



Presence of a functional vitamin D receptor does not correlate with vitamin D3 phenotypic effects in myeloid differentiation

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Abstract

Although VDR is expressed in all the acute myeloid leukemia cell populations studied, most of these leukemias do not exhibit any phenotypic response when exposed to VD. To determine whether VD resistance is related to an altered VDR function, we performed an analysis of VDR expression, phosphorylation, DNA binding capacity and transactivation activity in several leukemic myeloid cell lines arrested at different levels of maturation. Our results indicate that VD induces a clear phenotypic effect, i.e. terminal monocytic differentiation, only in leukemic cells of M2/M3 (intermediate myeloblasts) and M5 (monoblasts) types but not in erythroid precursor cells, early leukemic myeloblasts (M0/M1 type) and promyelocytes (M3 type). VDR expression and function are evident in all the nuclear extracts obtained from the different myeloid cell lines after 12 h of VD treatment, but VD activation of monocytic differentiation is limited to a narrow differentiation window characterized by the M2 type myeloid cellular context.

Keywords: monocytic differentiation, vitamin D receptor, 1,25 dihydroxyvitamin D₃, Acute Myeloid Leukemia, transcriptional factors, DNA binding proteins

Abbreviations: AML, acute myeloid leukemia; DMSO, dimethyl sulfoxide; ATRA, all-trans-retinoic acid; VD, 1,25-dihydroxyvitamin D₃; TPA, 12-O-tetradecanoylphorbol 13-acetate; APL, acute promyelocytic leukemia; VDRE, vitamin D responsive element; RXR, retinoid X receptor; CIAP, calf intestinal alkaline phosphatase; EMSA, electrophoretic mobility shift assays; hOC, human Osteocalcin; HMSE-1, human monocyte/macrophage serine esterase 1; FAB, French American British; FCS, fetal calf serum; RT-PCR, reverse transcription-polymerase chain reaction; MGG, May Grunwald Giemsa; PMSF, phenylmethylsulfonylfluoride; NP-40, Nonidet

P-40; PAGE, polyacrylamide gel electrophoresis, HRP, horseradish peroxidase; CAT, chloramphenicol acetyltransferase; ROS, Rat Osteosarcoma; β GAL, β galactosidase

Introduction

Acute myeloid leukemias (AML) are characterized by blast cells which are unable to mature into functional, terminally differentiated cells. A small percentage of leukemic blast cells remains in the proliferative pool in the bone marrow microenvironment and constitutes the growth fraction, but the large majority of these cells, especially in the peripheral compartment, are mainly arrested in the G1 phase of the cell cycle (Ferrari *et al*, 1986, 1989), have a long survival rate, and are not capable of efficiently activating programmed cell death (Torelli *et al*, 1994). Inducing leukemic cells to differentiate and therefore undergo apoptosis, provides an opportunity to modify the course of the malignancy.

The FAB classification of acute leukemias, based on morphology and cytochemical analysis of specific enzymes (Bennett *et al*, 1976), and the immunological classification based on the immunophenotype (Drexler, 1987) have clearly shown that the maturation arrest of AML blast cells occurs at different stages along the differentiation pathway. Several myeloid cell lines have been established from blast cell populations obtained from patients with AML (Miller and Koeffler, 1990) and despite the fact that these cell lines have a high proliferative capacity, they maintain the differentiation block characteristic of the parental leukemic cells from which they were derived. Therefore these leukemic cell lines are an interesting *in vitro* model for studying the pathways and for identifying the genes involved in myeloid differentiation (Lubbert *et al*, 1991).

Among the several compounds which have been identified which can overcome the differentiation block of acute leukemic blast cells, the most extensively studied are dimethyl sulfoxide (DMSO), butyrate, all-trans-retinoic acid (ATRA), 1,25-dihydroxyvitamin D₃ (VD) and 12-O-tetradecanoylphorbol 13-acetate (TPA) (Lea, 1992). VD and ATRA are differentiation inducers active on myeloid hematopoietic cells not only *in vitro* but also in physiological microenvironments (Pols *et al*, 1990; De Luca, 1991). It has been shown that 'differentiation windows' exist (Francis and Cunningham, 1991), within which the cells are sensitive to the different inducers, since the differentiation capacity of leukemic myeloid cells is related both to the level of maturation arrest and to the proliferative rate (Ferrari *et al*, 1990). An important example of differentiation therapy in AML is provided by ATRA which is a powerful inducer of granulocytic differentiation in acute promyelocytic leukemia (APL). In fact, when administered as a single agent, ATRA

can induce complete clinical remission in patients with APL (Castaigne *et al*, 1990; Warrell *et al*, 1991) even if subtypes of this leukemia might be spontaneously unresponsive to treatment due to the presence of mutation in the RAR α gene (Dore and Momparler, 1996), to a loss of expression of the intact pml/RAR α protein (Dermime *et al*, 1993), or acquire resistance to a second ATRA-induction therapy (Delva *et al*, 1993).

VD and VD analogues (Imai *et al*, 1995) have been shown to be active *in vitro* and *in vivo* models, raising the possibility of their use in the differentiation treatment of human malignancies (Kasukabe *et al*, 1987; Kelsey *et al*, 1992). Although the concentrations of VD that optimally induce differentiation *in vitro* models are at least three orders of magnitude higher than normal physiological serum levels, the hormone acts synergistically with cytokines and growth factors *in vitro*, suggesting that it may play a role in the control of normal hematopoiesis (Trinchieri *et al*, 1987; Okabe-Kado *et al*, 1991; Kelsey *et al*, 1992; Duits *et al*, 1992). It is well known that VD exerts its action by binding to a specific nuclear receptor (VDR) with high affinity. VDR regulates gene expression in target cells by binding to a specific DNA responsive element (VDRE), via heterodimerization with Retinoid X receptor (RXR) (Hsieh *et al*, 1995; Jin *et al*, 1996; Lemon and Freedman, 1996).

To better evaluate the relationship between VDR function and VD phenotypic effects and therefore to get useful information on the nature of the differentiation block which occur in AML blast cells, we have studied VDR expression, DNA binding and transactivation activity in several myeloid leukemic cell lines arrested at different levels of maturation.

Results

Differentiation effects of VD on myeloid cell lines arrested at different levels of maturation

Monocytic differentiation has been evaluated after five days of treatment with 10^{-7} M VD, by monitoring morphology, cell adhesion capacity and CD14 protein induction. As shown in Figure 1, immature myeloblastic cell lines such as K562 (myelo-erythroblastic), KG-1a and KG-1 (M0-M1 types), and the more differentiated promyelocytic cell line NB4 (M3 type) are not responsive to the differentiation effects of VD, since no induction of CD14 protein occurs after 5 days of VD treatment (Zhang *et al*, 1994). The more mature myeloblastic cell lines, such as AML-1 and HL-60 (M2-M3 types), monoblastic THP-1 (M5 type) and U937 (histiocytic) cell lines, and spontaneous blast cells of M5 type, are sensitive to VD since a clear induction of CD14 is detected. The levels of expression of CD45 protein (Thomas, 1989) remain unmodified before and after VD treatment, indicating good cell viability. Furthermore, no morphological and functional changes are evident in the unresponsive myeloid cells, while responsive cells develop the adhesion capacity and assume monocyte morphology after 5 days of VD treatment (data not shown).

VDR expression and phosphorylation

Western blot analysis reveals an immunoreactive VDR band in the nuclear extracts of all cell lines (Figure 2A) and of AML cells (M5 type) obtained from a patient (Figure 2C, lane 12). VD treatment is crucial for VDR detection, since nuclear extracts obtained from untreated cells do not contain VDR detectable by Western blot analysis (data not shown and

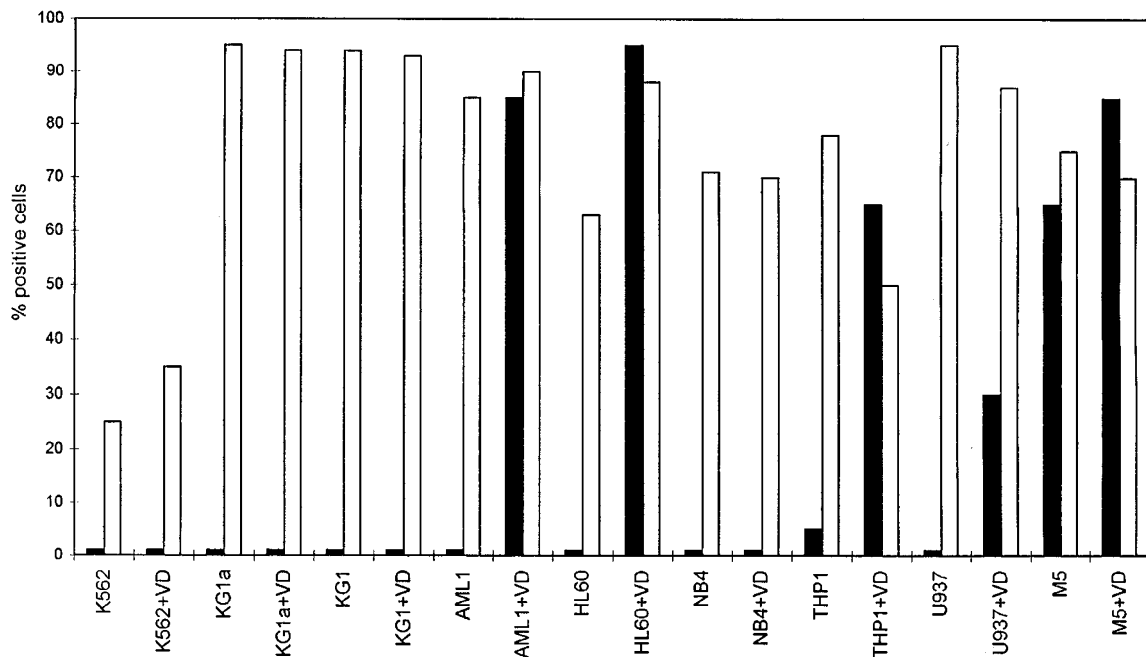


Figure 1 Flow cytometric analysis of CD14 (black bar) and CD45 (open bar) antigens expression in leukemic myeloid cell lines induced to monocytic differentiation with VD. The expression levels of these antigens are reported as percentage of positive cells. The cell lines studied are indicated at the bottom of the histogram. Three different sets of experiments were performed and the variability of positive cells, in each experiment, was lower than 10%. VD treatment was carried on for 5 days.

Figure 2C, compare lanes 11 and 12). The results shown in Figure 2A, allow two main observations: (a) a wide variability in VDR levels exists among the cell lines studied, in particular K562 and KG-1a appear to contain very little VDR; (b) the apparent molecular weight of VDR is not homogeneous in different cell types. Since it is known that VDR is a phosphoprotein (Brown and DeLuca, 1990; Suggs *et al*, 1992), we have investigated whether phosphorylation might be the basis for this size variability. Indeed, calf intestinal alkaline phosphatase (CIAP) treatment of KG-1 nuclear extracts leads to the detection of a VDR band (Figure 2B, lane 10) which is approximately 1 Kd smaller than in the untreated nuclear extract (lane 9). We estimated a MW of 52 Kd and 53 Kd for CIAP-treated and untreated control respectively. Quite interestingly the more phosphorylated VDR forms were present in cell lines which are not responsive to VD (Figure 2A, lanes 1, 2, 3 and 6).

DNA binding activity of VDR

Since all cell lines, either responsive or unresponsive to VD treatment contain VDR, albeit with different abundance and phosphorylation levels, we investigated the functional ability of the VDR present in nuclear extracts to bind its cognate DNA responsive element (VDRE). Oligonucleotides containing a VDRE like DR3, or rat and human osteocalcin DR-3 type responsive elements (Umesono *et al*, 1991; Ozono *et al*, 1990) were used as probes in electrophoretic mobility shift assays (EMSA). The presence of VDR in shifted complexes was assessed by using a monoclonal antibody (Ab)

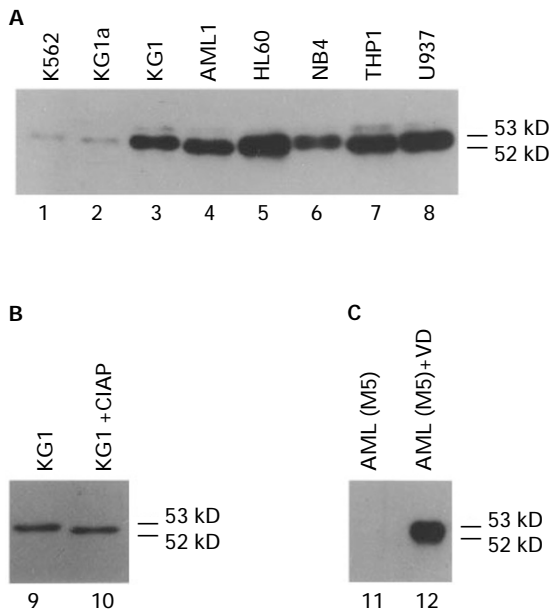


Figure 2 Western blot analysis of VDR expression in nuclear extracts of different leukemic myeloid cells after 12h VD treatment (A, lanes 1 to 8). The leukemic myeloid cell lines are indicated at the top of the panel. (B) shows the Western blot analysis comparing VDR protein size in CIAP untreated (lane 9) and treated (lane 10) KG1 nuclear extracts obtained after 12 h of VD treatment. (C) VDR protein expression in nuclear extracts of spontaneous AML-M5 type blast cells before (lane 11) and after (lane 12) 12h VD treatment. Molecular weights of VDR protein are reported on the right side of each panel.

specifically directed to VDR. Figure 3 shows that, using DR3 oligonucleotide, a major complex containing VDR (indicated by the arrow) is present in all cell lines and in

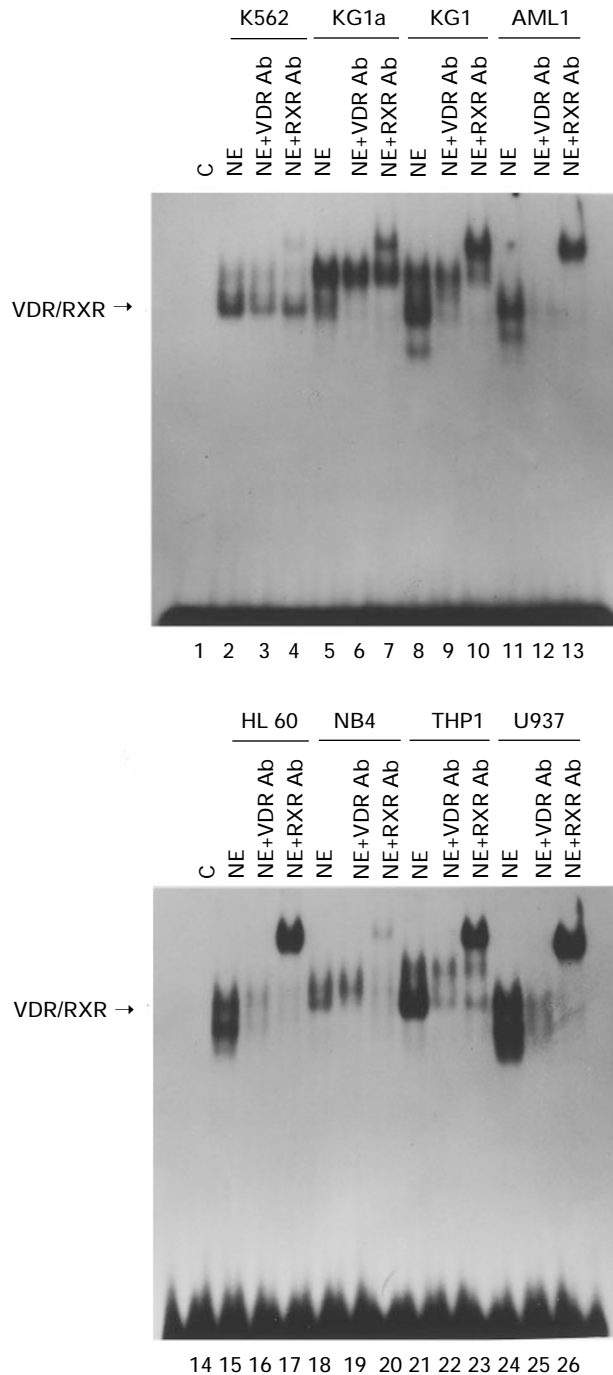


Figure 3 EMSA analysis on nuclear extracts obtained from myeloid cell lines after 12h VD treatment using 32 P-labeled DR3 probe: lanes 1 and 14, DR-3 unincubated oligonucleotide; lane 2, K562 cells; lane 5, KG1a cells; lane 8, KG1 cells; lane 11, AML1 cells; lane 15, HL60 cells; lane 18, NB4 cells; lane 21, THP1 cells; lane 24, U937 cells. To evaluate the VDR containing complexes, an anti VDR monoclonal Ab was added to the different nuclear extracts (lanes 3, 6, 9, 12, 16, 19, 22, 25). The presence of RXR protein was assessed by an antibody against RXR α , β , γ (RXRAb) (lanes 4, 7, 10, 13, 17, 20, 23, 26). The arrow indicates the major gel shift complex containing VDR/RXR heterodimer.

spontaneous leukemic blast cells of M5 type (data not shown). As it is expected from the use of a DR3 type probe, the VDR containing complex also contains a member of the Retinoid X receptor (RXR) family. In fact RXR is known to heterodimerize with VDR in binding this type of VDRE (Umesono *et al*, 1991). In myelo-erythroid precursor K562 cells, the major retarded complex is evident (Figure 3, upper panel, lane 2), which however contains very little VDR/RXR heterodimer. In fact, only a small reduction of the complex is detected with anti VDR Ab (lane 3) and consequently a faint supershifted band is observed with anti RXR Ab (lane 4). This result is compatible with the low VDR protein abundance in the nuclear extract shown by the immunoblot analysis. In the early myeloblastic cell lines KG-1a and KG-1, which are not responsive to VD, two major complexes are detected (Figure 3, upper panel, lanes 5 to 10), but only the lower one (indicated by the arrow) contains VDR/ RXR heterodimers as judged by the effect of the anti VDR Ab (lanes 6 and 9), and the anti RXR Ab (lanes 7 and 10). A very similar EMSA pattern is observed in NB4 and THP-1 nuclear extracts (Figure 3, lower panel, lanes 18 to 23). Finally nuclear extracts from KG-1, AML-1, HL-60 and U937, in addition to the main retarded complex, also contain other faster mobility complexes which are VDR/RXR related and probably depend on the presence of different RXR isoforms (Hsieh *et al*, 1995) or to proteolysis of either VDR and/or RXR in the nuclear extracts. The same results were obtained using the rat and human osteocalcin natural VDRE (data not shown).

Ability of endogenous VDR to activate transcription of VDRE-dependent promoters

As shown in Figure 4, hormone dependent transcriptional activation of the reporter gene chloramphenicol acetyltransferase (CAT) occurs in all transfected cell lines, independent

of their ability to differentiate after VD treatment. The extent of VD-induced transcriptional activation was quantified by scintillation counting, expressed in arbitrary units and compared with CAT activities detectable in the same transfected cell lines but not VD treated. The mean of three different experiments was analysed and the variability of CAT positivity in the different experiments was lower than 0.5 arbitrary unit. The transcriptional activation varies slightly between the different cell lines, consisting of a fourfold increase in KG-1 and HL-60, a sixfold increase in NB4 and a sevenfold increase in THP-1 and K562.

Transcriptional induction of genes associated with monocytic differentiation after a short treatment with VD

To monitor the early stages of activation of the genetic program underlying monocytic differentiation, we have investigated, with the highly sensitive RT-PCR technique, the expression of genes associated with monocytic differentiation after 12 h treatment with VD. Among the primary VD responsive genes, i.e. genes carrying a functional VDRE, we have studied the expression of the hOC (Breen *et al*, 1994) and the p21^{waf-1} gene (Liu *et al*, 1996). Among the early responsive gene associated with the monocytic differentiation program we have studied the expression of CD14 (Zhang *et al*, 1994) and HMSE-1 (Uphoff *et al*, 1993). Results shown in Figure 5A, indicate that CD14 mRNA is induced by VD treatment only in AML-1, HL-60 (M2/M3 types of AML), THP-1 and U937 cells (M5 types of AML), that is in all the VD sensitive cell lines (Figure 5A, lanes 7–10, and 13–16), but is not expressed in myelo-erythroid precursors (K562), M0/M1 early myeloblastic (KG-1a and KG-1) and in M3 promyelocytic cells (NB4), i.e. in the VD unresponsive cell lines (Figure 5A, lanes 1–6 and 11, 12). HMSE-1 mRNA is not detected in VD

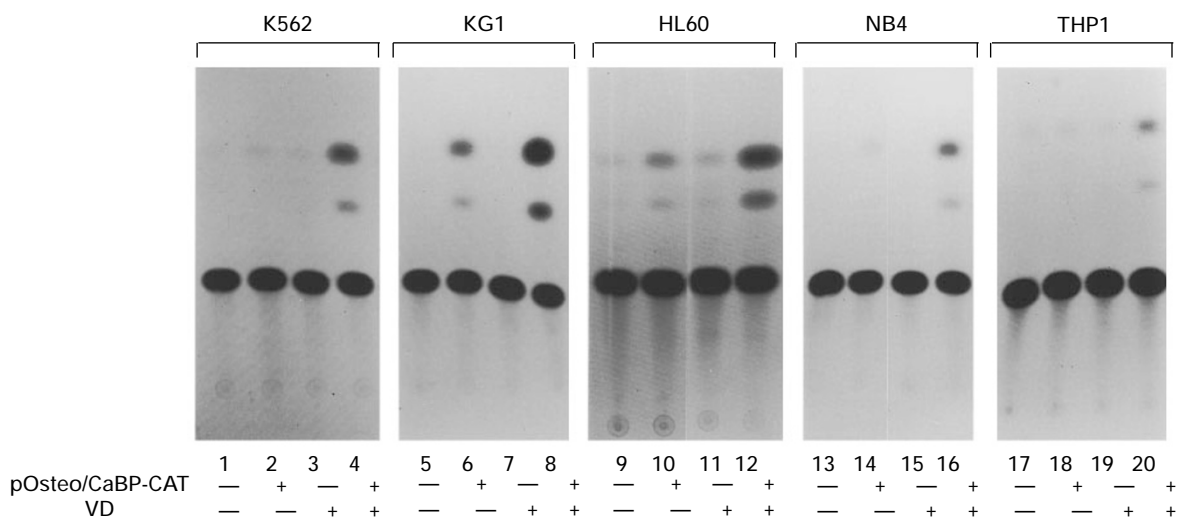


Figure 4 Cat assay from cell extracts of K562, KG1, HL60, NB4 and THP1 cell lines, evaluated after transfection with pOsteo/CaBP-CAT. Each assay was performed electroporating the cells without DNA (negative controls) in the absence (lanes 1, 5, 9, 13 and 17) or in the presence of VD (lanes 3, 7, 11, 15 and 19), or with plasmid DNA, in the absence (lanes 2, 6, 10, 14 and 18) or in the presence of VD (lanes 4, 8, 12, 16 and 20). Normalization of the CAT assay was performed using different amounts of cell lysates based on the β -Gal activity of the co-transfected pMCV β plasmid. The transcriptional activation varies slightly between the different cell lines, consisting of a fourfold increase in KG-1 and HL-60, a sixfold increase in NB4 and a sevenfold increase in THP-1 and K562.

non-responsive cell lines (Figure 5B, lanes 1 to 6), whereas is constitutively expressed and not modulated by VD treatment in VD responsive cell lines (Figure 5B, lanes 7, 8, 11 to 16) with the only exception of HL-60 cells where a clear induction is detected after VD treatment (Figure 5B, compare lanes 9 and 10). The p21^{waf-1} mRNA is constitutively expressed in all cell lines (Figure 5C, lanes 1 to 8 and 11 to 14) with the exception of HL60 cells, where it is not detected (Figure 5C, lanes 9 and 10), and of U937, where a clear induction is observed after VD-treatment (Figure 5C, lanes 15 and 16). The hOC gene, which is a primary response gene in VD-dependent osteoblast differentiation (Hauschka *et al*, 1989), is clearly expressed in all the cell populations studied even if with different abundance (Figure 5D, lanes 1 to 16), but it is not related to monocytic differentiation. No modulation of β -2 microglobulin expression is detectable in the myeloid cell lines studied independently on VD treatment (Figure 5E, lanes 1 – 16).

Discussion

AMLs are characterized by the clonal expansion of a precursor myeloid cell and by a differentiation block which occurs at different levels of maturation as characterized by the FAB and immunological classification (Bennett *et al*,

1976; Drexler, 1987). It is thus likely that the growth advantage of acute leukemia blast cells is realized not only through an increased proliferation rate, but also through the maturation arrest which leads to a prolonged survival and to a decreased rate of apoptosis (Torelli *et al*, 1994). Therefore, the induction of these neoplastic cells to differentiate and undergo apoptosis provides an opportunity to modify the course of the malignancy (Castaigne *et al*, 1990; Warrell, *et al*, 1991). Among the natural hematopoietic differentiation agents ATRA and VD are the most extensively studied *in vitro* and *in vivo* (Pols *et al*, 1990; De Luca, 1991). It is well known that the activity of these compounds is mediated by specific nuclear receptors which bind their cognate DNA responsive elements mostly as heterodimers with RXR and so activate gene transcription (Hsieh *et al*, 1995; Umesonon *et al*, 1991; Ozono *et al*, 1990). The biological effect is the activation of genetic programs which lead to a decrease of proliferative activity mainly due to the induction of differentiation. However, AML blast cells, blocked at different levels of maturation, are not responsive to hematopoietic growth factors with differentiation activity alone, *in vitro* and *in vivo*, but can be sensitive to VD in particular when cytokines are also present (Trinchieri *et al*, 1987; Kelsey *et al*, 1992; Collins *et al*, 1977). It has been shown that receptor binding of VD occurs efficiently in immature myeloid cells (Feldman *et al*, 1993). To get insights into the correlations between genotypic and phenotypic effects of VD, we have compared the effect of VD in different myeloid cell lines either responsive or unresponsive to this hormone. The phenotypic effects of VD have been monitored by studying the morphology, adhesion capacity and expression of CD14 monocytic differentiation marker on the cell surface. The genotypic effects have been monitored by studying VDR expression and phosphorylation, VDR-dependent DNA binding and transactivation activities. Our results clearly indicate that early myeloblastic cell lines such as K562 (myelo-erythroblastic), KG-1a and KG-1 (M0-M1 type), and the more differentiated promyelocytic cell line NB4 (M3 type) are not responsive to the differentiation activity of VD, while the late myeloblastic AML-1 and HL-60 cells (M2/M3 types), and monoblastic U937 (histiocytic) and THP-1 cells (M5 type), are induced to differentiate by VD. These observations indicate that a narrow window exists during the maturation of myeloid cells which is permissive to monocytic differentiation induced by VD. In fact, a clear phenotypic effect can be observed only in cellular contexts characterized by M2/M3 type and in monocyte precursor cells, such as the M5 type of leukemia.

VDR is clearly detected in the nuclear extracts of all cell lines after 12 h of treatment with VD even though the receptor abundance and the level of phosphorylation vary considerably. The hyperphosphorylated receptor (53 kD) is mainly associated with a VD unresponsive phenotype. However, regardless of the phosphorylation levels, EMSA indicates that VDR, present in all nuclear extracts assayed, heterodimerizes with RXR and binds a VDRE-containing oligodeoxynucleotide. These data strongly indicate that, independently on VD phenotypic effects, VDR expression and DNA binding activity are maintained in all cell lines.

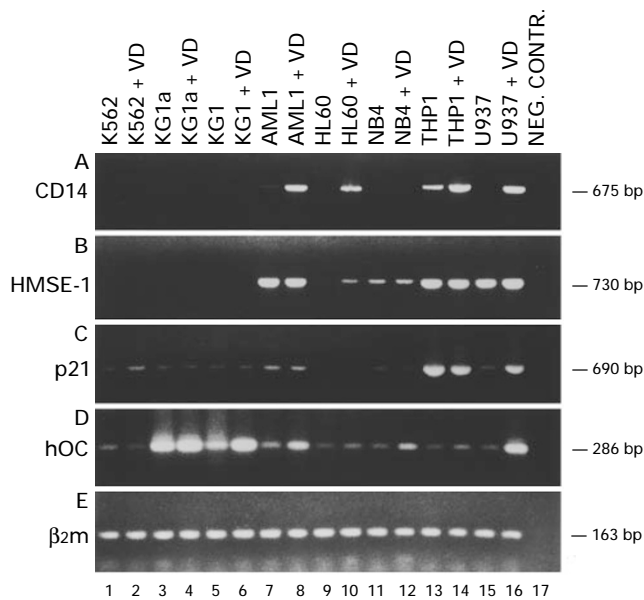


Figure 5 mRNA expression of CD14 antigen (A), HMSE (B), p21^{waf-1} (C), hOC (D) and β -2-m (E) evaluated by RT-PCR technique in myeloid cell lines, before and after 12 h VD treatment. Ethidium bromide stained agarose gels are shown. Cell lines and VD treatment indicated at the top of the figure, are: proliferating (lane 1) and VD-treated (lane 2) K562 cells; proliferating (lane 3) and VD-treated (lane 4) KG1a cells; proliferating (lane 5) and VD-treated (lane 6) KG1 cells; proliferating (lane 7) and VD-treated (lane 8) AML1 cells; proliferating (lane 9) and VD-treated (lane 10) HL60 cells; proliferating (lane 11) and VD-treated (lane 12) NB4 cells; proliferating (lane 13) and VD-treated (lane 14) THP1 cells; proliferating (lane 15) and VD-treated (lane 16) U937 cells; lane 17: negative control obtained performing RT-PCR amplification without cDNA template. The sizes of the amplified gene fragments are indicated on the right side of the figure.

This is confirmed by data showing that VD treatment increases transactivation of a VDRE-dependent reporter plasmid in K562, KG-1, HL-60, NB-4 and THP-1 cells.

Our data collectively indicate that in myeloid cells, arrested at different stages of differentiation, either responsive or non responsive to VD, VDR is expressed and is active in DNA binding and transcription promotion. However, since multiple factors such as chromatin structure, nucleosome organization and gene-nuclear matrix interactions, i.e. the cellular context, contribute to regulate gene transcription *in vivo*, we have studied the ability of VD to activate transcription of endogenous genes regulated by VDRE and of genes associated with terminal monocytic differentiation. Among the selected primary response genes, the hOC gene, which is mainly associated with osteoblast differentiation (Hauschka *et al*, 1989), and the p21^{waf-1} gene are not indicative of endogenous transactivation by VDR/RXR heterodimers since they are not strictly associated with monocyte differentiation. Instead, CD14 mRNA is clearly induced only in the cell lines with monocytic differentiation capacity and is not expressed in VD-unresponsive cell lines. The main feature of HMSE-1 mRNA expression is that it is not expressed in VD non responsive cells.

In summary, it appears that myeloid precursors at all stages of differentiation are equipped with the tools necessary for triggering the VD-dependent genetic program eventually leading to terminal differentiation. In other words, they contain a VDR which is fully active in binding its DNA site and in promoting transcription. This observation is particularly interesting since it is the first experimental evidence that early myeloblasts can transcribe nuclear receptors which undergo post-translational modification such as phosphorylation and nuclear translocation after VD treatment, and that in these cells, the nuclear abundance of VDR and RXR is sufficient to allow heterodimerization. Furthermore, in early myeloblast nuclei, VDR has functional DNA binding and transactivation domains. This VDR functional behavior was observed in all the myeloid cellular populations studied from early to late myeloblasts and monoblasts, independently of their VD dependent differentiation capacity, and seems to be a basic equipment of immature myeloid cells. Therefore, the lack of response to VD, which occurs at the early stages of differentiation, is likely to depend on steps located downstream of the VDR/DNA interaction. The analysis of VD-induction of primary response genes has not helped to clarify this point, since no clearcut correlation could be made between VD induction of these genes and the achievement of a terminally differentiated phenotype. This might not be surprising since myeloid-specific, VD primary response genes are not known at present. Therefore the discrepancies that we have observed between VDR function and VD induction of monocytic phenotype, might be the effect of the peculiarity of the different myeloid cellular contexts. Our data suggest that a narrow differentiation window permissive to monocytic differentiation exists in myeloid precursor cells at the M2/M3 maturation level which is not exclusively dependent on VDR function.

Materials and Methods

Cell cultures and differentiation

K562 (Lozzio and Lozzio, 1975), KG-1 (Koeffler and Golde, 1978), KG-1a (Koeffler *et al*, 1980), HL-60 (Collins *et al*, 1977), THP-1 (Tsuchiya *et al*, 1980) and U937 (Sundstrom and Nilsson, 1976) cell lines were obtained from the American Tissue Culture Collection (Rockville, MD) and from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). NB4 promyelocytic cell line (Lanotte *et al*, 1991) has been obtained from Dr. Lanotte and AML-1 cell line was a kind gift of Dr Valtieri (Lange *et al*, 1987). Cells were grown in RPMI 1640 (Gibco, Paisley, UK), supplemented with 10% heat-inactivated FCS (Boehringer, Mannheim, Germany), 2 mM L-Glutamine, in 5% CO₂ atmosphere at 37°C, and were passed twice a week. Cells were induced to differentiate by treatment with 10⁻⁷ M VD (Hoffman-Laroche, Basel, Switzerland) for 12 h or 5 days, depending on the different experiments performed. A blast cell population was obtained by leukapheresis from a patient with M5 type of leukemia before any pharmacological treatment. The blast cells were purified and characterized as already described (Ferrari *et al*, 1993). Monocytic differentiation was monitored primarily by studying the expression of CD14 (Zhang *et al*, 1994) and CD45 (Thomas, 1989) antigens by direct immunofluorescence with specific antibodies (anti CD45/CD14, Becton Dickinson, San Jose, CA, USA), and by RT-PCR (see RNA analysis in Materials and Methods) on CD14 and human monocytic specific esterase (HMSE-1) mRNAs (Uphoff *et al*, 1993). Flow cytometric analysis was performed as described (Manfredini *et al*, 1993). Morphology was assessed by cytocentrifugation followed by May Grunwald Giemsa (MGG) staining. The immunophenotype was assessed by using a standard panel of monoclonal Ab as described (Drexler, 1987).

Nuclear extract preparation

Nuclear extracts were obtained from the different myeloid cells as already described (Hennighausen and Lubon, 1987) before and after 12 h of VD treatment. Briefly the cells were centrifuged, washed in PBS and re-suspended in 5 vol. of 0.3 M Sucrose, 10 mM HEPES-KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF), and 0.2 µg/ml each of antipain, leupeptin and pepstatin-A. Cells were then lysed in Dounce homogenizer by 10 strokes of B pestle, followed by two strokes in the presence of 0.15% Nonidet P-40 (NP-40). Lysis was monitored by phase contrast microscopy. The nuclei were washed twice in the same buffer without NP-40, recovered by centrifugation at 1200 × g for 10 min and then resuspended in 10 mM HEPES-KOH pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF. After four strokes with B pestle, the homogenate was stirred for 30 min at 4°C followed by centrifugation for 60 min at 100 000 × g. The nuclear extracts were finally dialyzed for 4 h at 4°C against 100 vol. of 10 mM HEPES-KOH pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, 0.5 mM PMSF, cleared by centrifugation at 25 000 × g for 15 min at 4°C, and stored at -80°C in small aliquots. Protein concentration was evaluated by the Lowry method and the quality of the extract by PAGE. Dephosphorylation was obtained by diluting the nuclear extracts four-fold in gel shift buffer and incubating at 30°C for 30 min in the presence of 50 units of calf intestine alkaline phosphatase (CIAP) (Lefebvre *et al*, 1995).

Western blotting

Western blotting was performed using the Burnette method with minor modifications (Burnette, 1981). 50 µg of nuclear extract were then

dissolved in 1× reducing loading buffer (50 mM Tris pH 8, 5% β-mercaptoethanol, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol), loaded onto 7.5–10% SDS–PAGE and electrophoresed in TGS buffer (25 mM Tris pH 8.3, 250 mM glycine, 0.1% SDS). The separated proteins were transferred at 4°C onto a nitrocellulose sheet by electroblotting in TGM buffer (25 mM Tris pH 8.3, 250 mM glycine, 20% methanol) for 2 h at 1 A. In order to monitor the electroblotting efficiency the membrane was stained in 0.2% Ponceau S/0.3% TCA and destained in 0.3% TCA. Membranes were pretreated with TBST blocking solution (10 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 20, supplemented with 4 mg/ml normal serum) for 1 h at r.t., incubated with the primary antibody (1:500) (1 h at r.t.) and then with the indicated HRP-conjugated secondary antibody (1:5000) (1 h at r.t.) in blocking solution. The 9A7γ rat monoclonal anti-VDR (ABR, affinity bioreagents, Golden CO) was used as primary antibody for the detection of VDR protein. The detection was carried out by the ECL chemiluminescence method (Amersham Life Science, Little Chalfont, England).

Gel shift assay

Oligonucleotides were synthesized with an automated solid phase DNA synthesizer (Applied Biosystem, Inc., Foster City, CA; Mod. 394) with the standard phosphoramidite chemistry and purified by PAGE followed by electroelution as described (Manfredini *et al*, 1993). The following single stranded oligomers and their complement were synthesized to be used as probes in the gel shift assay: 5'-TGCCCTGCACTGGGTGAATGAGGACACTACTGACC-3'; rat osteocalcin VDRE (Umesono *et al*, 1991); 5'-TCTAGGTGACTCACCGGGTGAACGGGGGCATTGCGAG, human osteocalcin VDRE (Ozono *et al*, 1990); 5'-AGCTTCAGGTCAAGGAGGTCAGAGAGC, DR3 (Umesono *et al*, 1991). Oligonucleotide annealing was performed as already described (Kadonaga and Tjian, 1986). The double stranded oligodeoxynucleotides were further purified by PAGE and electroelution, and, 5' end labeled (100 ng) as already described (Ferrari *et al*, 1994). The mixture was incubated at 37°C for 1 h followed by addition of 80 μl 50 mM EDTA, extraction with 1 vol of 1:1 phenol/chloroform and ethanol precipitation. Specific radioactivities ranged from 1 to 3 × 10⁸ c.p.m./μg DNA. The gel shift retardation assay was performed as already described (Ferrari *et al*, 1994). Briefly: nuclear extracts (15 μg protein) were incubated with 5 μg poly (I.C) (Pharmacia, Piscataway, NJ), for 20 min at room temperature in 1× a gel shift buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1 mM EDTA and 15% glycerol). The labeled oligonucleotide was then added (1 ng) and the incubation continued for a further 30 min (final volume 20 μl). When indicated, specific antibodies were added to the incubation mixture 10 min before the addition of the probe. The following antibodies were used: 9A7γ (rat monoclonal anti-VDR, ABR, affinity bioreagents, Golden CO); 4RX-1D12 (mouse monoclonal anti-RXRα,-β,-γ, kindly provided by Dr. P. Chambon). In all the gel mobility shift experiments the VDR specificity of the shifted bands was confirmed by the disruption of the band when anti-VDR monoclonal antibody was added. In several control experiments VDRE specificity was also confirmed by adding 100-fold excess cold VDRE oligonucleotides. The use of anti-RXRα monoclonal antibody has the ability to demonstrate a supershifted band when the RXRα is complexed with VDR in an heterodimeric conformation. All the antibodies were added to the samples at 1:20 dilution. The reaction mixtures were then electrophoresed on a 5% polyacrylamide gel in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA), prerun for 1 h at room temperature. Finally the gel was fixed, vacuum dried, and exposed to X-ray films (2–4 h).

RNA analysis

Total cellular RNA was extracted using a modification of the guanidinium-cesium chloride centrifugation technique (MacDonald *et al*, 1987), and digested with RQ1-DNAse (Promega, Madison, WI) to avoid DNA contamination as already described (Grande *et al*, 1995). RT–PCR reactions were carried out using a modification of a previously described technique (Ferrari *et al*, 1993). Briefly 3 μg of total RNA extracted from each sample was reverse transcribed using 400 U of M-MLV Reverse Transcriptase (GIBCO, BRL, Gaithersburg, MD) and 1 μg of OligodT15 primer (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) for 1 h at 42°C in 1× RT buffer (50 mM Tris pH 8.3, 60 mM KCl, 1.5 mM MgCl₂, 10 mM DTT, 1 mM dATP, dCTP, dGTP, dTTP, 20 U RNAsin, 0.1 mg/ml BSA), in a total volume of 30 μl. The cDNA was then heated at 95°C for 3 min and stored at 4°C. 1 μl of cDNA was amplified adding 2.5 U of Taq polymerase (Boehringer, Mannheim, Germany), 0.5 μg of specific direct primer (DP) and reverse primer (RP) in a total volume of 50 μl, in 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs. The amplification was carried out using 20–40 cycles under the following conditions: denaturation at 95°C for 45 s; annealing at 55°C for 2 min; extension at 72°C for 3 min. 10 μl of each sample was then electrophoresed on a 2% agarose gel. RT–PCR analysis was performed using oligodeoxy-nucleotide primers specific for CD14 antigen (Ferrero and Goyert, 1988), HMSE-1 (Zschunke *et al*, 1991), p21^{waf-1} (el Deiry *et al*, 1993), human osteocalcin (hOC) (Kiefer *et al*, 1990) and β2 microglobulin (β2m) (Suggs *et al*, 1992). The oligonucleotides used as direct primer (DP) or reverse primer (RP) were: CD14-DP, 5'-TCCAGAGCCTG-TCCGGAGCTCAGA-3'; CD14-RP, 5'-GCGTTCGCCAGTCCAGGATTGTCA-3'; HMSE1-DP, 5'-CCTCCTATGTGCACCCAAGAT-3'; HMSE1-RP, 5'-GCATCCCATCAATCACAGTGC-3'; p21^{waf-1}-DP, 5'-AGTTCCTTGTGGAGCCGAGCTGGG-3'; p21^{waf-1}-RP, 5'-TCCAGGACTGCAGGCTT-CCTGTGGG-3'; hOC-DP, 5'-GAGCCCTCACAC-TCCTCGCCCTATT-3'; hOC-RP, 5'-GTAGAAGCGCCGATAGGCCTCCTGA-3'; β2m-DP, 5'-CTCGCGCTACTCTCTCTTCT-3'; β2m-RP, 5'-TCCATTCTTCAGTAAGTCAACT-3'.

Cell transfection

We used the following plasmids in the transfection experiments: pMCVβ (Clontech Laboratories Inc, Palo Alto, CA, USA), a plasmid capable of expressing *E. coli* β-galactosidase under the control of cytomegalovirus immediate early promoter/enhancer; pOsteo/CABP-CAT containing the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the VDRE of the human osteocalcin gene (–511/–480) and the minimal promoter region of the chicken Calbindin gene (Ferrari *et al*, 1990). VD treatment of Rat Osteosarcoma (ROS) cells transfected with this plasmid causes a 6–8 fold induction of CAT reporter activity as compared to untreated cells. The obtained β-GAL activity values were used in all transfection experiments to normalize for transfection efficiency. K562, KG-1, HL60, NB-4 and THP-1 cells were maintained in RPMI 1640 medium with 5% fetal calf serum (FCS). 2.5 × 10⁷ cells were transfected by electroporation with 30 μg of pOsteo/CABP-CAT plasmid and 3 μg of pMCVβ plasmid. Electroporation was performed at 250 V, 960 μF (Gene Pulser Apparatus, Bio Rad). Transfected cells were cultured in RPMI medium containing 15% FCS with or without VD for 48 h and then harvested for CAT assay. In relationship to the transfection efficacy, as determined by the β-Galactosidase activity of the cotransfected plasmid, normalization was performed using different amounts of cellular extracts in the CAT assay. This assay was performed and quantified by scintillation counting as already described (Alam and Cook, 1990).

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