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Characterization of vitamin D insensitive prostate cancer cells

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Abstract

The antitumor effects of 1,25-dihydroxyvitamin D_3 (calcitriol) are being exploited for prevention and treatment of prostate cancer (CaP). These studies examined antitumor effects of calcitriol in primary cell cultures derived from transgenic adenocarcinoma of mouse prostate (TRAMP) mice chronically treated with calcitriol (20μg/kg) or vehicle 3x/week (MWF) from 4 weeks-of-age until palpable tumors developed. This is a report on the response of 2 representative control (vitamin D naïve, naïve) and calcitriol-treated (vitamin D insensitive, VDI) cells to calcitriol. VDI cells were less sensitive to calcitriol based on less cell growth inhibition and less inhibition of DNA synthesis as measured by MTT and BrdU incorporation assays. Similarly, VDI cells were also less sensitive to growth inhibition by the vitamin analog, 19-nor-1α,25-dihydroxyvitamin D_2 (paricalcitol). There was no change in apoptosis following treatment of naïve and VDI cells with calcitriol. Vitamin D receptor (VDR) expression was up-regulated by calcitriol in both naïve and VDI cells. Calcitriol induced the vitamin D metabolizing enzyme, 24-hydroxylase (cyp24) mRNA and enzyme activity similarly in naïve and VDI cells as measured by RT-PCR and HPLC respectively. In summary, VDI cells are less responsive to the antiproliferative effects of calcitriol. Understanding vitamin D insensitivity will further clinical development of vitamin D compounds for prevention and treatment of CaP.

Keywords

1,25-Dihydroxyvitamin D3; Calcitriol; Vitamin D Insensitivity; TRAMP; Prostate cancer

INTRODUCTION

Prostate cancer (CaP) is one of the leading causes of cancer and cancer-related mortality in American men [1]. 1,25-Dihydroxyvitamin D_3 (calcitriol) is currently being developed for CaP prevention and treatment. Calcitriol has antitumor activities in CaP [2–6]. The mechanisms of action of calcitriol in CaP include induction of cell cycle arrest [7], apoptosis [8,9] and differentiation [2,10]; and inhibition of invasion [11] and metastasis [4]. Clinical trials have been conducted in men with CaP using calcitriol alone or in combination with other antitumor agents [12–14]. Calcitriol is inactivated and degraded by 24-hydroxylase (cyp24) [15]. Thus availability of calcitriol in CaP cells is inversely proportional to cyp24 activity [16].

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Evidence of insensitivity to the antiproliferative effects of calcitriol has been described in some malignant cells. Following chronic exposure to calcitriol, MCF-7 human breast cancer cells become resistant to the growth inhibitory effects of calcitriol and other vitamin D analogs [17,18]. The vitamin D resistant chronic myelogenous leukemia cell line, $JMRD_3$, is less sensitive to growth inhibition by calcitriol compared to parental RWLeu-4 cells [19]. Development of vitamin D insensitivity and the molecular mechanisms of vitamin D insensitivity in CaP is poorly understood. These studies are a report on primary cell cultures established from the transgenic adenocarcinoma of mouse prostate (TRAMP) model that were used to study vitamin D insensitivity in CaP. The TRAMP model was developed using the minimal rat probasin promoter to target expression of SV40 early genes (T, t) specifically to the prostatic epithelium [20]. These mice progressively develop disease in the prostate ranging from prostatic intraepithelial neoplasia (PIN) to poorly differentiated cancer and metastasis. Prostate tumors of calcitriol-treated (vitamin D insensitive, VDI) and vehicle-treated (vitamin D naïve, naïve) mice were digested to generate primary cell cultures. This is a report on the response of a representative pair of naïve and VDI primary cells to calcitriol.

MATERIALS AND METHODS

Chemicals and reagents

Calcitriol and paricalcitol (Abbott Laboratories, Chicago, IL) were dissolved in 100% ethanol and stored at −80ºC. Calcitriol was always handled with indirect light. Test compounds were diluted in DMEM media ((Invitrogen, Frederick, MD) before use.

Animal studies

Experimental uses of laboratory animals were in accordance with IACUC and NIH guidelines. TRAMP animals were in the C57BL/6 X FVB 50:50 strain background. Breeding colonies were maintained at the Roswell Park Cancer Institute animal facilities. Four week-old TRAMP mice were treated chronically with vehicle (control) or 20μg/kg calcitriol interperitoneally (i.p.) 3x/week (MWF) until they developed tumors.

Cell culture

Primary cell cultures were generated by dissociating poorly differentiated prostate tumors of control mice (vitamin D naïve, naive) and calcitriol-treated mice (vitamin D insensitive, VDI). Prostatic tumors were minced and digested with 2.5mg/ml Collagenase Type II (Sigma-Aldrich, St. Louis, MO), 2.5mg/ml Collagenase Type IV (Sigma) and 1mg/ml DNase I (Sigma). Naïve and VDI cells were cultured in DMEM media supplemented with 10% FBS (Hyclone, Logan, UT), 10^{-8}M dihydrotestosterone (Sigma), 5μg/ml insulin (Sigma) and 25U/ml penicillin/streptomycin (Invitrogen) at 37ºC in a humidified atmosphere containing 5% CO_2. VDI cells were cultured with media containing 10^{-8}M calcitriol to maintain selective pressure. The response of 2 representative naïve and VDI cells to calcitriol are presented in this report.

Cell proliferation assays

Naïve and VDI cells were seeded in 96 well plates overnight. Cell growth was measured by treating cells with increasing concentrations of calcitriol or paricalcitol for 96h. Ethanol (0.04%) was used as vehicle control. MTT (Sigma) was added to each well and absorbances were measured at 570nm with an ELISA plate reader (Molecular Devices, Sunnyvale, CA). The concentration that inhibited cell growth by 30% (GI_{30}) was determined using Calcusyn software (Biosoft, Ferguson, MO). DNA synthesis was measured using a bromodeoxyuridine (BrdU) ELISA kit (Roche, Indianapolis, IN) according to manufacturer's instructions. Briefly, sub-confluent cells were treated with calcitriol (100nM) or vehicle (0.004% ethanol) for 96h.

Cells were labeled with BrdU and incubated with anti-BrdU antibody. BrdU incorporation was detected using a colorimetric substrate solution and absorbances (370nm) were measured as described above.

Annexin V staining

Apoptosis was measured using the Annexin V-PE Apoptosis Detection Kit (BD PharMingen) according to manufacturer's instructions. Briefly, sub-confluent cells were treated with vehicle or 100nM calcitriol for 96h. Cells were harvested, stained with annexin V and 7-AAD; and analyzed by flow cytometry. Cells undergoing early apoptosis stain positive for annexin V, while necrotic cells and cells undergoing late apoptosis stain positive for annexin V and 7-AAD.

Western blot analysis

Sub-confluent cells were treated with vehicle or calcitriol (100nM) for 24h. Cells were lysed, proteins were resolved by SDS-PAGE and transferred overnight to a PVDF membrane. Membranes were blocked for at least 1h in 5% milk and incubated overnight with the following primary antibodies: VDR (Santa Cruz, Santa Cruz, CA) and actin (Calbiochem, San Diego, CA). Membranes were incubated with HRP-conjugated secondary antibodies and proteins were detected using a chemiluminescence reagent.

RT-PCR

Sub-confluent cells were treated with vehicle or 100nM calcitriol for 96h. RNA was isolated from cells using TRIzol Reagent (Invitrogen) and RNAqueous-4PCR kit (Ambion, Austin, TX) according to manufacturer's protocol. cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen). RT-PCR was performed using primers (5′-3′) for mouse cyp24(forward): TGGGAAGATGATGGTGACCC; cyp24(reverse): ACTGTTCCTTTGGGTAGCGT.

Cyp24 activity assay

Sub-confluent cells were treated with vehicle or 100nM calcitriol for 24h. Cells were harvested and resuspended in cyp24 buffer (0.19M sucrose, 25mM sodium succinate, 2mM $MgCl_2$, 1mM EDTA Na_4, 20mM HEPES; pH 7.4). Protein concentration was determined and samples were incubated for 30min with cyp24 substrate, [26,27-^{3}H]-25-hydroxyvitamin D_3 (Perkin Elmer, Boston, MA). Vitamin D metabolites were extracted using tetrahydrofuran (Fisher Scientific, Fair Lawn, NJ) and ethyl acetate (Fisher). Samples were evaporated to dryness and resuspended in HPLC mobile phase (94% hexane and 6% 2-propanol). Radioactive metabolites were separated by HPLC and the amount of radioactivity in each fraction was measured by scintillation counting.

Statistical analysis

ANOVA was used to determine the significance between treatment groups relative to vehicle control using the Statview software (SAS Institute Inc., Cary, NC).

RESULTS

Antiproliferative effects of vitamin D compounds in naïve and VDI cells

Compared to naïve cells, VDI cells were less sensitive to inhibition of cell growth following treatment with calcitriol for 96h (Fig. 1A). Calcitriol (3.6nM) inhibited growth of naïve cells by 30%; however, there was no effect on growth of VDI cells. To determine whether VDI cells are cross-resistant to other vitamin D compounds, cells were treated for 96h with the vitamin

D analog, paricalcitol. VDI cells were less responsive to growth inhibition by paricalcitol than naïve cells which were inhibited by 30% with 44.0nM of paricalcitol (Fig. 1B).

Effects of calcitriol on cell proliferation and apoptosis

BrdU incorporation assay was performed to examine the effect of calcitriol (100nM) on DNA synthesis in naïve and VDI cells. Calcitriol inhibited DNA synthesis in naïve cells by ~40%, however, VDI cells were insensitive to the antiproliferative effects of calcitriol (Fig. 2A). To further study the response of naïve and VDI cells to calcitriol, apoptosis was measured by annexin V staining. Calcitriol did not induce apoptosis in either the naïve or VDI cells after 96h of treatment (Fig. 2B).

Effect of calcitriol on vitamin D receptor (VDR) expression, cyp24 mRNA levels and cyp24 enzyme activity

Immunoblot analysis was performed to determine if naïve and VDI cells induce expression of the VDR, the key mediator of the genomic response to vitamin D compounds. Calcitriol increased VDR expression in naïve and VDI cells (Fig 3A). This suggests that decreased sensitivity of VDI cells to calcitriol is not due to lack of VDR expression.

Increased expression of cyp24, is associated with decreased sensitivity of DU145 human prostate cancer cells to the antitumor effects of calcitriol [16]. Cyp24 expression was examined in naïve and VDI cells to evaluate contribution of altered cyp24 levels in vitamin D insensitivity. Following treatment with calcitriol (100nM) for 96h, cyp24 mRNA levels were increased in both naïve and VDI cells to comparable levels (Fig 3B). Cyp24 enzyme activity was measured in naïve and VDI cells by HPLC. Following treatment with calcitriol (100nM) for 24h, cyp24 activity was increased in naïve and VDI cells by ~3–5 fold (Fig. 3C). There was no difference in induction of cyp24 expression and activity in naïve and VDI cells.

DISCUSSION

Vitamin D resistance has been observed in some cancers and may limit the efficacy of vitamin D therapies in the clinic. Progression to advanced CaP is associated with insensitivity to vitamin D. There is a need to understand the molecular mechanisms that underlie insensitivity to vitamin D therapies in order to improve the clinical efficacy of vitamin D compounds. Previous studies have indicated that MCF-7 breast cancer cells and RWLeu-4 leukemia cells become insensitive to the antitumor effects of calcitriol following chronic treatment with calcitriol [17,19]. These studies demonstrate for the first time that chronic treatment of TRAMP mice with calcitriol results in development of vitamin D insensitive (VDI) CaP. VDI cells that were generated from these tumors were less sensitive to cell growth inhibition by vitamin D compounds (calcitriol and paricalcitol). Based on results obtained thus far, decreased sensitivity of VDI cells does not appear to be due to aberrant expression of the vitamin D inactivating enzyme, cyp24. Some of the molecular pathways deregulated in vitamin D resistant variants of MCF-7 breast cancer cells (MCF-7^{D3RES}) and RWLeu-4 leukemia cells ($JMRD_3$) cells include loss of cell cycle regulation [18,19] and resistance to induction of apoptosis in MCF-7^{D3RES} cells [17]. Similar to VDI cells, MCF-7^{D3RES} and $JMRD_3$ cells express functional VDRs [17,19], that are uncoupled from downstream antiproliferative pathways. Further studies are underway to dissect the role of metabolism by cyp24, deregulation of downstream molecular pathways (cell cycle and apoptosis) and VDR function/signaling in development of vitamin D insensitive/resistant CaP.

An *in vitro* model system that will facilitate future studies to elucidate the molecular mechanisms of insensitivity has been successfully created using the TRAMP model. Studying and understanding the difference between vitamin sensitive and insensitive CaP cells is critical

for rational design of future clinical trials with vitamin D compounds for prostate cancer prevention and treatment.

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ABBREVIATIONS

CaP
Prostate Cancer

TRAMP
Transgenic Adenocarcinoma of Mouse Prostate

MWF
Monday/Wednesday/Friday

VDI
Vitamin D Insensitive

HPLC
High Performance Liquid Chromatography

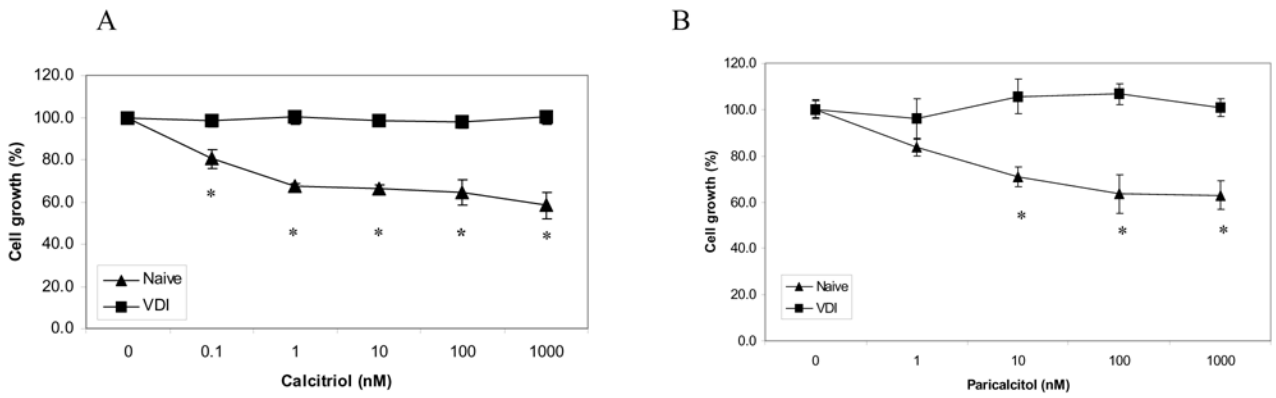

Figure 1.

VDI cells are less sensitive to growth inhibition. *(A)*, Naïve and VDI cells were treated with vehicle (control) or different concentrations of calcitriol for 96h. Cell growth was determined by MTT assay and results are reported as mean cell growth (%) of 3 replicate wells ± SD normalized to cell growth at time point zero. *(B)*, Cells were treated with different concentrations of paricalcitol for 96h and cell growth was measured by MTT assay. Results were analyzed as described above. *, $P < 0.005$ by ANOVA. Results are representative of at least 3 replicate experiments.

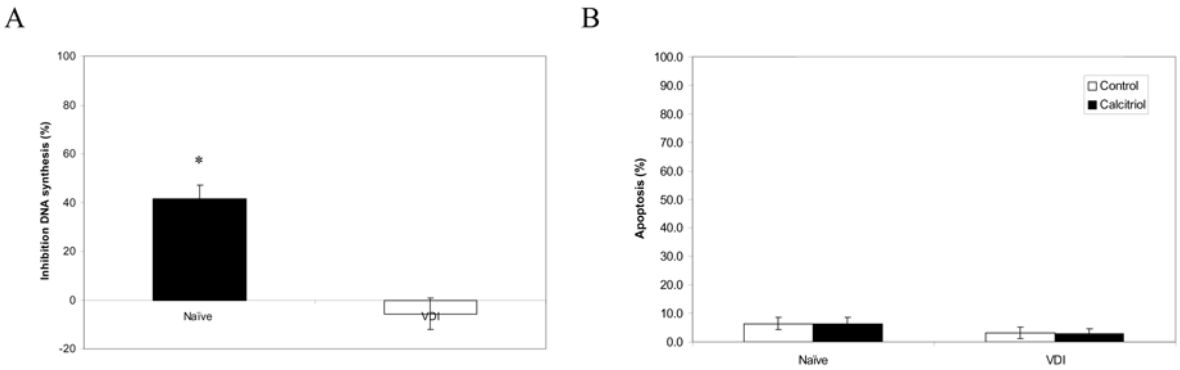

Figure 2.

Effects of calcitriol on cell proliferation and apoptosis in naïve and VDI cells. Naïve and VDI cells were treated with vehicle (control) or 100nM calcitriol for 96h. *(A)*, DNA synthesis was measured by BrdU incorporation assay. Results are reported as % inhibition of DNA synthesis relative to vehicle-treated cells. *, $P < 0.001$ by ANOVA. *(B)*, Apoptosis was measured by Annexin V/7-AAD staining. Results are reported as % apoptosis (Annexin V and Annexin V/7-AAD cell populations). Results are representative of at least 3 replicate experiments.

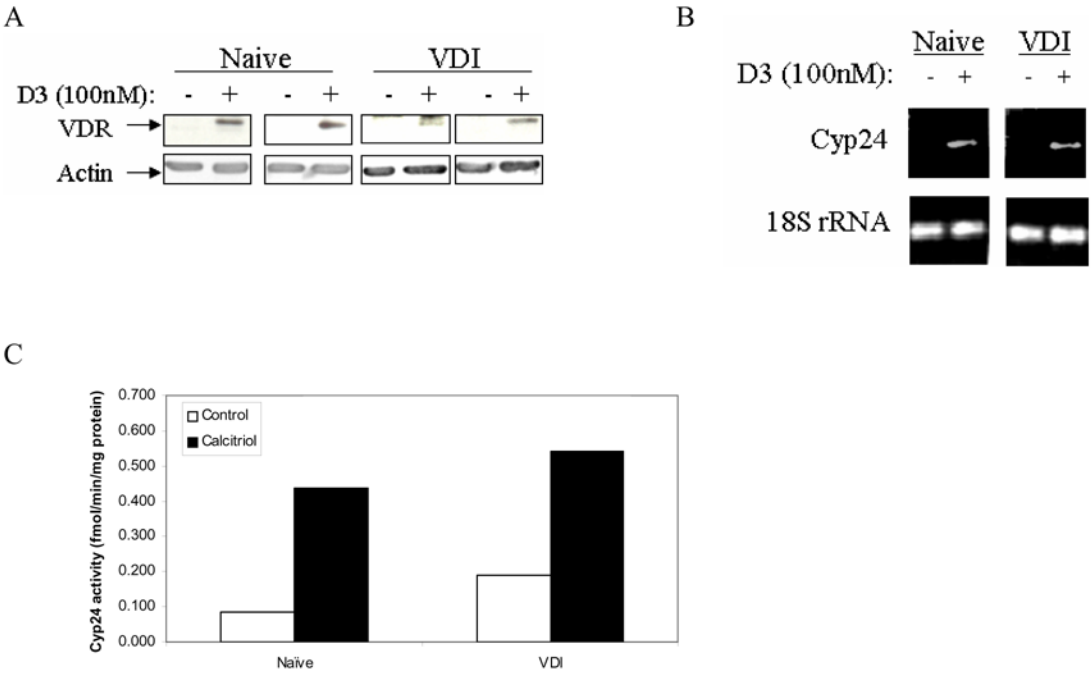

Figure 3.

Calcitriol induces VDR and cyp24 expression in naïve and VDI cells. *(A)*, Cells were treated with vehicle or calcitriol (100nM) for 24h. Western blot analysis was performed with anti-VDR antibody *(B)*, Cells were treated with vehicle or 100nM calcitriol for 96h. Cyp24 mRNA levels were detected by RT-PCR. *(C)*, Cells were treated with 100nM calcitriol for 24h. Cyp24 enzyme activity was measured by HPLC. Results are reported as fmol/min/mg protein. Results are representative of 2 replicate experiments.