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

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Retinoids play a critical role in embryonic morphogenesis, epidermal cellular growth, and hematopoiesis. Selective vitamin A (retinol) deprivation induces anemia in humans and rats that is reversible by readministering retinol. Retinoids enhance the clonal growth of normal human myeloid precursors and inhibit the clonal growth of fresh leukemic cells and cell lines from patients with acute myelogenous leukemia (AML) in vitro. Retinoids have been reported either to stimulate or inhibit the clonal growth of normal erythroid precursors depending on culture conditions. Retinoids also promote granulocytic differentiation of HL-60 cells, a human M2-derived cell line, and fresh acute promyelocytic leukemia (APL) cells. Eleven leukemic samples, including three from patients with chronic myelogenous or chronic myelomonocytic leukemia, were relatively refractory to both retinoids. 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. 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. 9-cis-RAs to induce 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 the 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. A mixture of 9-cis-retinal (Aldrich, Milwaukee, WI) and poly(ethylene glycol) (PEG) was established from a patient in CML blast crisis, and has an early effect on the differentiation of leukemic cells (28). K562 was established from a patient with undifferentiated chronic myelogenous leukemia (CML) in blast crisis. All cells were maintained in T flasks with a modified minimum essential medium (Flow Laboratories, Inc., McLean, VA), 10% fetal bovine serum (FBS; J. 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 (CMMoL). 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 had a PML-RAR fusion gene, identified by molecular analysis. Leukemic cells from CML patients had the Philadelphia (Ph1)1 chromosome. Cells were isolated by Ficoll-Hypaque centrifugation, and the mononuclear cells were harvested and washed twice in medium.

Retinoids. A modified oxidation procedure was modified for the synthesis of 9-cis-RA. A mixture of 9-cis-retinal (Aldrich, Milwauk ee, 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 for 18 hours. The mixture was filtered and then dried (MgSO4). The residue was taken up in ether (50 mL) and washed with water (2 x 15 mL). After drying (MgSO4), the solution was removed under reduced pressure to give an orange oil (0.092 g), which was dissolved in ethyl acetate (95% EtOH) and filtered. The filtrate was concentrated at reduced pressure to give the crude acid as a yellow residue. The compound was characterized using IR, 1H NMR, and elemental analysis.

RESULTS

Leukemic cell lines (2 x 106/mL) were incubated with retinoid in medium and 15% FBS for 1 week at 37°C in a humidified atmosphere of 5.5% CO2 in air. Viability was assessed by trypan blue dye exclusion.

Clonogenic assay in soft agar culture. Cells (5 x 103) from the AML lines were plated in a 35-mm petri dish (Becton Dickinson and Co., Lincoln Park, NJ) with 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 x 106) freshly obtained from patients were plated in 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 x 106) were plated in 0.9% methylcellulose with 30% FBS, 10% bovine serum albumin (Sigma), 2 μM recombinant human erythropoietin (Amgen), 1 x 10-8 M L-monomethionylglycine (Sigma), and retinoid solution. After incubation for 14 days, erythroid colonies were scored. For CFU-GM culture, a total of 2 x 106 cells/mL were suspended in 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 x 106/mL) were incubated with retinoid in medium and 15% FBS for 1 week at 37°C in a humidified atmosphere of 5.5% CO2 in air. Fresh leukemic cells (1 x 106/mL) from patients were also cultured with retinoid in 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, cytospin slides were stained with Giemsa and assessed with a light microscope. The NBT reduction was assessed 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 with direct immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated mouse antihuman CD11b (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 FACScan 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 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
effects of 9-cis-RA and all-trans-RA on differentiation of HL-60. The HL-60 cells (2 x 10^5/mL) were cultured (7 days) with either 9-cis-RA (●) or all-trans-RA (△) and differentiation was determined by NBT reduction (A) and CD11b expression (B). Each point in (A) represents the mean (±SD) of three experiments performed in triplicate. Each point in (B) represents the mean of two experiments.

Fig 3. Effects of 9-cis-RA and all-trans-RA on differentiation of HL-60. The HL-60 cells (2 x 10^9/mL) were cultured (7 days) with either 9-cis-RA (●) or all-trans-RA (△) and differentiation was determined by NBT reduction (A) and CD11b expression (B). Each point in (A) represents the mean (±SD) of three experiments performed in triplicate. Each point in (B) represents the mean of two experiments.

growth in the presence of increasing amounts of 9-cis-RA and all-trans-RA (5 concentrations, 10^-6 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 ED50 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 ED50 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 x 10^{-8} 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^-9 mol/L 9-cis-RA and 10^-7 mol/L all-trans-RA (Fig 6A). Also, 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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Abbreviation: >, 50% inhibition of growth not reached at 10^-6 mol/L retinoid.
* Chronic phase.
† Lymphoid blast crisis.
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 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 for patients with AML 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.
of leukemic cells from patients and cell lines. The difference in their activity varied widely between patients, from slight to greater than 100-fold. (2) The range of activities of the two retinoids was similar. For example, clonal proliferation of AML cells from patients no. 6, 7, 8, 14, 15, 16, and 21, and each of the CML and CMMoL patients were not affected by either retinoid. However, 3 patients (no. 5, 12, and 13) had leukemic cells that were sensitive to the inhibitory effects of 9-cis-RA, but were resistant to the action of all-trans-RA. Interestingly, even leukemic cells from patients with the same disease subtype (eg, APL) varied in the relative potency of action of 9-cis-RA as compared with all-trans-RA. (3) Both 9-cis-RA and all-trans-RA can inhibit the clonal proliferation of leukemic cells without necessarily inducing the cells to differentiate. In fact, the only fresh AML cells that underwent excellent differentiation were from patients with M3 AML. We had previously noted that all-trans-RA could inhibit clonal growth of leukemic cell lines without inducing differentiation. (4) Both 9-cis-RA and all-trans-RA can stimulate
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. 49,50 For example, HL-60 cells must express RARs to be induced to differentiate by all-trans-RA. 51 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. 35,36 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-RXR 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 RXRβ. 51 (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 RAREs. 52,53 RXR also interacts directly with and enhances the transcriptional activity of receptors for 1,25-dihydroxy-vitamin D3 and thyroid hormone. 52,53

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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