Int J Clin Exp Pathol 2016;9(3):3879-3888
www.ijcep.com /ISSN:1936-2625/IJCEP0022327

Original Article

Decrease in serum levels of interleukin-1β, interleukin-5 and interleukin-9 after vitamin D repletion in patients with chronic lymphocytic leukemia

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Received December 20, 2015; Accepted February 27, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Background: Vitamin D (VD) deficiency in chronic lymphocytic leukemia (CLL) is associated with a worse prognosis, a shorter time to start the treatment and the overall survival. VD deficiency is the first potentially modifiable prognostic factor in CLL that may interfere with the production of cytokines. However, there is the lack of studies concerning the VD repletion and its influence on interleukins in CLL patients. The aim of our prospective study was to assess the influence of cholecalciferol repletion on serum interleukins levels in CLL patients. Materials and methods: The study comprised 18 CLL patients. A six-month-long interventional study was conducted in CLL subjects with serum 25-OH-D_3 concentrations at the level of <30 ng/ml. Patients were supplemented with cholecalciferol. Interleukins levels were assessed at the beginning of the study and after a six-month-long supplementation of cholecalciferol. Baseline measurements of interleukins were compared to those in apparently healthy controls. Results: CLL patients were characterized by significantly increased levels of IL-1β, IL-4, IL-5, IL-6, IL-9, IL-12, IL-13 and IL-17, in comparison with healthy controls. During the VD supplementation a decrease in IL-1β, IL-1ra, IL-5, IL-9 and IL-17 serum levels was found. The decrease in IL-1β, IL-5 and IL-9 was observed in the subgroup of CLL patients who were not receiving chemotherapy, while in IL-17 in CLL patients on chemotherapy. Conclusion: The VD repletion may exert favorable influence on interleukins levels in VD deficient CLL patients.

Keywords: Chronic lymphocytic leukemia, vitamin D, interleukin

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the western hemisphere and its incidence is ever-growing. CLL has been defined as the accumulation of monoclonal neoplastic B lymphocytes [1] as a result of an imbalance between the proliferation and apoptosis of leukemic cells [2]. Diverse microenvironmental stimuli confer a growth advantage on these cells and extend their survival [3]. The most explicit manifestation of the impact of microenvironment on B-CLL is the rapid, spontaneous apoptosis of leukemic cells when cultured *ex vivo* [4]. Co-cultured stromal cells and certain cytokines can prevent this spontaneous apoptosis [5]. Signals delivered by microenvironment do not only favor clonal expansion of leukemic cells but can also cause drug resistance [6]. Numerous soluble factors and cell-to-cell interactions involved in the survival of neoplastic B-cells have been found [7]. Leukemic cells interact with several types of stromal cells, such as bone marrow stromal cells [8], nurse-like cells [9], T lymphocytes [10] and follicular dendritic cells [11]. Cytokines derived from stromal cells are well established factors, which support survival and growth in CLL cells [12].

IL-1, comprising IL-1α and IL-1β, is a pleiotropic cytokine, which plays a significant role in proin-

flammatory immune responses, triggered by binding with the IL-1 receptor (IL-1R) [13]. IL-1 receptor antagonist (IL-1ra), as a competitive inhibitor of IL-1 signaling, regulates activity of IL-1α and IL-1β [14]. IL-1β was found to be associated with the overall survival (OS) in CLL patients [15]. The recently found correlation between increased IL-1β concentration and significantly longer OS was somehow surprising, as the cytokine had been previously reported as a factor independently promoting *in vitro* CLL cell growth and survival [15]. IL-1β stimulates differentiation of naive T cells into Th17 cells [16], which secretes IL-17. Vitamin D insufficient young adults were found to have higher serum concentrations of pro-inflammatory cytokines, such as IL-1β [17].

IL-5 is a T helper 2 (Th2) cytokine that stimulates normal B-cell growth and differentiation, as well as increases immunoglobulin secretion, particularly IgA [18]. IL-5 suppresses cell-mediated immune responses crucial for an effective antitumor activity [19], thus, increased concentrations may support tumor growth. IL-5 is one of the best differentiators between CLL and healthy groups - in CLL levels of IL-5 are significantly higher [15]. Overexpression of IL-5 increases the risk of CLL transformation in mouse B cells [20]. The expansion of transcripts for IL-5 required the presence of CD5+ in CLL B [21]. Binding NFAT (nuclear factor of activated T cells) with an IL-5 promoter is crucial for IL-5 expression in human T cells [22]. In view of the significant role of NFAT in the transcriptional regulation of IL-5, agents which target NFAT might be valuable clinically, particularly in those diseases where the over expression of IL-5 is associated with pathology. $1,25(OH)_2D_3$ has been described to inhibit NFAT complex formation in activated normal T cells [23].

Th9 cells are a subset of Th cells that express IL-9 but no other Th cell lineage-specific cytokines [24]. IL-9 belongs to the IL-2 family, which is involved in the development and activation of lymphocytes. IL-9 binds with a heterodimeric receptor composed of the cytokine-specific α-chain (IL-9Rα) and the γ-chain [25] and activates the JAK-STAT pathway [26]. IL-9 is a multifunctional Th2 cytokine that was shown to stimulate the proliferation of thymic lymphomas and inhibit dexamethasone-induced apoptosis [27]. In CLL cytokine alterations, favoring regulatory T cells (Tregs) and Th2 response with inhibition of Th1 differentiation, have been described [28]. The dysregulated expression of IL-9 can be identified in biopsies and serums from patients with various hematologic malignancies [29] and reveals an unfavorable course of the disease [30].

IL-17, a proinflammatory cytokine secreted primarily by CD4+ Th17 cells [16], can mediate both pro- and antitumor actions [31] and may impact CLL growth, survival, or both. Because the Th17 cells produce high amounts of IL-17A, most Th17-mediated effects are attributed to this cytokine [32]. IL-17 signals through IL-17R and activates mitogen-activated protein kinases and nuclear factor κB (NF-κB) [33]. IL-17 induces IL-6 and IL-8 [34]. A lack of Th17 cells might contribute to the suboptimal immune response in non-Hodgkin's lymphoma [35]. Many of the IL-17 inflammatory actions can primarily benefit the host, but with the modify tumor microenvironment, IL-17 may promote tumor growth [31]. $1,25(OH)_2D_3$ is a regulator of Th cells and has been shown to inhibit IL-17 secretion by the Th17 cells [36].

Vitamin D deficiency is also associated with cardiovascular [37], and autoimmune diseases [38], as well as various types of cancer, infections and other diseases [39]. The majority of the known biological actions of $1,25(OH)_2D_3$ are mediated through the Vitamin D receptor (VDR) [40]. In the recent years, a resurgence of the interest in this vitamin has been spurred due to an expanded identification of VDR in various cells. The VDR expression was found in immune cells, such as antigen-presenting cells (monocytes, macrophages, dendritic cells), activated CD4+and CD8+T lymphocytes [41, 42]. Moreover, the immune system influences the capacity for a regulating activity of 1-α-hydroxylase in macrophages and dendritic cells [43]. Vitamin D plays a role in maintaining the balance between Th1 and Th2 profiles. $1,25(OH)_2D$ appears to inhibit Th1 cells and, under certain conditions, might support a shift to the Th2 phenotype [44]. Vitamin D deficiency may favor the disproportional production of Th17 and Th9 cells at the expense of regulatory IL-10 producing T cells [45]. Altogether these findings suggest paracrine immunomodulatory properties of vitamin D.

Additionally, higher concentrations of 25-OH-D_3 were found to be associated with a reduced risk of CLL [46]. Living in an area with higher ambient UVR was found to be associated with a reduced risk of NHL, especially DLBCL and CLL [47]. Furthermore, after the vitamin D supplementation CLL remissions have been reported [48]. Shanafelt et al. revealed shorter OS and time-to-treatment (TTT) in CLL patients with vitamin D insufficiency [49]. Association of vitamin D deficiency with poor prognosis was confirmed by Molica S. et al. [50] and Aref S. et al. [51]. Finally, vitamin D substitution enhances rituximab efficacy [52].

The aim of our prospective study was to assess the effect of cholecalciferol repletion on serum interleukins levels in CLL patients.

Materials and methods

Patients and samples

Eighteen patients with CLL who were not treated during the previous year and ten patients without CLL were enrolled between November 2013 and July 2015. The control group subjects compromised age-matched hospitalized patients, who met the same inclusion and exclusion criteria as the study group. The major exclusion criteria were malignancies, acute inflammatory diseases, history of renal, liver or heart failure and hypercalcemia. The protocol was approved by the Local Bioethics Committee and written informed consent was obtained from each participant. Blood specimens were collected after an 8h overnight fast during routine visit. Serum and plasma samples were collected and stored in liquid nitrogen till assessment of the cytokines concentration. All laboratory tests (25-OH-D_3, total calcium, ionized calcium, phosphate, alkaline phosphatase, creatinine, 24 h urine calcium excretion, intact parathormon (iPTH), complete blood count, IgG, IgA, IgM, LDH, β2-microglobulin, D-dimer) were repeated in the study group after 6 months of the VD supplementation. Secondary hyperparathyroidism was defined as iPTH level above manufacturer's reference range (15-68.3 pg/ml) but with a normal concentration of ionized calcium.

The decision concerning the incorporation of chemotherapy or the "watchful waiting" strategy was made according to the International Workshop on Chronic Lymphocytic Leukemia guidelines [53].

25-OH-D_3 measurements

The serum 25-OH-D_3 levels were measured on Cobas E422 Roche by electrochemiluminescent immunoassay (ECLIA) with the inter-assay variability below 10.3%.

Serum cytokines measurements

Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to measure cytokines in serum samples. Cytokines were quantitated using the Bio-Plex 200 System based on xMAP suspension array technology (Bio-Rad Laboratories Inc., Hercules, CA, USA). All procedures were followed according to the manufacturer's manual. Standard curves for each cytokine were created using standard solution with known concentrations of recombinant human cytokine of interest and performed during the same run as the subjects' serum analyses. Limits of detection (LOD) for measured cytokines were as follows: IL-1β - 0.6 pg/ml, IL-1ra - 5.5 pg/ml, IL-2 - 1.6 pg/ml, IL-4 - 0.7 pg/ml, IL-5 - 0.6 pg/ml, IL-6 - 2.6 pg/ml, IL-7 - 1.1 pg/ml, IL-9 - 2.5 pg/ml, IL-10 - 0.3 pg/ml, IL-12(p70) - 3.5 pg/ml, IL-13 - 0.7 pg/ml, IL-15 - 2.4 pg/ml, IL-17 - 3.3 pg/ml.

Intervention

VD deficiency was defined as a serum 25-OH-D_3 level <30 ng/ml. Subjects in the study group were divided into 3 categories according to 25-OH-D_3 levels: mild deficiency (20-30 ng/ml), moderate deficiency (10-19.9 ng/ml) and severe deficiency (<10 ng/ml). Patients with mild deficiency received 2000 IU/d of cholecalciferol, patients with moderate deficiency received 4000 IU/d and patients with severe deficiency received 6000 IU/d of cholecalciferol.

Patients qualified for the "watchful waiting" strategy received cholecalciferol and were defined as group 1. Subjects allocated in group 2 received cholecalciferol supplementation and chemotherapy. All patients in group 2 received rituximab, with an addition of fludarabine and cyclophosphamide in two cases (R-FC), cladribine and cyclophosphamide in two subjects (R-CC), cyclophosphamide, vincristine

Table 1. Characteristics of the patients with CLL and patients in the control group. Interleukins concentrations, age, BMI, β2-microblobulin and lymphocytes in bone marrow are presented as medians with an interquartile range (1-3 Q)

Characteristic	CLL [N=18]	Control [N=10]	*p* value
Age, years	67 (63-73)	62.5 (57-66)	0.13
Male, n (%)	9 (50%)	4 (40%)	0.68
BMI, kg/m^2	28 (25.3-30.8)	30.6 (28.0-34.0)	0.065
Concomitant diseases, n (%)			
Type 2 diabetes	4 (22%)	1 (10%)	0.45
Coronary artery disease	6 (33%)	1 (10%)	0.19
Hypertension	12 (67%)	5 (50%)	0.41
Binet stage A, n (%)	7 (39%)		
Binet stage B, n (%)	8 (44%)		
Binet stage C, n (%)	3 (17%)		
Genetics, n (%)			
del 11q	4 (22%)		
del 13q	9 (50%)		
del 13q (as a sole mutation)	6 (33%)		
del 17p	1 (6%)		
Trisomy 12	2 (11%)		
Previous chemotherapy	7 (39%)		
Time from diagnosis, years	2 (0-7)		
β2-microblobulin, mg/l	3.3 (2.6-5.2)		
Lymphocyte in bone marrow, %	51 (41-77.5)		
IL-1b, pg/ml	10.3 (9.6-11.5)	9.0 (8.0-10.0)	<0.05
IL-1Ra, pg/ml	349.9 (333.2-424.5)	333.2 (291.5-374.8)	0.16
IL-4, pg/ml	9.6 (7.6-10.4)	7.7 (6.7-8.9)	<0.05
IL-5, pg/ml	79.9 (71.7-90)	69.5 (60.3-79.4)	<0.05
IL-6, pg/ml	2.6 (2.6-4.8) Below detection range in 11 pts	2.6 (2.6-2.6) Below detection range in 10 pts	<0.05
IL-9, pg/ml	26.2 (12.9-43.1)	9.1 (2.5-22)	<0.01
IL-12, pg/ml	9.6 (3.5-27.4) Below detection range in 10 pts	3.5 (3.5-3.5) Below detection range in 10 pts	<0.05
IL-13, pg/ml	1.0 (0.7-6.7) Below detection range in 8 pts	0.7 (0.7-0.7) Below detection range in 9 pts	<0.05
IL-17, pg/ml	171.6 (144.4-184.6)	136 (109.3-145.7)	<0.05

and prednisone in two patients (R-CVP), and bendamustine in two cases (R-B). One patient with 17p deletion after 4 cycles of R-CC was given idelalisib.

Safety issues

The two major concerns related with the VD supplementation: hypercalcemia and hypercalciuria were evaluated by quantification of ionized calcium and 24 h urine calcium excretion. Hypercalcemia was defined as ionized calcium levels above 1.35 mmol/l and hypercalciuria as urinary calcium excretion over 5 mmol/24 h, or urinary calcium-to-creatinine ratio higher than 0.4 mg/mg.

Statistical analysis

Differences in variables between the groups at the beginning of the study, as well as after 6 months into the supplementation were examined by Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance. The change of serum, plasma and urine examinations were compared using the Wilcoxon signed-rank test for paired data. The value of limit of detection (LOD) was used when interleukin measurement was lower than LOD. All analyses were performed using the statistical package Statistica, version 10 (StatSoft, Inc.). P<0.05 was considered significant in all analyses.

Results

Characteristics of study subjects

The characteristics of the subjects are shown in **Table 1**. Serum levels of IL-2, IL-7, IL-10 and IL-15 were below detection range in all controls and in 16, 15, 12 and 17 of the 18 CLL patients respectively and therefore were excluded from

Table 2. Comparison of the baseline and a 6-month post supplementation characteristic in each group. Interleukins concentrations are presented as medians with an interquartile range (1-3 Q)

	Group 1 (VD) [N=8]		P	Group 2 (VD+CTH) [N=8]		P	Group 1 + Group 2 [N=16]		P
	Baseline	After 6 months		Baseline	After 6 months		Baseline	After 6 months	
IL-1β pg/ml	10.3 (9.8-12.8)	8.9 (8.3-9.6)	0.01	10.0 (8.9-11.2)	8.9 (8.7-9.5)	0.16	10.2 (9.5-11.4)	8.9 (8.5-9.5)	0.005
IL-1ra pg/ml	350 (329-424)	333 (296-375)	0.11	342 (333-424)	333 (317-358)	0.18	342 (333-424)	333 (304-358)	0.03
IL-5 pg/ml	83.6 (68.9-91.5)	72.8 (66.0-80.5)	0.049	76.7 (71.7-83.7)	67.2 (60.3-77.7)	0.03	77.8 (71.7-90.0)	70.6 (62.6-80.5)	0.003
IL-9 pg/ml	24.9 (12.9-60.9)	16.3 (10.2-43.3)	0.02	22.6 (12.3-42.6)	13.7 (8.3-20.3)	0,093	24.1 (12.3-47.9)	14.5 (8.8-21.8)	0.003
IL-17 pg/ml	164 (135-174)	148 (142-164)	0.99	172 (139-190)	121 (114-138)	0.04	168 (135-175)	140 (116-157)	0.11

analysis. CLL patients were characterized by significantly increased levels of IL-1β, IL-4, IL-5, IL-6, IL-9, IL-12, IL-13 and IL-17.

After chemotherapy in group 2 five patients obtained a partial remission of the disease and three patients achieved a complete remission. In group 1 the disease remained stable.

Vitamin D status and the cholecalciferol supplementation

In the group of the 18 analyzed CLL patients, only two patients had 25-OH-D_3 level within the optimal range (30-80 ng/ml) and the remaining 16 subjects were enrolled into further intervention. Eight patients had mildly reduced (insufficient) 25-OH-D_3 level, seven moderate and one a severe deficiency. In total, almost 90% had suboptimal levels of 25-OH-D_3. The mean 25-OH-D_3 level at the baseline for the study subjects was 19.8 ± 6.9 ng/ml (range 7.6-29.7). Sixteen patients received cholecalciferol supplementation in a mean dose of 3125 ± 1218 IU. Supplementation resulted in a significant increase of 25-OH-D_3 in study subjects ($P<0.001$), as well as in both subgroups ($P<0.01$). On completion of the study, the mean 25-OH-D_3 level was 38.45 ± 14 ng/ml (range 18.9-70), one patient had a moderate VD deficiency. Four subjects (25%) had a mild VD deficiency and eleven (69%) had optimal VD levels.

Serum iPTH concentration significantly decreased ($P<0.001$) after 6 months of the VD supplementation and almost all patients (94%) achieved normal iPTH level. None of patients developed hypercalciuria, as well as hypercalcemia during the study. Patients did not complain of constipation.

Changes of interleukins levels after the cholecalciferol repletion

During the VD supplementation some significant changes (decreases) in IL-1β, IL-ra, IL-5, IL-9 and IL-17 serum levels were found (**Table 2**). The decrease of IL-1β, IL-5 and IL-9 was observed in CLL patients on the VD supplementation exclusively, while of IL-17 in CLL patients on CTH and the VD supplementation. Changes in serum levels of IL-4, IL-6, IL-7, IL-10, IL-12, IL-13 and IL-15 were not statistically significant.

Discussion

VD deficiency in CLL is associated with worse prognosis, shorter time to start treatment and the overall survival. It is the first potentially modifiable prognostic factor in CLL [51], which can modify the production of various cytokines that play an important role in the course of CLL [15]. In this prospective study we found that the VD repletion is associated with IL-1β level decrease. This is in agreement with the previous report, which showed that a seasonal variation in the VD status is associated with a decreased production of IL-1β during the summer [54]. Prognostic significance of IL-1β remains a questionable issue: some researchers found lower mRNA levels of IL-1β in CLL cells obtained from patients with progressive disease [55], whereas others [56] reported a higher expression of this cytokine in subjects with an unfavorable course of the disease. IL-1β was recently found to be correlated with positive prognostic markers and increased survival [15]. Additionally, a reduced expression of IL-1β was found in CLL cells obtained from patients with shorter survival and at higher Rai stages [57]. On the other hand, the expression of IL-1β is increased in the presence of ZAP70

[58], which is a well-known marker of a poor prognosis. This inconsistency could illustrate a changeable expression of cytokines during the course of the disease, as well as both pro- and antitumor activities in diverse contexts [59].

The VD repletion was also found to suppress the production of IL-5. It is a Th2 cytokine overexpressed in CLL [60], which can support tumor growth by suppressing cell-mediated immune response [19], and is related with shorter TFT [15], and a worse response to lenalidomide [61]. However, *in vitro* studies revealed that IL-5 increases a spontaneous apoptosis of CLL cells [62]. Our results, which show that the VD repletion decreases IL-5, are consistent with the previous in vitro findings concerning asthma [63] and may lead to a favorable effect on the course of CLL.

IL-9 was among the interleukins, which serum levels were increased in CLL and suppressed by the VD repletion. The increased serum IL-9 levels in CLL patients was previously found and was associated with adverse prognostic factors, such as ZAP-70 expression, β2-microglobulin expression, IGVH status and Rai & Binet classification [64]. The suppressive effect of VD on IL-9 levels was previously shown *in vitro* [63]. The beneficial consequences of IL-5 suppression related to the VD supplementation in CLL remains unknown.

We have demonstrated the increased levels of IL-17 in CLL patients, like some [65], but not all studies [60]. In general, higher IL-17 levels are considered beneficial. IL-17 activates immune cells and favors antitumor activity of immune system. It was shown, that low IL-17 levels correlate with a progression of CLL and a need of chemotherapy [60]. We have demonstrated that not the VD itself, but rather chemotherapy suppresses the production of IL-17, which is in line with the previous studies, demonstrating a decrease in Th17 cell number level after a therapy with rituximab [66] and chlorambucil [65], but not lenalidomide [67].

Furthermore, patients with CLL were found to have higher serum levels of IL-6, IL-12 [15], IL-1Ra, IL-13 and IL-15 [60], which is consistent with our findings. The levels of these interleukins did not change after the VD repletion, which is in agreement with other studies [68].

The results of our study demonstrate that the VD repletion in CLL patients may not only optimize musculoskeletal health [69], but also affect the function of the immune system (resulting in the suppression of the production of some interleukins) and potentially improve the clinical course of the disease. The VD deficiency can be easily determined by blood testing and treated according to the available recommendations for general population. Large, multicenter, preferentially randomized studies are necessary to demonstrate the optimal VD levels in CLL patients.

The main limitation of the study was the number of the enrolled patients, high variability of interleukins levels and the limited sensitivity of the method used in the detection of some interleukins. Further studies are needed to evaluate the effect of the cholecalciferol supplementation on the levels of other interleukins.

In conclusion, the VD repletion may have a favorable effect on interleukins levels in VD deficient CLL patients.

Acknowledgements

The study was support by grant to M. K. from Medical University of Silesia (KNW-2-044/D/4/N). M. K. received a scholarship as part of the DoktoRIS-Scholarship Program for Innovative Silesia co-financed by the European Union under the European Social Fund.

Disclosure of conflict of interest

None.

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