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Induction of differentiation by 1α-hydroxyvitamin D_5 in T47D human breast cancer cells and its interaction with vitamin D receptors

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Abstract

The role of the active metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), in cell differentiation is well established. However, its use as a differentiating agent in a clinical setting is precluded due to its hypercalcaemic activity. Recently, we synthesised a relatively non-calcaemic analogue of vitamin D_5, 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), which inhibited the development of carcinogen-induced mammary lesions in culture and suppressed the incidence of chemically induced mammary carcinogmas in rats. In the present study, we determined the differentiating effects of 1α-$(OH)D_5$ in T47D human breast cancer cells and compared its effects with $1,25(OH)_2D_3$. Cells incubated with either 10 or 100 nM of the analogues inhibited cell proliferation in a dose-dependent manner, as measured by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay. Similar growth-inhibitory effects were also observed for $MCF10_{neo}$ cells. Both vitamin D analogues induced cell differentiation, as determined by induction of casein expression and lipid production. However, $MCF10_{neo}$ cells failed to respond to either vitamin D analogue and did not undergo cell differentiation. Since the cell differentiating effect of vitamin D is considered to be mediated via the vitamin D receptor (VDR), we examined the induction of VDR using reverse transcriptase–polymerase chain reaction (RT-PCR) in both cells. The results showed that, in T47D cells, both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ induced VDR in a dose-dependent manner. Moreover, both analogues of vitamin D upregulated the expression of vitamin D response element-chloramphenicol acetyl transferase (VDRE-CAT). These results collectively indicate that 1α-$(OH)D_5$ may mediate its cell-differentiating action via VDR in a manner similar to that of $1,25(OH)_2D_3$.

Keywords: Vitamin D; Breast cancer cells; Differentiation; T47D; MCF10

1. Introduction

The research on vitamin D_3 and related compounds is currently at its apex. A vast amount of evidence has been collected, implicating the essential involvement of vitamin D metabolites in several cellular processes. The active metabolite 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) and related compounds suppress the development and progression of breast cancer and other carcinomas *in vivo* [1,2], inhibit the metastatic spread of tumour cells [3–5], and promote differentiation of breast cancer cells [6–8]. However, the calcaemic side-effects of $1,25(OH)_2D_3$ have prevented its application as a pharmaceutical agent. In recent years, considerable attention has been given to the development of vitamin D_3 analogues capable of inducing cell differentiation without systemic hypercalcaemia [8–10]. Many structural modifications are known to enhance several-fold the differentiating potency of vitamin D_3 analogues in normal (usually keratinocyte) or malignant (usually leukaemia) cell lines. Little attempt, however, has been made to evaluate vitamin D analogues of other series such as vitamin D_2, D_4, D_5 and D_6. This structural classification is based on the differences encountered in the side chain. Earlier studies reported that vitamin D_5 was the least toxic of vitamins D_2 through to D_6 [11].

During the past 2 years, we have been studying the role of 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), an analogue of vitamin D_5 (24-ethyl-vitamin D_3), on breast cancer cell differentiation. We have characterised its calcaemic

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activity in vitamin D-deficient Sprague–Dawley rats [12]. The analogue $1\alpha(OH)D_5$ was synthesised from sitosterol acetate and was found to be less calcaemic than vitamin D_3. It was observed that $1\alpha(OH)D_5$ was effective against the development of carcinogen-induced mammary lesions in mouse mammary gland organ cultures [12]. In a more recent study, we observed that $1\alpha(OH)D_5$ inhibited incidence and tumour multiplicity of N-methyl-N-nitrosourea-induced mammary adenocarcinoma in rats (data not shown). These results clearly demonstrate that this vitamin D analogue might be a good candidate in the prevention of mammary carcinogenesis. In the present study, we evaluated the effects of $1\alpha(OH)D_5$ on cell differentiation and proliferation in oestrogen receptor (ER)-positive T47D breast cancer cells and compared the effects of the D_5 analogue with the active metabolite of vitamin D_3, $1,25(OH)_2D_3$. Moreover, we compared the effects of vitamin D analogues between ER+ T47D cells and ER- $MCF10_{neo}$ cells. Both cell lines are negative for functional p53 [13].

It is well known that the nuclear activity of vitamin D_3 is based on the interaction of the vitamin D active metabolite, $1,25(OH)_2D_3$, with the vitamin D receptor (VDR) [14]. The VDR is a nuclear receptor that belongs to the superfamily of ligand-dependent transcription factors and is expressed in all the vitamin D target tissues. VDR mediates its action by conjugating with the Retinoid X Receptor (RXR) [15–17]. The VDR-RXR dimer, once formed, is capable of recognising the vitamin D response element (VDRE) in the promoter region of the gene. The VDRE is composed of direct repeats of 6 DNA bases separated by 3-base intervening sequences [18]. Vitamin D appears to play an important role in stabilising and transactivating the VDR/RXR–VDRE complex [19,20]. Its interaction with VDR, therefore, represents the central step in the transmission of a signal to the transcription machinery, resulting in activation or suppression of transcription of genes leading ultimately to differentiation. We recently showed that the normal human breast epithelial cells lacking functional VDR do not respond to vitamin D to induce cell differentiation. However, transient transfection of VDR in these HBL-100 cells resulted in increased association of VDR-VDRE, as measured by the CAT reporter assay [21]. In the present study, we compared the effects of $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ on the transactivation of VDR–VDRE in T47D cells.

2. Materials and methods

2.1. Cells

The breast epithelial cell line, $MCF10_{neo}$, and human breast cancer cell line, T47D, were obtained from the American Type Culture Collection (Rockville, MD, USA). The $MCF10_{neo}$ cells were maintained in minimum essential medium with Earl's salts (MEME) medium supplemented with 10% fetal bovine serum (FBS), whereas T47D cells were maintained in RPMI supplemented with 0.2 I.U. bovine insulin/ml and 10% FBS. The monkey renal cancer CV-1 cells were maintained in MEME with 10% FBS supplement.

2.2. MTT assay

The cells were seeded in a 96-well/plate at a density of 500 cells/well in 100 μl/well of cell culture medium supplemented with 10% steroid-stripped serum. 24 h after seeding, the cells were incubated with 10 and 100 nM concentrations of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, respectively. The medium was changed every 3 days. After 7 days, the cultures were used for the dimethylthiazolyl-2,5-diphenyltetrazolium Bromide (MTT) assay. MTT (5 mg/ml in phosphate buffered serum (PBS)) was added to the wells (15 μl/well) and incubated at 37°C for 2 h. The stop solution (20% sodium dodecyl sulphate (SDS) in 50% N,N-dimethylformamide) was then added (100 μl/well) and incubated for an additional 2 h. The plates were scanned at 590 nm OD, and the results for each treatment group were averaged.

2.3. Immunohistochemistry

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS and fixed in 10% formalin for 5 min. The fixed cells were further incubated in cold methanol for 3 min and acetone for 2 min. After blocking the cells with a protein block/normal goat serum (BioGenex, San Ramos, CA, USA), they were incubated with casein antibody (100 μg/ml) (Accurate Chemical and Scientific Corp. Westbury, NY, USA) for 2 h. The cells were then incubated with secondary anti-mouse biotinylated antibody for 30 min, followed by streptavidin–peroxidase complex and 3,3′-diaminobenzidine (DAB) solution as chromogen. Appropriate controls were performed to rule out non-specific staining with secondary antibody.

2.4. Lipid assay

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS, and fixed by incubating in cold methanol for 3 min and propylene glycol for 2 min. The cells were, at this point, stained with Oil Red O' for 30 min and rinsed in isopropyl alcohol then de-ionised water. Haematoxylene staining for 30 s and Scott solution rinse completed the assay.

2.5. RNA isolation and RT-PCR

The cells were incubated with 1, 10 or 100 nM $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 3 days. The medium

from the tissue culture flasks was removed and the cells were treated with RNA-Zol B (Tel-Test Inc., Friendswood, TX, USA). RNA were isolated according to the manufacturer's instruction and quantified spectrophotometrically. Reverse transcription (RT) and PCR were carried out using Advantage RT for PCR and Advantage cDNA PCR kit (Clontech Inc., Palo Alto, CA, USA). Primer sequences for VDR were selected and custom-synthesised by Oligos Etc. The sense primer was 5′-GGA GTT GCT GTT TGT TTG AC, and the antisense primer was 5′-CTT CTG TGA GGC TGT TTT TG. The primer for the housekeeping gene *G3PDH* was purchased from ClonTech. The touchdown PCR procedure was employed with minor modifications [22]. The first strand cDNA was heated at 94°C for 1 min followed by denaturation at 94°C for 45 sec, annealing at 68–66–64–62–60°C for 45 sec each time and extension at 72°C for 2 min, for 26 cycles. The final cycle was followed by a 7-min extension step at 72°C to ensure that the amplified DNA was double stranded. The absence of contaminant was routinely checked by RT-PCR assays of negative control samples (sterile buffer, provided in the kit). The PCR products were separated on 1.5% agarose gel at 64 volts for 3 h, stained with ethidium bromide and visualised by ultraviolet (UV)-transillumination.

2.6. Transient transfection

The reporter construct VDRE-tk-CAT was prepared by inserting a copy of VDRE into the *Bam*HI site of the pBLCAT$_2$ as previously described [23]. For transfection, 1×10^5 CV-1 cells were plated in 24-well plates. Transfections were carried out using the calcium phosphate precipitation procedure. Briefly, 100 ng of VDRE-tk-CAT reporter plasmid, 250 ng of β-galactosidase (β-gal) expression vector, and 500 ng of VDR expression vectors were mixed with carrier DNA (pBluescript) to 1 μg of total DNA per well. The CAT activity was normalised for transfection efficiency by the corresponding β-gal activity.

3. Results

3.1. Effect of 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ on cell proliferation

The breast epithelial cells MCF10$_{neo}$ and breast cancer cells T47D were incubated with the vitamin D analogues for 7 days in culture. After this, the effects of vitamin D analogues were evaluated by the MTT assay. The results indicated a 31% and 50% growth inhibition for MCF10$_{neo}$ at 10 and 100 nM of 1,25(OH)$_2$D$_3$ concentrations, respectively, as compared with 50% and 72% inhibition with 1α(OH)D$_5$ at 10 and 100 nM, respectively (Fig. 1a). The ER-positive, T47D cells showed a 29% and 52.5% growth inhibition after being exposed for 7 days to 1,25(OH)$_2$D$_3$ at 10 and 100 nM, respectively. Unlike MCF10$_{neo}$ cells, T47D cells did not exhibit increased growth suppression when exposed to 1α(OH)D$_5$. Both analogues suppressed growth of T47D cells by approximately 30% and 50% at low and high concentrations, respectively (Fig. 1b). These results suggest that both 1α(OH)D$_5$ and 1,25(OH)$_2$D$_3$ are comparable in producing antiproliferative effects in breast cancer cells.

3.2. Induction of differentiation of breast cancer cell lines

Since one of the major recognised functions of vitamin D is induction of cell differentiation, we evaluated the effects of both analogues on the induction of differentiation in both cell lines. As markers of cell differentiation, we used casein and lipid. Casein expression was measured by immunocytochemistry using casein antibodies. Results showed that, for T47D cells, casein was expressed in less than 10% of the control cells.

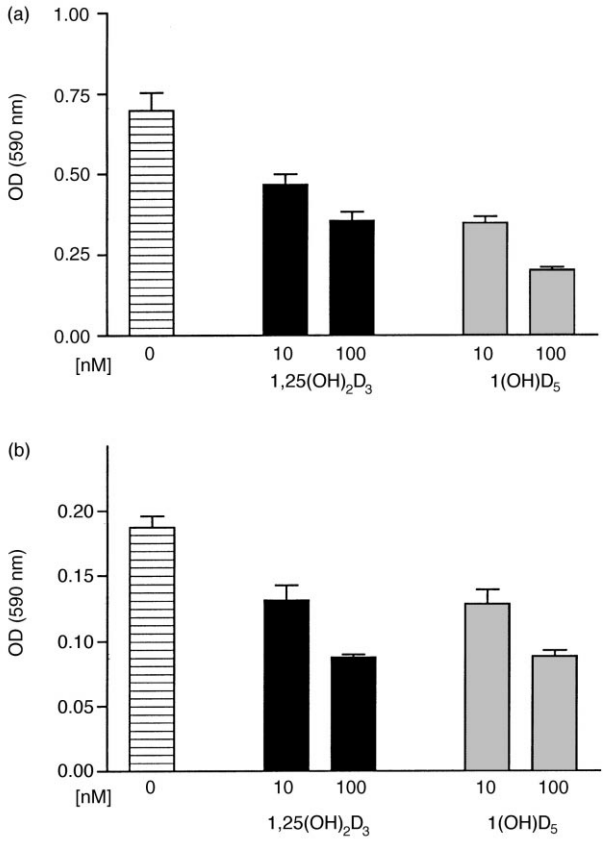

Fig. 1. Effects of 1,25(OH)$_2$D$_3$ and 1α (OH)D$_5$ on the proliferation of MCF10$_{neo}$ cells and T47D cells. The MTT assay was carried out using duplicate cultures and the experiments were repeated three times. The error bars represent the standard deviation. (a) MCF10$_{neo}$ cells; (b) T47D cells.

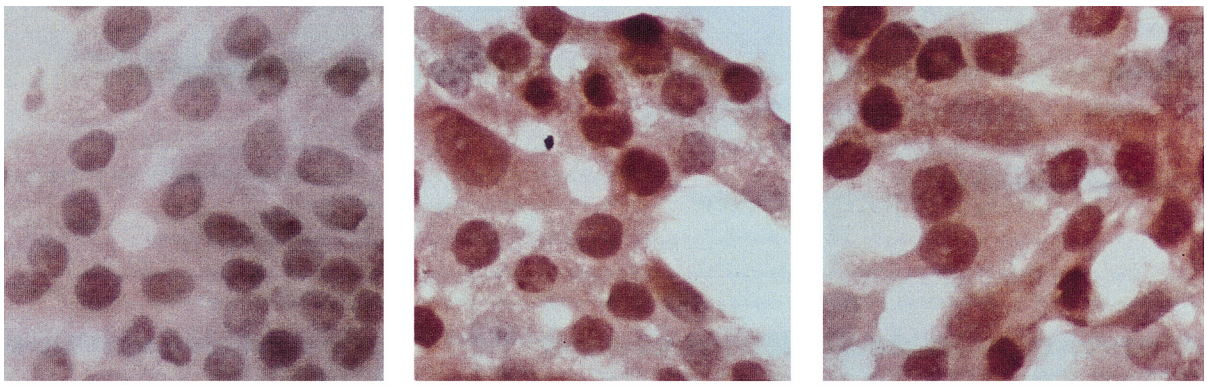

Fig. 2. Effect of vitamin D analogues on casein expression in T47D cells. Immunohistochemical staining for casein expression was carried out as previously described in the presence or absence of the vitamin D analogues.

After 7 days treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, the intensity and number of cells expressing casein increased to approximately 70 and 85% at 10 and 100 nM concentrations, respectively. No difference was noticed between the effects of D_3 or D_5 analogues (Fig. 2 and data not shown). Similarly, there was a dramatic increase in the expression of lipid production in T47D cells after 7 days of treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ (Fig. 3). These results indicated that both vitamin D analogues induce cell differentiation in T47D cells. In contrast, the $MCF10_{neo}$ cells, tested for the same markers of differentiation, did not show any presence or induction of either casein or lipids in the control cells or in cells exposed to vitamin D_3 or D_5 (data not shown).

3.3. Transactivation of VDRE

The VDRE transactivation activity of the vitamin D analogues was determined using the *CAT* reporter gene containing VDRE (VDRE-tk-CAT). In order to compare the activity of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ for transactivating the *VDRE* reporter gene, we selected monkey renal cancer cells (CV-1). These cells lack a functional VDR, so one can evaluate the binding activity of vitamin D analogues only in the transiently transfected VDR. The active metabolite of vitamin D, $1,25(OH)_2D_3$, should not show any increase in CAT activity if the cells are transfected only with VDRE-tk-CAT. As shown in Fig. 4, neither vitamin D_3 nor vitamin D_5 analogues could induce CAT activity, indicating a lack of endogenous VDR in these cells. However, when 500 ng VDR (Fig. 4b) was co-transfected with VDRE and the cells were incubated with 10 or 100 nM of $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$, there was enhanced expression of the *CAT* reporter gene. These results clearly indicate that both analogues of vitamin D can bind to the VDR and the complex can bind to the VDRE to initiate signal transduction. However, the extent of VDRE-reporter transactivation was 7- to 8-fold greater when the transfected cells were incubated with $1,25(OH)_2D_3$ at 10 nM and nearly 2-fold greater at 100 nM, respectively, compared with $1\alpha(OH)D_5$ at the same concentrations. This is consistent with the observed

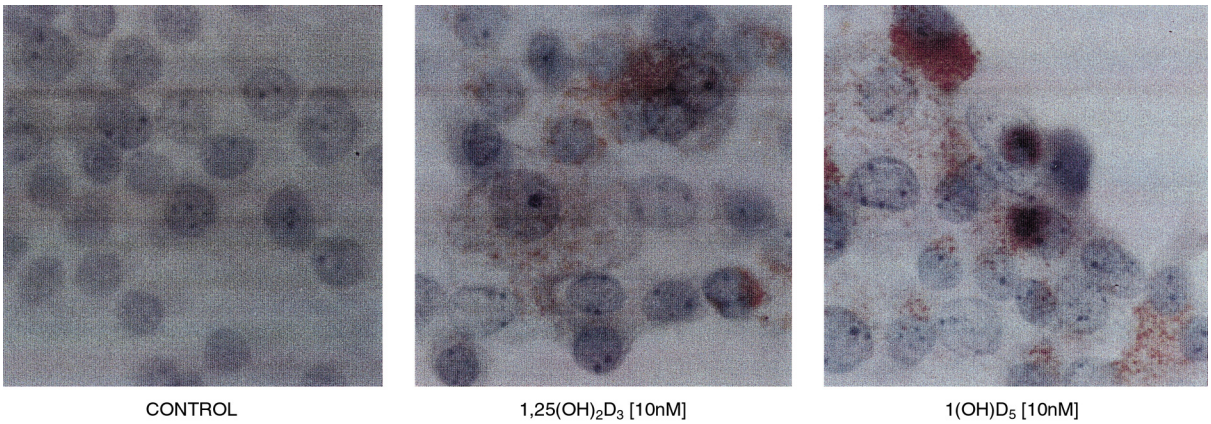

Fig. 3. Effects of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on lipid expression in T47D cells. A lipid assay was carried out as previously described in the presence or absence of vitamin D analogues.

finding that a log molar higher concentration of $1(OH)D_5$ is needed to obtain an equivalent response to that observed with $1,25(OH)_2D_3$.

3.4. Induction of VDR mRNA as determined by RT-PCR

Experiments were carried out to determine if VDR mRNA is induced by the vitamin D analogues in T47D and $MCF10_{neo}$ cells. Total RNA from the cells was isolated and reverse-transcribed. The cDNA was amplified using Taq polymerase and separated on 1.5% agarose gel. As shown in Fig. 5, the housekeeping gene *G3PDH* (C) was identical for all the cDNAs, indicating an equal loading of the gels. The VDR separated as a 420 bp fragment on the gel. As shown in Fig. 5(a), in T47D cells, there was a basal level of expression of VDR; however, incubation of cells for 3 days with either 10 or 100 nM of $1,25(OH)_2D_3$ increased the VDR expression in a dose-related manner. Similar results were also obtained with $1\alpha(OH)D_5$, as shown in Fig. 5(a). In contrast, $MCF10_{neo}$ cells expressed the basal level of VDR in the cells; but, there was no induction of VDR message by the vitamin D analogues (Fig. 5b). These results indicate that the lack of induction of differentiation by vitamin D in $MCF10_{neo}$ cells may be related to a lack of induction of VDR in these cells by vitamin D analogues.

4. Discussion

The effects of vitamin D analogues as differentiating agents and inhibitors of cell proliferation for breast cancer cells have been reported [1,7]. It is generally believed that the cells expressing VDR often respond to vitamin D analogues, whereas cells such as MDA-MB-231, which are ER- and express low or non-detectable

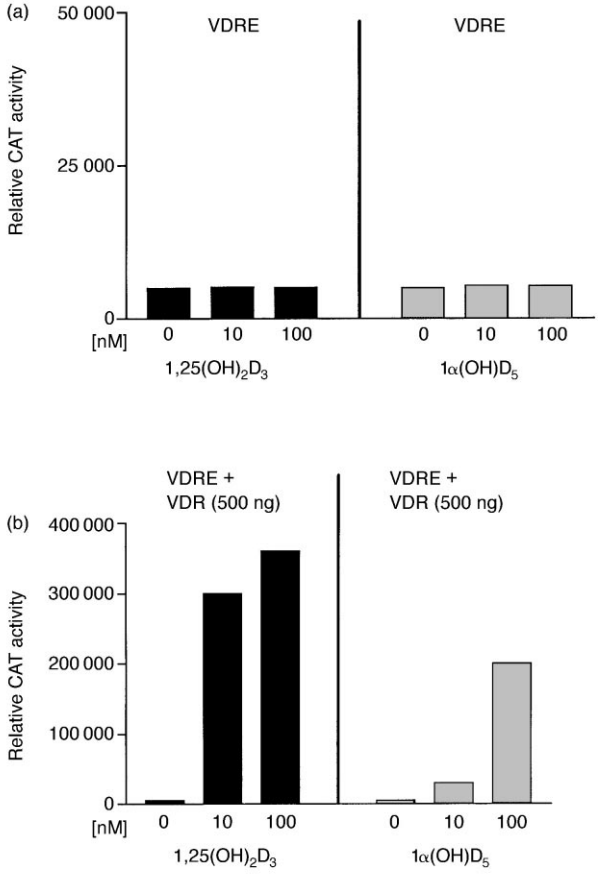

Fig. 4. Effects of $1,25(OH)D_3$ and $1\alpha(OH)D_5$ on the transactivation of the VDRE-conjugated reporter gene. Transient transfections of CV-1 cells with either VDRE-tk-CAT alone (a) or with VDR (b) was carried out by the calcium phosphate precipitation procedure. The cells were incubated with 10 and 100 nM vitamin D analogues for 3 days. CAT activity was measured spectrophotometrically. The experiments were repeated twice.

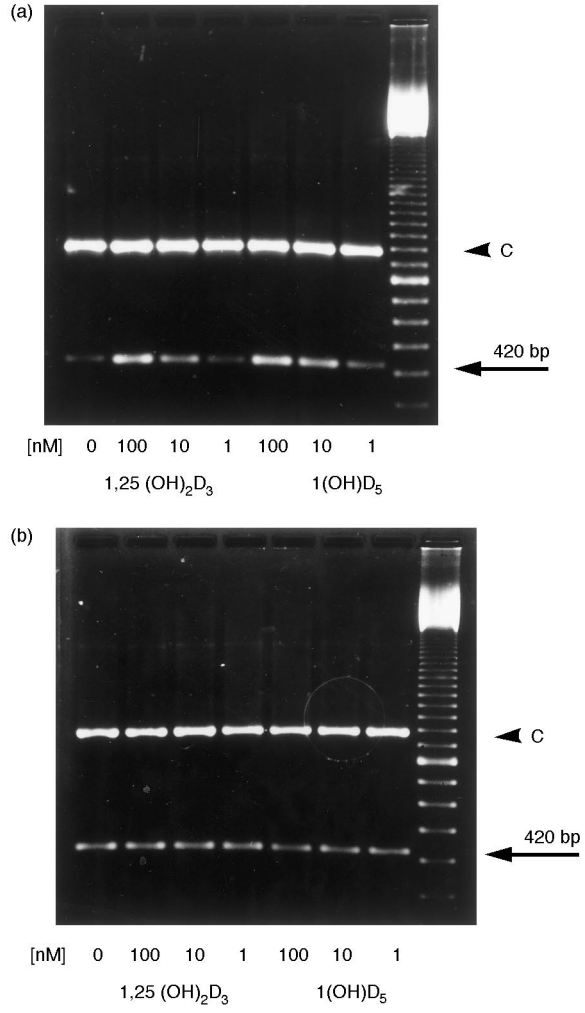

Fig. 5. Effects of vitamin D analogues on the expression of VDR mRNA in $MCF10_{neo}$ and T47D cells. Cells were incubated with various concentrations of analogues for 3 days in culture as previously described. VDR expression was measured by RT-PCR in (a) T47D cells; (b) $MCF10_{neo}$ cells. C, control housekeeping gene.

levels of VDR, do not respond to active vitamin D analogue(s) [24]. The VDR-mediated transcription regulatory genes include *TGFβ*, *EGF*, *c-myc* [25,26], and cell cycle regulators. The effects of various vitamin D analogues on programmed cell death have been evaluated in a variety of breast cancer cell lines. Consistently, MCF-7 cells which are ER+, VDR+ and positive for wild-type p53 exhibit apoptosis in response to vitamin D [27,28]. Although considerable literature exists for vitamin D-induced differentiation, its clinical application has been limited. This is due to its cytotoxicity at the concentration that induces differentiation. To this end, we have identified an analogue of the vitamin D_5 series which is non-calcaemic at the concentration at which 1,25-dihydroxyvitamin D_3 would induce hypercalcaemia. We previously reported that 1α-hydroxyvitamin D_5 inhibits carcinogen-induced development of mammary lesions in culture [12]. We also reported that it induces VDR and TGFβ in mammary epithelial cells. In this report, we addressed the question, ''Does 1-hydroxyvitamin D_5 induce cell differentiation of breast cancer cells to the same extent as the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3?''. T47D and $MCF10_{neo}$ cells were selected for the present study, since T47D cells are ER- and progesterone (PR)-positive and $MCF10_{neo}$ cells are negative for both ER and PR. Both analogues of vitamin D, $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, inhibited cell proliferation to the same extent and induced differentiation as determined by the increased expression of differentiation markers.

The $MCF10_{neo}$ cells were originally derived from normal breast tissue and the epithelial cells were subsequently immortalised. The $MCF10_{neo}$ cells are ER-VDR+ and stably transfected with ras. The cells are tumorigenic in athymic mice. Since both T47D and $MCF10_{neo}$ have similar VDR and p53 status and differ only in their ER status, we compared the response of T47D ER+ and $MCF10_{neo}$ cells to two analogues of vitamin D. The $MCF10_{neo}$ cells, like T47D cells, exhibited a suppression of cell proliferation; however, no induction of differentiation was noticed. This, therefore, raised the question of whether induction of VDR is essential for cell differentiation. We evaluated the induction of VDR mRNA by these two vitamin D analogues. The results showed that $MCF10_{neo}$ cells constitutively expressed VDR-mRNA. However, there was no induction of the VDR message by either of the vitamin D analogues. In contrast, there was a dose-dependent increase in the expression of VDR mRNA in the T47D cells by both vitamin D_3 and D_5 analogues. These results suggest that there may be a positive association between the differentiation of cells by vitamin D and the induction of vitamin D-induced mRNA of VDR. Alternatively, the antiproliferative effects may be mediated by p53 although this is most unlikely in this case as both $MCF10_{neo}$ and T47D cells do not have functional p53 [10] and yet they respond to antiproliferative activity of vitamin D analogues. These results suggest that the antiproliferative effects and differentiating effects of vitamin D analogues may be independent of the cellular p53 status. These results are consistent with a recent report indicating the non-involvement of p53 in vitamin D-mediated differentiating/cell growth suppressing functions in breast cancer cells. Thus, it is not clear what mechanism may be operative for the suppression of cell growth by vitamin D analogues. If both antiproliferative effects and cell differentiating effects are mediated by VDR, then it is possible that the constitutive level of VDR will be sufficient to mediate vitamin D's effects in suppressing cell proliferation but that induction of new VDR mRNA may be necessary for cell differentiation.

Comparison between the action of a natural ligand of vitamin D, $1,25(OH_2)D_3$, and a vitamin D_5 analogue was also made in terms of their ability to transactivate a VDRE-reporter *CAT* gene. We selected VDR-negative CV-1 cells for these studies so that the endogenous VDR would not interfere with the interpretation of data. Since CV-1 cells are truly VDR-negative, they do not respond to incubation with $1,25(OH_2)D_3$ and do not transactivate the VDRE-CAT reporter. Since both T47D and $MCF10_{neo}$ cells express basal levels, to different extents, of VDR, the vitamin D analogue-induced transactivation of the CAT reporter may vary between these two cells and will compromise comparing the two analogues of vitamin D. Results showed that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ bind VDR and interact with VDRE. It was noted that, with 500 ng VDR transfection into CV-1 cells, $1,25(OH)D_3$ at 10 nM induced the reporter expression by more than 150-fold compared with the induction by $1\alpha(OH)D_5$ at the same concentration. The results indicate that, at equimolar concentrations, $1,25(OH)_2D_3$ is more potent in transactivating the *VDRE* reporter gene than $1(OH)D_5$. This is consistent with the earlier findings that, in mouse mammary gland organ cultures, the D_5 analogue is required at a log molar higher concentration to achieve similar effects to those observed with $1,25(OH)_2D_3$. The advantage, however, is that the D_5 analogue does not induce unwarranted toxicity which is often associated with 1,25-dihydroxyvitamin D_3. These studies collectively indicate that the vitamin D_5 series of agents mediate their action via the same VDR-mediated mechanism that is operative with the active metabolite of vitamin D_3.

Acknowledgements

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References

1. Colston KW, Chander SK, Mackay AG, Coombes RC. Effect of synthetic vitamin D analogues on breast cancer cell proliferation *in vivo* and *in vitro*. *Biochem Pharmacol* 1992, **44**, 693–702.
2. Danielpour D, Kadomatsu K, Anzano MA, Smith JM, Sporn MB. Development and characterization of nontumorigenic and tumorigenic epithelial cell lines from rat dorsal lateral prostate. *Cancer Res* 1994, **54**, 3413–3421.
3. Colston KW, Berger U, Coombes RC. Possible role for vitamin D in controlling breast cancer cells proliferation. *Lancet* 1989, **28**, 188–191.
4. Bower M, Colston KW, Stein RC, *et al*. Topical calcipotriol treatment in advanced breast cancer. *Lancet* 1991, **337**, 701–702.
5. Eisman JA, Barkla DH, Tutton PJM. Suppression of *in vivo* growth of human cancer solid tumor xenografts by $1,25(OH)_2D_3$. *Cancer Res* 1987, **47**, 21–25.
6. Sato T, Tagusagawa K, Asoo N, Konno K. Effects of 1-α-hydroxyvitamin D_3 on metastasis of rat ascites hepatoma K-231. *Br J Cancer* 1984, **50**, 123–125.
7. Elstner E, Linker-Israeli M, Said J, *et al*. 20-epi-vitamin D_3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. *Cancer Res* 1995, **55**, 2822–2830.
8. Norman AW, Zhou JH, Henry HL, Uskokovic MR, Koeffler H. Structure–function studies on analogues of 1 a,25-dihydroxyvitamin D_3: differential effects on leukemic cell growth, differentiation, and intestinal absorption. *Cancer Res* 1990, **50**, 6857–6864.
9. Frappart L, Falette N, Lefebvre M, Bremond A, Vauzelle JL, Saez S. *In vitro* study of effects of 1,25-dihydroxyvitamin D_3 on the morphology of human breast cancer cell line BT 20. *Differentiation* 1989, **40**, 63–69.
10. Mathiasen IS, Colston KW, Binderup L. EB 1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells. 1. *J Steroid Biochem Mol Biol* 1993, **46**, 365–371.
11. Napoli JL, Fiivzzani MA, Schnoes HK, DeLuca HF. Synthesis of vitamin D_5: its biological activity relative to vitamins D_3 and D. *Arch Biochem Biophys* 1979, **197**, 119–125.
12. Mehta RG, Moriarty RM, Mehta RR, *et al*. Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1a-Hydroxivitamin D_5. *J Natl Cancer Inst* 1997, **89**, 212–218.
13. Mithasen IS, Laderman U, Jattela M. Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by bcl-2 but does not involve capases or p53. *Cancer Res* 1999, **59**, 4848–4856.
14. Haussler MR. Vitamin D receptors: nature and function. *Ann Rev Nutr* 1986, **6**, 527–562.
15. Yu VC, Delsert C, Andersen B, *et al*. RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 1991, **67**, 1251–1266.
16. Kliewer SA, Umesono K, Mangeldorf DJ, Evans R. Retinoid X receptors interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signaling. *Nature* 1992, **355**, 446–449.
17. Ross TK, Moss VE, Prahl JM, DeLuca HF. A nuclear protein essential for binding of rat 1,25-dihydroxyvitamin D_3 receptor to its response elements. *Proc Natl Acad Sci USA* 1992, **89**, 256–260.
18. Umesono K, Murakami KK, Thompson CC, Evans R. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. *Cell* 1991, **65**, 1255–1266.
19. Ross TK, Darwish HM, Moss VE, DeLuca HF. Vitamin D-influenced gene expression via a ligand-independent, receptor-DNA complex intermediate. *Proc Natl Acad Sci USA* 1993, **90**, 9257–9260.
20. Cheskis B, Freedman LP. Ligands modulate the conversion of DNA-bound vitamin D_3 receptor (VDR) homodimers into VDR-Retinoid X receptor heterodimers. *Mol Cell Biol* 1994, **14**, 3329–3338.
21. Agadir A, Lazzaro G, Zheng Y, Zhang X-K, Mehta RG. Resistance of HBL100 human breast epihthelial cells to vitamin D action. *Carcinogensis* 1999, **20**, 577–582.
22. Hecker KH, Roux KH. Touch down RT-PCR for better resolution in BioTechniques 1996, **20**, 478–485.
23. Wu Q, Li Y, Liu R, Agadir A, Lee -M, Liu Y, Zhang X-K. Modulation of retinoic acid sensitivity in lung cancer cells by a dynamic balance nur77 and COUP-TF orphan receptors and their heterodimerization. *EMBO J* 1997, **16**, 1656–1669.
24. Buras RR, Schmaker LM, Brenner RV, Shabahang M, Nauta RJ, Evans SR. Vitamin d receptors in breast cancer cells. *Breast Cancer Res Treat* 1994, **31**, 191–202.
25. Darwish H, DeLuca HF. Recent advances in the molecular biology of vitamin action. *Prog Nucl Acid Res Mol Biol* 1996, **53**, 321–344.
26. Simpson RU, Hsu T, Wendt MD, Taylor JM. 1,25-Dihydroxyvitamin D_3 regulation of c-myc protooncogene transcription. *J Biol Chem* 1989, **264**, 19710–19715.
27. Welsh J, VanWeelden K, Flanagan L, Nolan E, Narvaez CJ. The role of vitamin D_3 and antiestrogens in modulating apoptosis of breast cancer cells and tumors. *Subcell Biochem* 1998, **30**, 245–270.
28. Simboli-Campbell M, Navaez CJ, van Weelden K, Tenniswood M, Welsh J. Comparative effects of $1,25(OH)_2D_3$ and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells. *Breast Cancer Res Treat* 1997, **42**, 31–41.