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Influence of vitamin D on cell cycle, apoptosis, and some apoptosis related molecules in systemic lupus erythematosus

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ABSTRACT

Objective(s): Genetic and environmental factors are involved in the pathogenesis of systemic lupus erythematosus (SLE). Autoreactive lymphocytes are cleared through apoptosis and any disturbance in the apoptosis or clearance of apoptotic cells may disturb tolerance and lead to autoimmunity. Vitamin D has anti-proliferative effects and controls cell cycle progression. In this study we investigated the effects of vitamin D on cell cycle and apoptosis induction in lupus patients.

Materials and Methods: Isolated peripheral blood mononuclear cells (PBMCs) from 25 SLE patients were cultured in the presence of 50 nM of 1,25(OH)2D3; then one part of the cells were stained with FITC labeled Annexin V and PI and were analyzed for apoptosis determination. For gene expression assessment of FasL, Bcl-2 and Bax, RNA was extracted from one another part of the cells, cDNA was synthesized and gene expression analysis was performed using Real time PCR. An additional part of the cells were treated with PI and the cell cycle was analyzed using flowcytometer.

Results: The mean number of early apoptotic cells in vitamin D treated cells decreased significantly (18.48±7.9%) compared to untreated cells (22.02±9.4%) (P=0.008). Cell cycle analysis showed a significant increase in G1 phase in vitamin D treated cells (67.33±5.2%) compared to non treated ones (60.77±5.7%) (P =0.02). Vitamin D up-regulated the expression levels of Bcl-2 by (18.87 fold increase), and down-regulated expression of Bax (23%) and FasL (25%).

Conclusion: Vitamin D has regulatory effects on cell cycle progression, apoptosis and apoptosis related molecules in lupus patients.

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease in the pathogenesis of which genetic and environmental factors play an important role. Breakdown of tolerance and generation of autoantibodies against self antigens and augmentation of inflammatory responses are implicated in the pathogenesis of SLE (1).

Autoreactive B and T lymphocytes are eliminated through apoptosis in the course of self tolerance (2). Any disturbance in the process of apoptosis or impairment in the clearance of apoptotic cells could lead to the accumulation of apoptotic bodies, generation of autoantibodies (3), and exacerbation of inflammatory responses (4).

In different studies an association of vitamin D deficiency with predisposition to SLE was reported (5, 6), and in some studies in mice models of lupus it was shown that vitamin D consumption improved the disease progression (7) and increased the number of Regulatory T cells (8). Human studies have also showed that vitamin D deficiency is correlated with disease activity in lupus patients (6, 9). Vitamin D could promote the production of anti inflammatory mediators (10), and is considered to be implicated in immune regulation (11). It could regulate the growth of normal and cancer cells (12) and appears to be involved in controlling agitated cells proliferation (13). The mechanism of anti-proliferative effects of vitamin D is not yet well known (14), but controlling the expression of some genes that are involved in proliferation, differentiation, angiogenesis, and cell death (15) are supposed. Vitamin D can also affect cell cycle progression (16, 17).

As impaired apoptosis is one of the possible mechanisms involved in the development and exacerbation of symptoms in systemic lupus erythematosus, and because vitamin D ameliorates the disease symptoms in SLE we aimed to study the regulatory effect of vitamin D on the apoptosis and cell cycle progression as well as the expression levels of FasL, Bcl-2 and Bax in lupus patients.

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Materials and Methods

Participants

Twenty five SLE patients (21 women and 4 men, with a mean age of 34.4 ± 9.3 years) participated in this study. All SLE patients fulfilled at least four of the revised SLE criteria of the American College of Rheumatology (1997 revised criteria) for the classification of SLE (Hochberg, 1997). Each lupus patient was evaluated by a rheumatologist according to the following inclusion and exclusion criteria:

Inclusion criteria:

a- Patients who were newly diagnosed (before starting treatment).

b- Patients in remission who took only a maximum dose of 10 mg/day prednisolone.

c- Patients with active major organ involvement were sampled before changing treatment strategy and starting cytotoxic therapy.

Exclusion criteria:

a- Patients in remission who took hydroxyl-chloroquine.

Patients in remission who took higher than 10 mg/day of prednisolone.

b- Patients who took any cytotoxic drugs.

c- Patients with overlap syndromes.

d- Patients with concomitant malignancy.

e- Patients with infection.

f- Female patients who were pregnant.

Informed written consent was taken from all subjects prior to blood sampling. The Ethic Council of Mashhad University of Medical Sciences (Mashhad, Iran) approved all protocols used with these subjects in this study.

PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (GIBCO, USA) density gradient centrifugation. Cells were washed in phosphate buffered saline (PBS), and then resuspended and adjusted to 5×10^6 cells/ul in PBS.

Assessment of cell cycle by Flowcytometry using propidium iodide staining

The distribution of DNA content in cell cycle was determined using propidium iodide (PI) as a DNA-binding dye. Briefly, lymphocytes (5×10^5 cells) were incubated with vitamin D (50 nM) for 24 hr, (the optimal dose of vitamin D and time of incubation was optimized in our lab in previous studies). Then harvested cells were washed twice with cold phosphate-buffered saline (PBS), incubated with 250 µg/ml RNAse A (GenetBio Co., Nonsan, Korea) for 30 min at 37 ˚C and subsequently incubated with PI (Sigma Chemical Co., MO, USA) solution (100 µg/ml PI, 0.1% Triton X-100 and 0.1% sodium citrate) in dark for 30 min at 4 ˚C. The percentage of DNA content (1×10^4 events) in cell cycle area was determined using a FACSCalibur Flowcytometer (Becton Dickinson, Carlsbad, CA) and the data were subsequently analyzed using FSC Express 3.0 software. Cultured cells without vitamin D were used as controls.

Detection of apoptosis using annexin-V/propidium iodide (PI) dual staining

Death pattern was quantified using fluoroscein isothiocyanate-labeled Annexin V (Annexin V-FITC) and propidium iodide (PI) staining kit (Abcam, Cambridge, MA). The combination of Annexin V-FITC with PI has been used widely to distinguish cells in early and late apoptosis stages. During early apoptosis, phosphatidyl serine, which is usually located in the inner membrane of the cells, is transported into the outer portion of the membrane that can be detected by its strong affinity for annexin V-FITC, a phospholipid binding protein. The dead cells can be detected by binding PI to the cellular DNA, where the cell membrane has been totally crashed. In this study, PMBCs (5×10^5/well) were treated with vitamin D (50 nM) for 24 hr. After that, cells were washed with PBS, resuspended in binding buffer and incubated at room temperature for 5 min in dark with Annexin-V FITC and PI solutions. The cells were then analyzed within 1 hr using a FACSCalibur Flowcytometer (Becton Dickinson, Carlsbad, CA). In each case, a minimum of 1×10^4 events were analyzed. Results were reported as percentage of Annexin V^+ (early apoptosis), PI^+ (necrosis), Annexin V^+PI^+ (double positive; late apoptosis), or Annexin V^-PI^- (double negative; non-stained) cell population. Cells with no treatment were used as control.

Gene expression analysis of FasL, Bcl-2 and Bax by Real time-PCR

Total RNA was extracted using Tripure (Roche, Mannheim, Germany), employing manufacturer's instructions. cDNA was synthesized and the expression level of the genes of FasL, Bcl-2 and Bax were assessed using specific primers and probes by Real time PCR method. A comparative CT method ($2^{-\Delta\Delta CT}$) was used to quantify gene expression levels. All data was expressed as mean transcript expression fold-change over untreated samples (controls), normalized to GAPDH (internal control).

Statistical analysis

All data are reported as mean±SD. Data of apoptosis, cell cycles and gene expression were analyzed using Student's t-test. *P*-values less than 0.05 were considered as significant.

Results

Twenty five SLE patients (21 women and 4 men, with a mean age of 34.4±9.3 years) participated to current study. Anti-dsDNA was positive in 57.8% of patients and arthritis was the most common disorder (52.6%), followed by skin involvement

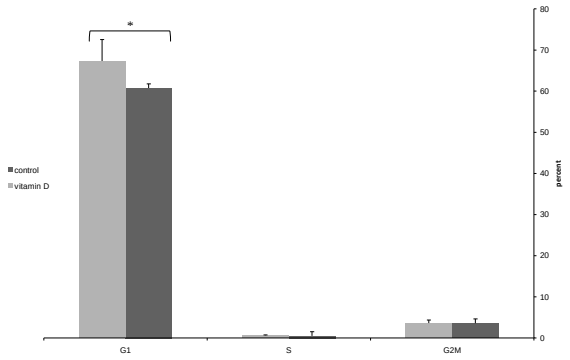
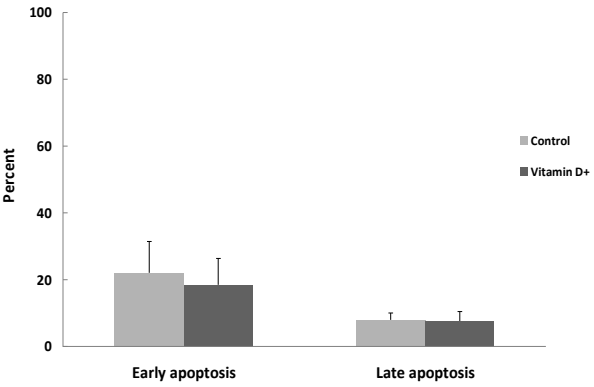


Figure 1. Comparison of cell cycle progression in 50 nM of 1,25(OH)2D3 treated (D+) and in non treated (D-) PBMCs (control) of systemic lupus erythematosus patients. Vitamin D treatment significantly increased the number of cells in G1 phase G1 (Gap1; growth phases of cell cycle), S (Synthesis phase of cell cycle), G2M (Gap2 and Mitosis phases of cell cycle). **P*-value<0.05



Finger 2. Comparison of the effects of 50 nM of 1,25(OH)2D3 treatment on the early and late apoptosis in vitamin D treated (D+) and in non treated (D-) PBMCs (control) of systemic lupus erythematosus patients. Vitamin D treatment (for 24 hr) of PBMCs of SLE patients (D+) significantly decreased the percent of cells in early apoptosis (Annexin V-FITC+) compared to control (untreated) cells. There was no difference in the late apoptotic cells between vitamin D treated and non treated cells. **P*-value< 0.05

(50.5%), kidney damage (35.4%), anemia (19.3%), and thrombocytopenia (13.9%).

Flowcytometry assessment of cell cycle

To further explore the influence of vitamin D on PBMCs of SLE patients, cells were cultured as indicated above, and analyzed for DNA content by flowcytometr. The mean number of cells in the G1 phase in vitamin D treated PBMCs was significantly higher (67.33±5.2%) compared to untreated cells (control group) (60.77±5.7%) (*P*-value 0.02) (Figure1). There was not any significant difference in the number of cells in G2M and S phase between vitamin D treated cells and the control group.

Influence of vitamin D on apoptosis

To determine if any alteration in cell viability induced by vitamin D might be attributable to apoptosis, Annexin-V and PI staining was conducted. Our results showed that vitamin D dramatically decreased early apoptotic population of lymphocytes (18.48±7.9%) (Annexin V^+ cells) compared to untreated cells (22.02±9.4%) (*P*-value 0.008) (Figure 2).

Gene expression of FasL, Bcl-2 and Bax

Quantitative Real-time PCR analyses of the expression of FasL, Bcl-2 and Bax in vitamin D treatment of the cells of SLE patients showed that transcript expression of Bcl-2 (18.87±9.23), Bax (0.77±0.04) and FasL (0.75±0.03) underwent fold-change relative to the levels in untreated cells. This translated to up-regulated expression of Bcl-2(18.87 fold increase), and down-regulted expression of Bax (23%) and FasL (25%) due to culturing in the presence of 50 nM vitamin D (Figure 3).

All data was expressed as mean transcript expression fold-change over untreated samples (controls), normalized to GAPDH (internal control).

Statistical analysis

All data are reported as mean±SD. Data of cell cycles and gene expression were analyzed using Student's t-test. *P*-values less than 0.05 were considered as significant.

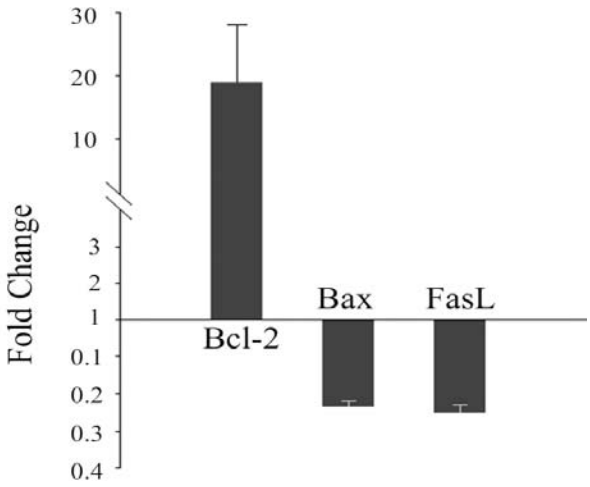


Figure 3. Real-time PCR results using comparative CT method to quantify gene expression levels. Data expressed as mean transcript expression fold-change over untreated samples (controls) normalized to GAPDH. Treatment of PBMCs of SLE patients with 50 nM of 1,25(OH)2D3 (for 24 hr) significantly increased the expression of Bcl-2, and decreased the expression of FasL and Bax

Discussion

Immunomodulatory role of vitamin D has been the subject of much interest in recent literature (18, 19), but the precise mechanisms of this modulation needs further studies. An inhibitory effect of vitamin D on cell proliferation was previously reported (13), but it was not well characterized whether this property is exerted by arrest in cell cycle, apoptosis or both. We studied the effects of vitamin D treatment on apoptosis, cell cycle progression and expression of FasL, Bax and Bcl-2 on PBMCs of lupus patients.

Findings of our study showed that vitamin D treatment decreased the apoptosis rate in PBMCs of SLE patients, increased the DNA content in G0/G1, down regulated the expression rate of FasL and Bax, while up regulated the expression of Bcl-2.

Importance of impaired apoptosis in SLE development was frequently reported in previously published literature (20, 21), and we found that vitamin D by decreasing the expression of FasL and Bax and by increasing the expression rate of Bcl-2 which is an antiapoptotic molecule, could control the apoptosis rate in lupus patients. Results of current study showed that anti apoptotic and anti proliferative effects of vitamin D is also regulated partly by cell cycle arrest in G1 and in parallel to our findings, Ohnishi *et al* showed that cell cycle arrest of vitamin D is mediated by inhibiting several key proteins which regulate the G1/S phase and up-regulates gene expression of P53 (22). Chen *et al* reported an anti proliferative effect for vitamin D on B cells differentiation and autoantibody production (23).

Apoptosis plays a controversial role in systemic lupus erythematosus. Impaired apoptosis and decreased elimination of autoreactive lymphocytes in the developmental phase of immune system could lead to the loss of self tolerance, and some studies showed increased rate of apoptosis in lymphocytes of SLE patients (24). Regulatory T cells are a subset of lymphocytes which are highly susceptible to apoptosis (25), and role of increased apoptosis in the reduction of regulatory T cells was demonstrated in a number of studies (25). In a previous investigation in our group we showed that consumption of vitamin D increased the number of regulatory T cells and down-regulated the expression of IL-6 in animal models of lupus (8). Several studies suggested a profound role for IL-6 in the reduction of the number and activity of regulatory T cells (7). Decreased expression or function of regulatory T cells was correlated with active lupus (25). Results of this study indicates that 1,25(OH)2D3 regulates the processes related to the cell cycle, and apoptosis, and considering the data of our previous study, it is highly suggested that some beneficial effects of vitamin D on increasing the number of regulatory T cells might be achieved through its influence on the apoptosis and cell cycle arrest which needs further studies.

Lupus is a disease with few treatment regimens. Vitamin D as an immunomodulator has shown potential benefits in lupus patients. By decreasing the apoptosis rate in hyper activated cells and by inducing arrest in cell cycle progression, vitamin D can fine-tune the balance and probable reestablishment of tolerance. More studies are needed to better understand the real mechanisms and the ideal dose of vitamin D needed for beneficial effects on lupus patients.

This was a cross sectional study, and further longitudinal works in future will provide more data about the immunomodulatory and anti apoptotic effects of vitamin D in systemic lupus erythematosus.

Conclusion

Our results showed that vitamin D as an immunomodulator decreased the apoptosis rate in PBMCs of lupus patients and induced arrest in cell cycle progression. Presence of 1,25(OH)2D3 decreased the expression rate of FasL and Bax and increased the expression of Bcl-2 in lupus patients.

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