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Resistance to 1,25D-Induced Differentiation in Human Acute Myeloid Leukemia HL60-40AF Cells Is Associated With Reduced Transcriptional Activity and Nuclear Localization of the Vitamin D Receptor

Edward Garay, Robert Donnelly, Xuening Wang, and George P. Studzinski*

Department of Pathology and Laboratory Medicine, UMDNJ-New Jersey Medical School, Newark, New Jersey

Abstract

The anti-neoplastic effects of 1,25-dihydroxyvitamin D₃ (1,25D) are well documented in numerous tumor cell systems and animal models of cancer. However, despite this pre-clinical success, the clinical use of 1,25D is currently impeded by the dose-limiting hypercalcemia, and the risk of development of resistance to 1,25D. In this study, we investigated the mechanism of resistance to 1,25D of HL60-40AF cells, a model of drug-resistant acute myeloid leukemia, derived from HL60 cells by cultivation in the presence of 1,25D. The data indicate that transcriptional activity of vitamin D receptor (VDR) in 40AF cells increases only briefly when the cells are treated with 1,25D, despite greater basal cellular levels of VDR protein in the resistant than in the 1,25D-sensitive cells. Analysis of the 40AF VDR mRNA sequence indicated alterations in the 5' untranslated region (UTR), but coding domain variations were not observed. When resistance to 1,25D-induced differentiation of 40AF cells was reversed by a combination of 1,25D with potentiators of differentiation (plant derived antioxidants and a p38MAPK inhibitor), an increase in the level of nuclear VDR, as well as an increase in CYP24 mRNA expression was observed. These data suggest that decreased ability of 1,25D to induce VDR nuclear localization and the consequent VDR target gene transcription may be an important reason for the resistance of 40AF cells to 1,25D. Further, our data show that VDR localization and phosphorylation can be increased by combining 1,25D with potentiators of differentiation. Analysis of the mechanisms that underlie the reduction and potentiation of 1,25D-mediated changes in VDR activity may lead to the identification of new cellular targets that enhance 1,25D-induced monocytic differentiation.

The use of 1,25-dihydroxyvitamin D₃ (1,25D) has been studied in many models of disease and mineral homeostasis. In addition to its role in regulating calcium/phosphorus serum levels and maintaining skeletal integrity, 1,25D is known to have anti-proliferative and differentiation-inducing effects in numerous cancer models such as neoplasms originating from breast, prostate, colon, as well as, epidermal, hepatic, and hematopoietic tissues (Cross et al., 2001; Bikle, 2004; Pourgholami and Morris, 2004; Beer et al., 2005; Luong and Koeffler, 2005). In the pathological spectrum of acute myeloid leukemia (AML), 1,25D inhibits proliferation and induces in vitro differentiation of murine and human myeloid leukemia cells along the monocytic/macrophage lineage (Tanaka et al., 1982), and can extend the survival of animals in murine models of AML (Honma et al., 1983; Zhou et al., 1990; Sharabani et al., 2006).

Importantly, the differentiation-inducing activity of 1,25D is robustly potentiated by combining it with plant-derived antioxidants (Sokoloski et al., 1997; Danilenko et al., 2003) and/or an inhibitor of the p38MAPK pathway such as SB202190 or SB203580 (Wang et al., 2000). For instance, in a recent translational study using ex vivo blood samples a subset of AML cells was observed to differentiate in response to 1,25D or its analogs, when combined with these potentiators of differentiation (Wang et al., 2005). Despite such promising preclinical results, the in vivo use of 1,25D is limited by the induction of hypercalcemia and the potential for development of resistance to the anti-neoplastic properties of 1,25D (Lasky et al., 1994; Narvaez et al., 1996; Chen et al., 2003).

Resistance to chemotherapeutic agents has been a disappointing development in the management of certain cancers. In myeloid leukemias acquired chemotherapeutic resistance is observed frequently, including chronic myeloid leukemia positive for the chromosomal t (9;22) abnormality (Deininger et al., 2000). In this clinical presentation, alterations in the Bcr-Abl hybrid protein have been observed to limit the activity of Imatinib (Gorre et al., 2001; Weisberg and Griffin, 2001), and reduce the disease-free survival of patients (Branford et al., 2002). In the context of differentiation therapy of acute promyelocytic leukemia positive for t (15;17), acquired resistance to the differentiation-inducing properties of all trans retinoic acid (ATRA) is observed in a significant proportion of relapsed patients, and has been associated with mutations in the binding pocket of the retinoic acid receptor (for review see Gallagher, 2002). Although no clinical case of acquired 1,25D or deltanoid-resistance in the treatment of cancer has been reported at the time of this study, numerous in vitro models of 1,25D-resistance have been reported and the cause of 1,25D-resistance has been attributed to various molecular mechanisms (Prufer et al., 2002; Banwell et al., 2006; Byrne and Welsh, 2007).

The molecular mechanisms involved in the differentiation-inducing actions of 1,25D in AML are not clearly understood, although it is known that 1,25D is a ligand for VDR, which, when activated, functions as a transcription factor (Mangelsdorf et al., 1995). VDR is a member of the nuclear receptor family that targets the promoter region of genes that contain a vitamin D receptor element (VDRE). When bound to its ligand, 1,25D, VDR can homodimerize (Carlberg et al., 1994) or heterodimerize with an isoform of the Retinoid X Receptor (RXR), and initiate the transcription of VDRE-containing target genes (Haussler et al., 1995; Prufer et al., 2000; Wang et al., 2006). The expression of a functional VDR is required for the biological activity of 1,25D (Christakos et al., 2003), or of structurally similar deltanoids (Peleg and Posner, 2003). Of note, clinical resistance to the biological actions of 1,25D has been observed in patients with vitamin D-dependent rickets type II, and one of the primary mechanisms observed to contribute to this phenotype are mutations in the VDR coding domain (Hughes et al., 1991; Malloy et al., 1999; Nguyen et al., 2002).

In the present study we utilized the previously developed model of acquired resistance to 1,25D-induced differentiation of AML, HL60-40AF (40AF) cells (Wajchmann et al., 1996) to investigate the underlying basis for 1,25D-insensitivity. We found that although VDR is expressed in the resistant cells and has a wild-type coding region, it has reduced transcriptional activity that may be explained by its decreased nuclear localization in the presence of 1,25D used as a single agent.

Materials and Methods

Reagents and antibodies

1,25D was a kind gift from Dr. Milan Uskokovic (Bioxell, Nutley, NJ). Antibodies MY4-RD1 and MO1-FITC, which recognize CD14 and CD11b surface differentiation markers, and isotype controls were from Beckman Coulter (Fullerton, CA). Primers for CYP24 and Acidic Riboprotein Subunit 0 (ARP0) were synthesized by the Molecular Resource Facility of

UMDNJ (Newark, NJ). RNase-free DNase was obtained from Qiagen (Valencia, CA). Inhibitor of p38 MAPK, SB202190, was obtained from Calbiochem (La Jolla, CA), carnosis acid from Alexis Pharmaceuticals (Lausen, Switzerland), silibinin and curcumin from Sigma-Aldrich (St. Louis, MO). Complete protease inhibitor cocktail and DNase-free RNase were from Hoffmann-La Roche (Nutley, NJ).

Antibodies used for Western blotting (WB) were as follows: anti-VDR (C-20 and N-20), anti-RXR α (D-20), anti-Crk-L (C-20), and anti-Calreticulin (H-170) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA), anti-histone H3 (H0164) from Sigma-Aldrich, and anti-CYP24 was a kind gift from Dr. Morey Campbell, University of Birmingham Medical School. Anti-rabbit and anti-goat antibodies linked to HRP were purchased from Cell Signaling Technologies (Danvers, MA).

Cell cultures

HL60 cells, derived from a patient with promyeloblastic leukemia (Gallagher et al., 1979), were obtained for these studies from the ATCC. HL60-G (Xu et al., 1992) and HL60-40AF cells derived from HL60-G cells by cultivation in the presence of 1,25D (Wajchmann et al., 1996) are differentiation-sensitive and resistant subclones of HL60 cells, respectively. These cells were propagated as a suspension culture in RPMI 1640 medium supplemented with 10% bovine calf serum (Hyclone, Logan, UT). The cells were kept at standard cell culture conditions, that is, humidified atmosphere of 5%CO₂ at 37°C. The cell number and viability were determined by hemocytometer counts and trypan blue (0.4%) exclusion, and routine microbiology testing for mycoplasma was performed by selective culture techniques. For all experiments, the cells were suspended in fresh medium containing 1,25D or the equivalent volume of ethanol as a vehicle control. Each experimental condition was repeated at least three times.

Determination of markers of monocytic differentiation and cell cycle parameters

Aliquots of 1×10^6 cells were washed twice with $1 \times$ phosphate-buffered saline (PBS), then placed on ice with 0.3 μ l MY4-RD1 and 0.3 μ l MO1-FITC in a total volume of 10 μ l PBS for 45 min to analyze the expression of CD14 and CD11b, respectively. After incubation the cells were washed two times with ice-cold $1 \times$ PBS and then suspended in 0.5 ml of $1 \times$ PBS and analyzed using EPICS XL Flow Cytometer (Beckman Coulter). The acquisition parameters were set for an isotype control. Data analysis was performed using EPICS XL software. For assessment of non-specific esterase (NSE), also known as monocyte specific esterase, smears were made by resuspending 2×10^6 cells in 100 μ l $1 \times$ PBS and spread onto slides. The air-dried smears were fixed in formalin-acetone mixture buffer for 30 sec, then washed with distilled H₂O and stained for 45 min at room temperature with the following solution: 8.9 ml of 67 mM phosphate buffer (pH 7.6), 0.6 ml of hexazotized pararosaniline, 1 mg/ml alpha-naphthyl acetate, and 0.5 ml ethylene glycol monomethyl ether. The percentage of NSE-positive cells was determined by counting 100 total cells in triplicate.

To determine with $1 \times$ PBS cell cycle distributions aliquots of 1×10^6 cells were washed twice and fixed in 75% ethanol at -20°C overnight, washed twice with PBS and incubated with 1 μ g/ml of RNase at 37°C for 1 h. The cell pellet was washed twice and resuspended in a propidium iodide (10 μ g/ml) in PBS solution. The cells were analyzed using an EPICS XL Flow Cytometer (Beckman Coulter) and the cell cycle distribution was determined by Multicycle Software Program (Phoenix Flow System, San Diego, CA).

PCR of VDR mRNA coding domain and quantitative RT-PCR (qRT-PCR)

Aliquots of 5×10^6 cells were harvested and washed twice with ice-cold $1 \times$ PBS. Total cellular RNA was extracted with RNeasy Mini kit (Qiagen) and treated with RNase-free DNase

according to the manufacturer's protocol. RNA purity and final concentration were determined by spectrophotometric reading at 260 and 280 nm, and 1 µg of total RNA was reverse transcribed with MuLV Reverse Transcriptase primed with oligo-d(T) according to the manufacturer's protocol (Applied Biosystems, Foster City, CA) and used for VDR mRNA sequencing and quantitative PCR (qRT-PCR).

The VDR mRNA coding domain was sequenced by using PCR primers (31a, 33a, and 33b) described by Hewison et al. (1993), and primer 1273 (5' GGTGTGTCAATCCTGGTATC 3') which was developed with Primer Express software using cloned human VDR cDNA (NCBI #J03258) as a template. Primers were designed to expand overlapping regions of the VDR mRNA coding domain, see Figure 4, Panel B. The parameters for PCR were as follows: 1 cycle of 95°C for 10 min followed by 35 cycles of (1) 95°C for 1 min, (2) 58°C for 1 min, and (3) 72°C for 2 min. The products were separated on a 1% agarose gel and bands that corresponded to an 804 bp product (primers: 31a and 1273) and a 645 bp product (primers: 33a and 33b) were excised, cDNA extracted with QIAquick Gel Extraction kit (Qiagen) and submitted for direct sequencing.

Relative quantification of target cDNA was performed using a Roche Lightcycler instrument with Faststart DNA Master^{plus} Syber Green I kit (Hoffmann-La Roche) and gene specific primers. The parameters for PCR were as follows: 40 cycles of (1) 95°C for 10 sec, (2) 62°C for 10 sec, and (3) 72°C for 10 sec followed by an incremental increase (0.1°C/sec) of temperature from 75 to 95°C to analyze the melting curve of the products. A final primer concentration of 150 nM was used: (1) CYP24 upstream (5' CAAACCGTGGAAGGCCTATC 3') and downstream (5' AGTCTTCCCCTTCCAGGATCA 3'), (2) ARP0 upstream (5' AGATGCAGCAGATCCGCAT 3') and downstream (5' GTGGTGATACCTAAAGCCTG 3') (Vaisanen et al., 2005). The Ct value, the cycle number at which signal fluorescence surpassed fluorescence background noise, was recorded, and relative fold values were calculated based on the equation $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t^{\text{treated}} - \Delta C_t^{\text{calibrator}}$ (untreated or vehicle treated samples) and $\Delta C_t = C_{t\text{CYP24}} - C_{t\text{ARP0}}$.

Western blotting (WB)

Western blotting was performed using whole cell extracts except as indicated. The cells were harvested and washed twice with ice-cold 1 × PBS. The washed cell pellets were solubilized with a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin), followed by centrifugation at 16,000g for 15 min. The protein concentrations of the extracts were determined by using Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) and then mixed with (2:1) 3 × SDS sample buffer [150 mM Tris-HCl, pH 6.8, 30% glycerol, 3% SDS, 1.5 mg/ml bromophenol blue dye, and 100 mM dithiothreitol (DTT)] and incubated for 5 min at 100°C. Thirty-five micrograms of protein cell extracts was separated on 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). The membranes were blocked with 5% milk in TBS/0.1% Tween-20 for 1 h, incubated overnight with primary antibodies, and then blotted with a horseradish-linked secondary antibody for 1 h. The protein bands were visualized using a chemiluminescence assay system (Pierce Biotechnology, Rockford, IL). The optical density of each band was quantitated using an ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA).

Subcellular cell fractionation

Cytosolic and nuclear fractions were prepared by the procedure of Andrews and Faller (1991). Briefly, 2×10^7 cells were harvested and washed twice with ice-cold PBS, and resuspended in 0.2 ml ice-cold hypotonic cell extract buffer [10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCL, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl (PMSF), and

Complete Protease Inhibitor cocktail]. The cells were kept on ice for 10 min (HL60-G) and 1 min (40AF) to allow them to swell, vortexed for 10 sec, and microfuged at 4°C at 16,000g for 30 sec. The supernatant was saved as the cytoplasmic extract, and the pellet was resuspended in 30 µl of nuclear extraction buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and Complete Protease Inhibitor cocktail) placed on ice for 20 min, and centrifuged at 4°C at 16,000g for 2 min. The supernatant was saved as the nuclear extract.

Potato acid phosphatase assay

Cytoplasmic and nuclear protein extracts (100 µg) were incubated with 0.25 U of potato acid phosphatase (PAP; Sigma–Aldrich), in PIPES/DTT buffer (50 mM PIPES, pH 6.0, and 1 mM DTT) at 30°C for 10 min. To control for PAP enzyme activity, 100 mM NaPO₄ was added to inhibit dephosphorylation of protein. Following PAP treatment, 3× SDS buffer was immediately added to mixture and heated at 100°C for 5 min, 35 µg of protein was separated on 10% polyacrylamide gel, and the Western blot procedure described above was followed.

Immunoprecipitation (IP)

Immunoprecipitation was performed by using the Seize[®] Primary Mammalian Immunoprecipitation kit (Pierce Biotechnology) as described before (Wang and Studzinski, 2006). Briefly, 25 µg of an anti-VDR (N-20) antibody was coupled to 100 µl of AminoLink Plus coupling gel in a spin column and then incubated overnight at 4°C with a 200 µl aliquot of nuclear protein extract (75 µg). Next, the coupling gel was washed three times and the samples were eluted from the column and run on a 10% SDS–PAGE gel, transferred to a nitrocellulose membrane, and probed with specified antibodies. The protein bands were visualized with ECL^{plus} chemiluminescence assay system (Amersham Biosciences).

Statistical methods

Significance of the differences between mean values was assessed by a two-tailed Student's *t*-test. The effects of SB202190 and plant antioxidants (PAOx) on 1,25D action were assessed on the following measures: (1) CD11b and CD14 expression, (2) CYP24 mRNA expression, measured as $\Delta Ct = Ct_{CYP24} - Ct_{ARP0}$; the *P* values are reported in the text and in the figure legends. All computations were performed with Microsoft EXCEL software on an IBM-compatible personal computer.

Results

1,25D induces markers of monocytic differentiation and inhibits the cell cycle progression of HL60-G cells, but not of 1,25D-resistant HL60-40AF cells

The induction of markers of monocytic differentiation by 1,25D in human myeloid leukemia cells has been widely studied in HL60 cells, a model of human myeloid leukemia (Gallagher et al., 1979), and this laboratory developed a 1,25D-induced differentiation-sensitive subline designated HL60-G (Xu et al., 1992), and a 1,25D-resistant subline, HL60-40AF cells (Wajchmann et al., 1996). Monocytic differentiation can be recognized by the expression of surface markers, for example, CD14 and CD11b (Griffin et al., 1981, 1983), and by the presence of the cytoplasmic enzyme, NSE, also termed monocyte specific esterase (Yam et al., 1971; Danilenko et al., 2001). In the current study, as expected, a significant dose-dependent increase in these markers was evident in HL60-G cells treated with 1,25D when compared to EtOH-treated cells [$P < 0.001$ (**), $P < 0.05$ (*)], but in contrast 40AF cells demonstrated little, if any, sensitivity to 1,25D administered to the cells at a wide range of concentrations (Fig. 1, Panel A).

1,25D-induced inhibition of the cell cycle progression can be demonstrated by an accumulation of cells in the G1-phase, with an accompanying decrease of cells in the S-phase. Analysis of the HL60-G cell cycle indicated a dose-dependent increase in the percentage of cells in the G1-phase, and a decrease in S-phase cells (Fig. 1, Panel B) [$P < 0.001$ (**)] when compared to EtOH-treated cells]. Treatment of 40AF cells with increasing concentrations of 1,25D did not affect the distribution of cells in the cell cycle compartments, further demonstrating their resistance to 1,25D (Fig. 1, Panel B). Of note, analysis of the cell cycle in 40AF cells left untreated, or treated with vehicle control (0.04% EtOH), indicated a lower percentage of cells in the G1-phase and a greater percentage of S-phase cells than in the parental HL60-G cells (Fig. 1, Panel B), consistent with the increased rate of cell proliferation of 40AF cells when compared to the parental HL60-G (Wang et al., 1998).

Nuclear receptor proteins which mediate 1,25D signaling are expressed in HL60-G and HL60-40AF cells

Nuclear receptor proteins, required for 1,25D-induced signaling (Pike and Shevde, 2005) were analyzed by WB, and both VDR and RXR α were found to be expressed in 1,25D-sensitive and 1,25D-resistant cell types (Fig. 2, Panel A). Interestingly, at basal state, the level of VDR protein in 40AF cells exceeded the level of VDR protein in 1,25D-sensitive HL60-G cells at basal state. Further, the basal expression of RXR α , the principal heterodimeric partner of VDR in HL60 cells (Haussler et al., 1995; Wang et al., 2006), was also higher in 40AF than in HL60-G cells (Fig. 2, Panel A).

Analysis of Western blot protein bands by optical density measurements indicated that treatment with increasing concentrations of 1,25D induced a robust dose-dependent increase of the levels of VDR protein in HL60-G cells [$P < 0.05$ (*) when compared to EtOH-treated HL60-G cells] but no apparent increase in 40AF cells (Fig. 2, Panel B). This is in keeping with the lack of VDR protein induction following treatment of 1,25D-resistant MCF-7 breast cancer cells (Jensen et al., 2002).

When the levels of RXR α protein expression were analyzed, a dose-dependent increase in RXR α protein levels was observed in HL60-G cells [$P < 0.05$ (*) when compared to EtOH-treated HL60-G cells], similar to the increase in VDR protein levels. In contrast, a decrease in the level of RXR α protein was observed in 40AF cells following treatment with a high concentration of 1,25D (100 nM) [$P < 0.05$ (¥) when compared to EtOH-treated 40AF cells], as shown in Figure 2, Panel C. Thus, both components of the heterodimer necessary for optimal transcription of 1,25D target genes are present in the resistant 40AF cells, but the exposure to 1,25D does not promote an increase in total cellular VDR or RXR α proteins in these cells.

VDR target gene CYP24 is transcribed in time- and dose-dependent manner in HL60-G and HL60-40AF cells treated with 1,25D

The primary action of the ligand-bound VDR is to initiate the transcription of genes that contain a VDRE in their promoter regions (Whitfield et al., 2005). One well-studied VDR target gene is CYP24, the gene that encodes steroid 24-hydroxylase (Ohyama et al., 1994; Chen and DeLuca, 1995). To assess if VDR and VDR-RXR α heterodimers are functional in 40AF cells, we determined by qRT-PCR the expression of CYP24 mRNA following an exposure of the cells to 1,25D. In HL60-G cells not exposed to 1,25D, CYP24 mRNA was detected at very low levels; however, when these cells were treated with 1,25D there was a progressive increase in CYP24 mRNA which continued for at least the first 48 h of exposure to 1,25D [$P < 0.001$ (**)] and $P < 0.05$ (*) when compared to baseline HL60-G cells] (Fig. 3, Panel A). On the other hand, CYP24 mRNA was easily detected in untreated 40AF cells, but the same exposure to 1,25D induced an increase in CYP24 mRNA levels principally during the first 6 h of treatment, while a more prolonged exposure to 1,25D resulted in only marginal further increases in the

levels of CYP24 mRNA in these cells [$P < 0.001$ (¥¥) and $P < 0.05$ (¥) when compared to baseline 40AF cells] (Fig. 3, Panel A). Thus, basal levels of CYP24 mRNA in 40AF cells, which developed resistance by cultivation in the presence of 1,25D, are higher than the parental HL60-G cells, but the rate of increase following exposure to 1,25D in 40AF cells, although transiently exceeds the rate of increase in HL60-G cells, is less marked over time.

Similarly, when the cells were treated for 48 h with increasing concentrations of 1,25D (1–100 nM), dose-dependent increases in CYP24 mRNA and protein levels were observed in both HL60-G and 40AF cells, but were less marked in 40AF than in HL60-G cells [$P < 0.001$ (***) and $P < 0.05$ (*) when compared to untreated HL60-G cells and $P < 0.05$ (¥) when compared to untreated 40AF cells] (Fig. 3, Panels B and C). These results show that although VDR is present and functional in 1,25D-resistant 40AF cells, the ability to upregulate its expression (Fig. 2, Panels A and B), and transcriptional activity (Fig. 3, Panels A and B) is reduced. Combined, our data suggest that the resistance to 1,25D-induced differentiation of 40AF cells may result, at least in part, from reduced 1,25D-induced transcriptional activity of VDR or VDR-RXR α heterodimers.

Direct sequencing of 40AF VDR mRNA indicates alterations in the 5' UTR, but not in the coding domain of the VDR gene

The reduced rate of transcriptional activation of CYP24 gene in 40AF cells by 1,25D (Fig. 3, Panels A and B) raises the possibility that in 40AF cells the functionality of VDR is compromised by a mutation. Multiple variations in the VDR gene have been reported in the literature, and have been linked to a decrease in the physiological activity of 1,25D (Haussler et al., 1997; Malloy et al., 1997; Nguyen et al., 2004). These alterations are typically located in the coding regions of the VDR mRNA (Kristjansson et al., 1993; Malloy et al., 1999; Nguyen et al., 2002). To analyze the VDR coding sequence of the 40AF cell VDR mRNA we utilized multiple sets of PCR primers that expand overlapping regions of the VDR mRNA coding domain (Fig. 4, Panel A). The PCR products were sequenced, and the resulting sequences were aligned and compared to the similarly obtained sequence of HL60-G cells and the published Genbank (accession #J03258) human VDR cDNA sequence (Fig. 4, Panel C). No variation was observed in the 40AF cell VDR mRNA coding sequence as compared to the Genbank or HL60-G cell VDR mRNA coding sequences (Fig. 4, Panel C). However, we noted several variations in the 40AF cell 5' UTR compared to the published sequence. These included cytosine base insertions between bases #80–81 and #102–103. In addition, a nucleotide base mismatch T > C was noted at residue #117 in the wild-type start codon and induces a change in the site for translation initiation to the next in frame ATG and results in a shortened VDR primary structure (Gross et al., 1996). This variation, the FokI polymorphism, has been observed in population studies of the promoter region of human VDR, and leads to the translation of a VDR protein that has greater transcriptional activity than full-length VDR (Arai et al., 1997). However, only the #102–103 cytosine insertion is not already present in the parental HL60-G cells. Thus, the VDR mRNA is not significantly altered or selected during the development of 1,25D-resistance, and the protein coding domain sequence is identical to the HL60-G and to the published human VDR sequence, indicating that a change in the primary structure of the VDR protein is unlikely to be the basis for the resistance.

Reversal of resistance to 1,25D-induced differentiation by enhancers of 1,25D activity correlates with the increased expression of CYP24, a VDR-target gene

To investigate further what causes the resistance of 40AF cells to 1,25D-induced differentiation, we utilized the enhancers of 1,25D action previously described by this laboratory. These enhancers comprise the pyridinyl imidazole compound, SB202190 (SB), which inhibits p38 MAPK (Wang et al., 2004), and a group of PAOx such as carnosic acid (Danilenko et al., 2001), sibilinin, and curcumin (Danilenko and Studzinski, 2004). Each of

these, used as a single agent, potentiates the 1,25D-induced differentiation of HL60 and other myeloid leukemia cells (Sharabani et al., 2006), but has little, if any, effect on 40AF cell differentiation (data not shown). However, when one of the PAOx is combined with SB and 1,25D, this “differentiation cocktail” partially reverses the resistance of 40AF cells to the induction of differentiation (Wang et al., 2004) and significantly increased the expression of markers of differentiation [$P < 0.001$ (***) when compared to vehicle-treated HL60-G cells and $P < 0.001$ (¥¥) and $P < 0.05$ (¥) when compared to vehicle-treated 40AF cells] (Fig. 5, Panel A). We now also show that the functionality of VDR, as demonstrated by the expression of CYP24 mRNA, is increased in 40AF cells by the addition of SB and PAOx enhancers to 1,25D (Fig. 5, Panel B). This experiment was performed over the period of 96 h to allow full expression of differentiation markers in the differentiation-sensitive HL60-G cells which were used here as a positive control. It can also be noted that the 1,25D-induced level of CYP24 mRNA expression in 40AF cells was similar to the level observed at shorter time points (Fig. 3, Panel A), suggesting that the initially increased levels of CYP24 reached a new equilibrium and remained relatively constant in the presence of 1,25D.

Resistance to differentiation is not due to increased nuclear localization of calreticulin, an inhibitor of nuclear hormone receptor activity

We examined the possibility that in spite of relatively high levels of cellular VDR in 40AF whole cells (Fig. 2, Panel A), its transactivation activity is reduced by the presence of a nuclear inhibitor, and that this contributes to the 1,25D resistance of these cells. One such inhibitor is calreticulin, which is known to inhibit the activity of nuclear hormone receptors, including 1,25D-induced VDR transactivation (Dedhar et al., 1994; Wheeler et al., 1995; DeFranco, 2001). We first tested if our fractionation procedure separates nuclei from the cytosol and membranes. The nuclear protein, histone H3, was indeed found almost exclusively in the nuclear fraction, with only occasional contamination of the cytoplasm (C/M) fraction (Fig. 6, Panel A). In contrast, a mitochondrial enzyme, CYP24 (Henry, 1992), was found predominantly in the C/M fraction (Fig. 6, Panel B). We then determined in four consecutive experiments the levels of calreticulin in these cells fractions, and found remarkable reproducibility of this procedure (Fig. 6, Panel C). In agreement with previous reports (Opas et al., 1991; Rojiani et al., 1991; Dedhar et al., 1994; Wheeler et al., 1995; Clark et al., 2002) we found a large amount of calreticulin in nuclear fractions of both cell types, and in the 1,25D-sensitive HL60-G cells a decrease in calreticulin levels following exposure to 1,25D or differentiation cocktail. Importantly, the levels of calreticulin in the nuclei of 1,25D-resistant 40AF cells were lower than the levels of this inhibitor observed in the nuclei of HL60-G cells, making it an unlikely cause for the resistance to 1,25D.

Treatment of 40AF cells with the differentiation cocktail enhances phosphorylation of VDR and its nuclear localization

When we examined the migration and intensity of bands visualized by an antibody to the N-terminal region of human VDR on Western blots of nuclear extracts from HL60-G and 40AF cells we noted a doublet of bands (Fig. 7, Panel A). The more rapidly migrating band (VDR-n2) was apparent in both C/M and nuclear fractions, but the more slowly migrating band (VDR-n1) was detected only in the nuclear fractions, and its intensity correlated with 40AF cell differentiation, being faint in untreated or 1,25D-treated cells, but intense in cells treated with the differentiation cocktail DCS (Fig. 7, Panel A). The VDR identity of both bands was suggested by their co-migration with a similar doublet that was visible in nuclear extracts of MCF-7 human breast carcinoma cells (Fig. 7, Panel B), which are known to have abundant VDR (Buras et al., 1994). This was confirmed by the absence of these bands when the VDR antibody was blocked by the immunizing peptide (Fig. 7, Panel B). Previous reports described similar VDR doublets in other cell types, including rat osteosarcoma (ROS) cells (Masuyama and MacDonald, 1998), human fetal osteoblast (hFOB) and human intestinal-like Caco-2 cells

(Ismail et al., 2004), and human mammary carcinoma (MCF-7) cells (Jensen et al., 2002; Narvaez et al., 2003). In these reports, the lower band in the VDR doublet was considered to be the target of proteosomal degradation, and evidence was also provided that the faster migrating VDR form is transcriptionally active (Ismail et al., 2004). However, an important caveat is that these reports also noted marked differences between cell types as regards VDR protein modifications and its subcellular localization.

The nature of the two nuclear VDR bands in our system was therefore further investigated by incubating the nuclear extracts with PAP. As shown in Figure 7, Panel C, treatment of MCF-7 and HL60-G nuclear extracts reduced the intensity of the VDR-n1 band while increasing the intensity of the VDR-n2 band. These data strongly suggest that the VDR-n1 is a phosphorylated form of the VDR protein, and are in agreement with the previous demonstration by the Welsh group that the upper band in the VDR doublet seen in 1,25D-sensitive and resistant MCF-7 cells is phosphorylated VDR (Narvaez et al., 2003).

An alternative approach used to demonstrate the presence of phosphorylated VDR in the nuclei of differentiated HL60-G and 40AF cells is illustrated in Figure 7, Panel D. VDR in nuclear extracts from DCS-treated HL60-G and 40AF cells was immunoprecipitated with an anti-VDR antibody, and Western blot analysis with an anti-phosphoserine antibody indicated that the nuclear VDR found in these samples was phosphorylated on serine residues (top panel). This is in agreement with the known sites of VDR phosphorylation (Hsieh et al., 1991; Jurutka et al., 2002; Hsieh et al., 2004). Reprobing with an anti-VDR antibody confirmed the presence of co-migrating VDR (bottom panel). Collectively, these data indicate that the nuclear localization and phosphorylation of VDR is deficient in 40AF cells exposed to 1,25D as a single agent. The lack of sufficient phosphorylated VDR in the nuclei of the 1,25D-resistant cells is a novel and a likely contributor to such a resistance.

Discussion

Several laboratories, including ours, have developed 1,25D-resistant sublines of human leukemic cells, but the mechanisms of the acquired resistance traits have not been clearly established. Suda's group proposed that 1,25D-resistant variants of HL60 cells are due to mutations in VDR (Kuribayashi et al., 1983), while Lasky's group found that the VDR was not mutated in the 1,25D-resistant variant of chronic myelogenous leukemia cell line, RWLeo-4 (Lasky et al., 1994), and suggested that some process subsequent to VDR activation is altered in the resistant cells (Iwata et al., 1996). As indicated above, in cell types derived from solid tissues, resistance to 1,25D has been attributed to the susceptibility of the faster migrating VDR form to proteosomal degradation (Masuyama and MacDonald, 1998; Jensen et al., 2002), but the relevance to leukemia cells is not clear.

In our study, we demonstrate that there is a novel variation in the resistant 40AF cells that is not present in the sensitive HL60-G cells, a single base insertion in the 5'-non-coding region that maps to exon 1 of the VDR gene. However, sequence analysis of the VDR mRNA coding region predicts the expression of full length, non-mutated VDR. While further studies are needed to explore the relevance of this alteration in 5' UTR on VDR activity, it is unlikely to represent a major cause of the resistance to 1,25D.

The primary action of ligand-bound VDR is to promote gene activation and to induce transcription of target genes. Therefore, the ability of 1,25D to increase the expression of CYP24 in both HL60-G and 40AF cells suggests the presence of transcriptionally competent VDR in both types of cells. However, our data suggest that there is impaired rate of CYP24 mRNA induction by 1,25D in 40AF cells (Fig. 3, Panels A and B), although VDR is quite

abundant in 40AF cells, suggesting that there are other alterations in 40AF cells that limit the function of VDR.

One such factor that contributes to the development of resistance could be the diminishing ligand availability, although we have no direct proof for this. The resistant 40AF cells express CYP24 in their basal state, so the 24-hydroxylation which initiates the degradation of 1,25D can start immediately on its addition, while the sensitive HL60-G cells require a considerable period of time and ligand concentration to increase CYP24 expression to detectable levels (Fig. 3, Panels A and B), so high levels of 1,25D can build up in the cell and reach the threshold necessary for the induction of differentiation.

Another simple explanation for the observation that VDR expressed in 40AF cells has reduced transcriptional activity is that there is a requirement for its activation by phosphorylation, and if this is reduced, VDR is retained in the cytoplasm. Thus, although the whole cell content of VDR in 40AF cells is relatively high, this is not relevant to its transcriptional activity, and a block to nuclear localization of VDR, may also block the differentiation pathways, as reported before in 1,25D-resistant THP-1 cells (Humeniuk-Polaczek and Marcinkowska, 2004). Indeed, we observed a reduction in nuclear localization of the phosphorylated forms of VDR in 40AF cells following 1,25D-treatment alone (Fig. 7, Panels A and B), which can be partially overcome by an addition of known differentiation enhancers (plant antioxidants and p38MAPK inhibitors). The mechanisms by which these enhancers activate pathways that lead to the phosphorylation of VDR, pathways dampened by the prolonged exposure to 1,25D, are subject of ongoing investigation in our laboratory. We anticipate that an understanding of the mechanisms that induce resistance and those that lead to its reversal will have important implications for development of novel cancer therapies.

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Fig. 1.

HL60-40AF cells are markedly resistant to differentiation-inducing and anti-proliferative activity of 1,25D. HL60-G and 40AF cells were treated for 48 h with increasing concentrations of 1,25D. A: 1,25D significantly increased the expression of markers of monocytic differentiation, CD11b, CD14, and NSE, in 1,25D-sensitive HL60-G cells, when compared to EtOH-treated cells, $P < 0.001$ (**), $P < 0.05$ (*), but not in the 1,25D-resistant 40AF cells. B: 1,25D induces an accumulation of HL60-G cells in G1 and a decrease of cells in the S-phase when compared to EtOH-treated cells, $P < 0.001$ (**). In contrast, 1,25D has no detectable effect on the cell cycle of 40AF cells. Note also that a higher percentage of cells are in the S-phase and a lower percentage are in the G1 phase in 40AF cells than in HL60-G cells. EtOH

(Et) = 0.04% ethanol. Bars represent mean values $\pm$ SEM (n = 5). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2. VDR and RXRα protein levels are increased by 1,25D in HL60-G but not in 40AF cells. HL60-G and 40AF cells were treated for 48 h with increasing concentrations of 1,25D. **A:** Western blots showing an increase in VDR and RXRα protein in HL60-G cells, but not in 40AF cells. Note also that basal protein levels are higher in 40AF cells than in HL60-G cells. Crk-L was used as a loading control. **B:** Measurements of the optical density (OD) of the VDR protein bands indicate that treatment with 1,25D increases the levels of VDR protein in HL60-G, but not in 40AF cells. **C:** A similar analysis of RXRα protein levels indicates that 1,25D-induces an increase of RXRα protein in HL60-G cells, but a slight decrease in 40AF cells. EtOH (Et) = 0.04% ethanol, bars represent mean values $\pm$ SEM ($n = 3$), $P < 0.05$ (*) when compared to EtOH-treated HL60-G cells, $P < 0.05$ (¥) when compared to EtOH-treated 40AF cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 3.

1,25D-induced expression of the VDR target gene CYP24 is lower in 40AF than in HL60-G cells. **A:** Time-dependent progressive increase in CYP24 mRNA levels in HL60-G and 40AF cells treated with 10 nM 1,25D for the indicated time intervals. Total cellular RNA was extracted and CYP24 mRNA was detected by qRT-PCR and relative fold increase was calculated. Data points are plotted as the log value of the fold increase to allow comparison between cell lines. The table below the graph provides fold increase values ($\pm$ SEM) to emphasize the large differences between the HL60-G and 40AF cells that were not easily evident on a log scale. Note that the basal level of CYP24 mRNA is higher in 40AF cells than in HL60-G cells, but after a prolonged exposure to 1,25D the increase in mRNA levels by

1,25D is less pronounced in 40AF cells than in HL60-G. These data are expressed relative to untreated HL60-G cells at baseline. B: Cells were treated with increasing concentrations of 1,25D for 48 h and total cellular RNA was extracted and measured for CYP24 mRNA levels by qRT-PCR. 1,25D induced a dose-dependent increase in CYP24 mRNA in both HL60-G and 40AF cells; however, the 1,25D-induced CYP24 transcription in 40AF cells was at least tenfold less marked than in HL60-G cells at 48 h. These data are expressed relative to untreated HL60-G cells. C: A Western blot illustrating the 1,25D concentration-dependent increases in the expression of CYP24 protein in HL60-G and 40AF cells after 48 h of treatment with 1,25D. Data in Panels A and B are expressed relative to untreated HL60-G cells at baseline. UNT = untreated and Et = 0.04% ethanol. * signifies $P < 0.05$, ** $P < 0.001$ when compared to baseline HL60-G cells, and ¥ signifies $P < 0.05$, ¥¥ $P < 0.001$ when compared to baseline 40AF cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 4.

Variations in the 5' UTR, but not in the VDR coding domain are present in 1,25D-resistant 40AF cells. A: Schematic representation of human VDR mRNA. Total, DNase-treated, cellular RNA was collected from HL60-G and 40AF cells and subjected to reverse transcription primed with oligo d(T). Translation of full length VDR proceeds from the first ATG encountered by the ribosome located at nucleotide (nt) #116 of the cloned VDR cDNA sequence (NCBI #J03258) to the TGA stop codon located at nt#1396. B: Primers were used to expand the overlapping regions of the VDR mRNA coding domain, and cDNA products were subjected to direct sequencing. An 804 bp product (primers: 31a and 1273) ranged from nt#72 to 876 of the cloned VDR cDNA sequence and encompassed a portion of the 5' UTR, the ATG start codon, and a portion of the coding domain. A 645 bp product (primers: 33a and 33b) ranged from nt #780 to 1425 of the cloned VDR cDNA sequence and included a portion of the coding domain, the TGA stop codon and a portion of the 3' UTR. C: No variation was observed in the 40AF cell VDR mRNA coding domain sequence when compared to published (NCBI #J03258), or HL60-G cell VDR mRNA coding domain sequences. Variations in the 40AF cell 5' UTR (1) were noted (arrows) when compared to the published sequence (3) and to the HL60-G cell 5' UTR (2) and included cytosine base insertions (bold) between residues #80–81 and #102–103, as well as a nucleotide mismatch T → C (underlined) at #117 in the wild-type start codon.

Fig. 5.

Reversal of resistance of 40AF to 1,25D-induced differentiation is associated with increased expression of CYP24. HL60-G and 40AF cells were treated for 96 h with 10 nM 1,25D (D3) in combination with potentiators of 1,25D-induced differentiation, SB202190, and one of three plant-derived antioxidants, carnosic acid (DCS), silibinin (DSS), or curcumin (DCuS). A: FACS analysis of monocytic surface markers indicates a significant increase in CD11b and CD14 expression on HL60-G and 40AF cells treated with these triple combinations. Bars represent mean values + SEM (n = 5). B: The levels of VDR-induced transcription were determined by qRT-PCR of CYP24 mRNA levels and relative fold values were calculated as described in the Materials and Methods section. Treatment with triple combinations significantly enhanced the expression of VDR-target gene, CYP24 in 40AF cells when compared to vehicle-treated samples. Levels of CYP24 mRNA induced by DCS-treatment of HL60-G cells are shown as a positive control. ** signifies $P < 0.001$ when compared to vehicle-treated HL60-G cells, and ¥ signifies $P < 0.05$, ¥¥ $P < 0.001$ when compared to vehicle-treated 40AF cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 6. 1,25D-treated HL60-G and 40AF cells have similar levels of nuclear calreticulin, a potential inhibitor of VDR-induced transcription. HL60-G and 40AF cells were fractionated into cytoplasmic and nuclear protein-enriched extracts and analyzed by Western blotting. A: Histone H3 was found predominantly in the nuclear fraction, validating the cell fractionation procedure. B: The mitochondrial CYP24 protein was found mainly in the cytoplasmic fraction, further validating the procedure. C: Four consecutive Western blots demonstrate the consistency of the fractionation procedure used in this study, and show somewhat lower levels of nuclear calreticulin in 40AF than in HL60-G cells. This excludes inhibition of VDR activity by calreticulin as a cause for 1,25D-resistance in 40 AF cells.

Fig. 7.

1,25D-induced differentiation of HL60-G cells and reversal of resistance to 1,25D in 40AF cells are associated with increased levels of phosphorylated nuclear VDR. HL60-G and 40AF cells were treated with 1,25D (10 nM) or a combination of 1,25D (10 nM), carnosis acid (10 μ M) and SB202190 (5 μ M), termed DCS-treatment, for 96 h to induce maximal differentiation of HL60-G cells. A: Western blot analysis of cytoplasmic and nuclear fractions with anti-VDR antibody indicates the presence of a protein doublet, consisting of a more slowly migrating (VDR-n1) and a more rapidly migrating band (VDR-n2) in the nuclear extracts of HL60-G and 40AF cells. An increase in the ratio of nuclear VDR-n1 to VDR-n2 intensity was observed in both HL60-G and 40AF cells treated with DCS, as well as a decrease in the intensity of cytoplasmic VDR-n2 in 40AF cells. These values are shown below the Western blot. B: Co-migration with MCF-7 nuclear extract (upper panel), and co-incubation of anti-VDR antibody

with the blocking peptide (lower panel), both confirm that the bands VDR-n1 and VDR-n2 are specific for VDR protein. C: PAP treatment of nuclear extracts from MCF-7 cells, which express VDR at high levels (Buras et al., 1994; Narvaez et al., 2003) demonstrate a decrease in the ratio of nuclear VDR-n1 to VDR-n2 intensity. A decreased ratio of VDR-n1 to VDR-n2 intensity was observed when nuclear extracts from HL60-G cells treated with 1,25D were subjected to hydrolysis by PAP and can be seen to co-electrophorese with the MCF-7 bands. The optical density ratio of nuclear VDR-n1/VDR-n2 is shown below the blot. D: Cells were treated with DCS for 96 hrs and the nuclear protein extract (75 μ g) was immunoprecipitated with the anti-VDR (N-20) antibody (25 μ g). Two samples of the eluate (sample 1 = 20 μ l and sample 2 = 40 μ l) were run on 10% polyacrylamide gel. Western blotting with an anti-phosphoserine antibody (top panel) shows the presence of a phosphorylated protein the mobility of which coincides with VDR (bottom panel).