Effects of Novel Retinoic Acid Compound, 9-cis-Retinoic Acid, on Proliferation, Differentiation, and Expression of Retinoic Acid Receptor-α and Retinoid X Receptor-α RNA by HL-60 Cells

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Retinoic acid modulates proliferation and differentiation of a wide variety of normal and leukemic cells through two distinct families of transcriptional factors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). A stereoisomer of retinoic acid, 9-cis-retinoic acid, is a high-affinity ligand for RXR and binds efficiently to RAR. In contrast, all-trans-retinoic acid interacts 40-fold less efficiently with RXR as compared with RAR. To clarify the biologic role of retinoic acid compounds (all-trans, 9-cis-, and 13-cis-retinoic acid) in hematopoietic cells, we studied their effects on clonal growth, differentiation, and expression of RAR-α and RXR-α genes in HL-60 cells. At very low concentrations (10^-10 to 10^-12 mmol/L), each retinoid enhanced clonal growth of HL-60 cells. These concentrations of the retinoids had no capacity to induce differentiation of leukemic cells as measured by ability either to reduce nitroblue tetrazolium and to express CD11b antigens, suggesting that retinoids at very low concentrations may stimulate proliferation of leukemic cells rather than induce their differentiation. These findings may help explain why patients with acute promyelocytic leukemia may relapse while receiving retinoids. With continuous therapy, retinoids are metabolized rapidly with increased urinary excretion, lowering their plasma levels to a range that may stimulate proliferation without inducing differentiation of leukemic cells. In contrast, we found that at higher concentrations (≥10^-11 mmol/L) each retinoid inhibited clonal growth, reduced c-myc RNA levels, and induced differentiation of HL-60 cells. 9-cis-retinoic acid was a slightly more potent inducer of differentiation than all-trans-retinoic acid; the mechanism for this increased potency and its clinical potential requires additional studies. Steady-state levels of RAR-α mRNA in HL-60 cells were not affected by either 9-cis- and all-trans-retinoic acid. In contrast, 9-cis-retinoic acid, but not all-trans-retinoic acid, reduced RXR-α mRNA accumulation in a dose-dependent manner.

Mechanism of actions of these retinoids on hematopoietic cells is still unknown. Cytoplasmic retinoic-acid-binding protein (CRABP) was hypothesized as a biologic mediator of retinoic acid. However, CRABP is undetectable in HL-60, KG-1, or leukemic blasts from patients. Retinoic acid probably modulates the transcription of a variety of genes associated with cellular proliferation and differentiation by binding retinoic-acid receptors (RARs) and retinoid X receptors (RXRs), which are members of the steroid/thyroid hormone receptor superfamily of nuclear transcription factors. The three types of RARs (RAR-α, -β, -γ) and the three types of RXRs (RXR-α, -β, -γ) are ligand-inducible trans regulators that modulate the transcription of target genes by interacting with cis-acting DNA retinoic-acid response elements.

To date, of the natural retinoids, all-trans-retinoic acid 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. All-trans-retinoic acid has poor binding to RXR-α, and therefore is not the RXR ligand. Recently, another retinoic-acid compound has been discovered known as 9-cis-retinoic acid, which is a stereoisomer of retinoic acid and is produced in cultured cells and identified in the liver and kidney. It is an RXR ligand. Cotransfection experiments showed that RARs are activated by either all-trans- or 9-cis-retinoic acid at a ligand concentration of 5 × 10^-8 mmol/L, whereas RXRs are activated at least 40-fold more efficiently by 9-cis-retinoic acid as compared with all-trans-retinoic acid. These data suggest that 9-cis-retinoic acid may be more potent than all-trans-retinoic acid in modulating hematopoiesis. We have compared the abilities of 9-cis-retinoic acid and all-trans-retinoic acid to induce differentiation and inhibit proliferation of AML cell lines and fresh leukemic cells from 28 patients, and the data suggested a
Fig 1. Liquid and soft-gel cultures: Effects of all-trans-, 9-cis-, and 13-cis-retinoic acid on HL-60 proliferation. (A) Effects of retinoids on proliferation of HL-60 cells in liquid culture. HL-60 cells (2 $\times$ 10$^6$) were cultured in the presence of various concentrations of retinoids for 4 days with 1 $\mu$Ci of $^3$H-thymidine. Each point represents the mean ± SD of $^3$H-thymidine incorporation in quadruplicate cultures. (B) Effects of retinoids on clonal growth of HL-60 cells. HL-60 cells (1 $\times$ 10$^3$/mL) were cultured with various concentrations of retinoids in soft-gel culture and colonies (220 cells) were counted after 12 days of incubation. Results are expressed as the percentage of clonal growth in plates containing retinoids compared with number of colonies in control dishes without retinoids. Data represent mean of triplicate cultures. The SD was within 10% of the mean.

moderately enhanced potency of 9-cis-retinoic acid. 27 To clarify the biologic role and mechanisms of action of various retinoic acid compounds (all-trans-, 9-cis-, and 13-cis-retinoic acid) in hematopoietic cells, we studied their effects on clonal growth, differentiation, and expression of RAR-α and RXR-α RNA in HL-60 cells at a wide variety of ligand concentrations (10$^{-15}$ to 10$^{-6}$ mmol/L).

MATERIALS AND METHODS

Cells. To eliminate the influence of endogenous retinoic acids in the fetal bovine serum (FBS), 2,3 the HL-60 cells (promyelocytic leukemia cell line) 30 were maintained in serum-free culture medium (Cosmedium 001; Cosmo Bio Co, Tokyo, Japan) with 100 U/mL penicillin and 100 μg/mL streptomycin, or the cells were cultured in α-modified Eagle’s medium (α-MEM) ( Gibco-BRL, Gaithersburg, MD) with charcoal-stripped FBS (Sigma Chemical Co, St Louis, MO) in a humidified atmosphere with 5% CO₂. Although serum albumin can bind retinoic acids, Cosmedium 001 is free of albumin. Thus, this medium should be free of retinoids.

Chemicals. All-trans- and 13-cis-retinoic acid were purchased from Sigma, and 9-cis-retinoic acid 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 diluant had no effect on proliferation of the cells.

Assays for cellular proliferation. HL-60 cells (2 $\times$ 10$^5$) were incubated with various concentrations of retinoids (all-trans-, 9-cis-, and 13-cis-retinoic acid) in Cosmedium 001 for 4 days in 96-micro well plates (Flow Laboratories, Irvine, CA). One microcurie per well of H-thymidine (6.7 Ci mmol$^{-1}$; New England Nuclear, Boston, MA) was added for the last 4 hours of incubation. Cells were washed twice in phosphate-buffered saline, precipitated in 5% trichloroacetic acid (TCA; 30 mmol/L Na₂HPO₄) at 4°C for 1 hour, filtered onto glass microfilter membrane (Whatman, Hillsboro,
Dissolved by adding 0.1 mL of dimethylsulfoxide (Sigma) and measured cytospin preparations and by nitroblue tetrazolium (NBT). For clonal growth in plates containing retinoids as compared with the cells (Fig 1A). Similar clonogenic growth curves were also observed. At very low concentrations ($10^{-13}$ to $10^{-11}$ mmol/L), each retinoid (all-trans-retinoic acid, 13-cis-retinoic acid, 9-cis-retinoic acid) increased $^3H$-thymidine incorporation by the HL-60 cells; at higher concentrations ($10^{-10}$ to $10^{-8}$ mmol/L), they inhibited $^3H$-thymidine incorporation in a dose-dependent manner. The inhibitory effect of 9-cis-retinoic acid was significant at these higher concentrations (Fig 1A). Similar clonogenic growth curves were also observed. At very low concentrations ($10^{-13}$ to $10^{-11}$ mmol/L), each retinoid (all-trans-retinoic acid, 13-cis-retinoic acid, 9-cis-retinoic acid) increased $^3H$-thymidine incorporation by the HL-60 cells; at higher concentrations ($10^{-10}$ to $10^{-8}$ mmol/L), they inhibited $^3H$-thymidine incorporation in a dose-dependent manner. The inhibitory effect of 9-cis-retinoic acid was significant at these higher concentrations (Fig 1A).

Effects of retinoids on differentiation of HL-60 cells. Induction of differentiation of HL-60 cells into mature granulocytes by the retinoids was assessed by morphology (Fig 2), and expression of CD11b (Fig 3A), and expression of CD11b...
Fig 3. Comparison of the differentiation-inducing activities of all-trans-, 9-cis-, and 13-cis-retinoic acid. (A) NBT reduction activity. Cells were cultured with either all-trans-, 9-cis-, or 13-cis-retinoic acid for 4 days and differentiation was determined by NBT reduction. Results are expressed as the mean percentage of control dishes that contained no retinoid (mean ± SD from three experiments). (B) Expression of CD11b antigens in HL-60 cells. Cells were treated for 4 days with either all-trans-, 9-cis-, or 13-cis-retinoic acid and then analyzed by FACS (see Materials and Methods). Data represent the mean of triplicate experiments. The SD was within 10% of the mean.

antigens (Fig 3B). Exposure of cells to 10⁻⁸ mmol/L of either all-trans- or 9-cis-retinoic acid for 4 days resulted in more than 80% of HL-60 cells differentiating toward mature granulocytes (Fig 2). At very low concentrations (10⁻¹⁵ to 10⁻¹² mmol/L), ability to reduce NBT and to express CD11b antigens was unchanged compared with control cells (Fig 3). At higher concentrations (≥10⁻⁹ mmol/L), the retinoids induced differentiation of HL-60 cells. The 9-cis-retinoic acid was more potent than all-trans- and 13-cis-retinoic acid as measured by NBT activity, but both 9-cis- and all-trans-retinoic acid had similar abilities to induce expression of CD11b antigens.

Modulation of c-myc mRNA expression by retinoids in HL-60 cells. Studies have shown that exposure of HL-60 cells to all-trans-retinoic acid decreased accumulation of c-myc mRNA. We compared the abilities of 9-cis- and all-trans-retinoic acid with modulate expression of c-myc mRNA in HL-60 cells (Fig 4A and B). HL-60 cells were cultured for 4 days in the presence of various concentrations (10⁻¹⁵ to 10⁻⁶ mmol/L) of either retinoid. 9-Cis-retinoic acid was slightly more potent than all-trans-retinoic acid in decreasing levels of c-myc mRNA (Fig 4A and B). Accumulation of c-myc mRNA decreased by 50% after the cells were exposed to about 8 × 10⁻¹⁰ mmol/L 9-cis-retinoic acid for 4 days (Fig 4C). In contrast, ~2 × 10⁻¹⁰ mmol/L all-trans-retinoic acid was required to achieve the same decrease in accumulation of c-myc mRNA (Fig 4C).

Effects of retinoids on RAR-α and RXR-α mRNA expression in HL-60 cells. The modulation of RAR-α and RXR-α mRNA levels in HL-60 cells by various concentrations of retinoids was determined by Northern blot analysis (Fig 5A and B). Steady-state levels of RAR-α mRNA in HL-60 cells were not affected by a wide range of concentrations (10⁻¹⁵ to 10⁻⁶ mmol/L) of all-trans-retinoic acid (Fig 5A), 9-cis-retinoic acid (Fig 5B), and 13-cis-retinoic acid (data not shown). In addition, levels of RXR-α mRNA in HL-60
Fig 4. Modulation of expression of c-myc mRNA by all-trans- and 9-cis-retinoic acids in HL-60 cells. Cells were treated with various concentrations of all-trans- (A) and 9-cis-retinoic acid (B) for 4 days. Total RNA was extracted and analyzed by Northern blot technique and hybridized with [32P]-labeled c-myc cDNA, as described in Materials and Methods (20 μg per lane). Staining of ribosomal RNA in ethidium bromide gels confirmed integrity of RNA and showed comparable RNA loading in each lane. (C) Densitometric reading of the relative levels of c-myc transcriptions in (A) and (B) compared with that of undifferentiated cells (defined as 100%).
cells did not change after exposure to either all-trans- or 13-cis-retinoic acid (Fig 5A and data not shown). But interestingly, 9-cis-retinoic acid ($10^{-9}$ to $10^{-6}$ mmol/L) reduced RXR-α mRNA accumulation in a dose-dependent manner (Fig 5B).

**DISCUSSION**

All-trans-retinoic acid and its analogs enhance normal human hematopoiesis, inhibit leukemic cell proliferation, and induce differentiation of leukemic blast cells. In this study, we showed for the first time that retinoids at very low concentrations can stimulate proliferation, but do not induce differentiation of leukemic cells; and at higher concentrations, the retinoids can both induce differentiation and inhibit proliferation of leukemic cells. The 9-cis-retinoic acid was slightly more potent than all-trans- and 13-cis-retinoic acid in these latter effects. For example, each retinoid (all-trans-, 9-cis-, and 13-cis-retinoic acid) enhanced clonal growth of HL-60 cells at $10^{-6}$ to $10^{-3}$ mmol/L with $10^{-15}$ mmol/L of retinoids increasing clonal growth by 2- to 2.5-fold. In contrast, the retinoids at $10^{-11}$ to $10^{-9}$ mmol/L inhibited clonal growth and induced differentiation of HL-60 cells.

One potential problem of this and similar studies is the effects of endogenous retinoids on the results, especially when studying very low concentrations of retinoids ($10^{-15}$ to $10^{-13}$ mmol/L). To eliminate the effects of endogenous retinoic acids in culture system, we used serum-free media that contained no albumin for our liquid-culture studies and used charcoal-stripped FBS for our soft-gel culture analysis. Studies have shown that charcoal treatment removed greater than 90% of unconjugated steroids measured. Therefore, our soft-gel culture system was not completely free of endogenous retinoids. Moreover, a detection limit by specific high-performance liquid chromatography (HPLC) is 3 ng/mL ($=10^{-8}$M) for all-trans-retinoic acid. Therefore, we could not determine residual levels of retinoids in the charcoal-stripped FBS. Nevertheless, no active forms of retinoic acids (all-trans- and 9-cis-retinoic acid) were present in our liquid culture assays.

How retinoic acid regulates various aspects of proliferation and differentiation remains incompletely defined. Candidate molecules for mediating the biologic effects of retinoic acid in hematopoietic cells are the retinoic-acid receptors. Hematopoietic cells express several RAR and RXR receptors. The biologic effects of retinoic acid appear to be mediated not through a single receptor, but through multiple receptors that are structurally similar to other members of the steroid-thyroid-receptor superfamily of nuclear transcriptional factors. The RXR-α, -β, and -γ receptors exhibit considerable sequence homology in their ligand- and DNA-binding domains. The RXR appears to define a separate family of retinoic-acid receptors. 9-cis- and all-trans-retinoic acid can directly bind the RAR family with similar affinity. 9-cis-retinoic acid can directly bind and activate RXR-α; in contrast, all-trans-retinoic acid interacts 40-fold less efficiently with RXR-α than with RAR-α. Genes important in hematopoietic proliferation and
differentiation that are directly regulated by either RAR or RXR have not yet been identified. Potentially, RXR response elements may more efficiently control expression of several genes important in differentiation explaining the enhanced potency of 9-cis-retinoic acid as compared with all-trans-retinoic acid. Another possibility is that 9-cis-retinoic acid is more stable in vitro than all-trans-retinoic acid, and therefore provides the cell with more active compound. This would not explain why at lower concentrations the two retinoids are nearly equally potent in stimulating proliferation. Further studies are required to resolve this issue.

Our studies may have relevance to patients with APL. Recent studies have shown that a high proportion of patients with APL achieve complete remission after treatment with all-trans-retinoic acid. However, one serious problem of this exciting treatment is that the remissions are of brief duration and relapses of leukemia occur despite continuous retinoic-acid treatment. Several possible mechanisms for this acquired retinoic-acid resistance have been suggested, including either new mutations in retinoid receptors or alterations in either the absolute amount or binding affinity of CRABP. Recent studies have shown that clinical relapse and resistance to continuous treatment with all-trans-retinoic acid in APL is associated with progressive reduction of plasma concentrations of this retinoid. Our findings, in concert with the pharmacokinetic studies, suggest an explanation for early relapse of patients maintained on retinoic acid. Namely, with continuous therapy, all-trans-retinoic acid is rapidly metabolized with a 10-fold increase in urinary excretion of catabolized retinoids, lowering plasma levels of active retinoids. Therefore, these patients may only be achieving a concentration of retinoid that stimulates proliferation without inducing differentiation of leukemic cells.

We found that steady-state levels of RAR-α mRNA in HL-60 cells were not affected by 4 days’ exposure of 10^-11 to 10^-6 mmol/L of either all-trans- or 9-cis-retinoic acid. These results may suggest that the levels of expression of RAR-α mRNA are not related to either cellular proliferation or differentiation. Also, levels of RXR-α mRNA did not change in HL-60 cells after exposure to either all-trans- or 13-cis-retinoic acid. However, expression of RXR-α mRNA was downregulated by 10^-9 to 10^-6 mmol/L of 9-cis-retinoic acid in a dose-dependent fashion. The RXRα form heterodimers with RARs and other receptors in the steroid/thyroid superfamily and these interactions can enhance transcriptional activation of their response elements. Our results suggest that the loss of the partner for RAR-α may result in differential gene regulation through the retinoid pathway. These differences in regulation of RAR and RXR mRNA expression might be of physiologic significance. Further studies are required to clarify the mechanism and the biologic significance of expression of these retinoid receptors.

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Effects of novel retinoic acid compound, 9-cis-retinoic acid, on proliferation, differentiation, and expression of retinoic acid receptor-alpha and retinoid X receptor-alpha RNA by HL-60 cells

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