

Vitamin D Supplementation and Regulatory T Cells in Apparently Healthy Subjects: Vitamin D Treatment for Autoimmune Diseases?

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ABSTRACT: **Background:** Epidemiological data show significant associations of vitamin D deficiency and autoimmune diseases. Vitamin D may prevent autoimmunity by stimulating naturally occurring regulatory T cells.

Objectives: To elucidate whether vitamin D supplementation increases Tregs frequency (%Tregs) within circulating CD4+ T cells.

Methods: We performed an uncontrolled vitamin D supplementation trial among 50 apparently healthy subjects including supplementation of 140,000 IU at baseline and after 4 weeks (visit 1). The final follow-up visit was performed 8 weeks after the baseline examination (visit 2). Blood was drawn at each study visit to determine 25-hydroxyvitamin D levels and %Tregs. Tregs were characterized as CD4+CD25++ T cells with expression of the transcription factor forkhead box P3 and low or absent expression of CD127.

Results: Forty-six study participants (65% females, mean age \pm SD 31 \pm 8 years) completed the trial. 25(OH)D levels increased from 23.9 \pm 12.9 ng/ml at baseline to 45.9 \pm 14.0 ng/ml at visit 1 and 58.0 \pm 15.1 ng/ml at visit 2. %Tregs at baseline were 4.8 \pm 1.4. Compared to baseline levels we noticed a significant increase of %Tregs at study visit 1 (5.9 \pm 1.7, $P < 0.001$) and 2 (5.6 \pm 1.6, $P < 0.001$).

Conclusions: Vitamin D supplementation was associated with significantly increased %Tregs in apparently healthy individuals. This immunomodulatory effect of vitamin D might underlie the associations of vitamin D deficiency and autoimmune diseases. Hence, our finding provides a rationale for further studies to investigate vitamin D effects on autoimmunological processes.

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Vitamin D deficiency has been associated with several adverse health consequences including autoimmune diseases [1-3]. This is of particular interest when considering the high prevalence of hypovitaminosis D, which affects almost half the world's population. Reduced sunlight exposure is mainly responsible for this pandemic of vitamin D deficiency because approximately 80–90% of circulating vitamin D originates from ultraviolet B-induced vitamin D production in the skin, whereas dietary vitamin D intake plays only a minor role. Vitamin D from both sources is hydroxylated to 25-hydroxyvitamin D in the liver and is then further hydroxylated by 1 α -hydroxylase to 1,25-dihydroxyvitamin D (1,25[OH]2D), which is the most active vitamin D metabolite. It was recently discovered that apart from the kidney, various organs and cells – such as those of the immune system – also express 1 α -hydroxylase [1,4]. The vitamin D receptor, which regulates about 3% of the human genome, is also expressed in immune cells, suggesting physiologic immunomodulatory effects of the vitamin D endocrine system [1-5].

Reduced 25(OH)D levels, which are used to classify the vitamin D status, have been observed in several autoimmune diseases [2,3,6-10]. In addition, hypovitaminosis D as well as low vitamin D intake have been identified as a risk factor for the development of autoimmune diseases, such as type 1 diabetes mellitus or multiple sclerosis [1-3]. In mouse models for autoimmune diseases, vitamin D was shown to prevent type 1 diabetes and experimental autoimmune encephalitis [3]. Accumulating evidence supports the notion that vitamin D could prevent these autoimmune diseases as well as allograft rejection, by increasing the frequency or the effects of naturally occurring regulatory T cells [2,3]. Tregs, which are critical for maintaining immune tolerance, are characterized by high surface expression of CD4 and the interleukin-2 receptor (CD25), low or absent expression of the IL-7 receptor (CD127), and by expression

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Tregs = regulatory T cells
25(OH)D = 25-hydroxyvitamin D

IL = interleukin

of the transcription factor forkhead box P3 [11-14]. In animal and cell culture studies, tolerogenic dendritic cells are induced by active vitamin D treatment and promote the induction of Tregs, which are suggested to prevent autoimmune diseases due to their immunosuppressive activity [5,15-21]. In human renal transplant recipients, 1,25(OH)2D treatment over 6 months significantly increased the percentage of CD4+CD25+ T cells of total peripheral lymphocytes [22]. These data suggest that vitamin D supplementation may increase circulating Tregs in humans. This issue has, to our knowledge, not been addressed in previous studies. We therefore conducted a pilot trial to examine whether vitamin D supplementation in apparently healthy individuals increases the percentage of Tregs within 20,000 circulating CD4+ T cells.

PATIENTS AND METHODS

We investigated 50 apparently healthy subjects aged at least 18 years, who were recruited at our outpatient clinic (Department of Internal Medicine, Division of Endocrinology and Nuclear Medicine, Medical University of Graz, Austria). In addition, we invited colleagues of our department as well as their friends and family members to participate in our pilot trial. Exclusion criteria were hypercalcemia (serum calcium > 2.65 mmol/L), pregnancy, participation in other interventional clinical trials, and any disease requiring medical treatment. The study was conducted in the outpatient clinic of our department from February to June 2009. Written informed consent was obtained from all study participants. The study was performed in adherence to the Declaration of Helsinki and we obtained approval from the ethics committee at the Medical University of Graz, Austria.

In this uncontrolled, monocentric pilot trial we performed a baseline visit (visit 0) and two follow-up visits, 4 weeks (visit 1) and 8 weeks (visit 2) after the baseline examination. At each study visit blood was drawn after an overnight fast between 7 and 11 a.m. and a pregnancy test was performed in all female study participants. At baseline (visit 0) and at study visit 1, all subjects with missing exclusion criteria received 140,000 IU vitamin D3 orally (Oleovit D3[®], Fresenius Kabi, Austria). Anthropometric measurements (height and weight) were performed with the study participants wearing light clothes and no shoes. Body mass index was calculated as weight (in kilograms) divided by the height squared (in meters).

LABORATORY METHODS

Peripheral blood mononuclear cells were isolated within 24 hours using Histopaque-1077 Hybri Max (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation from sodium-heparinized blood samples and washed twice in Hank's buffered salt solution (Invitrogen, New Zealand). After counting and staining with 0.4% Trypan blue solution (Sigma-Aldrich) to examine the purity and viability, all samples were frozen in

fetal bovine serum (Invitrogen) containing 10% DMSO (Sigma-Aldrich) in a controlled-rate automated freezing device to -80°C and then stored in liquid nitrogen. All experiments were performed on thawed cells. An aliquot was counted and stained with 0.4% Trypan blue solution as described above. Cells were stained for surface antigens with the following fluorochrome-conjugated monoclonal antibodies, all purchased from BD Pharmingen (San Diego, CA, USA): anti-CD4 FITC (fluorescein isothiocyanate), anti-CD 25 PE-Cy7 (phycoerythrin cyanin 7) and anti-CD127 PE (phycoerythrin). After surface staining, cells were permeabilized with a special buffer (BD Pharmingen) and intracellular staining for the transcription factor FOXP3 was performed using anti-FOXP3-Alexa Fluor 647 monoclonal antibodies (BD Pharmingen) according to the manufacturer's instructions. To avoid having to measure signals developed from unspecific binding of FOXP3 antibody, an Alexa Fluor 647 isotype control (BD Pharmingen) was prepared for each sample. At least 20,000 CD4-positive events were acquired from each sample on a BD FACSCanto II and analyzed with FACS-Diva software (Vers 6.1.2). CD4+CD25++FOXP3+ cells with low or absent expression of CD127 were classified as Tregs.

25(OH)D was determined by means of a commercially available enzyme-linked immunosorbent assay (IDS, Bolden, UK) with an intra- and interassay coefficient of variation of 5.6 and 6.4%, respectively. C-reactive protein was measured by Tina quant CRP immunoturbidometric assay (Roche COBAS INTEGRA, Germany). Other laboratory measurements were performed by routine methods.

STATISTICAL ANALYSIS

Baseline characteristics are presented as means ± standard deviation for continuous variables and as percentages for categorical variables. Descriptive statistics and Kolmogorov Smirnov test were used to test for normality of the distribution of the continuous variables. 25(OH)D levels followed a skewed distribution and were thus logarithmically transformed before use in parametric procedures. We performed Pearson correlation analyses of %Tregs and 25(OH)D at each study visit. In addition, we used %Tregs and 25(OH)D values from all study visits for a correlation analysis. Paired Student's *t*-test was used to test for differences in %Tregs, 25(OH)D and serum calcium between the study visits. Statistical analyses were performed by SPSS version 16.0 (SPSS Inc, Chicago) and a *P* value below 0.05 was considered statistically significant.

RESULTS

Of the 50 study participants 46 completed the trial. Follow-up visits were performed 4.4 ± 0.5 (mean ± SD) weeks (visit 1) and 8.8 ± 1.0 weeks (visit 2) after the baseline examination.

FOXP3 = forkhead box P3

Table 1. Baseline characteristics

No. of patients	50
Females (%)	64
Age (yrs)	31 ± 8
Height (cm)	171.7 ± 8.8
Weight (kg)	68.8 ± 13.7
Body mass index (kg/m ²)	23.3 ± 4.3
C-reactive protein (mg/L)	2.2 ± 2.7
Serum calcium (mmol/L)	2.4 ± 0.99
25-hydroxyvitamin D (ng/ml)	24.1 ± 12.6

Continuous variables are presented as means ± standard deviation (SD)

After the baseline visit, one study subject was excluded because he was also participating in another interventional trial and another participant was excluded due to previously diagnosed type 1 diabetes mellitus. After study visit 1, we excluded a participant due to non-compliance with the study protocol and another one because of mild asymptomatic hypercalcemia (serum calcium 2.67 mmol/L, normal range 2.20–2.65). In this individual, hypercalcemia had resolved spontaneously by the time of the follow-up examination. We observed no clinically significant adverse event during the study.

Clinical and laboratory baseline characteristics of all 50 study participants are shown in Table 1. At baseline, 80% of our study subjects had 25(OH)D levels below 30 ng/ml, which indicates an insufficient vitamin D status. 25(OH)D levels increased from 23.9 ± 12.9 ng/ml (mean ± SD) at baseline to 45.9 ± 14.0 ng/ml at visit 1 ($P < 0.001$) and to 58.0 ± 15.1 ng/ml at visit 2 ($P < 0.001$). At visit 2, all study subjects had a sufficient vitamin D status (25[OH]D levels > 30 ng/ml).

%Tregs within 20,000 CD4+ cells were 4.8 ± 1.4 at baseline. Compared to baseline values %Tregs were significantly increased at visit 1 (5.9 ± 1.7, $P < 0.001$) and at visit 2 (5.6 ± 1.6, $P < 0.001$). %Tregs were significantly lower at visit 2 compared to visit 1 ($P = 0.011$). Pearson correlation coefficients of %Tregs and 25(OH)D levels were 0.223 ($P = 0.120$) at baseline, 0.092 ($P = 0.530$) at visit 1 and 0.170 ($P = 0.259$) at visit 2. When %Tregs and 25(OH)D levels of all study visits were used for correlation analysis the Pearson correlation coefficient was 0.315 ($P < 0.001$).

CRP (normal range 0 to 8 mg/l) was 2.3 ± 2.8 at baseline, 2.0 ± 1.9 at visit 1, and 2.4 ± 2.7 at visit 2. There was no statistically significant difference in CRP levels between any of the study visits. In addition, there was no significant difference between serum calcium levels at baseline (2.37 ± 0.09) and at visit 1 (2.36 ± 0.11, $P = 0.486$). Compared to baseline,

however, we observed a significant decrease in serum calcium at visit 2 (2.30 ± 0.09, $P < 0.001$).

DISCUSSION

This 8 week pilot trial demonstrated that vitamin D supplementation of 140,000 IU at baseline and after 4 weeks was associated with a significant increase in %Tregs among apparently healthy subjects. This finding supports the hypothesis that vitamin D-induced stimulation of Tregs is a possible pathophysiologic mechanism by which vitamin D may prevent autoimmune diseases.

Tregs are critical for controlling immunological tolerance to self-antigens [11-14]. They originate in the thymus but may also derive from peripheral CD4+ T cells following antigen stimulation [11-14]. The current literature supports the concept that Tregs suppress autoreactive effector T cells at the site of inflammation and in the draining lymph nodes [12]. Their immunomodulatory effects involve cytotoxic actions on pathogenic T cells, which are mediated through cell-to-cell contact [12]. In addition, Tregs modulate cytokine profiles at the site of inflammation and secrete cytokines such as transforming growth factor-beta and IL-10, which exert anti-inflammatory actions [12-14]. Mounting evidence linking reduced activity of Tregs to risk of autoimmune diseases and graft-versus-host disease in transplant recipients has already stimulated research work aiming to evaluate and introduce the use of “Tregs therapy” in the treatment of autoimmune diseases and GVHD [13]. In this context, vitamin D may be useful as a therapeutic agent because it exerts immunomodulatory effects that may involve stimulatory actions on Tregs [1-5]. Towards this, previous studies suggest that the impact of vitamin D on dendritic cells results in the development of a tolerogenic dendritic cell type that is able to induce Tregs [5].

In our study we tested the effect of vitamin D supplementation on %Tregs. To the best of our knowledge we are the first to show that vitamin D intake at relatively high doses significantly increases %Tregs in the peripheral circulation. This finding is in line with previous data showing increased Tregs in the draining lymph nodes of mice that were treated with topical application of the active vitamin D analog calcipotriol [19].

Accumulating evidence from experimental studies underlines the importance of vitamin D for Tregs stimulation, but data from clinical studies in humans are sparse [15-18,22]. Previous data on a significant increase of CD4+CD25+ T cells after calcitriol treatment in renal transplant recipients are limited due to the missing determination of FOXP3, which is important to differentiate naturally occurring Tregs from other CD4+CD25+ T cells [22]. Hence, our study, which includes an accurate characterization of Tregs, significantly extends the current

CRP = C-reactive protein

GVHD = graft-vs-host disease

knowledge on this topic. Interestingly, %Tregs, which markedly increased after vitamin D intake, were significantly higher at visit 1 than at visit 2. Underlying mechanisms for this difference remain speculative. However, it could be hypothesized that there exists a U-shaped association of vitamin D status and Tregs with optimal stimulatory effects on Tregs at 25(OH)D levels close to those at visit 1 and fewer effects on Tregs at higher and lower 25(OH)D levels. It should also be pointed out that there was no significant correlation of %Tregs and 25(OH)D levels at any of our study visits. There was, however, a significantly positive correlation of %Tregs and 25(OH)D level when the values of %Tregs and 25(OH)D at all study visits were used for a correlation analysis. Importantly, there was no significant change in CRP levels between the study visits, suggesting that infections or other unspecific inflammatory stimuli did not contribute to the observed changes in %Tregs.

Beyond autoimmunity and GVHD, our finding of increased %Tregs after vitamin D supplementation may also have implications for cardiovascular diseases because transfer of Tregs to apolipoprotein E-deficient mice led to reduced atherosclerotic lesion formation and increased plaque stabilization [23]. In humans, patients with acute coronary syndromes had significantly reduced numbers of Tregs when compared to patients with stable angina pectoris who had a similar extent of coronary atherosclerosis [23]. Hence, immunomodulatory effects of vitamin D might be relevant for several chronic diseases and this may hypothetically underlie the increased mortality in persons with a poor vitamin D status [24,25].

The main limitation of our work is the lack of a placebo group in this uncontrolled pilot trial. It should also be noted that we measured circulating Tregs and it is still unclear whether an increase of these peripheral Tregs is also associated with an increase of Tregs effects at the site of local inflammation. Apart from this, it still remains to be elucidated whether changes also occur in the immunosuppressive activity of Tregs after vitamin D supplementation. This could be measured in cell culture experiments by demonstrating reduced activity of Tregs (autoreactive effector T cells) in the presence of Tregs, and it remains an interesting research question for future studies [14].

In conclusion, our study results suggest that vitamin D supplementation increases %Tregs in apparently healthy subjects. This proposed immunomodulatory effect of vitamin D might be a key mechanism by which vitamin D exerts protective effects against autoimmunity. Our data might therefore serve as a rationale for further placebo-controlled trials to substantiate the beneficial effects of vitamin D supplementation on immunological processes related to dysfunctions of Tregs.

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