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The Role of Vitamin D Derivatives and Retinoids in the Differentiation of Human Leukaemia Cells

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ABSTRACT. The capabilities of 1α, 25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$),§ and two novel vitamin D analogues, EB1089 and KH1060, to induce the differentiation of two established leukaemia cell lines, U937 and HL-60, were assessed alone or in combination with the retinoid compounds, 9-*cis* retinoic acid (9-*cis* RA) and all-*trans* retinoic acid (ATRA). The vitamin D derivatives acted to increase the differentiation of U937 and HL-60 cell cultures in a dose-dependent manner, as determined by nitroblue tetrazolium (NBT) reduction, with EB1089 and KH1060 being more effective than the native hormone. As an additional index of leukaemic cell differentiation, induction of expression of the phenotypic cell surface antigen, CD14, and the β_2-integrins, CD11b and CD18 by the vitamin D and retinoid compounds were monitored using fluorescence activated cell sorting (FACS) analyses. Following 96-hr treatment of U937 and HL-60 cells with 5×10^{-10} M of the vitamin D derivatives, a striking increase in CD14 antigen expression was apparent, indicating the promotion by these compounds of a monocyte/macrophage lineage of cells. CD11b and CD18 antigen expression were also raised above control levels. In contrast, both retinoid compounds used at the higher concentration of 1×10^{-8} M were not effective inducers of CD14 antigen expression. However, CD11b and CD18 were both readily increased in U937 and HL-60 cell cultures. Treatment of U937 cell cultures with the vitamin D compounds and the retinoids resulted in cooperative effects on induction of differentiation, with correlation by both NBT reduction and FACS analyses of CD14 antigen expression. The presence of 9-*cis* RA or ATRA appeared to contribute to the further increase of CD14 in these cells. HL-60 cell cotreatment with these compounds also displayed enhanced cooperative effects in phagocytic function by NBT reduction. However, analysis of CD14 revealed a dramatic diminution in HL-60 cells treated with the combinations of the vitamin D derivatives and the retinoids. Assessment of HL-60 cell morphology treated with these combinations demonstrated the presence of a mixed population of monocytes and granulocytes. CD11b and CD18 antigen expression was also enhanced in both cell lines with cotreatment. The ability of EB1089 and KH1060 to induce leukaemic cell differentiation may provide an additional option for therapeutic use alone or together with other differentiation agents such as 9-*cis* RA or ATRA. BIOCHEM PHARMACOL **54**;5:625–634, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. vitamin D analogues; all-*trans* retinoic acid, 9-*cis* retinoic acid; leukaemia; differentiation

The concept of differentiation therapy, whereby immature cells may be stimulated to develop into their mature phenotype has, in recent years aroused considerable interest, with efforts being made to evaluate new differentiation agents for the treatment of myeloproliferative disorders, in which leukaemic blast cells are thought to exhibit maturation arrest. The vitamin A metabolite, all-*trans* retinoic acid (ATRA) has been highlighted as a promising candidate for differentiation therapy. Acting via the retinoic acid receptor (RAR), ATRA plays an important role in the growth and development of a variety of cells, inhibiting the proliferation and inducing the differentiation of the human promyelocytic leukaemia cell line, HL-60, into granulocytes, and acute myeloid leukaemia blast cells derived from patients [1–3]. The clinical use of ATRA has proved to be partially successful in inducing remissions in patients with acute promyelocytic leukaemia (APL) [4]. APL is a rapidly progressive disorder, characterised by a t(15;17) chromosomal translocation of the promyelocytic leukaemia (PML) gene present on chromosome 15 and the RARα gene on chromosome 17 [5]. The resulting chimeric PML-RARα protein is thought to behave in a dominant-negative

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§ *Abbreviations:* $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 9-cis RA, 9-cis retinoic acid; APL, acute promyelocytic leukaemia; ATRA, all-*trans* retinoic acid; DMSO, dimethyl sulfoxide; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; MFI, Mean Fluorescence Index; NBT, nitroblue tetrazolium; PE, phycoerythrin; PMA, phorbol myristate acetate; PML, promyelyocytic leukaemia; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; RXRE, retinoid X response element; TR, thyroid hormone receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

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manner by disrupting retinoic acid gene transcription or normal PML function [6].

Unfortunately, prolonged ATRA treatment often results in relapse and onset of a retinoid resistant condition by an as yet unidentified mechanism [7, 8]. Recently, a stereoisomer of ATRA, 9-*cis* retinoic acid, (9-*cis* RA) has been identified, which possesses similar biological effects to ATRA, and can mediate them via binding to RAR and a new class of receptor, the retinoid X receptor (RXR) [9–12]. 9-*cis* RA has also been proven to be an effective therapeutic agent in the treatment of APL, but like ATRA, is not able to reverse the onset of retinoid resistance [13]. To ensure, therefore, that optimal cell regulatory effects are achieved in clinical treatment, it looks increasingly likely that combinations of differentiation agents may have to be utilised together.

The physiologically active metabolite of vitamin D, 1α, 25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), inhibits the proliferation and promotes the differentiation of normal monocytes, leukaemia cells and activated T lymphocytes [14–18]. Immunodeficient murine models inoculated with myeloid cells, and treated with $1,25(OH)_2D_3$ *in vivo*, had a prolonged survival time with respect to untreated animals [19]. These interesting properties of $1,25(OH)_2D_3$ have led to its evaluation in the treatment of hyperproliferative disorders such as myelodysplastic syndrome [20]. Unfortunately, unacceptable rises in serum and urinary calcium levels usually accompany treatment, as has been previously reported [21], thus limiting the clinical usefulness of $1,25(OH)_2D_3$. New analogues of vitamin D have been developed in an attempt to separate the calcaemic actions from the effects on cell replication and differentiation. The most effective approach appears to be modification of the side chain of the vitamin D molecule. Two such analogues are EB1089 and KH1060, developed at Leo Pharmaceutical Products, Denmark. EB1089 has been shown to inhibit the proliferation of the human breast cancer cell line, MCF-7, *in vitro*, and promotes the regression of mammary tumours induced in rats by the carcinogen, nitrosomethylurea [22]. This compound has also been reported to induce the differentiation of megakaryoblastic leukaemia cells [23]. KH1060 is characterised by altered stereochemistry at the carbon 20 position, and is capable of inhibiting interleukin-1 induced mouse thymocyte replication [24], and inhibiting the progression of an autoimmune disease induced in a rat model by mercuric chloride [25].

The biological effects of $1,25(OH)_2D_3$, ATRA and 9-*cis* RA are mediated through their respective class of nuclear receptor, vitamin D receptor (VDR), RAR, and RXR. These receptors are members of the steroid, retinoic acid, and thyroid hormone receptor family [26, 27]. Formation of the ligand–receptor complex initiates the transcription of specific responsive genes, which then result in the synthesis of appropriate proteins, modulating cellular effects [28]. The elucidation of the molecular interactions between VDR, RAR, and RXR have indicated the possibility of different configurations of homo- and heterodimers [29, 30]. RXR is capable of forming heterodimers with VDR and RAR [31, 32]. Alternatively, VDR/RAR or VDR and thyroid hormone receptor (TR) heterodimers may also be formed [33]. Thus, depending on the nature of receptor dimerisation, diverse effects on transcriptional activity may occur, influencing the activation or repression of appropriate target genes.

In the present study we have evaluated the differentiative properties of $1,25(OH)_2D_3$, EB1089, and KH1060, using the human monocytic cell line, U937 and HL-60 cells as *in vitro* models of leukaemia, comparing them with the differentiative properties of 9-*cis* RA and ATRA. Our results demonstrate potent induction of leukaemic cell differentiation with these novel vitamin D analogues, with enhanced differentiative effects occurring in the presence of the retinoids.

MATERIALS AND METHODS

Compounds

$1,25(OH)_2D_3$, EB1089, and KH1060 were generously provided by Dr. Lise Binderup (Leo Pharmaceutical Products, Denmark). ATRA was purchased from Sigma Co. (Poole, UK). 9-*cis* RA was a generous gift from Dr. Milan Uskokovic (Hoffmann–La Roche, Nutley, NJ, USA). The vitamin D and retinoid compounds were dissolved in ethanol and stored at −20°C. For use in experiments the ethanol concentration did not exceed 0.1%. Nitroblue tetrazolium (NBT) and phorbol myristate acetate (PMA) were purchased from Sigma Co. (Poole, UK). The human fluorescein isothiocyanate (FITC) conjugated antibodies, CD14 and CD18, and the phycoerythrin (PE) antibody, CD11b were obtained from Becton Dickinson, Oxford, UK. Tissue culture reagents were supplied by ICN Flow (Irvine, UK). All other analytical grade reagents were purchased from Sigma unless otherwise stated.

Cellular Effects

The two leukaemic cell lines, U937 and HL-60, were routinely maintained in RPMI 1640 medium containing 2 mM glutamine and supplemented with 100U/mL penicillin, 100 μg/mL streptomycin and 10% foetal calf serum (GlobePharm, Esher, UK). For studies investigating the ability of the vitamin D compounds to induce cell differentiation, nitroblue tetrazolium (NBT) reduction assays were performed. Leukaemic cell cultures were seeded at $2–4 \times 10^4$ cells/mL in 24 well plates (Falcon), and dosed daily with the compound(s) of interest. At the end of 96 hr treatment the cell number was adjusted to 1×10^5 cells/mL, and incubated with 5 mg/mL NBT and 200 ng/mL PMA for 30 min at 37°C. The number of cells containing a blue-black formazan deposit were counted as NBT positive from a total of 200 cells.

Flow Cytometry

Cell surface antigen expression was determined by fluorescence activated cell sorting analysis (FACS). U937 and HL-60 cells were incubated for 96 hr with 5×10^{-10} M of the vitamin D compounds and/or 1×10^{-8} M of the retinoids. The cells were then washed with Hanks' buffer (without calcium or magnesium) containing 0.1% BSA and 20 mM HEPES, pH 7.3, and adjusted to 2×10^6 cells/volume. Aliquots of cell suspension (50 μL) were incubated with the appropriate human monoclonal FITC or PE conjugated antibody for 30 min at 4°C. Isotypic control antibodies, IgG_1-FITC and IgG_1-PE were also included. The cells were washed three times with Hanks' buffer at 400 × g for 5 min at 4°C, and then fixed in Hanks' buffer, containing 0.1% BSA, 1% formaldehyde, and 20 mM HEPES, pH 7.3. Fluorescence was read on a Becton Dickinson FACScan. Single parameter fluorescence histograms were generated (logE-01 scale), with cursors being set to ensure that cells incubated with isotypic control antibodies were not more than 2% nonspecifically stained. Data sets were recorded in real time, gating cells according to their side angle light scatter and fluorescence channel intensities. Results for these studies have been recorded as the mean fluorescence index, which is the product of the % fluorescence and the mean fluorescence intensity, with 10,000 cells being counted per treatment. To analyse changes in U937 and HL-60 cell morphology following treatment with the differentiation agents, cells were harvested and cytocentrifuged onto microscope slides. The slides were fixed and subjected to Wright-Geimsa staining before microscopic examination.

Statistical Methods

All comparisons were made using the unpaired Student's *t*-test or by three-way analysis of variance.

RESULTS

Leukaemia Cell Differentiation: Effects of the Vitamin D and Retinoid Compounds

Using NBT reduction assays as an index of cell differentiation, assessments were made of the ability of the vitamin D derivatives and the retinoids to induce U937 and HL-60 cell maturation. At concentrations starting from 5×10^{-12} M to 5×10^{-9} M, $1,25(OH)_2D_3$, EB1089, and KH1060 promoted U937 and HL-60 cell differentiation in a dose-dependent manner after 96-hr treatment (Fig. 1). In particular, EB1089 and KH1060 displayed differentiative effects in both cell lines that were at least 10-fold more potent than those of $1,25(OH)_2D_3$. In contrast, the number of NBT positive U937 cells induced to differentiate by 9-*cis* RA and ATRA in the same concentration range was low in comparison to the vitamin D compounds, suggesting that under these culture conditions the retinoids were not as effective at inducing U937 cell differentiation. However,

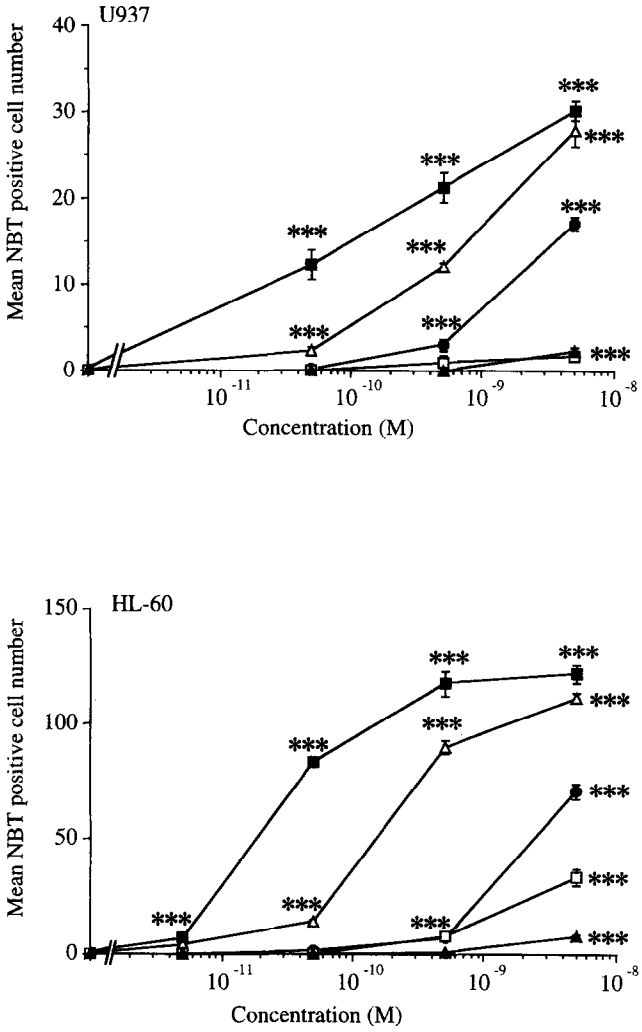

FIG. 1. Induction of leukaemic cell differentiation by the vitamin D derivatives. U937 and HL-60 cells were treated for 96 hr with $1,25(OH)_2D_3$, EB1089, or KH1060 with the indicated concentrations. Differentiation was determined by incubating 1×10^5 cells/mL with 5 mg/mL NBT and 200 ng/mL PMA for 30 min at 37°C. The number of cells containing blue-black formazan deposits from a total of 200 cells in random microscopic fields of view were counted. (●) $1,25(OH)_2D_3$, (△) EB1089, (■) KH1060, (□) 9-*cis* RA, and (▲) ATRA. ***$P < 0.005$.

9-*cis* RA and ATRA were both capable of stimulating HL-60 cell differentiation in a dose-dependent manner.

Cell Surface Antigen Induction by the Vitamin D Derivatives and the Retinoids

Cell surface antigen expression was measured by FACS. Table 1 illustrates the effects of the vitamin D and retinoid compounds on the expression of CD14, a monocyte associated antigen [34], and the β_2-integrins CD11b and CD18, used as granulocyte/monocyte differentiation markers [35, 36]. Following 96-hr treatment of both leukaemic cell cultures, a striking induction of CD14 by 5×10^{-10} M EB1089 or KH1060 was apparent, relative to control levels and the native hormone. Interestingly, EB1089 and

TABLE 1. Effects of vitamin D and retinoid compounds on leukaemia cell surface antigens

Treatment	Control MFI	5×10^{-10} M $1,25(OH)_2D_3$ MFI	5×10^{-10} M EB1089 MFI	5×10^{-10} M KH1060 MFI	1×10^{-8} M 9-*cis* RA MFI	1×10^{-8} M ATRA MFI
CD14						
U937	0.09 ± 0.05	0.06 ± 0.02	6.10 ± 3.51*	12.59 ± 7.27*	0.04 ± 0.03	0.03 ± 0.02
HL-60	0.90 ± 0.36	9.19 ± 0.39*	224.19 ± 28.08*	276.65 ± 45.76*	4.40 ± 2.89*	1.71 ± 0.58
CD11b						
U937	32.97 ± 2.21	48.06 ± 2.73*	128.34 ± 5.61*	155.88 ± 8.45*	62.91 ± 8.74*	44.03 ± 3.74*
HL-60	41.10 ± 16.48	29.26 ± 1.60	139.21 ± 44.14*	157.36 ± 47.33*	88.66 ± 39.42*	63.37 ± 29.76*
CD18						
U937	20.02 ± 3.59	25.40 ± 2.44	66.43 ± 4.56*	80.01 ± 5.74*	37.64 ± 4.35*	31.90 ± 3.53*
HL-60	34.80 ± 3.80	30.20 ± 1.78	56.69 ± 7.10*	62.69 ± 8.66*	42.65 ± 4.89*	41.89 ± 3.93*

U937 or HL-60 cell cultures were dosed for 96 h with either 0.1% ethanol control vehicle, or the indicated concentrations of the vitamin D compounds or 9-*cis* RA or ATRA. Treated cells were incubated with CD14, CD11b, or CD18 fluorescent antibodies and subjected to FACS analysis as described in Materials and Methods. Results are recorded as mean fluorescence index (MFI) ± SEM, which is the product of the % fluorescence and the mean fluorescence intensity. Values are representative of three independent experiments. * $P < 0.05$.

KH1060 were capable of promoting CD14 antigen expression by 100-fold and 210-fold in U937 and by 24-fold and 30-fold in HL-60 cells, respectively, when compared to the parent hormone. Treatment of the leukaemic cells with 1×10^{-8} M 9-*cis* RA or ATRA revealed the ability of these compounds to induce CD14 antigen expression relative to control cells. However, this induction was less effective than for that observed with the vitamin D compounds alone.

The expression of another class of cell surface antigens, the β_2-integrins, was also evaluated. It was observed that CD11b and CD18 antigen expression were increased in U937 and HL-60 cells following their treatment for 96-hr with the vitamin D compounds. EB1089 and KH1060 exhibited enhanced induction of these antigens relative to $1,25(OH)_2D_3$. Notably, induction of CD11b by EB1089 and KH1060 was increased fourfold in U937 cultures and threefold in HL-60 cell cultures compared to unstimulated cells. CD11b antigen expression was also increased by 9-*cis* RA and ATRA in both cell lines, with 9-*cis* RA being slightly more effective than ATRA. Induction of CD18 was upregulated threefold and fourfold in U937 cells by EB1089 and KH1060, respectively. Increases in CD18 antigen expression were also observed with the vitamin D and the retinoid compounds in HL-60 cell cultures. These immunophenotypic analyses demonstrate the enhanced differentiative effects of the vitamin D analogues when compared to the activity of $1,25(OH)_2D_3$.

Interaction of the Vitamin D Derivatives and the Retinoids

The potent differentiative effects of $1,25(OH)_2D_3$ and its analogues were next assessed in combination with 9-*cis* RA or ATRA. U937 and HL-60 cell cultures were exposed to 96-hr treatment with 5×10^{-10} M of the vitamin D compounds, in the presence or absence of 1×10^{-8} M 9-*cis* RA or ATRA. Cell differentiation was measured by the ability of the cell to reduce NBT. Figure 2 depicts the cooperative effects of the vitamin D and retinoid combinations in inducing U937 and HL-60 cell differentiation. In U937 cells, the combination of EB1089/9-*cis* RA stimulated an eightfold induction of cell differentiation, and KH1060/9-*cis* RA a fourfold induction relative to control cultures. Similarly, NBT reduction assay results of cotreatment of HL-60 cells with combinations of these compounds revealed augmentation of differentiation, with EB1089/9-*cis* RA and KH1060/9-*cis* RA promoting a 50-fold increase

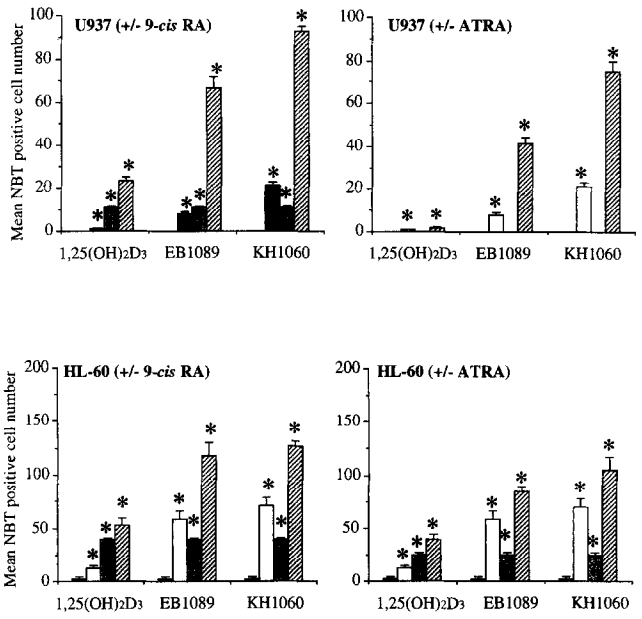

FIG. 2. Augmentation of leukaemic cell maturation by vitamin D derivatives and the retinoids. Assessment of U937 and HL-60 cell differentiation was made by NBT reduction following 96 hr treatment with 5×10^{-10} M of the vitamin D derivatives alone, or in the presence of 1×10^{-8} M of either 9-*cis* RA or ATRA, Control (solid column), 5×10^{-10} M of indicated vitamin D compound (open column), 1×10^{-8} M of indicated retinoid agent (dotted column), and vitamin D and retinoid compound (diagonal column). Results are representative of three separate experiments. *$P < 0.05$.

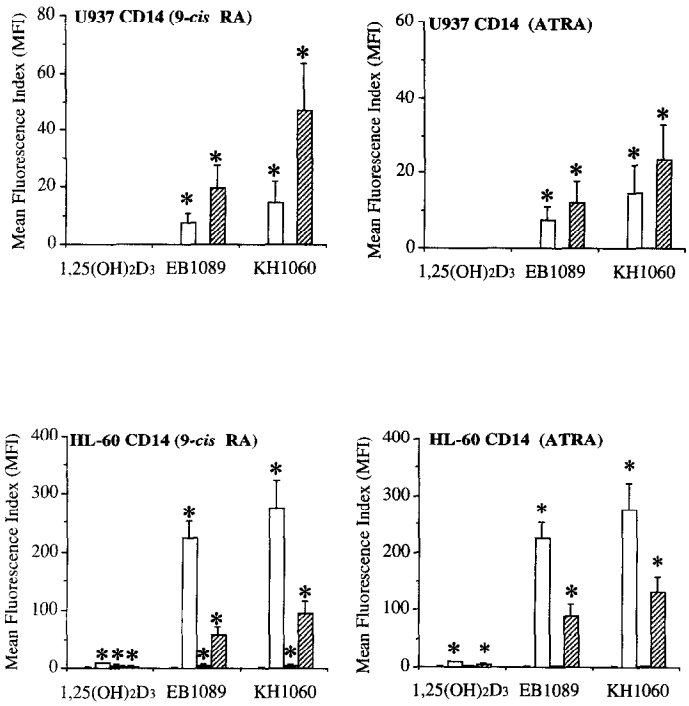

FIG. 3. Induction of CD14 by vitamin D derivatives and retinoids. U937 and HL-60 cells were treated with the vitamin D compounds in the presence or absence of 9-*cis* RA or ATRA. Induction of CD14 was measured by FACS analysis as described in Materials and Methods. Control (solid column), 5×10^{-10} M of indicated vitamin D compound (open column), 1×10^{-8} M of indicated retinoid agent (dotted column), vitamin D and retinoid compound (diagonal column). Results are representative of three separate experiments. *$P < 0.05$.

in cell maturation relative to unstimulated cultures. In both cell lines the cooperative effects of the vitamin D compounds with ATRA were slightly reduced to those effects shown in combination with 9-*cis* RA. Analysis of CD14 following cotreatment with the vitamin D derivatives and the retinoids also showed enhanced expression in U937 cell cultures (Fig. 3, top panel). These results correlate with the NBT data presented in Fig. 2. In stark contrast, CD14 expression in cotreated HL-60 cells was markedly reduced, suggesting an apparent decrease in the monocyte/macrophage cell population in the presence of the retinoid compounds (Fig. 3, bottom panel). In HL-60 cells treated with EB1089/9-*cis* RA or KH1060/9-*cis* RA, reductions in CD14 antigen expression of 74 and 65%, respectively were evident when compared to CD14 induced by the vitamin D analogues alone, with 60 and 52% decreases in CD14 observed with EB1089/ATRA- and KH1060/ATRA-treated cells, respectively. This profile of reduced CD14 induction was also found to be similar following 48 hr cotreatment (data not shown).

The reduction of CD14 antigen expression suggested that treated HL-60 cells may have either dedifferentiated or become directed along the granulocytic differentiation pathway. In the absence of a suitable immunophenotypic granulocytic differentiation marker, an independent index of differentiation status, Wright-Geimsa staining, was employed to investigate the morphological state of HL-60 cells when exposed to combinations of the vitamin D analogues and the retinoids. Figure 4 illustrates the representative morphology of HL-60 cells treated for 96 hr with 5×10^{-10} M KH1060 alone or in the presence of 1×10^{-8} M 9-*cis* RA. Cells treated with KH1060 alone exhibited monocytic characteristics (Fig. 4b), whilst those treated with 9-*cis* RA displayed a granulocytic phenotype (Fig. 4c). It was observed that cotreated cells consisted of a mixed population of monocytes and granulocytes (Fig. 4d).

FACS analyses also demonstrated augmentation of CD11b in both leukaemia cell lines, particularly U937 cultures, when cotreated with the differentiation compounds (Fig. 5). Similarly, increases in CD18 expression were apparent in cotreated U937 cell cultures, with induction above that of the vitamin D derivatives alone in HL-60 cells (data not shown). The enhanced induction of CD11b and CD18 in cotreated HL-60 cells further supported the suggestion that these compounds were inducing monocytic and granulocytic cell lineages, and not dedifferentiating back to the immature cell.

An assessment of the proportion of HL-60 cells treated with the vitamin D derivatives and/or 9-*cis* RA undergoing monocytic or granulocytic differentiation was made by dual colour immunofluorescence (Table 2). These results demonstrate that the percentage of cotreated HL-60 cells expressing both CD11b and CD14 (indicative of monocytic differentiation) is lower than cells expressing both of these antigens following treatment with the vitamin D derivatives alone.

DISCUSSION

In the present study, we have demonstrated the ability of two analogues of vitamin D, EB1089 and KH1060, alone, and in combination with the retinoids, 9-*cis* RA and ATRA, to induce the differentiation of two human leukaemic cell lines, U937 and HL-60. Cooperative effects on U937 cell differentiation were observed with cotreatment, suggesting that both 9-*cis* RA and ATRA potentiated the differentiative effects of the vitamin D derivatives. Concomitantly, CD14 and β_2-integrin expression, particularly CD11b, were also augmented. We have additionally demonstrated by NBT reduction that the dose-dependent induction of U937 cell differentiation by vitamin D derivatives is increased by at least 10-fold in the presence of 9-*cis* RA, with the dose response curve shifted to the left of the curve for the vitamin D compounds alone (our unpublished results). Coincubation of HL-60 cell cultures with these compounds increased their maturation and effector function, assessed by NBT assay. However, phenotypic analysis of CD14 antigen expression, induced by the vitamin D derivatives alone, was decreased in the presence of 9-*cis* RA or ATRA, while induction of the β_2-integrins was increased. Morphological studies of cotreated HL-60 cells have indicated that conflicting effects on cell maturation

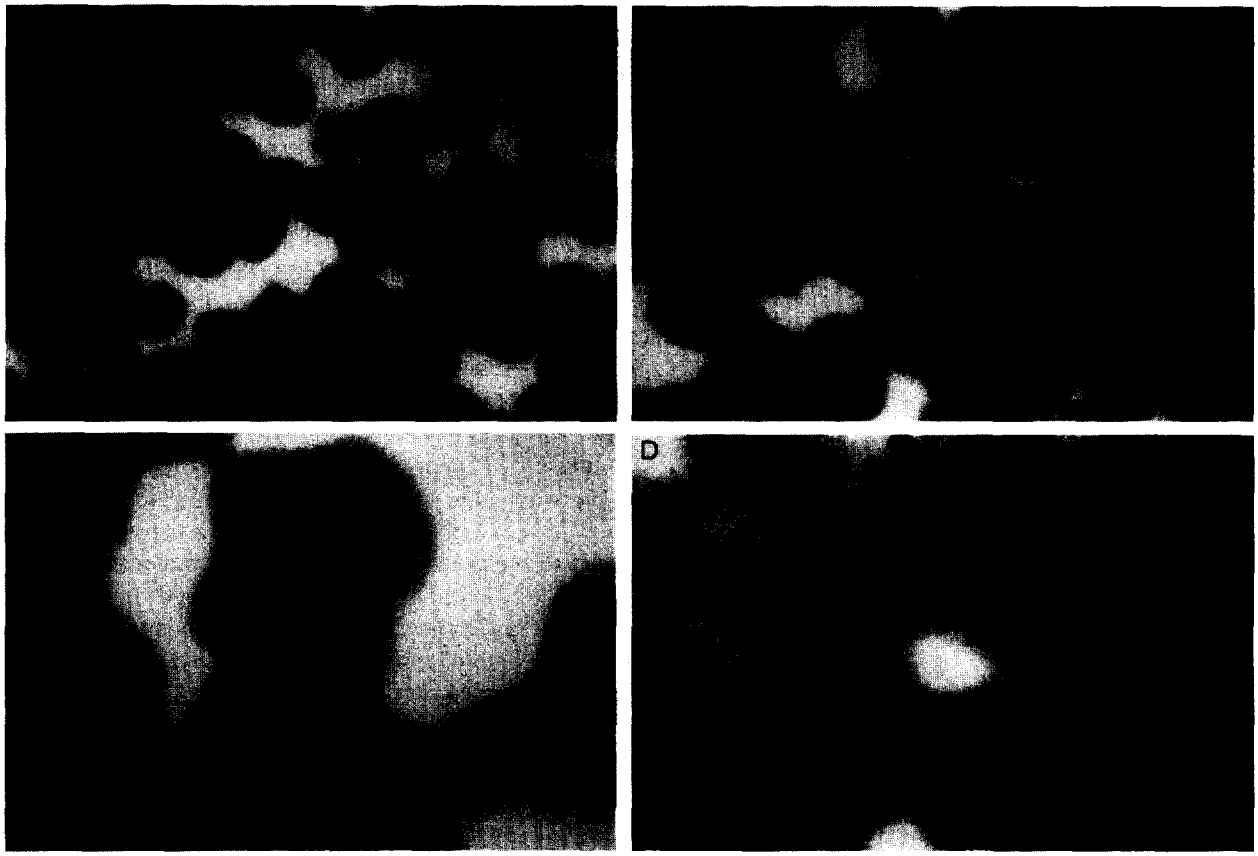

FIG. 4. Morphological assessment of HL-60 cell differentiation. HL-60 cells were treated for 96 hr with (a) ethanol vehicle, (b) 5×10^{-10} M KH1060, (c) 1×10^{-8} M 9-*cis* RA, or (d) KH1060 and 9-*cis* RA in combination. Cytospins of the treated cells were prepared on microscope slides and stained with Wright-Geimsa. Magnification ×460.

exist, with a population of cells favouring granulocytic differentiation under the influence of the retinoids rather than monocytes under the control of the vitamin D derivatives.

Our results demonstrating the cooperative effects of the vitamin D and retinoid derivatives in differentiating the U937 and HL-60 cell lines are in agreement with a number of other research groups [37–42]. A recent report by Doré and colleagues [42] has demonstrated the ability of vitamin D analogues to cooperate with ATRA in differentiating HL-60 cells, but unfortunately they did not investigate the phenotypic characteristics resulting from the actions of these compounds. Immunophenotypic studies performed by Verstuyf *et al.* [39], and Masciulli *et al.* [40] have yielded similar results to those described in this report, regarding increased CD11b antigen expression in HL-60 cells co-treated with $1,25(OH)_2D_3$ and the retinoids compared to the actions of each agent alone. These groups also evaluated induction of CD14, and found no reduction in the expression of this antigen following HL-60 cotreatment. Defacque *et al.* [38] have suggested that vitamin D induction of HL-60 cell cultures would give rise to predominantly monocytic differentiation even in the presence of ATRA. However, we have observed clear reductions of CD14 in HL-60 cells treated with $1,25(OH)_2D_3$, and, in particular, EB1089 and KH1060, in combination with 9-*cis* RA or ATRA, relative to the inducing effects of the vitamin D derivatives alone. This reduction is evident both in terms of decreases in the percentage number of cells expressing CD14 (% fluorescence) and the relative number of CD14 molecules being expressed per cell (mean fluorescence intensity). In conjunction with this phenotypic analysis, morphological assessment of cotreated HL-60 cells has revealed mixed populations of monocytes and granulocytes, as shown by ourselves (Fig. 4) and the aforementioned groups. The molecular mechanisms underlying the biological actions exhibited by the vitamin D derivatives and the retinoids have been partially elucidated, their effects being mediated through nuclear signalling pathways by regulation of the expression of target genes. Efficient binding of VDR and RAR to vitamin D (VDRE) and retinoic acid (RARE) specific response elements located within the promoter region of target genes may be accomplished by their heterodimerisation with RXR. Other studies have indicated that VDR may also form homodimers, which will recognise yet another class of distinct binding sites at the DNA level [30, 33]. Given the diversity in receptor configuration, a strong possibility exists of crosstalk between the retinoic acid and vitamin D signalling pathways, and detailed investigations concerning this area have been undertaken [43–45]. The expression of VDR, RARα, RARβ, and the α and β subtypes for RXR, have been established in U937 and

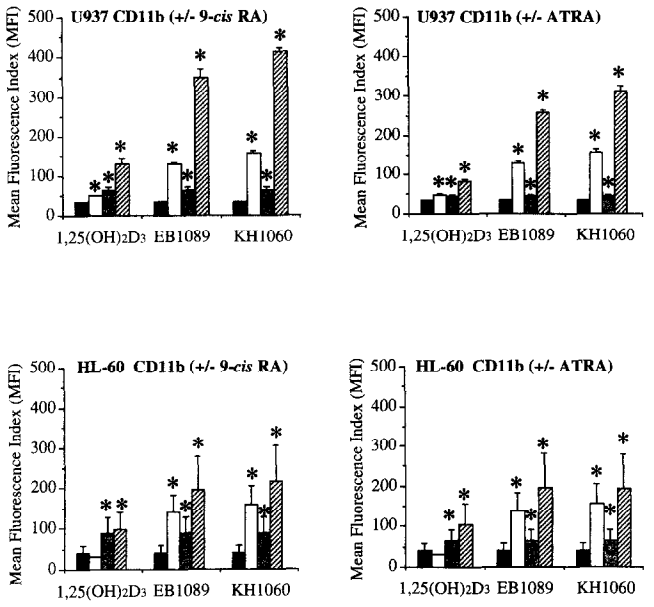

FIG. 5. Induction of CD11b by vitamin D derivatives and retinoids. U937 and HL-60 cells were treated with the vitamin D compounds in the presence or absence of 9-*cis* RA or ATRA. Induction of CD11b was measured by FACS analysis as described in Materials and Methods. Control (solid column), 5×10^{-10} M of indicated vitamin D compound (open column), 1×10^{-8} M of indicated retinoid agent (dotted column), and vitamin D and retinoid compound (diagonal column). Results are representative of three separate experiments. *$P < 0.05$.

HL-60 cell lines [3, 10, 41, 46, 47]. Granulocytic differentiation of HL-60 cells by ATRA has been shown to be mediated by RARα [3], and additional studies with synthetic retinoids have revealed that cellular responses may be achieved via interaction with RAR/RXR heterodimers rather than with RXR/RXR homodimers [48]. While enhancing their overall differentiation, the simultaneous exposure of vitamin D and the retinoids to HL-60 cell cultures generates monocytic and granulocytic subpopulations. An alternative mechanism for the differential phenotypic induction following cotreatment of these agents may require the simultaneous activation of VDR or RAR responsive genes, which contribute to monocytic or granulocytic lineage determination thus modulating effects on cell proliferation and differentiation. In respect of enhanced U937 cell differentiation observed with vitamin D and retinoid cotreatment, a possible molecular mechanism that might culminate in these cellular effects may involve the capability of VDR to form a heterodimeric partnership with RAR or RXR for efficient DNA binding and transcription of vitamin D responsive genes, thus implicating the retinoid ligands in augmenting the differentiative effects of vitamin D. In support of this suggestion, Defacque *et al.* [47] have noted a correlation between the maturational change in $1,25(OH)_2D_3$- and ATRA-treated U937 cells, and induction of RXRα protein expression, implying that the presence of RXRα may be necessary for the induction of monocyte specific genes by vitamin D. Furthermore, observations have been made indicating that the PML-RARα chimeric protein, when transfected into U937 cells, is capable of inhibiting $1,25(OH)_2D_3$-induced differentiation [49]. Another study, using Cos-1 cells transfected with a VDR responsive reporter gene, has demonstrated that PML-RARα may compete with VDR for heterodimerisation with RXRα, thereby sequestering this receptor protein, and preventing VDR from binding efficiently to its target gene [50]. Our studies show that the differentiative effects of ATRA and 9-*cis* RA in conjunction with the vitamin D compounds in the U937 and HL-60 cell lines are qualitatively similar, and would, therefore, suggest that RXR mediated activation may not be an exclusive mechanism through which these responses would be manifested, because ATRA does not bind to or activate RXR [9, 51, 52].

The interesting observation that in both the U937 and HL-60 cell lines, the induction of the β_2-integrins is enhanced by cotreatment of the vitamin D derivatives and the retinoids, suggests the existence of a common set of genes, responsive to VDR, RAR, or RXR homo- or heterodimeric complexes. A recent report has described the augmentation of U937 cell differentiation with $1,25(OH)_2D_3$ and 9-*cis* RA or ATRA, whereby synthetic VDRE and VDRE/RXRE sequences were activated by VDR

TABLE 2. Assessment by flow cytometry of the percentage of monocytic and granulocytic differentiation in HL-60 cells by vitamin D derivatives alone, and in combination with 9-*cis* retinoic acid

	Mean Fluorescence Index		% Fluorescence (Dual)		
	CD11b	CD14	CD11b+/CD14−	CD14+/CD11b−	CD11b+/CD14+
Control	19.00	0.24	72.50	0.00	0.40
5×10^{-10} M $1,25(OH)_2D_3$	16.25	1.87	52.70	0.10	5.30
5×10^{-10} M EB1089	29.06	29.87	19.70	5.70	51.40
5×10^{-10} M KH1060	35.77	44.84	12.20	7.50	60.10
1×10^{-8} M 9-*cis* RA	38.89	0.84	80.60	0.10	1.70
$1,25(OH)_2D_3$ + 9-*cis* RA	42.98	0.72	83.00	0.10	1.90
EB1089 + 9-*cis* RA	54.09	5.92	77.20	0.10	11.50
KH1060 + 9-*cis* RA	79.92	22.18	67.80	0.10	24.90

HL-60 cell cultures were dosed for 96 h with either 0.1% ethanol vehicle, or the indicated concentrations of the vitamin D compounds in the absence or presence of 9-*cis* RA. Treated cells were simultaneously incubated with CD14 (FITC) and CD11b (PE) fluorescent antibodies and subjected to dual flow cytometry. Results are recorded as mean fluorescence index, which is the product of the % fluorescence and the mean fluorescence intensity, and % fluorescence for the dual analysis.

homodimers and VDR/RXR heterodimers, respectively, the latter stimulating the expression of CD14 and CD11b antigens more effectively than the former [53]. It is worth noting, however, that effects of vitamin D on transcription of the CD14 gene are unlikely to be mediated directly through VDR. A recent report from Zhang *et al.* [54] has provided evidence that while $1,25(OH)_2D_3$ is capable of inducing the level of CD14 mRNA transcripts in U937 cells, VDR or VDR/RXR does not bind to the CD14 promoter. The authors suggest that $1,25(OH)_2D_3$ induces CD14 indirectly, and that the transcription factor Sp1 plays a critical role in this process [54]. While these and other studies may lead to a better understanding of receptor interactions, it is still unclear as to which classes of gene sequence may be directly or indirectly activated to elicit the observed cellular responses. The mode of administration of differentiation agents to leukaemic cells, simultaneous or sequential, has been evaluated by a number of research groups, because it might be argued that brief, rather than prolonged, exposure to inducing agents may be more beneficial therapeutically. Available data has revealed that pretreatment of U937 or HL-60 cell cultures, regardless of whether $1,25(OH)_2D_3$ or ATRA is added as the first agent, promoted differentiation, but that this was reduced in comparison to the cooperative effects on differentiation observed with agents added simultaneously [37, 40, 55]. The dosing regimen performed in our studies involved simultaneous addition of compounds on a daily basis. The majority of other studies detailing interactions of vitamin D and retinoid derivatives have used supraphysiological concentrations (typically 1×10^{-7} M $1,25(OH)_2D_3$ or 1×10^{-6} M retinoid compound) to ensure terminal leukaemic differentiation, which, if used therapeutically, would unfortunately have adverse effects on calcium handling *in vivo*. The use of vitamin D analogues in our studies demonstrate that induction of differentiation can be achieved with much lower concentrations, without necessarily influencing calcium homeostasis when extrapolated to the *in vivo* situation. Several reports have evaluated the calcaemic activities of a variety of vitamin D analogues, including EB1089, *in vivo* [56–58]. Studies by Pakkala *et al.* [58] revealed that EB1089, given intraperitoneally to Balb/c mice, was tolerated at the concentration of 0.25 μg/kg, and exhibited similar serum calcium levels to that of $1,25(OH)_2D_3$. Retinoid treatment in the clinical setting has similarly been reported to induce hypercalcaemia, and so any cotreatment with the vitamin D derivatives may potentiate this condition [59]. Additional studies need to be performed to establish optimal treatment regimens to limit the onset of hypercalcaemia.

In summary, two vitamin D analogues, EB1089 and KH1060, when compared to $1,25(OH)_2D_3$, are potent inducers of leukaemic cell differentiation and β_2-integrin expression *in vitro*. These compounds may have potential in differentiation therapy, particularly in combination with differentiation agents such as 9-*cis* RA or ATRA.

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