA alone directly binds to the RXR subfamily of receptors, suggesting that biologic differences may exist between these retinoids and these differences may be exploited clinically. Therefore, our study sought to define the biologic spectrum of activity of 9-cis-RA on normal and leukemic hematopoietic cells and to compare its activity with that of all-trans-RA. We found that: (1) In general, 9-cis-RA was more potent than all-trans-RA in inhibiting clonal growth and inducing differentiation.

Fig 4. Effects of 9-cis-RA and all-trans-RA on the clonal growth of APL cells from patients. Cells were cultured with 9-cis-RA [] or all-trans-RA (+), and colonies were counted after 14 days of soft gel culture. The methods used are described in the legend to Fig 1.
SAKASHITA ET AL

Fig 5. Effects of 9-cis-RA and all-trans-RA on the differentiation of fresh leukemic cells from patients. Cells (1 x 10^6/mL) were cultured (7 days) with either 9-cis-RA (A) or all-trans-RA (B) and differentiation was determined by NBT reduction. Each point represents the percentage of NBT-positive cells after exposure to retinoid minus the untreated control value.

Fig 6. Effects of 9-cis-RA and all-trans-RA on the clonal growth of normal human CFU-GM and BFU-E. Cells were cultured with 9-cis-RA (●) and all-trans-RA (○) and CFU-GM formation (A) and BFU-E formation (B) determined as described in Materials and Methods. Colony results are expressed as a mean percentage of control plates that contained no RA. Each point represents the mean results of three normal individuals, and each experimental point contained triplicate plates.
the clonal growth of normal CFU-GM and BFU-E. 9-cis-RA may be a little more potent than all-trans-RA in the enhancement of clonal growth of CFU-GM ($P < .05$).

The mechanisms behind why 9-cis-RA is slightly more potent than all-trans-RA require further study. Prior studies have shown the importance of the retinoic acid receptors in mediating the biologic activities of retinoids. For example, HL-60 cells must express RARs to be induced to differentiate by all-trans-RA. Both 9-cis-RA and all-trans-RA interact with the RAR family of receptors with similar potency, but only 9-cis-RA can interact efficiently with the RXR family of receptors. This may help explain the difference in potency of these two analogs. Hematopoietic cells may be capable of metabolizing all-trans-RA to 9-cis-RA, but this may not be as efficient as exogenously providing the ligand to the cells. Alternatively, all-trans-RA predominantly modulates genes that have RAR recognition elements, whereas 9-cis-RA modulates genes having both RAR and RXR recognition elements. Therefore, the 9-cis-RA–RXR complex might activate a unique set of differentiation-related genes that are not as efficiently transactivated by the all-trans-RA–RAR complex.

This study raises a series of additional questions requiring further studies. (1) What is the distribution of expression of RXR in various normal and leukemic hematopoietic cells? Preliminary data suggest that HL-60 cells express RXR8. (2) Does the PML–RAR chimeric protein, found only in APL cells, have a unique binding with 9-cis-RA and all-trans-RA? (3) What specific effects do the RXR and its ligand have, independent of RAR and its ligand, on induction of differentiation and inhibition of proliferation of leukemic cells? (4) What is the intracellular and extracellular metabolism of 9-cis-RA? (5) Do leukemic cells that develop resistance to all-trans-RA also develop resistance to 9-cis-RA? This is a clinically important question, because almost all APL patients relapse, even while receiving all-trans-RA. (6) Can 9-cis-RA synergize with other members of the steroid/thyroid hormone receptor superfamily? Recent evidence has shown that RXRs can form heterodimers with RARs and this interaction can enhance transcriptional activation of RAR–Es. RXR also interacts directly with and enhances the transcriptional activity of receptors for 1,25-dihydroxy-vitamin D3 and thyroid hormone.

In summary, our studies show that 9-cis-RA is moderately more potent than all-trans-RA in vitro; further in vivo studies are required to determine if similar comparative potencies occur and if 9-cis-RA has a broader range of activities than all-trans-RA.

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