Research article

RNAi-mediated knockdown of VDR surprisingly suppresses cell growth in Jurkat T and U87-MG cells

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Vitamin D receptor (VDR) is a nuclear receptor for 1,25-Dihydroxyvitamin D3. VDR is expressed in many types of cells and involved in different biological processes such as immunity and inflammation. In addition, the role for VDR has been indicated in different diseases including multiple sclerosis (MS). In this study, we investigated the effects of VDR knockdown on growth, apoptosis, cell cycle, and some inflammatory gene expressions in Jurkat and U87-MG cell lines.

1. Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) [1] which has a higher incidence at higher latitudes due to low exposure to sunlight and hence lower levels of vitamin D3 (25(OH)D3) [2]. Low levels of this vitamin have also been associated with higher susceptibility to different infections. Therefore, vitamin D plays an important role in immune regulation [3]. The receptor of vitamin D is vitamin D receptor (VDR) [4]. VDR is a transcriptional regulator belonging to the super family of nuclear receptors, which has interaction with specific DNA sequences [5]. This transcription factor is mainly distributed in cytoplasm, where it interacts with biologically active form of vitamin D, 1,25(OH)2D3, heterodimerizes with retinoid X receptor (RXR), and then translocates to nucleus. Then, in conjunction with several transcription factors, it interacts with vitamin D response elements (VDREs). Depending on the target genes, the VDR/RXR heterodimers induce or repress gene transcription (based on presence of co-activator or co-repressors) [6]. VDREs are found in the regulatory region of many genes such as osteocalcin, osteopontin, calbindin-D28K, calbindin-D9K, p21WAF1/CIP1, NF-KB, TGF-beta2, and vitamin D 24-hydroxylase [7]. VDR is expressed in many types of tissues and cells, such as the kinds of cells in the immune systems and cancer [8]. Studies have reported that vitamin D/VDR signaling regulates innate and adaptive immunity [9, 10]. For instance, vitamin D enhances IL-10 expression in dendritic cells [11] and induces it in T cells [12]. Further, vitamin D supplementation leads to increased IL-10 mRNA levels in vivo [13]. Also, vitamin D/VDR blocks NF-KB activation pathway [14]. Indeed, IL-10 and NF-KB are two regulators of several processes in immunity and inflammatory responses [15, 16]. Other downstream targets of VDR include components of the TGF-β signaling pathway. TGF-βs are the members of cytokines superfamily which interact with TGF-β receptors to regulate differentiation, cell growth and death, angiogenesis, immune response, and inflammation [17]. In addition to VDR roles in the immune system and inflammation, it has some functions in physiological and...
neurological development as well as protection against apoptosis [18]. Earlier studies demonstrated that VDR induces apoptosis and inhibits cell growth in the presence of its ligand, vitamin D [19].

Although a number of studies have investigated the probable roles of VDR in immunity, inflammation, neurobiology of MS, some molecular mechanisms of VDR in T cells and neuronal cells have remained controversial and unknown. Therefore, in this study, we analyzed the effects of VDR knockout on cell growth, apoptosis, and cell cycle in the human primary glioblastoma cell line (U87-MG cell line) and human T-cell leukaemia cell line (Jurkat cell line). Furthermore, we indicate that the down-regulation of VDR can alter the expression of some genes involved in inflammatory processes. Based on the previous studies, we assumed that VDR down-regulation in glioblastoma cell line and human T cell line would induce cell growth. However, we observed that silencing VDR expression inhibited cell growth indicating a novel role of VDR in inducing cell growth of neuronal cells and lymphocytes. In addition, we hypothesized that if VDR down-regulation would lead to enhanced apoptosis in these two cell lines, then in future studies, it could be used as a therapeutic target in MS.

2. Materials and methods

2.1. Cell culture

In this study, Jurkat E-6 and U87-MG cell lines were used. These cell lines were cultured in RPMI-1640 medium (Cat. 11879020, Invitrogen, USA), supplemented with 10% fetal bovine serum (Cat. 16000044, Invitrogen, USA), 2 mM glutamine, 100U/l penicillin and 100 U/l streptomycin. The cells were grown at 37 °C in an air/5% CO2 atmosphere at constant humidity.

2.2. VDR knockdown by short hairpin (sh) RNA vectors

In order to knock-down VDR, Jurkat T and U87-MG cells were transfected with four pGFP-V-RS shRNA clones targeting human VDR transcripts [shRNA1 (GI379488), shRNA2 (GI379489), shRNA3 (GI379490), shRNA4 (GI379491)] and a scrambled non-targeting control (TR30013) (OriGene Technologies, Inc. USA). Lipofectamine 2000 (Cat. 11668-019, Invitrogen) was used as a transfection reagent. Following the transfection, the cells emitted green light under fluorescent microscopy. Before transfection, 0.1 μM 1,25(OH)2D3 (cat. D1530, Sigma-Aldrich) was speciﬁed to knockdown VDR in normal and transfected Jurkat E-6 and U87-MG cells’ growth. These cells were seeded in 96-multiwell plates at a density of 2 × 104 per well and incubated for 24 and 48 h after shRNA transfection. Next, 20 μl of 5 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro- mide) (Cat. 11465007001, Roche Applied Science, Indianapolis, IN, USA) solution was added to each well. After 4 h of incubation, the medium was removed from the wells, and 150 μl of solubilization solution (Roche Applied Science, Indianapolis, IN, USA) was added to each well. Color intensity was measured at 490 nm by an enzyme linked immuno-sorbent assay plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Flow cytometric apoptosis assay

The level of apoptosis in Jurkat E-6 and U87-MG cells was measured using the Annexin V Apoptosis Detection Kit FITC (Cat. 88-8005, eBioscience, Inc., San Diego, CA, USA) at 24 and 48 h following transfection according to the manufacturer’s instructions. The cells were examined using FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) and Quest software (BD Biosciences).

2.6. Flow cytometric cell cycle analyses

The cells were harvested through trypsinization 24 and 48 h after transfection, washed four time with PBS. Next, the samples were stained with 50 μg/ml propidium iodide (PI; Cat. 88-8005, eBioscience, Inc., San Diego, CA, USA). The samples were incubated for an additional 30 min. Then, the cell cycle analysis was performed by using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The cell cycle distribution in different phases was interpreted using Quest software (BD Biosciences).

Table 1: Primer list of qRT-PCR.

| Gene names | RefSeq number | Primer sequences |
|------------|---------------|------------------|
| GAPDH | NM_002046.5 | F: 5-CTGCAAGAATGAGTACACGAC-3<sup>a</sup> | |
| IL-10 | NM_000572.2 | F: 5-CCCGCAGATCAGGGGCGAGTGT-3<sup>a</sup> | |
| TGF-β1 | NM_000660.5 | F: 5-GTATGAGTCTTCTCTGGACG-3<sup>a</sup> | |
| TGF-β2 | NM_00113599.2 | F: 5-ATCTAGGTTGAAAGTATGAACG-3<sup>a</sup> | |
| TGF-β RI | NM_004612.2 | F: 5-GGGAAGATTGGTGGAGAAGATTG-3<sup>a</sup> | |
| TGF-β RII | NM_001024847.2 | F: 5-GCTGCTCAGTTGCAAGAGG-3<sup>a</sup> | |
| VDR | NM_000376.2 | F: 5-GATGAGAATGAGGAAGGAGGACG-3<sup>a</sup> | |
| NF-KB | NM_003998.3 | F: 5-TACTCGACGGAGACGAGAATG-3<sup>a</sup> | |

2.2. RNA extraction and real-time PCR

Total RNA was extracted from cells and treated with DNase I (Sigma, USA) at 37 °C for 30 min cDNA was synthesized using the Revert AID<sup>®</sup>Reverse Transcriptase (Cat. EP0441, Fermentas, Canada) with 3 μg of purified total RNA, oligo dT, and random hexamer primers (MWG, Germany) in a total volume of 20 μl reaction mixture, according to the manufacturer’s instructions. For analyzing gene expression and testing the knockdown efficiency, qPCR was performed on Applied Biosystems 7500 Real Time PCR System (Applied Biosystem/MDS SCIEX, Foster City, CA, USA), with 2 μl of SYBR Green master mix (Cat. #RR430S Takara, Shiga, Japan), 10 ng cDNA, and 200 nM of forward and reverse primers up to a final reaction volumes of 20 μl, according to the manufacturer’s instructions. The primer sequences for qPCR are presented in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. Fold expression values were calculated using the 2<sup>ΔΔCt</sup> method. All experiments were performed in duplicate.

2.4. Determination of cell growth

An MTT assay was performed to detect the effect of VDR silencing on normal and transfected Jurkat E-6 and U87-MG cells’ growth. These cells

Quantitative PCR revealed that the VDR transcript expression was reduced in VDR shRNA-transfected groups. In Jurkat T cells, the

were used as 96-multiwell plates at a density of 2 × 104 per well and incubated for 24 and 48 h after shRNA transfection. Next, 20 μl of 5 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Cat. 11465007001, Roche Applied Science, Indianapolis, IN, USA) solution was added to each well. After 4 h of incubation, the medium was removed from the wells, and 150 μl of solubilization solution (Roche Applied Science, Indianapolis, IN, USA) was added to each well. Color intensity was measured at 490 nm by an enzyme linked immunosorbent assay plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

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2.7. Statistical analysis

In the current study, all experiments were assayed in duplicate and repeated at least three times in separate experiments. Statistical analyses were performed using statistical analysis software (GraphPad Software, La Jolla, CA, USA and SPSS Version 13.0 software, SPSS, Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD). Statistical analysis of the data was carried out using either an unpaired, two-tailed Student’s t-test or one-way ANOVA test. The differences were considered to be significant when P < 0.05.

3. Results

3.1. VDR mRNA expression after shRNA transfection

Quantitative PCR revealed that the VDR transcript expression was reduced in VDR shRNA-transfected groups. In Jurkat T cells, the
3.2. Suppression of Jurkat T and U87-MG cell growth

The effect of VDR silencing on the cell growth after transfection with the shRNA is displayed in Fig. 2. At 24 and 48 h post-transfection, the cell growth of VDR-shRNA transfected Jurkat T cells was significantly decreased by 48% (P < 0.05) compared with that in the Scramble-shRNA cells and in the untransfected cells (Fig. 2, A). Also, there was a difference in the cell growth among VDR-shRNA transfected U87-MG cells, Scramble-shRNA cells, and the untransfected cells (22%, P < 0.05) (Fig. 2, B).

3.3. Silencing VDR induces apoptosis of Jurkat T and U87-MG cells

Flow cytometry analysis revealed that suppression of VDR significantly enhanced cell apoptosis in Jurkat T cells (Fig. 3, A-C) as well as in U87-MG cells (Fig. 4, A-C), compared with Scramble-shRNA cells and the untransfected cells (control).

In Jurkat T cells, VDR-shRNA treatment promoted cell apoptosis by approximately 17%, while Scramble-shRNA cells and the untransfected cells only showed 1% and 2% cell apoptosis respectively (Fig. 3, D, p < 0.01). In U87-MG cells, VDR-shRNA treatment induced nearly 15% cell apoptosis compared to 9% and 10% cell apoptosis in two other groups (Fig. 4, D, p < 0.05).

3.4. Analysis of cell cycle following VDR down regulation

Cell cycle analysis using a flow cytometer indicated that following transfection with VDR-shRNA, the percentage of Jurkat T and U87-MG cells in all the phases was not significantly changed compared with Scramble-shRNA cells and the untransfected cells (P > 0.05) (data not shown).

3.5. Gene expression analysis after VDR silencing

In this study, the expression of IL-10, NF-KB, TGF-β1, TGF-β2, TGF-β RI, and TGF-β R II mRNAs was measured in Jurkat T and U87-MG cells at 24 h and 48 h post-transfection.

Analysis of qRT-PCR showed that the expression of IL-10 (0.06 fold, p = 0.004), NF-KB (0.02 fold, p = 0.001), TGF-β1 (0.1 fold, p = 0.008), TGF-β R I (0.15 fold, p = 0.012), and TGF-β R II (0.18 fold, p = 0.015) in Jurkat T cells transfected with VDR-shRNA significantly diminished compared to Scramble-shRNA cells and the untransfected cells (Fig. 5, A), but no significant difference was observed in these cells for TGF-β2 (p = 0.079).

Additionally, the expression of NF-KB (111 fold, p < 0.0001), TGF-β1 (8 fold, p < 0.001), TGF-β2 (12 fold, p < 0.001), TGF-β RI (90 fold, p < 0.001), and TGF-β R II (145 fold, p < 0.001) in U87-MG cells after transfection with the VDR-shRNA, was significantly increased compared with Scramble-shRNA cells and the untransfected cells (Fig. 5, B), but this difference was not significant for IL-10 (p = 0.082).

Also, the expression of these mRNAs was measured in Jurkat T and U87-MG cells before and after calcitriol (1, 25-Dihydroxyvitamin D3) treatment (without shRNA). In Jurkat T cells, the expression of IL-10 (p = 0.0057) and TGF-β1 (p = 0.0038) significantly enhanced after calcitriol treatment, but no significant difference was observed in these cells for TGF-β2 (p = 0.085), NF-KB (p = 0.071), TGF-β RI (p = 0.091), and TGF-β R II (p = 0.066) (Fig. 5, C). While in U87-MG cells, the expression of TGF-β1 (8 fold, p < 0.001) and TGF-β2 (12 fold, p < 0.001) was significantly reduced after vitamin D treatment, but this difference was not significant for IL-10 (p = 0.101), NF-KB (p = 0.095), TGF-β RI (p = 0.121) and TGF-β R II (145 fold, p < 0.001) (Fig. 5, D).

4. Discussion

Vitamin D receptor (VDR) is a ligand dependent transcription factor,
which belongs to the superfamily of nuclear receptors [20]. Studies have suggested that VDR is expressed in a wide range of tissues and cell types, and has very different functions in several processes such as cell proliferation and differentiation, apoptosis, immunity, and inflammation [21, 22]. Here, we have evaluated the effects of VDR knock down on cell growth, apoptosis, and cell cycles of two originally different cell lines, U87-MG cell line and Jurkat cell line. We decided to use U87-MG cells because this is a glioblastoma (astrocytoma) cell line originally derived from human brain [23]. Also, Jurkat is a type of T lymphocytes. T cells are one of cells in the immune system that their disruption have been reported in MS pathogenesis [24].

Previous studies have demonstrated that 1,25(OH)2D3 has anti-proliferative effects by its receptor, VDR, on immune and inflammatory cells [25]. However, there is no evidence of the effects of VDR in U87-MG and Jurkat cell lines. Our analysis revealed that silencing VDR using shRNA reduced the expression levels of VDR-mRNA dramatically in both Jurkat T cells, and U87-MG cell line. Diminished VDR-mRNA levels subsequently decreased cell growth significantly and enhanced apoptosis in both cells (P < 0.05), but it had no significant effect on cell cycle.

There is inadequate evidence about the function of VDR in the absence of vitamin D. In breast cancer cell, unliganded VDR has proven to control breast cancer cell growth [26] and in skin cells, it is necessary for...
the function of keratinocyte cells [27]. Our silencing VDR results propose a different function of VDR in U87-MG and Jurkat cell growth.

Conversely, the study of Salomon et al. (2014) in T98G, a glioblastoma multiform cell line, found that reduction in VDR levels by shRNA significantly increased the survival of T98G cells [28]. Elsewhere, the same results were obtained concluding that VDR has a negative effect on cancer and cancer cell lines and reduces their proliferation [21], but our study does not support this evidence. Trivedi et al (2017) suggested that in the absence of vitamin D, the cytoplasmic VDR induced breast cancer cell growth [26]. Indeed, a protein known as PTPH1 (protein tyrosine

Fig. 4. FITC-Annexin V analysis of cell apoptosis. U87-MG cells were untransfected (control) (A), transfected with scramble-shRNA (B) or transfected with VDR-shRNA (C). The percentage of apoptotic cells in U87-MG cells (D) was presented at 24 h and 48 h after transfection. Left lower quadrant: live cells; Left upper quadrant: dead cells; Right lower quadrant: early apoptotic cells; Right upper quadrant: late apoptotic cells. The results shown are from at least two biological replicates.

Fig. 5. Analysis of levels of mRNA expression in cell cultures following transfection with culture medium alone (control), scramble-shRNA, or VDR-shRNA at 24 h and 48 h after transfection or without transfection; (A) Down-regulation of IL-10, NF-KB, TGF-β1, TGF-β R I and TGF-β R II in Jurkat T cells after VDR knockdown; (B) Up-regulation of NF-KB, TGF-β1, TGF-β2, TGF-β RI and TGF-β R II in U87-MG cells after VDR silencing; (C) Up-regulation of IL-10 and TGF-β1 in Jurkat T cells after calcitriol treatment; (D) Down-regulation of TGF-β1 and TGF-β2 in U87-MG cells after calcitriol treatment; The mRNA Levels were analyzed by Real-Time PCR and mRNA expression was normalized with GAPDH. The results shown presented are from at least two biological replicates; -VD = without vitamin D; +VD = with vitamin D (1,25-Dihydroxyvitamin D3 or calcitriol).
phosphatase 1) which is a specific phosphatase for mitogen-activated protein kinase (MAPK), controls breast cancer cell growth by binding to VDR in the cytoplasm and stimulate it [29]. Therefore, the same mechanism may partially cause the VDR to promote growth in Jurkat and U87-MG cells in the absence of its ligand.

Our data demonstrate that VDR knock down enhanced apoptosis in Jurkat and U87-MG cells. This effect of VDR on apoptosis is presumably mediated by repression of the Wnt/β-catenin signaling pathway [30]. Wnt proteins regulate several processes including immunity, neuronal development, stem-cell proliferation, and tumorigenesis [31]. Furthermore, Wnt is a positive regulator of myelin gene expression in the myelination process [32].

Vitamin D deficiency is common in patients with MS [2, 3, 33]. Although several recent studies have indicated the relationship between vitamin D deficiency and increased risk of MS, molecular mechanisms and pathways have remained controversial. Therefore, other investigations are necessary for explaining them. Meanwhile, vitamin D signaling is mediated by binding 1,25(OH)2D3 to VDR. The expression of VDR has been reported in most immune cells as well as in neuronal cells [34]. Silencing VDR expression in the types of cells such as U87-MG and Jurkat T cells may help to elucidate the precise function of VDR in immune and neuronal cells, as well as in MS pathogenesis.

Although, in our previous studies we demonstrated that the expression of some inflammatory genes including VDR [35], IL-10 [13], TGF-βR I, TGF-βR II, and TGF-βR II [36] changed after supplementation with Vitamin D in PBMCs (peripheral blood mononuclear cell) from MS patients, to further clarify VDR function in U87-MG and Jurkat cell lines and elucidate molecular mechanisms of VDR, we evaluated the expression levels of NF-κB, IL-10, TGF-βR I, TGF-βR II, and TGF-βR II at mRNA levels in the mentioned cell lines by Real-Time PCR. In U87-MG cells, VDR knockdown dramatically increased NF-κB, TGFB1, TGFB2, TGFBRI, and TGFBRII mRNA levels. IL-10 expression level did not change significantly after VDR disruption.

On the other hand, in Jurkat cell line we found that NF-κB, IL-10, TGFB1, TGFBRI, and TGFBRII mRNA levels dropped significantly after VDR inhibition while TGFβ2 expression was not significantly altered after transfection.

Previous studies have shown that NF-κB, TGFB1, TGFB2, TGFBRI, and TGFBRII, and IL-10 are involved in immunomodulation and inflammation [37, 38, 39]. On the other hand, other studies have shown that VDR has immunomodulatory, pro-differentiative, and anti-proliferative effects, and VDR activation results in the activation of downstream gene in immune cells [40, 41]. Thus, we expect that VDR inhibition activates various signaling pathways involved in immunomodulation and inflammation such as NF-κB, TGFBeta, and so on. For example, VDR along with TGF-β mediates the expression of IL-10 and increases the generation of IL-10-producing CD4+ T cells [42].

Expression profiling results for U87-MG cell line have been concordant with this fact, but apoptosis and growth assay results indicate that activation of NF-κB and TGF-beta signaling pathways cannot lead to proliferation, and conversely induce apoptosis in these cells. Therefore, based on these results, we conclude that VDR inhibition causes apoptosis by other potential alternative pathways in this cell line. For instance, Yuan et al (2018) have reported that VDR activation leads to the activation of the ERK1/2 pathway in U87-MG cells which may result in apoptosis reduction [43].

In jurkat cell line transfected with VDR shRNA, cell growth was reduced significantly while, apoptosis was enhanced after the treatment. VDR knocking down has dramatic impacts on the reduction in expression level of NF-κB, TGFB1, TGFBRII, and IL-10. Our results indicated that VDR inhibition can diminish NF-κB and TGF beta signaling pathways directly or indirectly in Jurkat cells, which is not consistent with previous studies in other cell lines [44, 45, 46]. For example, IL-10 locus has a VDR-binding site in its promoter that VDR directly binds to this element and increased IL-10 expression [13]. On the other hand, there are several indirect pathways, which have indicated how VDR influences on the levels expression of IL-10 [13]. Also, studies of Zhang et al. (2008) suggested that nongenomic activation of a VDR/Pi3K/Akt survival pathway could have anti-apoptotic effects on osteoblast cell line [47], so silencing the VDR could increase apoptosis; all these results are consistent with our findings. Furthermore, study of mice with VDR deficiency did not manifest any increase of sporadic tumorigenesis [48]. On the other hand, the controversial results about the roles of VDR in apoptosis and the effects of VDR knock down on cell fate require further studies.

However, one limitation of this study is that the effects of VDR down-regulation on the expression of genes involved in apoptosis, cell growth, and cell cycle have not been investigated and these analyses are suggested for future studies.

5. Conclusion

In conclusion, these results indicate that VDR can play a fundamental role to regulate lymphocytes and neuronal cell growth without its ligand, vitamin D. However further studies are necessary to understand the function of VDR in the mentioned cells. Further, while VDR knocking down in two different cell lines of U87-MG and Jurkat cells had different effects on NF-κB and TGF-beta expression levels, its effects on cell growth and apoptosis were similar. This may suggest that these two different cell lines can show similar anti-proliferative effects by different downstream signaling pathways. Taken together, this information may be useful to design novel diagnostic and therapeutic methods for diseases associated with vitamin D deficiency.

Declarations

Author contribution statement

Z. Shirvani-Farsani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
M. Behmanesh: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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