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Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 221–225
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Breast cancer cell regulation by high-dose Vitamin D compounds in the absence of nuclear Vitamin D receptor☆

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Abstract

1α,25-dihydroxyvitamin D_3 ($1,25D_3$) inhibits growth and induces apoptosis in breast cancer cells in vivo and in vitro. To examine the role of the Vitamin D receptor (VDR) in mediating the actions of $1,25D_3$ at nanomolar and micromolar concentrations, mammary epithelial tumor cell lines generated in wild type (WT) and VDR knockout (VDRKO) mice were utilized. WT cells express VDR and are growth inhibited by $1,25D_3$ and synthetic analogs EB1089 and CB1093 at 1 nM concentrations, while VDRKO cells do not express VDR and are insensitive to Vitamin D compounds at concentrations up to 100 nM. In the current studies, we have confirmed and extended these previous observations. At nanomolar concentrations of $1,25D_3$ and all analogs tested, including EB1089, CB1093, MC1288, and KH1230, WT cells are growth inhibited and exhibit apoptotic morphology, while VDRKO cells show no growth inhibition or apoptosis. At concentrations of 1–10μM, however, $1,25D_3$ and synthetic analogs induce growth inhibition and apoptotic morphology in both WT and VDRKO cell lines. These data indicate that nanomolar concentrations of $1,25D_3$ and analogs mediate growth regulatory effects via mechanisms requiring the nuclear VDR, but that micromolar concentrations of Vitamin D compounds can exert non VDR-mediated effects.

Keywords: Vitamin D receptor; 1α,25-Dihydroxyvitamin D_3; Mammary tumor cells; Nanomolar; Micromolar

1. Introduction

The Vitamin D receptor (VDR) is a ligand-dependent transcription factor of the nuclear hormone receptor superfamily. The natural ligand for the VDR, 1α,25-dihydroxyvitamin D_3 ($1,25D_3$), is the biologically active form of Vitamin D_3. $1,25D_3$ treatment at nanomolar (nM) doses inhibits proliferation, maintains differentiation, and/or promotes apoptosis in breast cancer cells [1,2]. In some studies, cells that are insensitive to nM doses of $1,25D_3$ have been treated with higher (micromolar: μM) doses of $1,25D_3$ and/or Vitamin D analogs. At these doses, Vitamin D compounds also induce growth inhibiton, cell cycle alterations, and changes in expression and activity of target enzymes [3,4]. Although we have previously shown that the nuclear VDR is required for cellular responses to nM concentrations of $1,25D_3$, the mechanism by which higher concentrations of Vitamin D compounds elicit cellular effects remains unclear. Furthermore, a number of synthetic Vitamin D analogs with unique biological properties have been developed, and it is formally possible that some analogs could mediate effects in cells lacking the nuclear VDR. In these studies, we have used mammary tumor cells created from wild type (WT) and VDR knockout (VDRKO) mice [5] to examine the direct role of the VDR in mediating responses to Vitamin D compounds. WT cells express transcriptionally active VDR and are growth inhibited by $1,25D_3$, EB1089, and CB1093 at nM concentrations [6]. Cells derived from VDRKO mice do not express the VDR protein, lack VDR mediated transactivation in response to $1,25D_3$ and are unresponsive to nM concentrations of $1,25D_3$ [6]. Both WT and VDRKO cell lines exhibit comparable dose-dependent decreases in cell number following treatment with etoposide, indicating that VDRKO cells are able to initiate growth arrest and apoptosis in response to other stimuli [6]. These cell lines, which differentially express the VDR, represent a useful tool for assessing the role of the VDR in cell growth, apoptosis, and differentiation. In this study, we treated WT and VDRKO cells with $1,25D_3$ and several synthetic analogs to determine whether Vitamin D compounds might exert VDR independent cellular effects, and if so, the concentrations required to trigger these effects.

☆ Presented at the 12th Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

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doi:10.1016/j.jsbmb.2004.03.082

2. Materials and methods

2.1. Cell culture and growth assay

WT145 and KO240 cells, generated as described [6], were maintained in DMEM/F12 medium with 5% serum and passaged every 3–4 days. For assessment of growth, cells were plated in 24-well plates (4×10^3 cells per well) and treated with 1,25D_3, EB1089, CB1093, MC1288 or KH1230 [Leo Pharmaceuticals (Ballerup, Denmark)], or ethanol (EtOH) vehicle at the concentrations specified in the figure legends. After 96 h, cells were fixed with 1% glutaraldehyde for 15 min, incubated with 0.1% crystal violet for 30 min, destained with H_2O, and solublized with 0.2% Triton X-100. Absorbance at 590 nm, which is indicative of total adherent cell number, was determined on a Wallac Victor 2 microplate reader. Data are presented as percentage of control. Both cell lines were assayed in parallel, and all experiments were repeated at least twice.

2.2. Morphology

Phase contrast images were obtained of cells treated for 96 h with ethanol vehicle, 100 nM 1,25D_3, or 100 μM 1,25D_3 with an Olympus CK40 microscope equipped with a Spot RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI).

3. Results

3.1. Sensitivity of cell lines to nM concentrations of Vitamin D compounds

To assess the possibility that synthetic Vitamin D analogs may exert VDR-independent effects, WT and VDRKO cells were treated with 100 nM CB1093, EB1089, MC1288, KH1230 or 1,25D_3. As shown in Fig. 1, WT145 cells were growth inhibited by all Vitamin D compounds tested (1,25D_3, EB1089, CB1093, KH1230 and MC1288). In contrast, KO240 cells showed no growth inhibition following treatment with any of the Vitamin D compounds at concentrations up to 100 nM. In WT145 cells, all four Vitamin D analogs caused more pronounced growth inhibition than 1,25D_3, the natural VDR ligand, and MC1288 and KH1230 were the most potent analogs tested.

3.2. Sensitivity of cell lines to μM concentrations of Vitamin D compounds

We next assessed the possibility that higher concentrations of Vitamin D compounds could act in a VDR-independent manner. WT145 and KO240 cell lines were treated with 1–10 μM concentrations of 1,25D_3 and synthetic analogs,

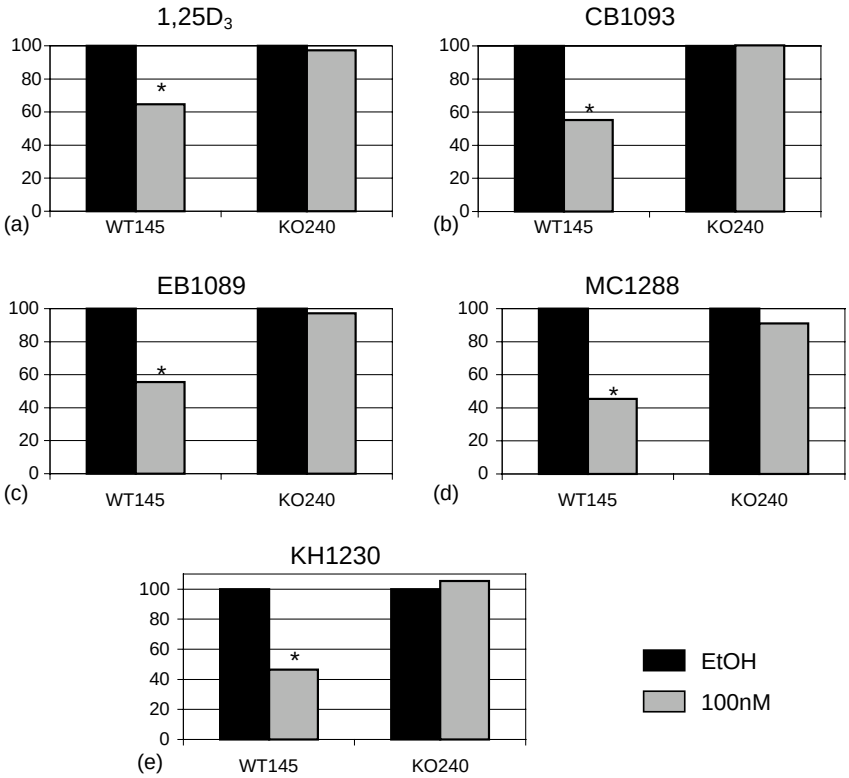

Fig. 1. Effect of nanomolar concentrations of Vitamin D compounds on growth of WT and VDRKO mammary tumor cell lines. WT145 and KO240 cell lines treated with100 nM 1,25D_3, CB1093, EB1089, MC1288, or KH1230 for 96 h were analyzed for relative cell number by crystal violet dye binding assay. Data (mean of six values) are expressed relative to ethanol (EtOH) treated vehicle controls; asterisks indicate statistical significance as determined by one-way ANOVA.

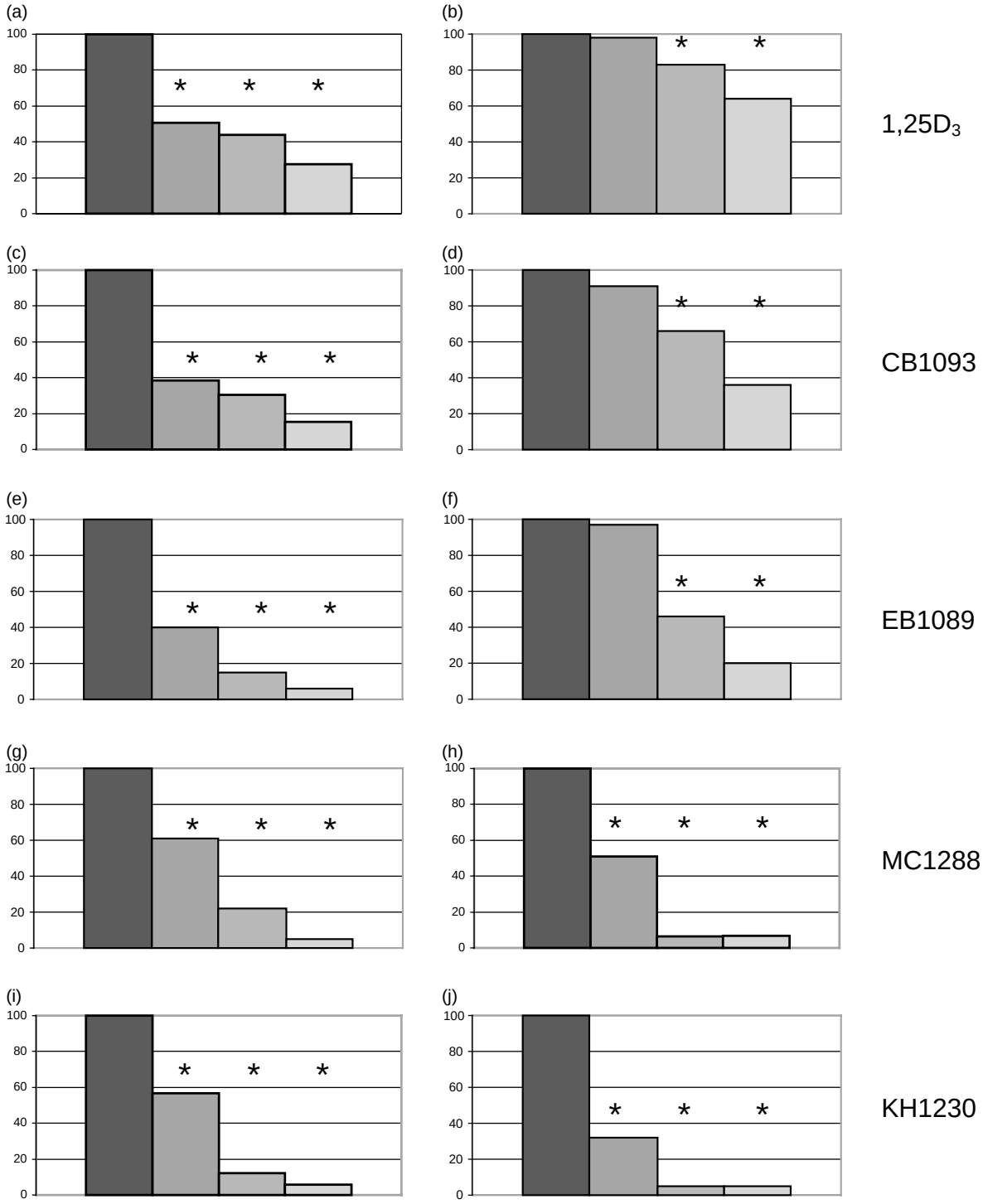

Fig. 2. Effect of micromolar concentrations of Vitamin D compounds on growth of WT and VDRKO mammary tumor cell lines. WT145 (a, c, e, g and i) and KO240 (b, d, f, h and j) cell lines were treated for 96 h with EtOH vehicle, 1, 5, or 10 μM $1,25D_3$, CB1093, EB1089, MC1288, or KH1230. Data (mean of six values) are expressed relative to ethanol (EtOH) treated vehicle controls; asterisks indicate statistical significance as determined by one-way ANOVA.

and growth was assessed. As shown in Fig. 2, WT145 cells were dose-dependently growth inhibited by all five compounds tested. In WT cells, all synthetic analogs were more potent than $1,25D_3$, with MC1288 and KH 1230 the most potent growth inhibitors. Surprisingly, KO240 cells lacking VDR were also growth inhibited by μM concentrations of $1,25D_3$ and all four analogs. Growth inhibition in KO240 cells by μM doses of the Vitamin D compounds was dose-dependent and followed the same trends in potency as observed in WT cells, i.e., all synthetic analogs were more

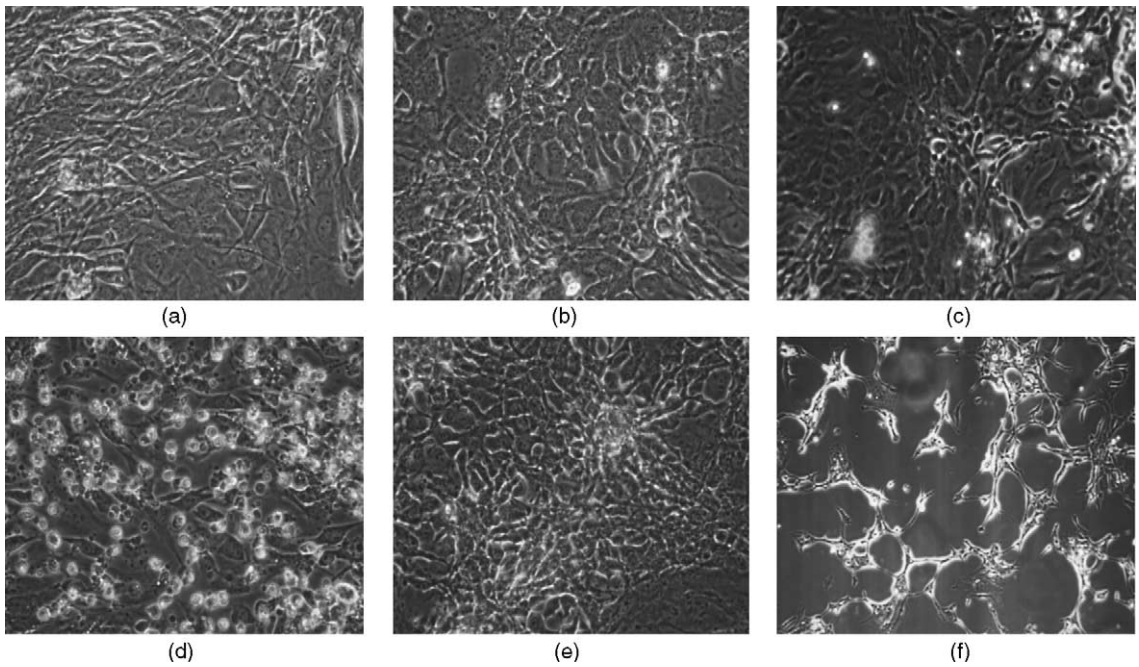

Fig. 3. Effect of $1,25D_3$ on morphology of WT and VDRKO mammary tumor cell lines. WT145 (a and d) and KO240 (b, c, e and f) cell lines were treated with EtOH vehicle (a, b, c), 100 nM $1,25D_3$ (d and e), or 10 μM $1,25D_3$ (f) for 96 h and photographed under phase contrast microscopy.

potent than $1,25D_3$, with MC1288 and KH 1230 the most potent growth inhibitors. In fact, KH1230 was more effective in growth inhibition of VDRKO cells than WT cells.

3.3. Morphology of cell lines following nM and μM 1,25D3 treatment

To determine whether Vitamin D compounds altered cellular morphology, WT and VDRKO cells treated for 96 h with EtOH, 100 nM or 10 μM $1,25D_3$ were observed by phase contrast microscopy. In response to 100 nM $1,25D_3$, WT145 cells exhibited cytoplasmic condensation and nuclear pyknosis (typical signs of apoptosis), whereas morphology of KO240 cells was unchanged (Fig. 3). However, both VDRKO cells (Fig. 3) and WT145 cells (not shown) showed morphological features of apoptosis, including cytoplasmic and nuclear condensation, following treatment with 10 μM $1,25D_3$.

4. Discussion

In these studies, we have demonstrated that nM concentrations of $1,25D_3$ and several structurally related synthetic analogs (EB1089, CB1093, MC1288 and KH1230) require a functional nuclear VDR to exert effects on growth and apoptosis of transformed mammary epithelial cells. This conclusion is based on the observation that WT145 cells, derived from a mammary tumor generated in a normal C57BL6 mouse, express VDR and are growth inhibited by Vitamin D compounds at nM concentrations, whereas KO240 cells, derived from a comparable tumor generated in a VDRKO mouse, are not. These data indicate that, despite their structural modifications and unique biological properties, the synthetic analogs tested likely mediate their growth regulatory effects on breast cancer cells via interaction with the nuclear VDR. This finding is consistent with data indicating that these analogs bind to and transactivate the VDR in vitro.

Surprisingly, we have also demonstrated that, at μM concentrations, $1,25D_3$ and the synthetic analogs have similar growth inhibitory effects in WT and VDRKO cells. This observation indicates that at these high concentrations, Vitamin D compounds elicit cellular effects in the absence of the nuclear VDR. Thus, effects reported in cells expressing VDR treated with μM concentrations of Vitamin D compounds [3,4] may reflect both VDR-dependent and VDR-independent mechanisms. Potential VDR-independent actions of Vitamin D compounds might include binding to alternative nuclear receptors, generation of toxic metabolites or byproducts, or direct interactions with components of cell signaling pathways. Further studies in WT and VDRKO cells will be required to distinguish between these possibilities. In addition, studies with xenografts from these cell lines [6] will be necessary to elucidate whether the concentrations of Vitamin D analogs which mediate tumor regression in vivo are high enough to mediate VDR independent effects.

In summary, we have demonstrated that while nM concentrations of $1,25D_3$ and synthetic analogs mediate growth regulation through a nuclear VDR-mediated mechanism, μM concentrations of Vitamin D compounds do not require the nuclear VDR to mediate effects on growth and apoptosis in breast cancer cells.

Acknowledgements

This work is supported by an IDEA award to JW from the Department of Defense Breast Cancer Research Program. MEV is supported by a Clare Booth Luce Scholar Award. The authors would like to thank Dr. Glendon Zinser for assistance with culture of the WT and VDRKO cell lines.

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