Antioxidants and doxorubicin supplementation to modulate CD14 expression and oxidative stress induced by vitamin D_3 and seocalcitol in HL60 cells

PATRICK BONDZA-KIBANGOU, CHRISTINE MILLOT, VICTORIA EL KHOURY and JEAN-MARC MILLOT

UFR de Pharmacie, Unité MéDIAN, UMR-CNRS- 6142, 1, Avenue du maréchal, Juin 51096 Reims Cedex, France

Received March 5, 2007; Accepted June 13, 2007

Abstract. 1α,25-dihydroxyvitamin D_3 (VD_3) and the EB1089 analog are well known for their roles in the modulation of proliferation and the differentiation of several malignant cells. In addition, VD_3 or EB1089 displayed a high disposal of oxidant features and the ability to cause release of reactive oxygen species (ROS). We attempted to enhance HL60 cell differentiation and to limit ROS generation, by the association of deltanoids with doxorubicin and the antioxidants catalase (CAT), superoxide dismutase (SOD) and N-acetyl cystein (NAC). Differentiation of HL60 cells into monocytic lineage was studied by expression of mRNA, protein CD14 and functional differentiation by the nitroblue tetrazolium assay. The 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) dye allowed to evaluate *in situ* ROS generation. When associated with 0.1 nM EB1089, 15 nM doxorubicin induced an increase of differentiated cell percentage from 29% to 87% and did not affect VD_3-treated cells. The association with doxorubicin also induced a significant increase of ROS release ($p<0.05$) versus VD_3 and EB1089-treated cells. These results correspond to additivity of individual effects of doxorubicin and deltanoids. Antioxidant agents (10 nM NAC, 50 U/ml SOD or 2000 U/ml CAT) were associated with 10 nM VD_3 or 1 nM EB1089 for 72 h. Compared to VD_3 and EB1089 treatments, associations with antioxidants induced a slight increase of differentiated cells and a significant increase of CD14 mRNA. The highest differentiation effect occurred in the case of the EB1089-NAC association. Antioxidants induced a decrease ($p<0.05$) in ROS release generated by VD_3 or EB1089 near the level of untreated cells. Thus, antioxidant agents demonstrated a protective effect against VD_3 and EB1089 oxidative cytotoxicity and an enhancement of the monocyte differentiation. Combinations of antioxidants with deltanoids could dissociate the oxidative stress and differentiation.

Correspondence to: Dr Christine Millot, UFR de Pharmacie, Unité MéDIAN, CNRS-UMR 6142, 1, Avenue du Maréchal, Juin 51096 Reims Cedex, France
E-mail: christine.millot@univ-reims.fr

Abbreviations: AFU, arbitrary fluorescence unit; CAT, catalase; Dox, doxorubicin; FCS, fetal calf serum; H_2-DCFDA, 2'-7'-dichloro-dihydrofluorescein diacetate; NAC, N-acetyl cysteine; PBS, phosphate buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase chain reaction; SOD, superoxide dismutase; VD_3, 1α,25$(OH)_2$-vitamin D_3

Key words: HL60 cells, 1α,25$(OH)_2VD_3$, EB1089, differentiation, oxidative stress

Introduction

1α,25-dihydroxyvitamin D_3 (VD_3) is well known for its regulatory role in the modulation of proliferation and differentiation in several normal and malignant cell types (1). *In vivo* and *in vitro* anticancer activities of VD_3 have been commonly attributed to cytostatic and cytotoxic effects on cancer cells. VD_3 induces apoptosis in breast cancer cells and decreases invasiveness of a number of cell lines *in vivo*. *In vitro* studies demonstrated that VD_3 inhibits cell growth and promotes cell differentiation in tumor cell types such as breast, colon, prostate, glioma, pancreas and neuroblastoma (2,3). Differentiation of immature myeloid cells into granulocytic-monocytic lineages resulted in the appearance of specific markers in presence of different agents (4). VD_3 induces HL60 cells to differentiate into mature myeloid cells with loss of proliferative capacity to acquire a monocyte-like phenotype (5). As hematopoietic stem cells show lower stage of differentiation in various lineages, their immunophenotype changes could be analyzed by different markers. In leukemic cells, the anti-proliferative capacity was accompanied by a marked increase in the expression of differentiation markers such as CD14 or CD11b (6). VD_3 binds to a vitamin D receptor (VDR) and to an uncharacterized membrane receptor that interacts with protein kinase C (PKC) and activates mitogen activated protein kinases (MAPKs) (7). Several studies have shown that HL60 cell differentiation requires the involvement of complex systems such as enzymatic translocation and activation (phospholipase C, phosphatidylinositol 3-kinase, protein kinase C). VD_3 effects involve fast activation pathways of kinase proteins especially towards the differentiation process of keratinocytes and myeloid cells.

Among synthesized analogs of VD_3, Seocalcitol (EB1089) is ~50-200 times more potent than VD_3 in most cancer cells with respect to inhibition of cell proliferation. This analog shows strong anti-proliferative and differentiating activities on cancer cells (1,2).

Several antineoplasic agents generate and release free radicals that cause severe cellular damage. Redox cycling leading to oxidative stress has been proposed as one of the mechanisms by which different agents cause damage, particularly at the DNA level and exert their cytotoxicity in different cancer cells (8). The final damage occurring in target cells will depend on the rate of ROS formation and on the efficiency of cellular defense mechanisms. The proliferative phenotype is essential for the cell response towards oxidative stress (9). VD_3 or EB1089 displayed a high disposal of oxidant features and consequently the ability to cause release of reactive oxygen species (ROS) (10,11). Non-toxic doses of VD_3 could induce active cell death within oxidative stress mechanism, via generation and release of ROS in MCF-7 cells. Our previous study has described the pro-oxidant activities of VD_3 and EB1089 and the relationship between oxidative stress induced by these two agents and cellular autofluorescence (12).

Doxorubicin (Dox) is a chemotherapeutic agent useful in treating various cancers. Dox is a quinone-containing anthracycline chemotherapeutic and is known to produce ROS in the heart (13). VD_3 enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage (14). This treatment can induce serious side-effects in various tissues, including brain, in addition to known cardiotoxic effects, which limit its successful use in chemotherapy.

Antioxidant supplementation to anticancer drugs decreases oxidative DNA damage in human cells. N-acetyl-cysteine (NAC), superoxide dismutase (SOD) and catalase (CAT) are strongly involved in cellular defences against the oxidative damage caused by anticancer drugs. NAC is thought to act by raising intracellular concentrations of GSH and by the direct scavenging of ROS (15,16). SOD represents the first line of defense of the cellular antioxidant system against the oxidative damage mediated by superoxide radicals, removing superoxides by catalysing the dismutation of two superoxide radicals to yield hydrogen peroxide (H_2O_2). This ROS is finally transformed into H_2O and oxygen by CAT (14).

As regards the myeloid differentiation and the ROS generation, this study aimed to compare VD_3 and EB1089 activities. To enhance differentiation and to limit ROS generation, deltanoids were supplemented with doxorubicin and antioxidants. These association results determined whether oxidative stress and monocyte differentiation could be dissociated.

Materials and methods

Cell lines and culture conditions. Human promyelocytic HL60 leukemia cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). These HL60 cells were grown in a 5% CO_2 atmosphere at 37˚C in RPMI-1640 (1X) medium (Bio-Wittaker, France) with L-glutamin, supplemented with 15% fetal calf serum (FCS) (Gibco-BRL, UK). Cell growth and viability were assessed utilizing the trypan blue exclusion technique (Sigma, France). Cells were routinely examined for mycoplasma contamination.

Chemicals. VD_3 was purchased from Sigma. EB1089 was a generously gift provided by Dr Lise Binderup from Leo Pharmaceuticals Products (Ballerup, Denmark). VD_3 was diluted in absolute ethanol at 10 μM stock solution. EB1089 was supplied as solution diluted in isopropanol (2-propanol) at $4x10^{-3}$ M. Dilutions were performed in absolute ethanol to give stock solutions of 100 μM. The aliquots of stock solutions from both compounds were stored at -20˚C, protected from light. The final ethanol concentration was 0.1% in the culture medium and had no effect on either cell growth or oxidative stress. Doxorubicin (Dox), used as hydrochloride, was prepared as 10^{-3} M stock solution (R. Bellon, France) in distilled water and stored at -20˚C. N-acetyl cysteine (NAC), superoxide dismutase (SOD) and catalase (CAT) were purchased from Sigma. NAC was dissolved in phosphate buffer saline (PBS) 1X to form a 1 M stock solution and the pH was adjusted to 7.3 with sodium hydroxide solution. Both SOD and CAT enzymes were also dissolved in PBS 1X to get stock solutions at 10^5 U/ml and $15x10^3$ U/ml, respectively. All antioxidant agents were freshly prepared and added to the cell culture medium 1 h before VD_3 or EB1089 incubation.

Determination of cell differentiation. HL60 cell differentiation was assessed by nitroblue tetrazolium reduction assay (NBT). This assay is based on the ability of mature cells (differentiated cells) to reduce tetrazolium salts into formazan beads, due to the superoxide anions they produce upon stimulation with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma). To measure the NBT reduction, $2x10^5$ cells were harvested by two centrifugations (400 g, 10 min) and incubated with 200 μl solution containing RPMI-1640, FCS, 1% NBT (Sigma) and 100 nM TPA for 30 min at 37˚C in the dark. Then, cells were examined by optical microscopy. The percentage of purplish cells, indicative of formazan beads presence, was assessed in comparison with untreated cells. At least 300 cells were counted for each experiment.

Measurement of ROS release by fluorescence spectroscopy. The 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) probe (Molecular Probes, France) has the ability to detect ROS production *in situ*. The cell-permeant form, H_2-DCFDA, rapidly diffuses through the cell membrane and then is hydrolyzed by intracellular esterases to an oxidative-sensitive form, dichlorodihydrofluorescein (H_2-DCF). In the cytosol, oxidation of H_2-DCF leads to a fluorescent compound, dichlorofluorescein (DCF) with a fluorescence intensity proportional to intracellular ROS (16). H_2O_2, a well known agent for its oxidative property, was used as a positive marker of oxidative stress.

This fluorescent dye allowed to determine ROS release in HL60 cells, untreated or treated VD_3 or EB1089. Briefly, after treatment, HL60 cells were washed and re-suspended at 10^6 cells/ml in RPMI-1640 without FCS and phenol red. Then, 10 μM H_2-DCFDA probe was added to each plate at a final volume of 2 ml. Cells were incubated for 45 min at 37˚C

in the dark. A second wash was made before the fluorescence analysis using spectrometer at 488 nm intensity excitation λ_{ex} and 516 nm emission λ_{em}. Results, in arbitrary fluorescence units (AFU), were expressed according to the ratio [(AFU-treated cells)/(AFU control cells)] x100.

Statistical analysis. The data, presented as mean ± SD, were obtained from at least three independent experiments. Significant differences were determined using the Student's t-test ($p<0.05$ was considered to indicate significant differences).

RNA extraction and RT-PCR analysis. HL60 cells were exposed to the following treatments: VD_3 (1-10-100 nM); EB1089 (0.1-1-10 nM) and antioxidant agents (NAC 10 mM, CAT 2000 U/ml, SOD 50 U/ml) for the indicated times. Total RNAs from $5x10^6$ cells were isolated using Tri Reagent™ (Sigma). For RT-PCR, aliquots (1 μg) of total RNA were incubated with 2 μl of a mix containing 10 mM dNTP (Boehringer, France), 2 μl buffer 10X, 1 μl of a 500 μg/μl random hexadeoxynucleotide primers (RH), 4 μl of 25 mM $MgCl_2$, 0.5 μl of human placenta ribonuclease inhibitor (HPRI: Amersham, France) at 40 U/μl plus 0.42 μl of 24 U/μl Moloney murine leukemia virus reverse transcription (M-MLV reverse transcriptase, Gibco-BRL, France) and RNase/DNase-free water to get a final volume of 20 μl. The samples were kept for 10 min at room temperature, then incubated 1 h at 42˚C and 5 min at 95˚C. RNAs were transcribed into cDNA which were stored at -20˚C, after adding 180 μl of DNAse/RNAse-free water. PCR were performed with 1/20 volume of the reverse transcription reaction for amplification. These amplifications were performed in a total volume of 50 μl containing 5 μl of buffer 10X, 1 μl of 10 mM dNTP, 4 μl of 25 mM of $MgCl_2$, 28.8 μl of DNase/RNase-free water, 5 U/μl of Taq DNA polymerase (Invitrogen Life Technologies, France) and 1 μg/μl of primers specific to CD14 and GAPDH genes as follows: CD14 (5'-TAAAGGAC TGCCAGCCAAGC-3' and 5'-AGCCAAGGCAGTTTGAG TGC-3'), GAPDH (5'-CTCTGCCCCCTCTGCTGATGC-3' and 5'-CCATCACGCCACAGTTTCCCG-3'). Amplification profiles included denaturation at 95˚C for 30 sec, primer annealing at 58˚C for 30 sec, and extension at 72˚C for 30 sec. All the amplifications were performed with 30 cycles for CD14 and GAPDH. After PCR, reaction mixtures were analyzed by a 2% agarose gel electrophoresis. In order to ensure total absence of any contamination negative controls, no RNA and no cDNA, were included in the RT-PCR reactions.

Determination of surface antigen by flow cytometry. HL60 cells, treated or not with VD_3 or EB1089 were incubated at various indicated times. Aliquots of 10^6 cells were collected to determine CD14 and CD71 expression by flow cytometric analysis. Cells were washed once in PBS, re-suspended in 200 μl of PBS 1X (supplemented with 2% of SVF and 0.01% NaN_2). Cell suspensions were gently mixed, protected from light, with 200 μl of Mo2-fluorescein isothiocyanate conjugated (FITC) anti-CD14 (Beckman Coulter, France) or 40 μl of YDJ.1.2.2.-Phycoerythrin (PE) conjugated anti-CD71 and incubated for 30 min at 4˚C. For each 10^6 cell density, 5 μl of anti-CD14 or MsIgM solution stock was diluted in 195 μl PBS 1X.

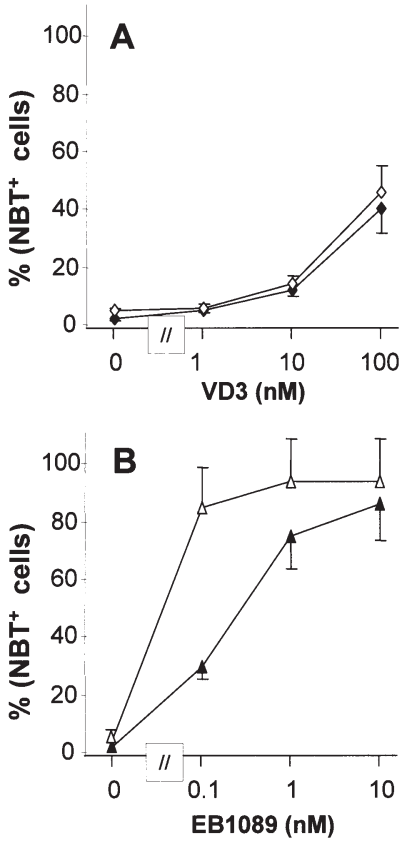

Figure 1. Differentiation induced by VD_3 or EB1089 associated with doxorubicin. (A) HL60 cells were exposed to VD_3 during 72 h (◆) or to 15 nM doxorubicin during 1 h, before exposure to VD_3 (◇). (B) HL60 cells were exposed to EB1089 for 72 h (▲) or to 15 nM doxorubicin for 1 h, before exposure to EB1089 (△). Cell differentiation was determined by the nitroblue tetrazolium reduction assay. Means ± SD correspond to analysis of 300 cells in 3 separate experiments.

After incubation, the cells were washed twice in PBS and re-suspended in 500 μl PBS 1X containing 1% paraformaldehyde and stored at 4˚C, always protected from light. As negative controls, FITC-labeled MsIgM for CD14 and PE-labeled IgG1 for CD71 were used to set threshold parameters. In each sample, at least 10000 cells were collected and analyzed by a FACS Calibur flow cytometer (Becton Dickinson, France).

Results

Modulation of functional differentiation. VD_3 and EB1089-induced differentiation of HL60 cells into monocytic lineage was studied by expression of mRNA, protein CD14 and functional differentiation using nitroblue tetrazolium assay (NBT). The effects of VD_3 and EB1089 were studied on HL60 cell differentiation, using the NBT assay. Fig. 1 displays the percentage of positive NBT (NBT^+)-treated cells, by increased concentrations of VD_3 (1-100 nM) or EB1089 (0.1-10 nM). VD_3 and EB1089 induced cell differentiation in a dose-dependent manner. A higher differentiating activity was observed for 1 nM EB1089 than for 1 nM VD_3. We examined

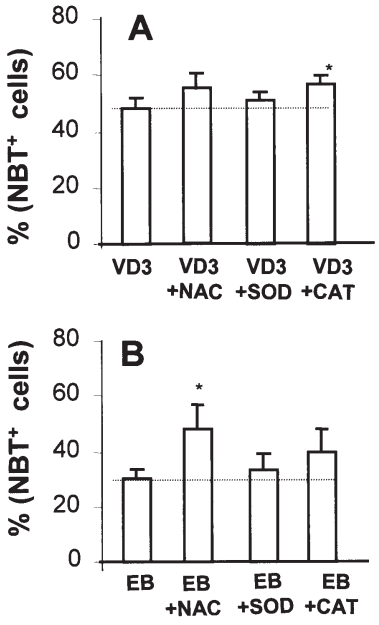

Figure 2. Differentiation induced by VD_3 or EB1089 associated with antioxidants. HL60 cells were treated with NAC (10 mM), SOD (50 U/ml) and CAT (2000 U/ml) for 1 h and then incubated with VD_3 (10 nM) (A) or EB1089 (0.1 nM) (B) for 72 h. Cell differentiation was determined by the nitroblue tetrazolium reduction assay. Values correspond to analysis of 300 cells in 3 separate experiments (Mean ± SD). *p<0.05, significantly different from control (Student's t-test).

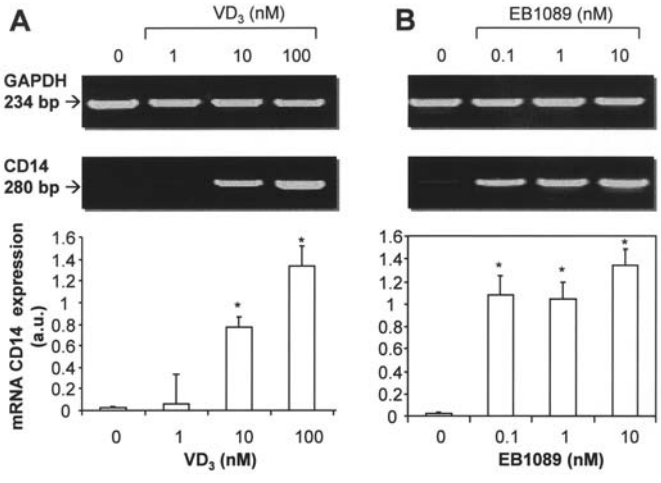

Figure 3. mRNA CD14 gene expression induced by VD_3 (A) or EB1089 (B). mRNA expression was measured by semi-quantitative RT-PCR after 72 h drug exposure. RT-PCR products (a.u. arbitrary units) were compared counting ubiquitary GAPDH transcripts. Each result corresponds to the average of 3 separate experiments (Mean ± SD). *p<0.05, significantly different from control (Student's t-test).

the doxorubicin capacity to increase the differentiation activity of VD_3 or EB1089. HL60 cells were incubated with 15 nM doxorubicin for 1 h before a continuous exposure with VD_3 (1-100 nM) or EB1089 (0.1-10 nM) for 72 h. The percentage of NBT^+ cells was unaffected by doxorubicin when associated with VD_3, as compared with VD_3 alone (Fig. 1). On the contrary, for 0.1 nM EB1089-treated cells,

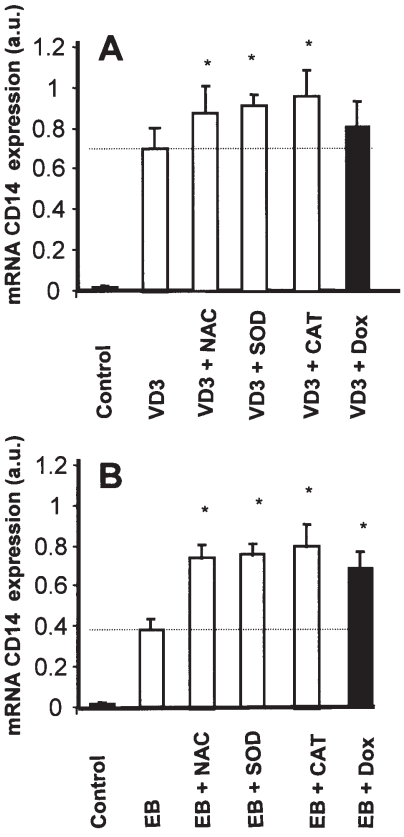

Figure 4. mRNA CD14 gene expression induced by VD_3 (A) or EB1089 (B) supplemented with antioxidants (□) or doxorubicin (■). HL60 cells were treated either with 10 mM NAC, 50 U/ml SOD, 2000 U/ml CAT or 15 nM doxorubicin for 1 h and then exposed to 10 nM VD_3 or 0.1 nM EB1089 for 72 h. Semi-quantitative RT-PCR results are expressed in arbitrary units (a.u.) and correspond to the average of 3 individual experiments (Mean ± SD). *p<0.05, significantly different from control (Student's t-test).

15 nM doxorubicin induced an increase of NBT^+ cell percentage from 29% to 87%. Note that 15 nM doxorubicin exposure has no effect on cell differentiation and viability. VD_3 or EB1089 were also associated with 10 nM NAC, 50 U/ml SOD or 2000 U/ml CAT (Fig. 2). Compared to VD_3 and EB1089 treatments, these associations induced a slight increase of NBT^+ cells. The highest effect occurred in the case of the EB1089-NAC association. Each antioxidant alone induced no growth inhibitory effect and <3% of NBT^+ cells (data not shown).

Modulation of mRNA CD14 and CD14 antigen expression. The mRNA CD14 antigen expression was analyzed during HL60 monocytic differentiation. During treatments with VD_3 or EB1089, mRNA levels for ubiquitary transcripts GAPDH did not vary significantly. After total RNA isolation and RT-PCR, mRNA CD14 levels were slight for untreated cells and 1 nM VD_3 (Fig. 3A). A significant increase of 40- and 50-fold in CD14 gene expression was observed for 10 and 100 nM VD_3 (Fig. 3A). The lowest 0.1 nM concentration of EB1089 induced a marked CD14 gene expression which was 40-fold higher than control cells (Fig. 3B). This result confirms the higher differentiating potential generated by EB1089, as compared with VD_3.

Table I. Percentage of CD14+ cells induced by VD_3 or EB1089, associated with NAC, SOD or CAT antioxidants.

		VD_3 10 nM CD14+ (%)	EB1089 0.1 nM CD14+ (%)	Control CD14+ (%)
	None	12±2	25±2	2±1
Antioxidant supplementation	CAT 2000 U/ml	16±1[a]	25±4	2±2
	NAC 10 mM	19±3[a]	29±2	1±1
	SOD 50 U/ml	20±3[a]	30±5	1±1

HL60 cells were treated with each antioxidant for 1 h and then with VD_3 (10 nM) or EB1089 (0.1 nM) for 72 h. Cell differentiation was determined by percentage of cell expressing the membrane antigen CD14 (CD14+), as determined by flow cytometry. Values correspond to the average of 3 separate experiments (Mean ± SD). [a]p<0.05, significantly different with no antioxidant supplementation (Student's t-test).

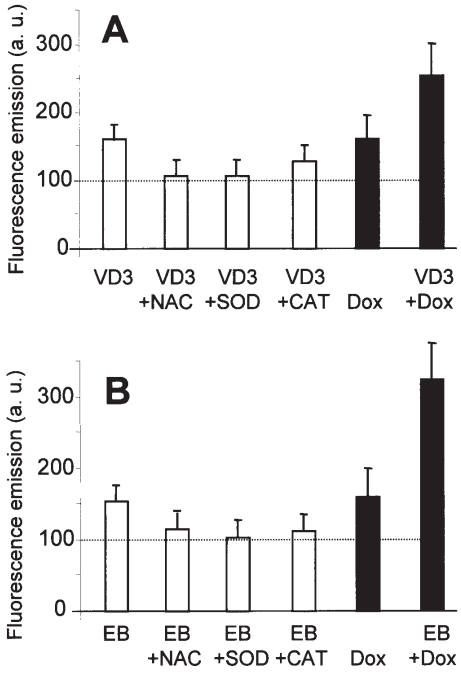

Figure 5. Oxidative stress induced by VD_3 (A) or EB1089 (B) supplemented with antioxidants or doxorubicin. HL60 cells were treated to 10 mM NAC, 50 U/ml SOD, 2000 U/ml CAT or 15 nM doxorubicin. Cells were labelled with H_2-DCFDA (10 μM) for 45 min. The fluorescent intensity is shown as the percentage of untreated cells. Results correspond to the average of 3 separate experiments (Mean ± SD).

The modulation of mRNA CD14 gene expression by NAC, SOD and CAT antioxidants is presented on Fig. 4. After a 72 h incubation of VD_3 or EB1089 with antioxidant agents (10 nM NAC, 50 U/ml SOD or 2000 U/ml CAT), CD14 gene expression showed a modulation of mRNA. Semi-quantification displays a significant increased expression of mRNA CD14 in co-treated cells (Fig. 4). In the absence of VD_3 or EB1089, each antioxidant was unable to stimulate significantly CD14 gene expression, as compared to untreated cells (data not shown). Moreover, cells were treated with VD_3 or EB1089, associated with 15 nM doxorubicin. The EB1089-doxorubicin association induced a significant increase of mRNA expression, as compared with EB1089 (0.1 nM) (Fig. 4B).

After a 72 h exposure to VD_3 or EB1089, the induction of the CD14 monocyte marker to immature HL60 cells was studied by flow cytometry (Table I). In the absence of a differentiation stimulation, only 2% of cells were CD14+. The VD_3 treatment induced 12% of CD14+ cells, that increased significantly to 19% and 20% when associated with NAC and SOD antioxidants, respectively.

Modulation of ROS release. It has been well described that some differentiation inducing agents such as VD_3 and the EB1089 analog display oxidant features and consequently ROS release. Evaluation of ROS was performed by the H_2-DCFDA fluorescent dye, whose emission intensity was expressed as a percentage of control cell intensity. VD_3- or EB1089-treated HL60 cells showed an increase of H_2-DCFDA fluorescence intensity ($p<0.05$), that corresponds to an oxidative effect.

The effects of NAC, SOD, CAT antioxidants to modulate ROS release in VD_3- or EB1089-treated cells was investigated (Fig. 5). Results are expressed as H_2DCFDA fluorescence intensity, with respect to untreated cells (100%). Cells were pre-treated for 1 h with each antioxidant before addition of VD_3 or EB1089. Antioxidants induced a decrease ($p<0.05$) in ROS release generated by VD_3 or EB1089 near the level of untreated cells. This suggests a protective role of these antioxidant agents against VD_3 and EB1089 oxidative cytotoxicity.

HL60 were treated with 15 nM doxorubicin to induce oxidative stress (Fig. 5). We investigated a possible additive effect of doxorubicin in combination with VD_3 or EB1089, in

modulating oxidative stress. The association with doxorubicin induced a significant increase of ROS release ($p<0.05$) versus 1 nM VD_3- and 0.1 nM EB1089-treated cells (Fig. 5). An additive ROS release ($p<0.05$) was observed in comparison with individual effects of doxorubicin and VD_3.

Discussion

The comparison of VD_3 with its analog has shown that EB1089 was more effective than the native hormone with 10-100 lower concentrations. Similar results have been described for U937 monocytic cells, MCF-7 carcinoma cells and HaCaT keratinocytes (17,18). These results are due to enhanced effects of EB1089 to cell cycle regulation through c-myc, c-fos, p21 and p53. In MCF-7 cancer cells, EB1089 induced a higher decrease of estrogen receptors than VD_3 (19). According to the drug used, HL60 differentiates either to granulocyte, monocyte or macrophage lineage. In a first step, HL60 cell differentiation was appraised by using the NBT test. The number of differentiated cells (NBT^+) increases for 48 h of the treatment. To detail VD_3 and EB1089 properties, the NBT test was completed by CD14 expression. CD14 antigen was highly expressed by monocyte and macrophage lineages. Following the treatment with 10 nM VD_3 or 0.1 nM EB1089 for 72 h, the mRNA CD14 and CD14 expressions strongly increased, associated with the abolishment of CD71 expression (data not shown). A time course study demonstrated that mRNA CD14 expression occurred after 6 h VD_3 treatment, whereas CD14 expression and NBT^+ cells were not observed after this time period.

VD_3 and EB1089 present oxidative properties displayed by ROS release which is dose- and time-dependent. We confirm that one biological mechanism of VD_3 and EB1089 is based on ROS induction (11). The comparison of EB1089 and VD_3 activities indicates that EB1089 is as active as VD_3, with 10-100 lower concentrations. VD_3 brought an increase in the cellular redox state as reflected in the ratio between oxidized and reduced glutathione and glyceraldehyde-3-phosphate dehydrogenase. In addition, the expression of the antioxidant enzyme Cu/Zn superoxide dismutase decreased (20). These reactive oxygen species are involved in the anticancer activity of vitamin D on its own and in its cross-talk with other anticancer modalities. It has been described that one mechanism of action leading to apoptosis by anticancer agent is based on ROS generation and release. The effect of VD_3 to the damage inflicted on breast cancer cells has been examined by the direct action of ROS. VD_3 sensitizes to ROS-induced death by affecting both caspase-dependent and -independent modes of cell death upstream to mitochondrial damage (21). This increased oxidative stress by VD_3 was manifested by glutathione depletion and was abolished by exposure to the thiol antioxidant NAC.

Recent evidence suggested that ROS and antioxidant enzymes could be associated with cell differentiation and disease (22). In this study, SOD, CAT, NAC antioxidants were associated with VD_3 and EB1089 to modulate their differentiation activity. After 72 h of exposure, NAC, SOD and CAT slightly increased the percentage of NBT^+ cells, CD14 expression and mRNA CD14 expression of VD_3 and EB1089 treated cells. These antioxidants highly reduced ROS generated by VD_3 and EB1089 drugs, suggesting a protective role of these antioxidant agents against latter VD_3 and EB1089-induced cytotoxicity. Thus, the modulation of the cell redox status influences the induction of differentiation. The NAC antioxidant, a sulfhydryl reductant, reduces oxidised glutathione, as well as being a precursor of intracellular cysteine and glutathione. Moreover, NAC regulates the conversion from proliferation to differentiation at a transcriptional level (23). The addition of antioxidants to VD_3 activated the JNK pathway as indicated by increased phosphorylation of c-jun and ATF-2. The potentiation of differentiation by antioxidants was inhibited by JNK inhibitor SP600125 (24). Thus, the JNK-AP1 pathway has an important role in the potentiation of VD_3-induced differentiation by antioxidants, and regulates expression of Egr-1 and c-fos (25). Antioxidants such as polyphenols, carotenoids, tocopherol and lipoic acid also potentiate myeloid differentiation by VD_3 or ATRA (26). In addition, the antioxidant carnosic acid, a phenolic terpen, increases the potency of VD_3 to subtype JNK pathways. Rather than their various chemical structures, the common property of these antioxidants to decrease ROS would play an important role to modulate differentiation.

In order to potentiate VD_3-induced differentiation, the association with subtoxic doxorubicin concentrations was evaluated. In presence of doxorubicin, differentiation effect (NBT^+ cells) of EB1089 was clearly enhanced. However, mRNA CD14 and CD14 expressions were not significantly modulated by doxorubicin. VD_3 was associated with other topoisomerase II inhibitors, such as etoposide and mitoxantrone to potentiate more clearly their differentiation activity (27). The modulation of oxidative stress by doxorubicin was also investigated. VD_3 synergistically enhanced the susceptibility of MCF-7 breast cancer cells to doxorubicin and menadione (14,28). In addition, the pre-treatment of cancer cells with VD_3 or EB1089 selectively increased their susceptibility to anticancer cytokines via ROS generation (15) or to all-*trans*-retinoic acid ATRA (29). Such combinations may provide useful therapeutic perspectives in the treatment of a variety of cancers.

In conclusion, the antioxidant supplementation inhibits oxidation and slightly increases cell differentiation, induced by VD_3 and EB1089. Combinations of antioxidants with VD_3 could dissociate oxidative stress and monocyte differentiation, and need further evaluation for cancer chemoprevention.

Acknowledgements

We are grateful to Dr Lise Binderup, Leo Pharmaceutical Products, for providing EB1089.

References

1. Mathiasen IS, Lademann U and Jaattela M: Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. Cancer Res 59: 4848-4856, 1999.

2. Hansen CM and Mäenpää PH: EB, a novel vitamin D analog with strong antiproliferative and differentiation-inducing effects on target cells. Biochem Pharmacol 54: 1173-1179, 1997.
3. Moore TB, Koeffler HP, Yamashiro JM and Wada RK: Vitamin D_3 analogs inhibit growth and induce differentiation in LA-N-5 human neuroblastoma cells. Clin Exp Metastasis 14: 239-245, 1996.
4. Trayner ID, Burstoff T, Etches AE, Mufti GJ, Foss Y and Farzaneh F: Changes in antigen expression on differentiating HL60 cells treated with dimethylsulphoxide, all-trans-retinoic acid, alpha-25-dihydroxyvitamin D3, or 12-O-tetradecanoyl phorbol-13-acetate. Leuk Res 22: 537-547, 1998.
5. White SL, Belov L, Barber N, Hodgkin PD and Christopherson RI: Immunophenotypic changes induced on human HL60 leukaemia cells by 1α,25-dihydroxyvitamin D_3 and 12-O-tetradecanoyl phorbol-13-acetate. Leukemia Res 29: 1141-1151, 2005.
6. James SY, Williams MA, Newland AC and Colston KW: Leukemia cell differentiation: cellular and molecular interactions of retinoids and vitamin D. Gen Pharmacol 32: 143-154, 1999.
7. Neri LM, Marchisio M, Colamussi ML and Bertagnolo V: Monocytic differentiation of HL-60 cells is characterized by the nuclear translocation of phosphatidylinositol 3 kinase and of definite phosphatidylinositol-specific phospholipase C isoforms. Biochem Biophys Res Commun 259: 314-320, 1999.
8. Morgan WA, Kaler B and Bach PH: The role of reactive oxygen species in adriamycin and menadione-induced glomerular toxicity. Toxicol Lett 94: 209-215, 1998.
9. Plantin-Carrenard E, Bernard M, Derappe C, Bringuier A, Vadrot N, Feldmann G, Foglietti MJ, Aubery M and Braut-Boucher F: Differential responses of proliferative and non-proliferative leukemia cells to oxidative stress. Free Rad Biol Med 39: 1-13, 2005.
10. Koren R, Hadari-Naor I, Zuck E, Rotem C, Liberman UA and Ravid A: Vitamin D is a prooxidant in breast cancer cells. Cancer Res 61: 1439-1444, 2001.
11. Narvaez CJ and Welsh JE: Role of mitochondria and caspases in vitamin D-mediated apoptosis of MCF-7 breast cancer cells. J Biol Chem 276: 9101-9107, 2001.
12. Bondza-Kibangou P, Millot C, Dufer J and Millot JM: Modifications of cellular autofluorescence emission spectra under oxidative stress induced by 1α,25 dihydroxyvitamin D_3 and its analog EB1089. Technol Cancer Res Treat 3: 383-391, 2004.
13. Joshi G, Sultana R, Tangpong J, Cole MP, St. Clair DK, Vore M, Estus S and Butterfield DA: Free radical mediated oxidative stress and toxic side effects in brain induced by the anticancer drug adriamycin: insight into chemobrain. Free Radic Res 39: 1147-1154, 2005.
14. Ravid A, Rocker D, Machlenkin A, Rotem C, Hochman A, Kessler-Icekson G, Liberman UA and Koren R: 1-25-dihydrovitamin D_3 enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage. Cancer Res 59: 862-867, 1999.
15. Koren R, Rocker D, Kotestiano O, Liberman UA and Ravid A: Synergistic anticancer activity of 1-25-dihydrovitamin D_3 and immune cytokines: involvement of reactive oxygen species. J Steroid Biochem Mol Biol 73: 105-112, 2000.
16. Plantin-Carrenard E, Braut-Boucher F, Bernard M, Derappe C, Foglietti MJ and Aubery M: Fluorogenic probes applied to the study of induced oxidative stress in the human leukemic HL60 cell line. J Fluorescence 10: 167-176, 2000.
17. Mathiasen IS, Colston KW and Bindreup L: EB1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells. J Steroid Biochem Mol Biol 46: 365-371, 1993.
18. Kissmeyer A, Binderup E, Binderup L, Mork Hansen C, Andersen NR, Makin HL, *et al*: Metabolism of the vitamin D analog EB1089: identification of *in vivo* and *in vitro* liver metabolites and their biological activities. Biochem Pharmacol 53: 1087-1097, 1997.
19. James SY, Mackay AG, Binderup L and Colston KW: Effects of a new synthetic vitamin D analogue, EB1089, on the oestrogen-responsive growth of human breast cancer cells. J Endocrinol 141: 555-563, 1994.
20. Ravid A and Koren R: The role of reactive oxygen species in the anticancer activity of vitamin D. Recent Results Cancer Res 164: 357-367, 2003.
21. Weitsman GE, Koren R, Zuck E, Rotem C, Liberman UA and Ravid A: Vitamin D sensitizes breast cancer cells to the action of H_2O_2: mitochondria as a convergence point in the death pathway. Free Rad Biol Med 39: 266-278, 2005.
22. Yang LY, Chen WI, Lin JW, Lee SF, Lee CC, Hung TI, Wei YH and Shih CM: Differential expression of antioxidant enzymes in various hepatocellular carcinoma cell lines. J Cell Biochem 15: 622-631, 2005.
23. Edlundh-Rose E, Kuperschmidt I, Gustafsson AC, Parasassi T, Serafino A, Bracci-Laudiero L, Greco G, Kranowska EK, Romano MC, Lundeberg T and Nilsson J: Gene expression analysis of human epidermal keratinocytes after N-acetyl L cystein treatment demonstrates cell cycle arrest and increased differentiation. Pathobiology 72: 203-212, 2005.
24. Wang Q, Salman H, Danilenko M and Studzinski GP: Cooperation between antioxidants and 1α25-dihydroxyvitamin D_3 in induction of leukaemia HL60 cell differentiation through the JNK/AP-1/Egr-1 pathway. J Cell Physiol 204: 964-974, 2005.
25. Wang Q, Harrison JS, Uskokovic M, Kutner A and Studzinski GP: Translational study of vitamin D differentiation therapy of myeloid leukaemia: effects of the combination with a p38 MAPK inhibitor and an antioxidant. Blood 19: 1812-1817, 2005.
26. Liu Y, Chang RL, CUI XX, Newmark HL and Conney AH: Synergistic effects of curcumin on all-trans retinoic-acid- and 1 alpha,25-dihydrovitamin D3-induced differentiation in human promyelocytic HL-60 cells. Oncol Res 9: 19-29, 1997.
27. Torres R, Calle C, Aller P and Mata F: Etoposide stimulates 1,25-dihydroxyvitamin D3 differentiation activity, hormone binding and hormone receptor expression in HL-60 human promyelocytic cells. Mol Cell Biochem 1-2: 157-162, 2000.
28. Sundaram S, Chaudhry M, Reardon D, Gupta M and Gewirtz DA: The vitamin D_3 analog EB 1089 enhances the antiproliferative and apoptotic effects of adriamycin in MCF-7 breast tumor cells. Breast Cancer Res Treat 63: 1-10, 2000.
29. Wang Q, Yang W, Uytingco MS, Christakos S and Wieder R: 1α25-Dihydroxyvitamin D_3 and all-trans-retinoic acid sensitised breast cancer cells to chemotherapy-induced cell death. Cancer Res 60: 2040-2048, 2000.