Leukemia (1997) 11, 393–400
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Alterations of differentiation, clonal proliferation, cell cycle progression and bcl-2 expression in RARα-altered sublines of HL-60

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All-*trans* retinoic acid (RA) induces granulocytic differentiation of acute promyelocytic leukemia cells both *in vivo* and *in vitro*. In the HL-60 wild-type (WT) early promyelocytic leukemia cell line, granulocytic differentiation appears to be directly mediated by the nuclear receptor RARα. An HL-60 subline resistant to RA (HL-60 R) contains a point mutation which results in a truncation of 52 amino acids at the COOH end of RARα. Cross-talk between differentiation, clonal inhibition of growth and apoptosis was studied using HL-60 WT, HL-60 R, and HL-60 R infected by a retroviral vector containing RARα (LX) as targets, which were cultured with various retinoids, vitamin D_3 analogs, HMBA, or DMSO. None of these compounds induced significant differentiation of HL-60 R and HL-60 LX, but they did induce differentiation of HL-60 WT. In contrast, retinoids inhibited the clonal proliferation of HL-60 WT, HL-60 R, and HL-60 LX. Vitamin D_3 analogs including KH1060 stimulated the clonal growth of HL-60 R; but they inhibited clonal growth of HL-60 WT and LX. Levels of Bcl-2 strongly decreased in HL-60 WT and LX after treatment by retinoids, while no change in expression occurred in HL-60 R. Neither KH 1060 nor 9-*cis* RA induced apoptosis of HL-60 R, but these agents did induce apoptosis in HL-60 LX WT. Taken together, we showed that HL-60 R has a global defect in its ability to be induced to differentiate by a variety of pathways, not merely the retinoid pathway. Furthermore, our HL-60 models showed that inhibition of proliferation and induction of apoptosis and differentiation can be dissociated. Clinically, these results suggest that several putative differentiation agents may have anti-cancer (antiproliferative) activities, even though they do not induce differentiation of the cancer cells.

Keywords: HL-60; resistance; differentiation; clonal growth

Introduction

Retinoids exert a wide range of biological effects predominantly related to cell proliferation and differentiation, including profound effects on hematopoeitic cells.[1] Retinoic acid (RA) enhances the clonal growth of normal human myeloid and erythroid precursors and inhibits the clonal proliferation of leukemic cells from patients with acute myelogenous leukemia (AML) as well as myeloid leukemia cell lines *in vitro*.[2–4] Moreover, treatment of acute promyelocytic leukemia (APL) with RA as a single agent leads to a high rate of complete remissions.[5,6] However, these remissions are generally not durable because RA fails to eliminate the malignant clone and because clinical resistance to RA develops.[7–13] The mechanism for this resistance is still unclear.

The biological effects of retinoids are mediated through their binding to the nuclear receptors: retinoic acid receptors (RARα,β,γ) and retinoid X receptors (RXR α,β,γ).[14–17] Both classes of receptors belong to the steroid/thyroid hormone superfamily and act as inducible transcription factors.[18] RARs bind all-*trans* RA as well as 9-*cis* RA, while RXRs bind only 9-*cis* RA.[19–21] Thus, RARs heterodimerize to form RAR/RXR complexes.[17,22–25] RXRs act as a coregulator, enhancing the binding of RA, 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], thyroid hormone, and peroxisome-activated receptors to their responsive elements via heterodimers.[22–27] Moreover, RARs may antagonize AP-1 function by either binding (directly or indirectly) to c-jun/c-fos to form an inactive complex or interacting with and sequestering another nuclear accessory factor that is required for AP-1-mediated transactivation.[28–30]

The problem of RA resistance in APL patients reflects the limits of differentiation-inducing therapy in general. Therefore, in an attempt to improve the therapeutic outcome of a single-agent, RA in the treatment of leukemia, we used RA and non-retinoid agents to examine their abilities to inhibit proliferation, and to induce differentiation and apoptosis. The RA-resistant subclone of the HL-60 myeloid leukemia cell line, designated as HL-60 R,[31–35] was used in our studies. These cells contain a mutation which results in a truncation of 52 amino acids at the COOH end of RARα resulting in resistance to RA-induced differentiation. Another model subline was HL-60 LX; these cells result from infection of HL-60 R with a retroviral vector containing RARα.[31,32] With these sublines of HL-60 as well as wild-type (WT) HL-60 cells as tools, we determined that inhibition of clonal proliferation could be disassociated from induction of differentiation and/or apoptosis of leukemic cells, and resistance to induction of differentiation was a global defect in the HL-60 R cells.

Materials and methods

Cell lines

HL-60 WT cells were obtained from the American Type Culture Collection (Rockeville, MD, USA). The retinoic acid resistant subline, HL-60 R, and the HL-60 R variant infected with a functional retinoic acid receptor RARα (HL-60 LX) were generated and characterized as described previously.[31,32] The HL-60* clone isolated by Gallagher *et al*,[33–35] was continuously grown in RA (10^{-7} M). All cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM l-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were incubated in a humidified incubator containing 5% CO_2 at 37°C. Cell viability was determined by trypan blue exclusion.

Cell treatment and differentiation

Differentiation of HL-60 cells was measured by reduction of nitroblue tetrazolium (NBT),[36] nonspecific α-naphthyl acetate acid esterase (NSE) (Sigma, St Louis, MO, USA)[36] and morphology using light microscopy of cytospin preparations

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Received 17 June 1996; accepted 5 November 1996

stained with Diff-Quick Stain Set (Baxter Heathcare Corporation, Miami, FL, USA) after 5 days cultivation of HL-60 cells in suspension with various compounds. The dimethylsulfoxide (DMSO) and the hexamethylbisacetamide (HMBA) were obtained from Sigma.

Retinoids and vitamin D_3 compounds

All-*trans*-retinoic acid (RA) (Sigma, Poole, UK); 9-*cis* retinoic acid (9-*cis*-RA); 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethenyl] benzoic acid (code name, LG1069) (kindly provided by Dr R Heymann, Ligand Pharmaceuticals, San Diego, CA, USA); 2-(3-4-dihydro-4,4-dimethyl-2H-1 benzopyran-6-yl)-2-(4-carboxyphenyl-1,3-dithiane (code name, SR11238) (kindly provided by Dr M Dawson, SRI International, Menlo Park, CA, USA; 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] code name, compound C); 1,25$(OH)_2$-16ene-23yne-D_3 (code name, compound V) (kindly provided by Dr M Uskokovic, Hoffman-La-Roche, Nutley, NJ, USA); 1,25$(OH)_2$-24α,26α,27α-tri-homo-22,24-diene-D_3 (code name EB 1089); and 20-epi-22-oxa-24a,26a,27a-tri-homo-1α,25-$(OH)_2$-D_3 (code name KH 1060) (kindly provided by Dr L Binderup, Leo Pharmaceutical Products, Ballerup, Denmark) were dissolved in ethanol. The 9-*cis* RA and LG1069 were dissolved in 50% ethanol, 50% DMSO.

Clonogenic assay in soft agar

HL-60 (2×10^3) cells were cultured in a two-layer soft agar system for 10 days according to previously described methods.[37]

Immunostaining for Bcl-2

Immunostaining for Bcl-2 was performed on HL-60 cells which had been grown in suspension culture with and without analogs (10^{-7} m) for 5 days. Intracellular Bcl-2 protein was detected with a murine monoclonal antibody (DAKO, Carpinteria, CA, USA). Antibody location for Bcl-2 was performed with 3,3′-diaminobenzidene hydrochloride (Sigma, 5 mg/10 ml) to which hydrogen peroxide (0.03%) was added just before use. Slides were counterstained with methyl green and mounted with permount.

Analysis of the cell cycle and apoptosis

After extensive washing with phosphate-buffered saline and fixation for 30 min on ice cold 80% methanol, 10^6 cells were incubated at 4°C in the dark with a solution of 50 μg/ml propidium iodide and RNase (100 unit/ml Sigma) (Becton Dickinson, Lincoln Park, NJ, USA). Analysis was performed immediately after staining using the Cell-Fit SOBR computer program. Apoptosis was studied in the cells by staining the cellular DNA with 5 μg/ml propidium iodide, analyzed 5–10 min in the dark, and then by flow cytometry as previously described.[38]

Results

Effects of retinoids and non-retinoid agents on differentiation of RA-resistant HL-60 cells

Prior studies showed that HL-60 wild-type (WT) cells are induced to differentiate down the granulocytic pathway by retinoids, HMBA and DMSO; and down the monocytic pathway by 1,25$(OH)_2D_3$ and related analogs and 12-*O*-tetradecanoylphorbol-13-acetate (TPA).[39,40] In this study, we examined the induction of differentiation of WT- and RA-resistant HL-60 cells by assessing their abilities to reduce nitroblue tetrazolium (NBT) (granulocytic and monocytic differentiation) and to acquire nonspecific esterase (NSE) activity (monocytic differentiation) and by morphological differentiation.

Several retinoids were examined for their abilities to induce differentiation. The RA (10^{-7} m), 9-*cis* RA (10^{-6} m) and their combination induced the differentiation of 60%, 70% and 75% of HL-60 WT, respectively, after 5 days of culture (Table 1, Figure 1a). The induction of differentiation was time and concentration dependent, with up to 85% of HL-60 WT cells becoming NBT-positive after exposure to 10^{-7} m RA for 7 days (data not shown). The RXR-selective ligand LG1069 (10^{-6} m, 5 days) did not induce differentiation of HL-60 WT cells (2% NBT-positive HL-60 WT cells). The 1,25$(OH)_2D_3$, another seco-steroid, and several of its analogs were also examined either alone or in combination with either 9-*cis* RA or RA (Table 1, Figure 1). The most potent analog was KH 1060 (10^{-7} m, 5 days), which resulted in 99% NBT-positive HL-60 WT; and the combination of 1,25$(OH)_2D_3$ and 9-*cis* RA was the most potent combination to produce differentiation of HL-60 WT (Table 1). In contrast, none of these compounds, either alone or in combination with RA or 9-*cis* RA induced significant differentiation (<7%) of HL-60 R down either the granulocytic or monocytic pathways (Table 1, Figure 1B). Other non-retinoid compounds which were known to induce differentiation of HL-60 WT,[41] such as HMBA (2×10^{-3} m) or DMSO (1.0, 1.25, 1.5% v/v) cultured 5 days were also studied; each failed to induce differentiation of HL-60 R cells (Table 1).

To investigate further the involvement of the truncated RARα on myeloid differentiation, we examined HL-60 R cells infected with the LXSN retroviral vector, harboring a complementary DNA insert that contained a complete coding sequence of RARα (HL-60 LX).[31] The NBT-positive cells increased in RA-treated HL-60 LX cells to 10% after culture with RA (10^{-7} m), 9-*cis* RA (10^{-6} m), or the combination of both (Table 1, Figure 1C). Nevertheless, the number of NBT-positive HL-60 LX cells was consistently less than the number of NBT-positive HL-60 WT cells induced by RA, 9-*cis* RA, or the combination of both (Table 1, Figure 1C). In addition, none of the non-retinoid agents could induce a significant level of differentiation of HL-60 LX (Table 1, Figure 1C).

We also analyzed the HL-60 RA cells (HL-60 R*) isolated by Gallagher *et al*;[33] these cells have the same mutation that was present in HL-60 R,[35] also resulting in the truncation of the last 52 amino acids of the COOH terminal end of the RARα. Neither RA (10^{-7} m), 9-*cis* RA (10^{-7} m) nor a combination of both (× 5 days) induced significant differentiation of HL-60 R*. In marked contrast to HL-60R, however, these cells were easily induced to differentiate by vitamin D_3 analogs and DMSO similar to HL-60 (Table 1, Figure 1D). For example, either 1,25$(OH)_2D_3$(10^{-7} m), KH 1060 (10^{-7} m), or DMSO

Table 1 Induction of differentiation of HL-60 sublines

Inducing agent[a]	*NBT (% positive)*				*NSE (% positive)*	
	HL-60 WT	*HL-60 R*	*HL-60 LX*	*HL-60 R**	*HL-60 WT*	*HL-60 R*
No Inducer	<1 ± 0.5	<1 ± 0.5	<1 ± 0.5	<1–0 ± 0.5	1 ± 0.5	1 ± 0.5
Retinoids						
RA (10^{-7} M)	60 ± 5	0 ± 0	10 ± 1	4 ± 1	0.5 ± 0	0 ± 0
9-*cis* RA (10^{-6} M)	70 ± 4	0 ± 0	10 ± 1	6 ± 1	0 ± 0	0 ± 0
LG1069 (10^{-6} m)	2 ± 1	0 ± 0	NE	NE	0 ± 0	0 ± 0
9-*cis* RA (10^{-6} M) + RA (10^{-7} M)	75 ± 10	0 ± 0	10 ± 1	7 ± 1	NE	NE
9-*cis* RA (10^{-6} M) + LG1069 (10^{-6} M)	72 ± 7	0 ± 0	NE	NE	NE	NE
Vitamin D_3 analogs						
$1,25(OH)_2D_3$ (10^{-7} M)	85 ± 6	0 ± 0	0 ± 0	90 ± 7	75 ± 4	0 ± 0
KH 1060 (10^{-7} M)	>99 ± 0	0 ± 0	0 ± 0	>99	99 ± 0	0 ± 0
EB 1089 (10^{-7} M)	90 ± 2	0 ± 0	NE	NE	80 ± 4	0 ± 0
V (10^{-7} M)	88 ± 2	0 ± 0	NE	NE	76 ± 2	0 ± 0
$1,25(OH)_2D_3$ (10^{-7} m) + RA (10^{-7} m)	71 ± 4	7 ± 2	NE	NE	NE	NE
$1,25(OH)_2D_3$ (10^{-7} m) + 9-*cis* RA (10^{-6} m)	90 ± 4	0 ± 0	NE	NE	72 ± 3.8	0 ± 0
Other compounds						
DMSO 1%	NE	0 ± 0	NE	NE	NE	NE
DMSO 1.25%	72 ± 1	0 ± 0	0 ± 0	70 ± 5	NE	NE
DMSO 1.5%	82 ± 4	0 ± 0	NE	NE	NE	NE
HMBA 2×10^{-3} M	33 ± 2	0 ± 0	NE	NE	NE	NE

[a]Cells were cultured for 5 days with a potential differentiation-inducing agent and differentiation was assayed by nitroblue tetrazolium (NBT) reduction and nonspecific esterase (NSE) assays. HL-60 R*: clone isolated by Gallagher *et al*.[33–35]
NE, not examined; RA, all-*trans* retinoic acid; 9-*cis* RA, 9-*cis* retinoic acid; LG1069, 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2 naphthyl)ethenyl] benzoic acid; KH 1060, 20-epi-22-oxa-24a,26a,27a-tri-homo-1α,25-$(OH)_2$-D_3; V, 1,25$(OH)_2$-16ene-23yne-D_3; EB 1089, 1,25$(OH)_2$-24α,26α,27α,-tri-homo-22,24-diene-D_3.
Results expressed as a mean ± s.d. of four experiments.

(1.00% v/v) (× 5 days) induced 90%, >99%, and 70% of HL-60 R* to become NBT positive, respectively (Table 1).

Effects of retinoids and non-retinoid agents on the clonal growth of RA-resistant HL-60 cells and RARα-infected HL-60 R cells

The effect of retinoids and non-retinoid agents was examined on the clonal growth of HL-60 R and those HL-60 R infected by the LXSN RARα retroviral vector (HL-60 LX). Surprisingly, the effective dose that inhibited 50% (ED_{50}) of HL-60 R colony formation was 3×10^{-7} m and 2×10^{-7} m for RA and 9-*cis* RA, respectively (Figure 2b). The ED_{50} for HL-60 WT was 8×10^{-8} m and 5×10^{-8} m for RA and 9-*cis* RA, respectively (Figure 2a). Furthermore, the RARα-infected HL-60 R (HL-60 LX) cells became hypersensitive to the clonal inhibitory activity of RA and 9-*cis* RA, (ED_{50}, 4×10^{-9} m and 2×10^{-9} m, respectively) (Figure 2c). In contrast, HL-60 WT and HL-60R dramatically diverged in their response to the vitamin D_3 compounds (Figure 2a and b). Both $1,25(OH)_2D_3$ and KH 1060 had an ED_{50} of about 1.8×10^{-8} m for HL-60 WT, and no colonies were present in dishes containing 10^{-6} m of either analog (Figure 2a). In contrast, the HL-60 R cells were stimulated to proliferate by $1,25(OH)_2D_3$ and KH 1060 (10^{-11}–10^{-6} m) (Figure 2b). However, the HL-60 LX became sensitive to clonal inhibition by the vitamin D_3 compounds (ED_{50}, 7×10^{-8} m and 8×10^{-10} m for $1,25(OH)_2D_3$ and KH 1060, respectively) (Figure 2c).

We also examined the activity of SR11238 a retinoid which does not induce cellular differentiation in various cell lines but has a strong anti-AP-1 function.[29] The SR11238 (10^{-7} m) inhibited 34% of the clonal growth of the HL-60 R cells; the same level of inhibition occurred with 10^{-7} m 9-*cis* RA (35%) and both were slightly more potent than RA which inhibited 22% of the HL 60 R colonies (Figure 3).

Cell cycle analysis

We investigated the effects of 9-*cis* RA and KH 1060 on the cell cycle of HL-60 WT, R, and LX. The HL-60 WT had a significant 10% and 18% increase in the number of cells ($P < 0.05$) in the G_0/G_1 phase after 4 days of exposure to either 9-*cis* RA (10^{-7} m) or KH 1060 (10^{-7} m), respectively (Table 2). The HL-60 R cells did not have a significant variation of the cell cycle in the presence of 9-*cis* RA but did have a significant (12%) decrease in the number of cells in G_0/G_1 ($P < 0.05$) after 4 days of treatment with KH 1060 (10^{-7} m). The HL-60 transfected with RARα (HL-60 LX) had no significant change of the number of cells in G_0/G_1 after culture with either 9-*cis* RA (10^{-7} m, 4 days) or KH 1060.

Expression of Bcl-2

The classical effects of retinoids on HL-60 cells are to inhibit their clonal growth and to induce their differentiation and apoptosis. An early event which underlies retinoid-induced apoptosis is the down-regulation of Bcl-2 expression.[42] Bcl-2 is a membrane-associated protein whose expression is associated with a suppression of apoptosis. We hypothesized that the ability of an analog to inhibit clonal growth would parallel its ability to modulate the level of Bcl-2 and the apoptotic

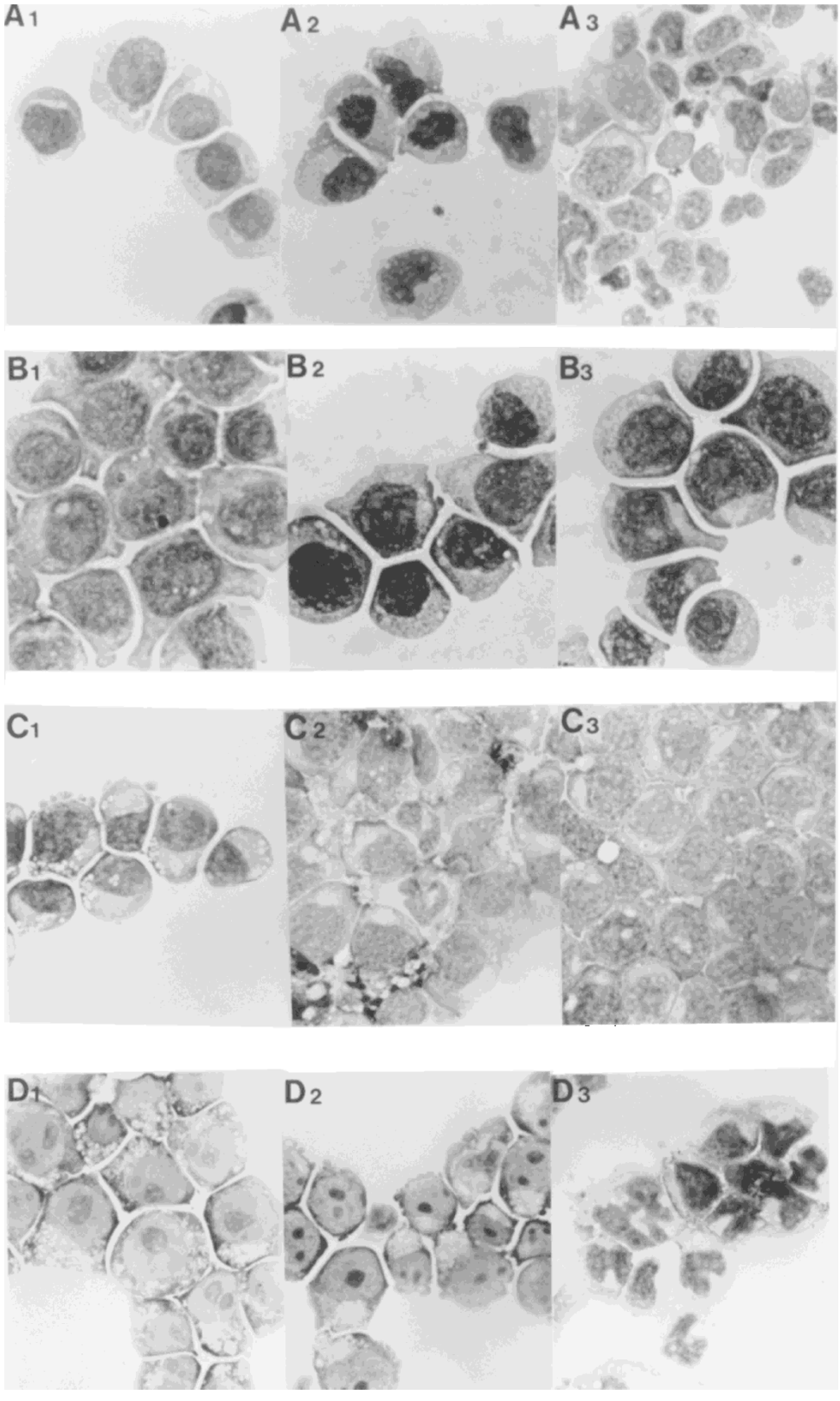

Figure 1 Effect of retinoids and vitamin D_3 analogs on differentiation of HL-60 sublines: HL-60 WT (A), HL-60 R (B), HL-60 LX (C) and HL-60 R* (D). Subpanel 1 shows control cells, subpanel 2 shows cells cultured with all-*trans* retinoic acid (10^{-7} m, 5 days), subpanel 3 shows cells cultured with KH 1060 (10^{-7} m, 5 days).

Table 2 Cell cycle analysis of HL-60 sublines cultured with a retinoid (9-*cis* RA) or a vitamin D_3 analog (KH 1060)

Cell lines	*Compounds*	*G_0/G_1 phase*	*S phase*
HL-60 WT	Control	52 (±1)	35 (±5)
	9-*cis* RA (10^{-7} M)	62 (±6)	25 (±10)
	KH 1060 (10^{-7} M)	70 (±10)	25 (±4)
HL-60 R	Control	51 (±2)	45 (±6)
	9-*cis* RA (10^{-7} M)	54 (±4)	38 (±10)
	KH 1060 (10^{-7} M)	39 (±2)	55 (±10)
HL-60 LX	Control	52 (±1)	41 (±3)
	9-*cis* RA (10^{-7} M)	58 (+11)	29 (±8)
	KH 1060 (10^{-7} M)	42 (±8)	43 (±6)

Cells were cultured with the analog for 4 days and then their cell cycle status was studied. Each point represents the mean (± s.d.) of at least three experiments.

pathway. Immunohistochemistry showed that HL-60 WT, R, LX displayed abundant Bcl-2 (Figure 4). After 5 days of culture with 9-*cis* RA (10^{-7} m), a strong decrease of Bcl-2 expression occurred in HL-60 WT and HL-60 LX, while 100% of HL-60 R continued to express Bcl-2. After 5 days exposure to KH 1060 (10^{-7} m), Bcl-2 signal strongly decreased in HL-60 WT while no change occurred in HL-60 R and HL-60 LX.

Induction of apoptosis

We analyzed the kinetics of induction of apoptosis by either 9-*cis* RA or KH 1060 on days 3–5 of culture of HL-60 WT, R and LX (Figure 5). Sixteen percent of HL-60 WT was apoptotic at day 5 of culture with 9-*cis* RA (10^{-7} m). HL-60 LX were slightly more sensitive; at days 4 and 5 of culture with 9-*cis* RA (10^{-7} m), the percent of apoptotic HL-60 LX cells was 13% and 28%, respectively. The KH 1060 (10^{-7} m, day 5) produced an equivalent increase of apoptosis in HL-60-WT and LX of about 13% for both. In contrast, HL-60 R did not undergo apoptosis in the presence of either 9-*cis* RA or KH 1060 (Figure 5).

Discussion

Retinoids exert a wide range of biological effects on normal and transformed cells. For example, in APL cells, retinoids induce differentiation, inhibit proliferation, and cause apoptosis. These biological effects can be mediated through multiple receptors which are structurally related to the steroid/thyroid receptor superfamily. This, in turn, results in regulation of expression of certain genes in the target cells. Evidence is now accumulating that the retinoid signaling pathway interacts with the non-retinoid pathway. For example, the VDR receptor acts not only through the VDR/RXR heterodimer but may also function through the RAR/VDR receptor pathway.[43]

Prior studies in the HL-60 model system,[44–46] as well as in fresh leukemic cells showed that RA cooperated with non-retinoid inducers such as either HMBA or DMSO to yield synergistic induction of differentiation. Moreover, concerning the NB4 promyelocytic leukemic cells, that are resistant to differentiation by a variety of non-retinoid inducers, a short pre-exposure to RA (30 min) abolished resistance to non-retinoids and potentiated their differentiation.[47] These results strongly support the presence of interconnections between the retinoid and non-retinoid signaling pathways. Therefore, the purpose

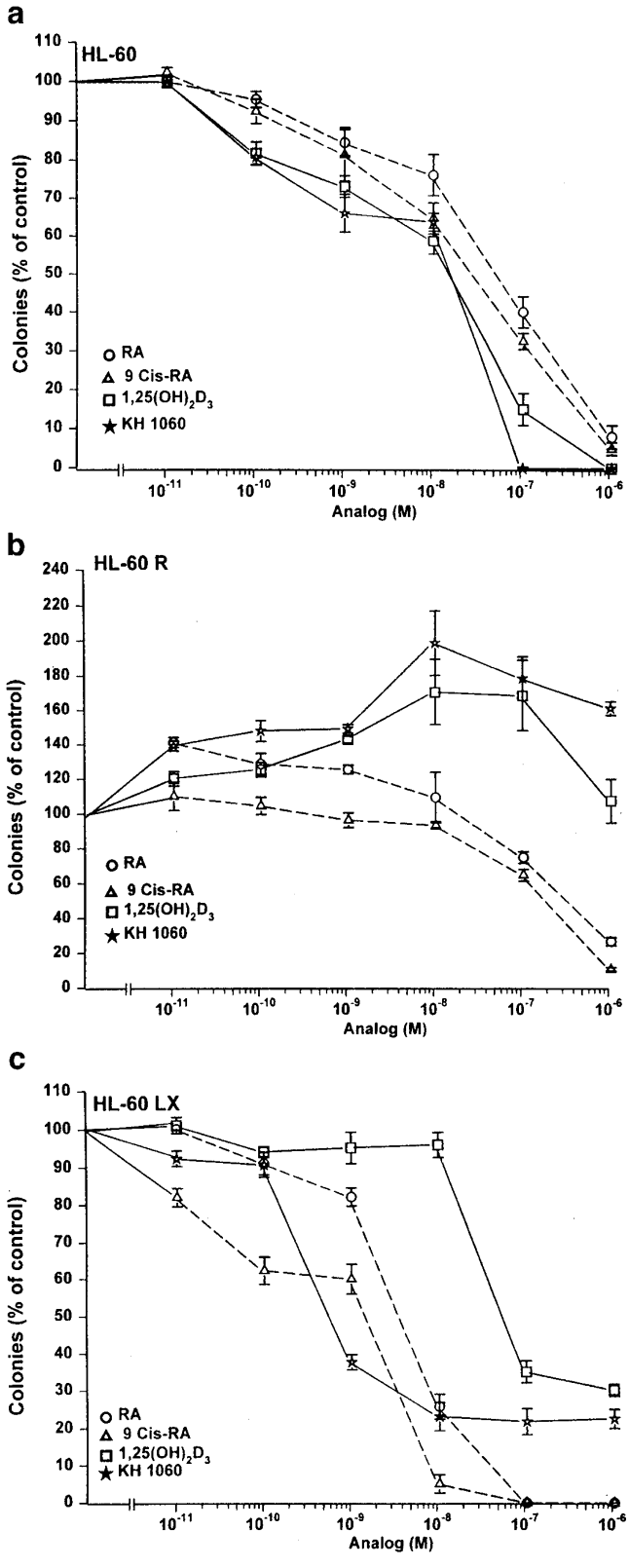

Figure 2 Effect of retinoids and vitamin D_3 analogs on differentiation of HL-60 sublines. The HL-60 WT (a), HL-60 R (b), and HL-60 LX (c) were cultured with various concentrations of either all-*trans* retinoic acid (RA), 9-*cis* RA, $1,25(OH)_2D_3$ or KH 1060. Results are expressed as percentage of control plates containing no compound. Each point represents the mean of at least three experiments performed in triplicate dishes.

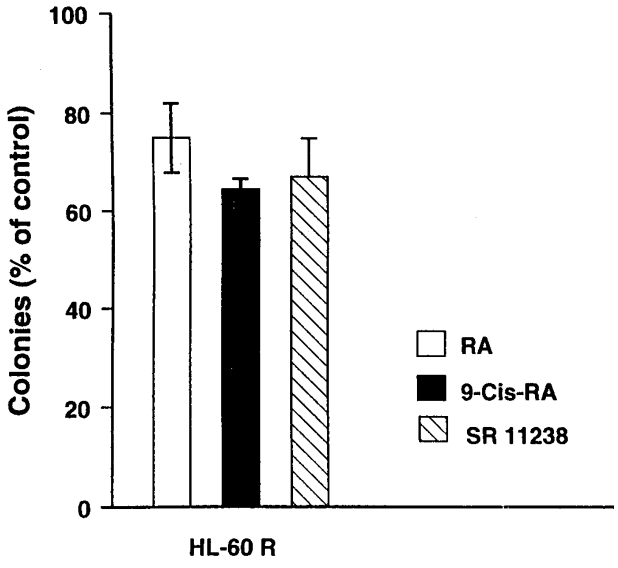

Figure 3 Effect of the anti-AP-1 analog SR 11238 on the clonal growth HL-60 R subline. The HL-60 R were cultured with all-*trans* retinoic acid (RA), 9-*cis* RA or SR 11238. Results are expressed as percentage of control plates containing no compound. Each point represents the mean of at least three experiments performed in triplicate dishes.

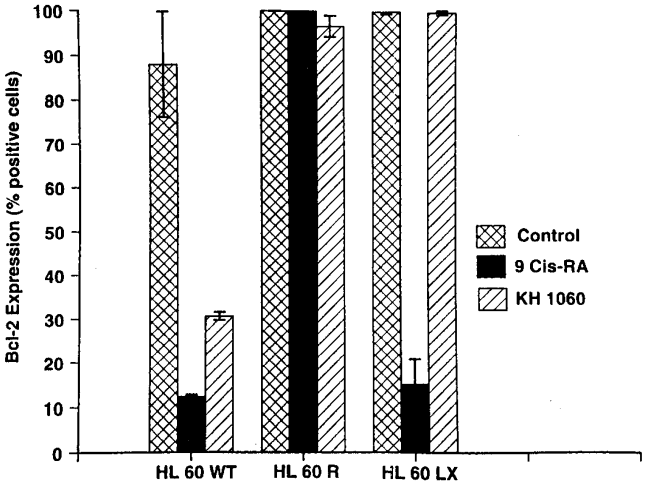

Figure 4 Bcl-2 expression in HL-60 sublines. The cells were treated with either 9-*cis* RA (10^{-7} m), KH 1060 (10^{-7} m), or diluant control for 5 days, and then analyzed for expression of Bcl-2. Results represent the mean of three independent experiments.

of this study was to analyze the effects of retinoid resistance of HL-60 on the cross-talk between differentiation, clonal growth, modulation of the cell cycle, expression of Bcl-2 and induction of apoptosis by retinoids and non-retinoid inducers.

Our studies indicated first that the pathway of differentiation was blocked in the HL-60 R subline exposed to either retinoids, vitamin D_3 analogs, or a combination of a retinoid and a vitamin D_3 analog, DMSO or HMBA. These results suggest that HL-60 R cells are carrying a defect which affects their differentiation at a step which is common to each of the pathways of differentiation. Presumably, RA, $1,25(OH)_2D_3$ and DMSO normally initiate differentiation through divergent routes which may converge in a common pathway. This global defect is further emphasized by noting that HL-60 LX,

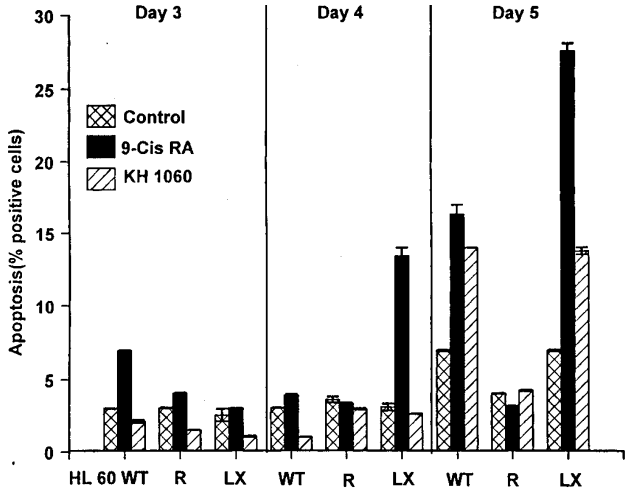

Figure 5 Induction of apoptosis in HL-60 sublines. The cells were cultured for 3, 4 and 5 days with either RA, 9-*cis* RA, or KH 1060 (10^{-7} m). Results are expressed in percentage of propidium iodide positive cells. Each point represents the mean of at least two experiments. RA, all-*trans* retinoic acid.

which has the RARα wild-type gene placed in it by retroviral infection,[31] was also poorly induced to differentiate by the inducers of HL-60 differentiation (Table 1, Figure 1). Our hypothesis that HL-60 R has acquired global resistance to inducers of differentiation, independent of the mutation of the RARα gene was also supported by HL-60 R*, derived by the Gallagher's group.[33–35] Although HL-60 R* was resistant to retinoids, it was induced to differentiate by vitamin D_3 compounds and DMSO (Table 1, Figure 1). Moreover, an antagonist of RARα, (Ro43-55) did not block the differentiation induced by $1,25(OH)_2D_3$ in HL-60 WT cells (data not shown).

The second finding in our study is that the pathway associated with induction of differentiation can be dissociated from the pathways which mediate inhibition of clonal growth and apoptosis. We showed that even though the HL-60R cells could not be induced to differentiate, their clonal proliferation was inhibited by retinoids at almost the same sensitivity as observed with HL-60 WT. The HL-60 LX became more sensitive to the inhibition of clonal growth mediated by retinoids than were both the HL-60 R and WT cells.

What is a possible explanation for the observation that retinoids inhibit clonal proliferation of HL-60 R even though they cannot induce differentiation of these cells? RARs are able to repress the ability of AP-1 to transactivate by a mechanism probably involving protein–protein interaction;[28,48,49] and this anti-AP-1 function is separable from the transactivational function of RAR and is associated with growth inhibition of transformed cells.[29,30] Thus, in the presence of a retinoid, the truncated RARα may still be able to generate an anti-AP-1 protein activity and thus may be able to mediate the inhibition of clonal growth of HL-60 R. Indeed, we examined the growth inhibition obtained by using the SR11238 compound. This retinoid does not induce differentiation but strongly inhibits the growth of various cancer cell lines; moreover, it displays a strong anti-AP-1 activity.[29] We showed that (10^{-7} m) SR11238 displayed a similar inhibition of clonal proliferation as (10^{-7} m) 9-*cis* RA and even slightly higher than (10^{-7} m) RA. This result is consistent with the growth inhibition pattern in T-47 D, and Calu-6 cells.[29] This suggests that the inhibition of the clonal proliferation observed in HL-60 R with either RA

or 9-*cis* RA may be mediated by the anti-AP-1 activity of these retinoids.

Interestingly, vitamin D_3 compounds increased the clonal growth of HL-60 R and inhibited clonal growth of HL-60 LX. This suggests that retinoids and vitamin D_3 compounds exert their inhibition of clonal growth by two pathways that are partially linked.

Of note, we have previously found that the early myeloblast cell line (KG-1) could not be induced to differentiate by either $1,25(OH)_2D_3$ or RA, but $1,25(OH)_2D_3$ stimulated and RA markedly inhibited their clonal growth.[37] Therefore, these early myeloblasts behave biologically similarly to HL-60 R cells. However, we have shown that the ligand binding region of the RARα gene of KG-1 cells was intact.[50] Therefore, these two myeloid leukemic cell lines (HL-60 R, KG-1) may have a common, unidentified genetic abnormality allowing these cells to respond similarly when exposed to these secosteroids.

Regulation of clonal growth of HL-60 cells results from a balance between differentiation, inhibition of proliferation (G_0/G_1 arrest) and apoptosis. Retinoids have been reported to regulate the expression of several genes which underlie molecular mechanism of apoptosis including Bcl-2 and transglutaminase I and II.[42,51] Prominent expression of Bcl-2 is often associated with suppression of apoptosis induced by a variety of chemotherapeutic drugs and other stimuli.[52–56] The HL-60 WT cells had a precipitous fall in Bcl-2 levels after exposure to 9-*cis* RA while levels did not change in HL-60 R. The reintroduction of a RARα in HL-60 R resulted in a strong downregulation of Bcl-2 in HL-60 LX cultured with 9-*cis* RA. This decreased expression of Bcl-2 in HL-60 LX cultured with 9-*cis* RA paralleled their increase of apoptosis. These results suggest the importance of RARα in the cell death pathway and are consistent with the studies in SPOC-1 cells in which overexpression of the truncated RARα (Δ 403) was associated with their inability to increase expression of transglutaminase II and to undergo apoptosis induced by RAR-specific ligands.[51] Our studies also showed that the retinoid-mediated fall of Bcl-2 and decrease in cell growth was independent of the differentiation pathway, because these HL-60 LX cells had very little capacity to differentiate.

Our results concerning the effects of the vitamin D_3 analog KH 1060 on the levels of Bcl-2 and induction of apoptosis were not easily understood. Exposure of HL-60 cells to KH 1060 decreased their levels of Bcl-2, increased apoptosis and decreased their clonal growth. The HL-60 R cells cultured with KH 1060 did not change their levels of expression of Bcl-2, did not induce apoptosis and did not cause a decrease in clonal growth. However, exposure of HL-60 LX to KH 1060 did not decrease their levels of Bcl-2, but did induce apoptosis and inhibit clonal growth of these cells. Thus, KH 1060 can inhibit cell growth without inhibiting expression of Bcl-2. The genetic pathway causing this disequilibrium between modulation of Bcl-2 levels, induction of apoptosis, and inhibition of cell growth is unclear at this time.

In conclusion, the pathway of differentiation of the HL-60 R subclone is profoundly altered as noted recently.[57] Nevertheless, we have found that these cells retain their ability to be inhibited in their clonal growth by retinoids, albeit not through the induction of apoptosis. These data suggest that inhibition of proliferation and induction of differentiation are not totally linked in myeloid leukemic cells. These results suggest that even though retinoids may not be able to induce differentiation of leukemic cells, they may still have clinical efficacy through their antiproliferative effects.

Acknowledgements

We thank Dr R Heymann for providing LG1069; Drs M Uskokovic and L Binderup for providing vitamin D_3 compounds; Dr M Dawson for providing SR11238; Dr R Gallagher for HL-60 R*; Dr J Said for Bcl-2 immunostaining analyses; the Cedars-Sinai Medical Center Flow Cytometry Core Facility for FACS analysis; and Gail Jao and Kelly Chung for preparation of the manuscript. This work was supported by NIH grants, the Concern Foundation, and the Parker Hughes Trust.

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