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

By Akiko Sakashita, Masahiro Kizaki, Seppo Pakkala, Gary Schiller, Nobuyoshi Tsuruoka, Ryuji Tomosaki, James F. Cameron, Marcia I. Dawson, and H. Phillip Koeffler

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 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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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; all-trans-retinoic acid (all-trans-RA) induces complete remission in APL patients.

Retinoids probably exert their effect by modulating the transcription of a variety of genes critical to cellular proliferation and differentiation. Upon entering the cell, retinoids must be transported to the nucleus to exert their activity. The cellular responses to RA are mediated by two families of transcription factors, which include the RA receptors (RARs) and the retinoid X receptors (RXRs), which belong to the steroid-thyroid hormone receptor superfamily. Each retinoid receptor subfamily consists of several receptor isoforms referred to as RAR α, β, γ and RXR α, β, γ. The binding of RA to its receptor stimulates the transcriptional regulatory activity of the protein. The retinoid-receptor complex interacts with specific sequences (RA response elements [RAREs]) of cellular genes. The consensus DNA sequences recognized by an RAR are represented by a tandem repeat of the sequence AGGTCA separated by five nucleotides. In contrast, the consensus sequence recognized by an RXR is composed of the same tandem repeats separated by only one nucleotide.

Retinol and its aldehyde derivative retinal are relatively poor inducers of differentiation and inhibitors of proliferation of leukemic cells. Substitution of a terminal carboxyl group and isomerization about the terminal double bond yield either all-trans-RA or 13-cis-RA, each of which is an effective modulator of hematopoiesis, being capable of inducing 50% of HL-60 cells to differentiate (ED50) at concentrations of 10^{-7} mol/L. Derivatization of the carboxyl group markedly reduces differentiating potency, confirming the functional importance of the carboxyl terminus. Among the naturally occurring retinoids, all-trans-RA had been considered the most potent modulator of hematopoiesis. It binds to RARs with high affinity and alters gene expression as a consequence of this direct ligand interaction. However, an indication that all-trans-RA is not the RXR ligand resulted from showing that all-trans-RA apparently does not bind RXR. These results suggested that cells might be able to metabolize all-trans-RA to an RXR-specific ligand. Recently, another naturally occurring stereoisomer has been discovered, known as 9-cis-RA. This retinoid is produced in cultured cells and is identified in the liver and kidney. The 9-cis-RA is a high-affinity ligand for RXRs; in conjunction with RXRa, it transactivates target genes up to 40-fold more.
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 reduced atmosphere of 5.5% CO2 in air. Viability was assessed by trypan blue dye exclusion.

Clonogenic assay in soft gel culture. Cells (5 x 10^5) from the AML lines were plated in a 35-mm petri dish (Becton Dickinson and Co., Lincoln Park, NJ) with a-medium, 15% FBS, 0.3% agar (Flow Laboratories), and retinoid solution. After incubation for 7 days at 37°C in a humidified atmosphere of 5.5% CO2 in air, viability was assessed by trypan blue dye exclusion.

As assay of proliferation and cell survival in liquid culture. HL-60 cells (2 x 10^5/mL) were incubated with either all-trans-RA or 9-cis-RA in a-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.

RESULTS

Effect 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,
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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 (ED50) 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 CD1lb (Fig 3B), and morphology (data not shown). 9-cis-RA ($10^{-5}$, $10^{-4}$, 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, CD1lb, 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 × 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.

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 < .0.5) more potent than all-trans-RA in the stimulation of CFU-GM at 10^-10 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

<table>
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<th>Patient No.</th>
<th>Leukemia Subtype</th>
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<th>all-trans-RA (nmol/L)</th>
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<tr>
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Abbreviation: >, 50% inhibition of growth not reached at 10^-6 mol/L retinoid.

* Chronic phase.
† Lymphoid blast crisis.
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\(^1\,\text{,}\,13\) as well as inhibiting the clonal proliferation of several myeloid leukemia lines.\(^9\) 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 found that differentiation occurred only in cells obtained from two patients with APL.\(^12\) A subsequent study confirmed the differentiation activity of RA on cells from APL patients.\(^4\) More recently, samples from 35 APL patients were cultured in the presence of all-trans-RA (\(10^{-6}\) mol/L) for 3 to 5 days.\(^4\) 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.\(^4\) Maximal granulocytic differentiation was achieved by \(10^{-7}\) 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,\(^4\) 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.\(^14\) 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,\(^35\) 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 (\(\square\)) or all-trans-RA (\(\blacklozenge\)), and colonies were counted after 14 days of soft gel culture. The methods used are described in the legend to Fig 1.
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.

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

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.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–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 RXRαβ.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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