Journal of Steroid Biochemistry & Molecular Biology 120 (2010) 11–21

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Down-regulation of NF-κB signals is involved in loss of 1α,25-dihydroxyvitamin D_3 responsiveness

Bo-Ying Bao[a,b,c,1], Huei-Ju Ting[a,1], Jong-Wei Hsu[a], Sayeda Yasmin-Karim[b], Edward Messing[a], Yi-Fen Lee[a,*]

[a] Department of Urology and Pathology & Laboratory Medicine, Rochester, NY 14642, USA
[b] Department of Chemical Engineering, University of Rochester Medical Center, Rochester, NY 14642, USA
[c] School of Pharmacy, China Medical University, Taichung 404, Taiwan

ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form 11 February 2010
Accepted 14 February 2010

Keywords:
Prostate cancer
Vitamin D-resistant
NF-κB

ABSTRACT

Vitamin D anti-tumor effect is often found reduced in the late stages of cancer. To uncover vitamin D resistance mechanism, we established a vitamin D-resistant human prostate cancer LNCaP cell line, LNCaP-R, by chronic exposure of cells to 1α,25-dihydroxyvitamin D_3 (1,25-VD). The vitamin D receptor (VDR)-mediated transcriptional activity was reduced in LNCaP-R, whereas VDR expression level and DNA-binding capacity were similar compared to parental cells (LNCaP-P). The expressions of the key factors involved in VDR transactivity, including CYP24A1 and VDR-associated proteins are all increased in LNCaP-R cells, and yet treatment with ketoconazole, P450 enzymes inhibitor, as well as trichostatin A (TSA), a histone deacetylase inhibitor, did not sensitize LNCaP-R cells response to vitamin D, suggesting that neither a local 1,25-VD availability, nor VDR-associated proteins are responsible for the vitamin D resistance. Interestingly, nuclear factor-kappaB (NF-κB) signaling, which is critical for 1,25-VD/VDR activity was found reduced in LNCaP-R cells, thereby treatment with NF-κB activator, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), can sensitize LNCaP-R vitamin D response. Together, we conclude that NF-κB signaling is critical for vitamin D sensitivity, and dysregulation of this pathway would result in vitamin D resistance and disease progression.

1. Introduction

Prostate cancer (PCa) is one of the most common types of cancer among American men. Despite the progress made in understanding the biology of this disease, the management of hormone refractory prostate cancer (HRPC) remains a significant clinical challenge. The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25-VD), has been shown to modulate growth, differentiation, and apoptosis of a variety of normal and transformed cells, such as breast, colon, myeloid, and prostate [1–4]. In clinical trials, 1,25-VD has been used as an adjuvant therapy for human PCa in combination with docetaxel, and has shown promising results in PSA response, time to progression, and survival in HRPC patients [5]. However, 1,25-VD responsiveness is frequently found to be lost during the late stages of cancer progression [6–9], and the detailed mechanisms remain to be determined.

Vitamin D resistance had been shown in many cell types, and numerous mechanisms have been found. 1,25-VD can be metabolized by 1,25-VD 24-hydroxylase (CYP24A1) to a less active form, and increased expression of CYP24A1 has been linked to reduced 1,25-VD sensitivity [10]. Because the major genomic actions of 1,25-VD are mediated by the vitamin D receptor (VDR), alterations on its sequence [11–13], expression [14,15], phosphorylation [16], and nuclear localization [16,17], have all been implied to be involved in vitamin D resistance. The 1,25-VD-bound VDR recognizes vitamin D response elements (VDREs) by forming a heterodimeric complex with the retinoid X receptor (RXR) in the promoters of vitamin D-responsive genes and regulates transcription. In rat osteosarcoma cells, accelerated and aberrant RXR degradation could cause resistance to the antiproliferative effects of 1,25-VD [18]. The classical hereditary vitamin D-resistant rickets patients who have normal VDR expression and function, might be caused by a constitutive overexpression of heterogeneous nuclear ribonucleoprotein (hnRNP) that competed

Abbreviations: 1,25-VD, 1α,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; VDRE, vitamin D response element; PCa, prostate cancer; SRC-1, steroid receptor coactivator-1; DRIP-205, VDR-interacting protein-205; NCoR1, nuclear receptor corepressor 1; SMRT, silencing mediator for retinoid and thyroid hormone receptors; TSA, trichostatin A; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NF-κB, nuclear factor-kappaB; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1.

* Corresponding author at: Department of Urology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 656, Rochester, NY 14642, USA.
Tel.: +1 585 275 9702; fax: +1 585 756 4133.
E-mail address: yifen_lee@urmc.rochester.edu (Y.-F. Lee).
[1] These authors contributed equally to this work.

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.02.030

with VDR/RXR binding to the VDRE [19,20]. Many coregulators have been identified to modulate the transcriptional activity of VDR. Coactivators, such as steroid receptor coactivator-1 (SRC-1) and VDR-interacting protein-205 (DRIP-205), contain histone acetylase activity that help chromatin remodeling and facilitate transcription. Corepressors, such as nuclear receptor corepressor 1 (NCoR1), silencing mediator for retinoid and thyroid hormone receptors (SMRT), ALIEN, and ligand dependent nuclear receptor corepressor (LCOR), recruit a complex with histone deacetylase (HDAC) activity and repress transcription of target genes. Our previous study showed that increased expression of the corepressors, NCoR1 and SMRT, result in reduced antiproliferative response to vitamin D [8]. In addition, a proteomics screening identified 14 differentially expressed proteins, which might dictate vitamin D resistance in breast cancer cells, such as down-regulation of caspases (caspase-7 and -14), and up-regulation of mitogenic signals [phospho-p38 and Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 2 (MEK2)] [21]. However, additional work is needed to determine their relevance to 1,25-VD resistance.

To understand the molecular mechanisms that cause vitamin D resistance, there have been several studies in the breast cancer field using vitamin D-resistant human breast cancer MCF-7 cell lines, MCF-7^{D3Res} and MCF-$7/VD^{R}$, as models [22–24]. Although lower VDR transcriptional activity and treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) could sensitize vitamin D-resistant MCF-7 variants to the effects of 1,25-VD [22–24], the detailed mechanisms underlying these effects remain unclear. In an attempt to gain more insight into the anticancer effects of 1,25-VD in PCa, we have selected a vitamin D-resistant subclone, LNCaP-R, by growing the parental LNCaP-P cells in the presence of 100 nM 1,25-VD for 3 months. In agreement with the studies of vitamin D-resistant MCF-7 variants, LNCaP-R cells express comparable levels of VDR and RXR, but the transcriptional activity of VDR and cell growth inhibition appeared to be less sensitive to 1,25-VD. In addition, we applied Ketoconazole (an inhibitor of P450 enzymes), trichostatin A (TSA), (a histone deacetylase inhibitor), and TPA to enhance VDR transcriptional activity in LNCaP-R cell. Our data demonstrated that only TPA downstream signals could potentiate 1,25-VD-induced VDR transactivity and cell growth inhibition in LNCaP-R cells. Dissecting the mechanisms involved in the differential responsiveness of 1,25-VD using these two sublines might provide us valuable information for understanding the basic underlying mechanisms of vitamin D resistance and the actions of vitamin D.

2. Materials and methods

2.1. Materials and plasmids

1,25-VD and EB1089 were generous gifts of Dr. Lise Binderup from Leo Pharmaceutical Products (Ballerup, Denmark). Antibodies to VDR, p65, IRB-α, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for phosphorylated-ERK1/2 (p-ERK1/2) was purchased from Cell Signaling Technology (Danvers, MA). TPA responsive element-luciferase (TRE-Luc) reporter construct was provided by Dr. Andrew M.-L. Chan from Mount Sinai School of Medicine, NY, NF-κB-Luc reporter construct was provided by Dr. Edward Schwarz from University of Rochester, and VDRE-Luc reporter construct was provided by Dr. Xianghuai Lu and Dr. Mark S. Nanes from Emory University School of Medicine, GA. The rCYP24A1-Luc construct has been described previously [8].

2.2. Cell culture and vitamin D-resistant subline selection

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection. Cells were maintained in 10% FBS supplemented RPMI-1640 (Gibco-BRL, Carlsbad, CA) containing penicillin (100 IU/mL) and streptomycin (100 mg/mL). The vitamin D-resistant variant, LNCaP-R, was developed by plating LNCaP cells in culture medium containing 100 nM 1,25-VD at a density of 2×10^5 cells/100 mm plate. Every the other day, the medium was replaced with fresh medium containing 100 nM 1,25-VD and cells were passaged every 8 days. Continued culture of surviving cells in 100 nM 1,25-VD for over 3 months resulted in a stable cell line that grew equally well in the presence or absence of 1,25-VD. In parallel with the LNCaP-R cells, LNCaP-P was also developed by exposing to equivalent amounts of ethanol vehicle. After the two sublines were established, the cells were grown in the culture medium without ethanol or 1,25-VD, and the responsiveness of the LNCaP-R cells to 1,25-VD was assessed regularly. LNCaP-R cells remained resistant to 1,25-VD for 6 months after establishment.

2.3. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 3000 cells/well. After overnight incubation, cells were treated with either ethanol vehicle (0.1%, v/v), 1,25-VD, EB1089, ketoconazole, TSA, or TPA at indicated concentrations. At the indicated time points, medium was replenished and cell proliferation was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma–Aldrich, St. Louis, MO) as described previously [25].

2.4. Flow cytometric analysis, real-time polymerase chain reaction (PCR), Western blot analysis, transient transfection, luciferase assay, and chromatin immunoprecipitation (ChIP) assay

The assays above were performed as described previously [25,26] or elsewhere [27]. The primers used in this study were described previously [8,25,27] and are listed in Table 1.

Table 1
Real-time PCR primer sequences.

Gene	Forward primer	Reverse primer
Protease M	5′-AAGGAGAAGCCAGGAGTC-3′	5′-TATTAAGCATCAGGGTCAGAG-3′
DRIP-205	5′-AAGCGGAAGAAGGCAGAC-3′	5′-GAGGAAGAGGAGGAAGAATGG-3′
SRC-1	5′-ATGGACAAATAATACAGTGACAG-3′	5′-AGGAGAAGGAGAAGAGTAAGG-3′
ALIEN	5′-CACGAGCCAAGATGTCTG-3′	5′-ACTCCGAATATAGGTCAATAGC-3′
LCOR	5′-CTCACCTCTGGACCTTAC-3′	5′-CTGCTCAGTAGTTCTTCAC-3′
hnRNPA1	5′-GGTGGCTATGGCGGTTCC-3′	5′-ACTTCTCTGGCTCTCCTCTCC-3′
hnRNPA2	5′-AGGAGGAAGAGGAGGATATGG-3′	5′-TCATTGGACCGTAGTTAGAAGG-3′
HDAC1	5′-GCCTAGTGCGGTGGTCTTAC-3′	5′-TCGTGTTCTGGTTAGTCATATTGG-3′
HDAC2	5′-GCTTGGAGGAGGTGGCTAC-3′	5′-ATTCTGGAGTGTTCTGGTTTGTC-3′
HDAC3	5′-TCTGGCTTCTGCTATGTC-3′	5′-AGGTGCTTGTAACTCTGG-3′
IκB-α	5′-CTCCACTCCATCCTGAAG-3′	5′-CCTCATCCTCACTCTCTG-3′
IκB-β	5′-TACTCCCGACACCAACCATACC-3′	5′-CCTCCTCACTCTCCTCTTCTTCC-3′