The Immunomodulatory effect of Vitamin D in Osteoimmunology

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ABSTRACT

Background and the purpose of the study: The aim of this study was to investigate whether Peripheral blood mononuclear cells from osteoporotic patients have a different expression profile of inflammatory and osteoimmunology markers. In addition, the in vitro anti-inflammatory properties of 1,25(OH)2D3 in osteoporotic patients was assessed. For this purpose, cytokines profile and RANKL/OPG system in osteoporotic patients in comparison with healthy controls was investigated.

Methods: Monocytes were isolated from peripheral blood of participants by the Ficoll density method, cultured and induced with vitamin D. Cells were harvested several times. RNA was extracted and then cDNA was synthesized. Inflammatory markers (IL-1β, IL- 6 and TNFα), bone metabolism markers (Osteoprotegrin and RANKL), vitamin D receptor (VDR) and β-actin genes were quantified by quantitative real-time reverse transcriptase.

Results and major conclusion: The expressions of the cytokines IL-6, TNF-α, IL-1, were all down regulated by 1,25(OH)2D3 in monocytes of all participants. Monocytes from healthy control in comparison to osteoporotic patients had a clear suppression pattern of inflammatory cytokines upon vitamin D3 incubation. Of noteworthy is that mRNA expressions of inflammatory cytokines in osteoporotic patients were clearly higher than controls. Results indicated that inflammation may have critical role in osteoporosis whereas osteoporotic patients have elevated pro- inflammatory profile. Vitamin D and its receptor may have Immunomodulatory effects in osteoimmunology. This regulatory function of VDR adds substance to the inflammatory theory behind osteoporosis pathogenesis.

Keywords: Vitamin D receptor, Osteoimmunology, Immunomodulatory , Cytokine, RANKL, Osteoporotegerin

INTRODUCTION

Bone remodeling is the result of activity of both osteoblasts and osteoclasts (1). The balance of these activities allows formation of strong bone (2). Bone remodeling varies by aging and different diseases. Immune responses can describe some of these changes. Osteoblasts and osteoclasts proliferation and differentiation are harmonized by a numeral of immune regulatory molecules (3). Cytokines have important roles in regulation of bone cells. The osteoblasts differentiation is geveened by TNF-α (4). Osteoblasts collagen production is reduced by IL-1, TNF-α and IFN-γ (5–8).

Osteoprotegrin (OPG) is a soluble decoy receptor that inhibits osteoclastogenesis (9) and bone mineralization has been changed by OPG overexpression in transgenic mice (10). Furthermore, OPG deficient mice were described as osteoporotic and found to have an excess of osteoclasts (11). Several immune and bone cells express the receptor activator of NFκ B ligand (RANKL). In inflammatory bone disease and in activated bone remodeling RANKL is highly expressed (10). Several investigations have indicated that OPG and RANKL balance are involved in bone homeostasis and osteoporosis pathogenesis (10-12). Also relation between the skeletal and immune systems may describe by RANKL/RANK/OPG system (13- 15).

This system may influence by numerous local and systemic factors, many of which like 1, 25 dihydroxyvitamin D_3 and parathyroid hormone (PTH) (16,17) are necessary for calcium homeostasis.

Also inflammatory cytokines such as TNFα and interleukin-1 (IL-1), IL-6 and IL- 11 have influence on RANKL expression (18-20). These cytokines are involved in physiological immune response but noticeable activities in some diseases caused by RANKL overproduction. This condition may be observed in age-related and postmenopausal bone loss

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and a variety of skeleton disorders with accelerated bone resorption (21).

The calciotropic hormones 1,25(OH)2D3 and PTH, as well as certain growth factors, cytokines, and prostaglandins, all regulate the expression of RANKL from stromal cells and osteoblasts (22). RANKL up-regulation can be induced by 1,25(OH)2D3 through its receptor (VDR) (23). However, in spite of wide interaction between bone homeostasis and the immune system, the process of cross talk and the biological inferences of this communications are inadequately investigated (2, 24).

The aim of this study was to investigate whether Peripheral blood mononuclear cells from osteoporotic patients have a different expression profile of inflammatory and osteoimmunology markers. In addition, the in vitro anti-inflammatory properties of 1,25(OH)2D3 in osteoporotic patients was investigated. For this purpose, cytokines profile and RANKL/OPG system in osteoporotic patients was determined and compared with those of healthy controls.

MATERIAL AND METHODS

Patient's characteristics

A total of 92 postmenopausal women (46 osteoporotic patients and 46 healthy controls) were included in this study. The osteoporotic patients were recruited from the bone mineral densitometry unit of the Endocrinology and Metabolism Research Center (EMRC) of the Tehran University of medical sciences and gave their informed consent along the rules of the ethics committee of the university. Patients and controls were free of infection and chronic disorders like endocrinology, gastrointestinal, malignancy and heart diseases at the time of sampling. Patient characteristics are summarized in table 1. In order to standardize the participant's profile, patients had a Z score in one of the measurement regions of the less than -1 and healthy control had Z score more than 1.

Peripheral blood mononuclear cells (PBMC's) isolated from blood (12 ml, heparin tube) was collected by venous puncture. Monocytes were isolated from peripheral blood through the Ficoll density method. In brief, blood was brought to a 1/1 solution with RPMI 1640 supplemented with Glutamax-I, 25mM HEPES (Cambrex Bio Science, Verviers, Belgium) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). Twenty-four ml of the solution was laid on top of 15 ml of a high density solution (LymphoprepTM, Axis-Shield PoCAS, Oslo, Norway) and centrifuged at a speed of 1500 rpm for 20 min. The middle layer containing peripheral blood mononuclear cells was harvested and washed twice.

Monocytes were cultured in RPMI 1640 supplemented with Glutamax-I, 25mM HEPES (Cambrex Bio Science, Verviers, Belgium) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) plus 15% FCS for 24 hrs. In parallel experiments, 1,25(OH)2D3 (10^{-7} M) (Sigma- Aldrich, St. Louis, MO) were added in some experiments at the beginning of culture.

Quantitative real-time RT-PCR

At each time point, 10^6 cells were harvested and RNA extraction performed using the High Pure RNA isolation kit (Roche Diagnostics). Inflammatory markers (IL-1β, IL-6 and TNFα) , bone metabolism markers (Osteoprotegrin and RANKL), vitamin D receptor (VDR) and β-actin genes were quantified by quantitative real-time reverse transcriptase (RT)- PCR as described in detail elsewhere. In brief, 10µl of extracted RNA was reverse transcribed using Revert Aid First Strand cDNA Synthesis Kit (Fermentase, EU). For subsequent Real Time PCR amplification a maximum of 2µl of each cDNA sample was used per 20 ml of Real Time PCR- mix. PCR reactions were performed in triplicate wells, in a Light Cycler (Roche Applied Science).

Data were obtained as cycle threshold (Ct) values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines and were used to determine differences in Ct values.

Results are represented as target copy number divided by β actin copy number. β Actin was used as housekeeping gene for sample normalization of this set of experiments after its assessment in a separate study which confirmed that its gene expression is not influenced by the imposed treatment and conditions of the study. All laboratory measurements were performed in Bio& Nano Unit of endocrinology and metabolism research center of Tehran university of medical sciences.

Statistical Analyses

The values are expressed as mean ± standard division. One sample Kolmogorov-Smirnov test was used to investigate the distribution characteristics of variables. Data for genes expression were log10 transformed in order to achieve the normality. For comparison of variables means in two groups, student t- test were employed.

Due to the fact that the data were not normally distributed non- parametric statistical namely, Mann-Whitney U- test to compare two groups at each time point of the study and Kruskal- Wallis tests to make comparisons between the three groups simultaneously. When compare the whole studied time period, the Kruskal-Wallis test was performed after calculation of the area under the curves. Statistical significance was considered, if a null hypothesis could be rejected at p value <0.05. The software SPSS for Windows was used for all statistical calculations.

RESULTS AND DISCUSSION

Totally 92 postmenopausal women (46 healthy and 46 osteoporotic patients) were recruited in

Table 1. Characteristic data of the population under study

Characteristic	Healthy control	Osteoporotic patients	P value
Age (years)	58.67 ±6.17	59.5 ±5.49	0.1
BMI (Kg/m^2)	29.96 ±4.1	30.02±4.47	0.7
Menarche age (years)	13.63 ±1.25	13.32±1.26	0.3
Hip BMD (gr/cm^2)	1.09±0.09	0.78 ±0.1	0.001
Spine BMD (gr/cm2)	1.25±0.13	0.96±0.1	0.001
Serum Osteocalcin (nmol/L)	6.03 ± 4.65	20.22 ±8.19	0.001
Serum Cross laps (pmol/L)	0.4 ± 0.21	1.03 ± 0.32	0.001
Serum IL1 (ng/mL)	0.57± 0.49	1.58± 1.36	0.01
Serum IL6 (ng/mL)	1.05 ± 0.99	2.63±2.08	0.02
Serum TNFα (ng/mL)	1.04± 0.66	2.85± 1.97	0.001

Values are expressed as mean ±SD, comparing of variables means in two groups performed by Student T test

the study. The characteristics of participants are summarized in table1. Osteoporotic patients had higher serum pro inflammatory cytokines (IL1, IL6 and TNFα) compared to the healthy group. Also serum Osteocalcin and cross laps concentration in osteoporotic patients were higher than healthy women (Table1).There were no significant differences in menarche age and body mass index between two groups but osteoporotic patients were older than the healthy control.

Serum concentration of IL6, TNFα, Osteocalcin and cross laps correlated significantly with age (IL6; p=0.01, r = 0.19, TNFα; p= 0.005, r = 0.21, Osteocalcin; p = 0.01, r = 0.18, cross laps; p = 0.001, r = 0.3). Serum concentration of IL6 correlated significantly with BMI (p = 0.001, r = 0.43).

In gene expression study, in freshly isolated monocytes from osteoporotic patients, elevated expression of IL-1β, TNF-α (Figure. 1A, p = 0.001 and Figure . 1C, p = 0.02, respectively) and border line result for IL- 6 (Figure 1 C, p = 0.05) could be noted, whereas RANKL expression levels was significantly elevated in osteoporotic patients (Figure 2 B, p=0.01). Also VDR was highly expressed in healthy control (Figure. 2A, p=0.01), whereas Osteoprotegrin expression was elevated in control group (Figure. 2C, p=0.01). To assess the effect of 1, 25(OH)2 D3 on gene expression, cells were exposed to 10^{-7} M 1,25(OH)2D3 for 0, 3, 8, and 24 hrs Total RNA was collected and used separately for cDNA synthesis. The cDNA was utilized as templates in the RT-PCR reactions. β-Actin was used as a housekeeping gene control.

The expression of the cytokines IL-6 (Figure. 3A), TNF-α (Figure. 3B), IL-1 (Figure. 3C), were all down regulated by 1,25(OH)2D3 in monocytes from all participants.

As it can be seen in figure. 1, monocytes from healthy control in comparison to osteoporotic patients had a clearly suppression pattern of inflammatory cytokines upon vitamin D3 incubation. Of noteworthy is that mRNA expressions of inflammatory cytokines in osteoporotic patients were clearly higher than controls (Figure. 1, $p < 0.05$). Osteoporotic patients had a higher expression in all time points, being significantly different from healthy controls ($p < 0.01$). Analysis over time, osteoporotic patients showed the higher pro-inflammatory cytokines expression, being different from controls (Figure. 1, $p < 0.001$).

As it can be seen in Figure . 1B, 1,25(OH)2D3 was capable of inducing RankL mRNA levels with a consistent time course.

Figure. 2A shows that VDR mRNA levels were up regulated in cells from healthy control whereas mRNA levels of osteoporotic patients remained at a basal level.

Baseline expression of VDR gene in monocytes from osteoporotic patients had significant correlation with baseline expression of TNF-α (p=0.03, r=19) and serum concentration of Osteocalcin (p=0.04, r=0.28). No correlation could be found between baseline expressions of VDR gene in monocytes from osteoporotic patients with baseline expression of RANKL, OPG genes.

In all time points in monocytes from all participants expression of VDR gene was correlated with RANKL/ OPG genes expression ratio (p=0.003, -0.22), also this ratio was correlated with Z score of hip (p=0.03, r = -0.15).

There are relations between the bone and immune system and they share many regulatory factors. Several cytokines are involved in bone resorption such as Interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-α (25). Expression of these cytokines enhance during estrogenic deficiency induced with menopause (26). There are several reports about the close relationship between inflammation and osteoporosis. Our results suggest that circulating monocytes are also expected to contribute to this inflammatory situation.

In this study it was shown that osteoporotic patients have elevated pro-inflammatory profile. This abnormal profile is mainly true in osteoporotic patients, confirming previous studies where osteoporotic patients presented higher plasma levels of cytokines

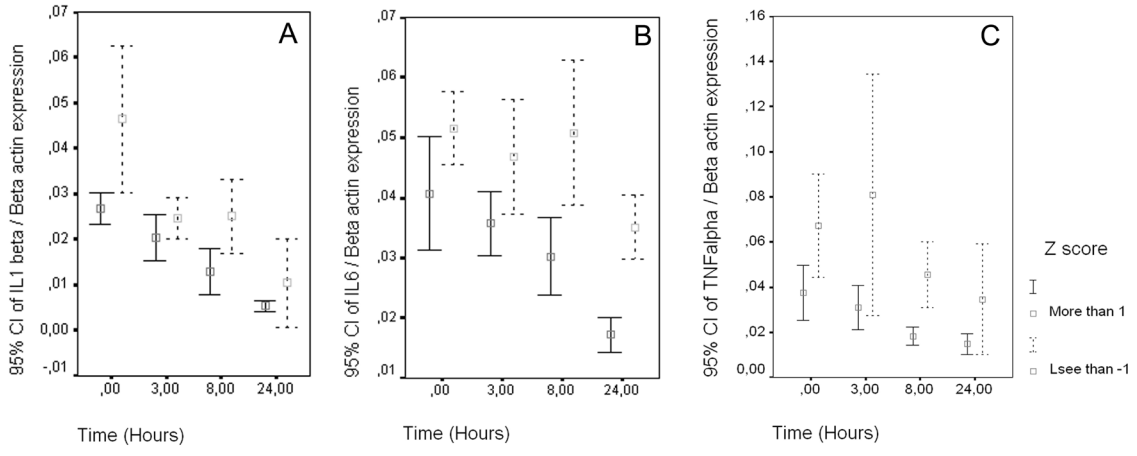

Figure 1. Expression of pro-inflammatory cytokines in healthy controls and osteoporotic patients. IL-1β [A] , IL-6 [B], and TNF-α [C] mRNA expression levels in freshly isolated Peripheral blood mononuclear cells [in time 0] and in Peripheral blood mononuclear cells incubated with vitamin D3 for 3, 8, 24 were quantified by real-time RT-PCR. Levels were normalized to β- actin. Data are represented as 95 Confidence Interval of transformed means by log10. Participants based on bone mineral densitometry data divided in two groups (Osteoporotic patients with Z score less than -1 and healthy group with Z score more than 1).

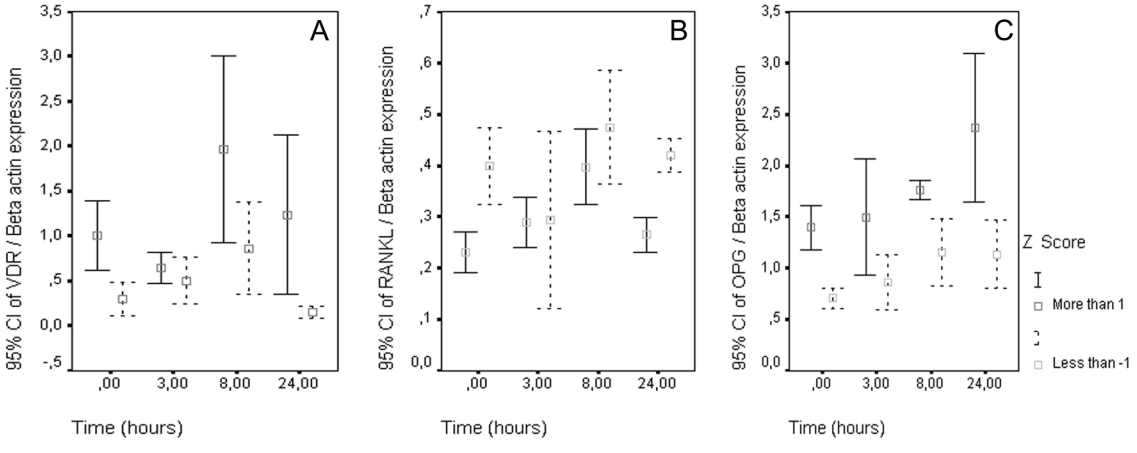

Figure 2. Expression of Vitamin D receptor and bone markers including RANKL and Osteoprotegrin in Peripheral blood mononuclear cells of the healthy controls and osteoporotic patients. VDR [A], RANKL [B], and Osteoprotegrin [C] mRNA expression levels in freshly isolated Peripheral blood mononuclear cells [in time 0] and in Peripheral blood mononuclear cells incubated with Vitamin D3 for 3,8,24 were quantified by real-time -PCR. Levels were normalized to β-actin. Data are represented as 95% Confidence Interval of transformed means by log10. Participants based on bone mineral densitometry data were divided in two groups (Osteoporotic patients with Z score less than -1 and healthy group with Z score more than 1).

such as TNF-α and IL-6. Gianni et al. reported that osteoporotic patients had high baseline expression of IL-6 and TNFα (27). Another study shows that TNF α involvement in the regulation of osteoclastogenesis and the bone remodeling and concludes that over expression of TNF α in osteoclasts progenitors (macrophages) may be concerned (28). In another study the downstream anti resorptive effects of TSH on the bone mediated by TNF α is described (29).

In the present study VDR expression PBMC's increased by induction of 1,25(OH)2D3.

In several studies, 1,25(OH)2D3 treatment increased VDR binding and VDR mRNA. 1,25(OH)2D3 treatment enhances the concentration of receptor that possibly improves the prolonged existence of the receptor (30). 1,25(OH)2D3 up-regulates VDR at the transcriptional level as well as by stabilization of the VDR protein after binding with the 1,25(OH)2D3 (30-33).

Thus, stabilization of VDR protein by 1,25(OH)2D3 is ligand- induced effect that decreases turnover of receptor. Also ligand induced stabilization of the VDR shows to be ubiquitous, and increased VDR binding has been observed in most target tissues which were examined (34, 35). Changes in VDR mRNA may be more tissue specific (36-39). Parathyroid hormone and estrogen up- regulate VDR expression (40).

In the present study it is clear that 1,25(OH)2D3 is a modulator of cytokine expression in monocytes.

Indeed, less effects were observed on cytokine expression when monocytes from osteoporotic patients were used, but important down- regulation of IL-6, TNF-a, IL-1 was observed when monocytes from healthy controls were induced with 1,25(OH)2D3. There is evidence that 1,25(OH)2D3 has modulatory effect on T cell-mediated immunity and also in other study immunosuppressant activities of the vitamin D hormone has been reported (41, 42). Similar immunosuppression was reported with analogs of vitamin D (43). Experimental study shows that 1,25(OH)2D3 inhibit production of both extra cellular and cell-associated immunoreactive IL1 alpha and IL1 beta and concluded that part of the ability of 1,25(OH)2D3 to inhibit T cell proliferation may be due to direct effects on monocytes by down-regulating of IL-1 production (44).

New cytokine that participate in osteoclastogenesis is considered by the receptor activator of NF-κB ligand (RANKL) (45). Our results indicated that RANKL gene expression in osteoporotic patients were higher than healthy control and this RNKL gene expression induced by 1,25(OH)2D3 which is known to induce RANKL up regulation primarily through actions initiated by the vitamin D receptor (25). Kitazawa and colleagues have reported a 1,25(OH)2D3 response in the mouse RANKL gene promoter and mapped this activity to a 16-bp vitamin D response element (VDRE) that was located 935 bp upstream of the transcriptional start site(46). RANKL expression can also be influenced by the glucocorticoides, stress hormones (47, 48), inflammatory cytokines such as TNF-α and IL-1 (20).

Osteoprotegerin (OPG) is a soluble member of the TNF receptor family that decoy receptor for RANKL that inhibits its interaction with RANK, thus preventing osteoclastogenesis (10). In this study OPG expression in the osteoporotic patients were lower than healthy controls. Also OPG gene expression induced by 1,25(OH)2D3. Mature osteoblasts play an inhibitory role in bone resorption, with active vitamin D metabolites acting through the VDR to increase OPG (49).

In the previous reports OPG were known as osteoclastogenesis inhibitory factor (9). which not only inhibits in vitro formation but also in vitro and in vivo bone resorption (9, 10).

CONCLUSION

In conclusion results of this study indicated that pro-inflammatory cytokine may have important role in osteoporosis. Also vitamin D and its receptor may interact with immune system to regulate bone turnover. This regulatory function of VDR adds substance to the inflammatory theory behind osteoporosis pathogenesis.

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