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Evaluation of the Relationship Between T Regulatory Cells and Vitamin D Levels in Chronic Spontaneous Urticaria

Kronik Spontan Ürtikerde T Regülatuvar Hücre Düzeyi ve D Vitamini Düzeyi İlişkisinin Değerlendirilmesi

Abstract

Objective: T regulatory (Treg) cells play a role in autoimmunity and vitamin D is one of the factors involved in regulation of Treg cells. In this study, it is aimed to evaluate the relationship between Treg cells and disease-related parameters and serum vitamin D levels in chronic spontaneous urticaria (CSU) patients.

Methods: The percentage of Treg cells were evaluated by flow cytometric analysis and serum 25 hydroxy vitamin D (25(OH)Vit D) levels were determined by commercial available ELISA kit.

Results: Thirty eight CSU patients and 30 healthy controls were included in the study. The percentage of CD4⁺CD25⁺ and CD4⁺FOXP3⁺ T cells in the patient group were found lower than in the control group (p=0.018, p=0.000). No difference was detected between groups in terms of the percentage of CD4⁺CD25⁺FOXP3⁺ T cells and the 25(OH) Vit D levels (p=0.192, p=0.218). There was no significant relationship between disease duration, weekly urticaria activity score, autologous serum skin test (ASST), serum 25(OH) Vit D levels and the percentage of Treg cells in CSU patients.

Conclusion: The percentage of CD4⁺CD25⁺ and CD4⁺FOXP3⁺ T cells in CSU patients were observed to be lower than control group independent from the serum 25(OH)Vit D levels, ASST positivity, disease duration and severity. This result suggests that Treg cells are one of the factors involved in the pathogenesis of CSU.

Keywords: Chronic spontaneous urticaria, T regulatory cells, vitamin D, urticaria activity score, autologous serum skin test, autoimmunity

Öz

Amaç: T regülatuvar (Treg) hücreler otoimmünitede rol alır ve D vitamini Treg hücre regülasyonunda rol alan faktörlerden biridir. Bu çalışmada kronik spontan ürtiker (KSÜ) hastalarında Treg hücreleri ile hastalık ilişkili parametrelerin ve serum vitamin D düzeyi ilişkisinin değerlendirilmesi amaçlanmıştır.

Yöntemler: Treg hücrelerinin yüzdeleri flow sitometrik analiz ile serum 25 hidroksi vitamin D (25(OH)Vit D) düzeyi ise ticari hazır ELISA kiti ile değerlendirildi.

Bulgular: Çalışmaya 38 KSÜ ve 30 sağlıklı kontrol dahil edildi. CD4⁺CD25⁺ ve CD4⁺FOXP3⁺ T hücre yüzdesi hasta grubunda kontrol grubuna göre düşük düzeyde bulundu (p=0,018, p=0,000). CD4⁺CD25⁺FOXP3⁺ T hücre yüzdesi ve serum 25(OH)Vit D düzeyi açısından gruplar arasında farklılık saptanmadı (p=0,192, p=0,218). KSÜ hastalarında hastalık süresi, haftalık ürtiker aktivite skoru, otolog serum deri testi (OSDT) ve serum 25(OH)Vit D düzeyi ile Treg hücre yüzdesi arasında anlamlı ilişki tespit edilmedi (p>0,05).

Sonuç: Çalışmamızda KSÜ hastalarında CD4⁺CD25⁺ ve CD4⁺FOXP3⁺ T hücrelerinin serum 25(OH)Vit D, OSDT pozitifliği, hastalık süresi ve şiddetinden bağımsız olarak kontrol grubuna göre düşük olduğu gözlemlendi. Bu sonuç Treg hücrelerinin KSÜ patogenezinde yer alan faktörlerden biri olduğunu düşündürmektedir.

Anahtar kelimeler: Kronik spontan ürtiker, regülatuvar T hücreleri, D vitamini, Ürtiker Aktivite Skoru, otolog serum deri testi, otoimmünite

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Introduction

Chronic spontaneous urticaria (CSU) is used to describe urticaria persists longer than 6 weeks by excluding the inducible chronic urticaria according to the European Academy of Allergology and Clinical Immunology (EAACI)/the Global Allergy and Asthma European Network (GA²LEN)/the European Dermatology Forum (EDF)/World Allergy Organization (WAO) guideline (1). CSU can be associated with autoimmune disorders and antibodies against high-affinity immunoglobulin E (IgE) receptor and IgE can be found in CSU patients (2,3). The role of tissue T cells in chronic urticaria pathogenesis has been investigated and CD4⁺ T cells is thought to play important role in the pathogenesis of the disease (4,5).

T regulatory (Treg) cells are one of the T cell subtypes playing a role in immune tolerance and the inflammatory response against self-antigens and are co-expressed as CD4, CD25 and FOXP3 (6,8). Treg cells are classified as CD4⁺CD25⁺, CD4⁺FOXP3⁺ and CD4⁺CD25⁺FOXP3⁺ (9). Treg cells have been reported to have lower percentages in the blood or defective function in allergic, autoimmune disorders and also chronic urticaria (10-15).

Vitamin D is considered to be an immune modulatory molecule and low vitamin D levels have been reported in autoimmune disorders (16). Vitamin D has been shown to inhibit T cell proliferation and regulate Treg cells differentiation and activation (17-19).

We aimed to evaluate the relationship between Treg cells and disease-related parameters and the vitamin D levels in CSU patients in this study.

Methods

A total of 38 CSU patients and 30 healthy volunteers were included in the study. All patients and volunteers were involved to the study after having received informed consent. This study was approved by Eskişehir Osmangazi University Clinical Research Ethics Committee (approval number: 04-2016). In accordance with the EAACI/GA²LEN/EDF/WAO guideline, patients diagnosed with CSU were included in the study (1). Subjects who were pregnant or lactating, were suffering from or treated for liver or kidney dysfunction, those with a history of systemic inflammatory disease, who had received systemic immunosuppression treatment within the past month, or with an accompanying dermatologic disease other than CSU were excluded. The demographic information of the patients and disease-related parameters; duration of urticaria, autologous serum skin test (ASST) positivity and weekly urticaria activity score (UAS7) were evaluated.

The Evaluation of Urticaria Activity Score

UAS was used to evaluate the disease activity in CSU patients. The number of raised plaques (0, none; 1, <10 plaques; 2, 10-50 plaques; 3, >50 plaques) and pruritus severity (0=no pruritus; 1=mild; 2=moderate; 3=severe) were scored. The UAS score was determined as the weekly UAS7 between 0 and 42 points (1).

Evaluation of Autologous Serum Skin Test

ASST evaluation was performed according to EAACI/GA²LEN/EDF/WAO (1). The result was accepted as positive if the diameter of the erythematous papule response to autologous serum was 1.5 mm or more larger than the response to physiological saline.

Collection of Blood Samples

Serum blood samples were collected between October and April, considering the effect of ultraviolet radiation on the vitamin D level. A total of 5 mL of venous blood was collected from the patients and controls. Two mL was placed in a tube containing tri-potassium ethylenediaminetetra-acetic acid for flow cytometric analysis. The other 3 mL was collected in a plain tube, centrifuged, and stored at -80 °C until it was used for assessment of vitamin D. The serum 25 hydroxy vitamin D (25(OH)Vit D) levels were assessed with a commercial ELISA kit.

Flow Cytometric Analysis

Cell surface markers on peripheral mononuclear cells were evaluated with CD4-peridinin-chlorophyll proteins (eBioscience) and CD25-fluorescein isothiocyanate (BD Bioscience: San Jose, California, USA) surface antigens. We then added 100 µL blood with the cell content adjusted to 1x10⁶. Incubation for 15-20 min at room temperature followed. The next step was a 10 min incubation with 2 cc of erythrocyte lysing solution at room temperature. The solution was then centrifuged at 1.800 rpm for 5 min. The supernatant was removed and washed with phosphate buffer saline (PBS). The pellet was vortexed after adding 1 mL freshly prepared fixation/permeabilization solution and kept in the dark for 30-60 min at 4 °C. It was then washed twice with 2 mL permeabilization solution. The supernatant was again removed and 20 µL (FOXP3) phycoerythrin (eBioscience) was added after keeping the solution in the dark at °C for 30 min. Permeabilization solution at a volume of 2 mL was then used to wash 2 times. The supernatant was taken for resuspension with PBS. We then counted 25.000-30.000 cells with FACSCalibur flow cytometry. Only the CD4⁺ helper T cells were gated for the analysis (Figure 1).

Statistical Analysis

The IBM SPSS Statistics 21.0 software program was used for data analysis. Continuous data are presented as mean ± standard deviation. Categorical data are presented as percentages (%). The Shapiro-Wilk test was used to analyze the compliance of the data with a normal distribution. The Mann-Whitney U test was used in the comparison of the groups not complying with a normal distribution. The presence of correlation between the data was evaluated with the Spearman correlation test. P<0.05 was accepted as the criterion for statistical significance.

Results

We included a total of 38 CSU (29 females, 9 males) patients and 30 healthy controls (21 females, 9 males) in the study.

The mean age was 43.7 ± 15.1 years in the CSU patient group and 46.2 ± 10.1 years in the control group. No significant difference was found between the patient and the control groups in terms of gender and mean age ($p=0.558$, $p=0.528$). The mean disease duration in CSU patients was 28.1 ± 38.5 (2-180) months. This duration was ≤ 1 year in 24 (63.1%), 1- ≤ 5 years in 11 (28.9%), 5- ≤ 10 years in 2 (0.05%) patients and longer than 10 years in 1 (0.03%) patient. Mean UAS7 of the CSU patients was 28.7 ± 8.1 . ASST was negative in 16 (42.1%) and positive in 22 (57.9%) of the patients. The percentage of CD4+CD25+ T cells was 4.1 ± 2.8 in the CSU patients and 5.4 ± 1.9 in the control group. The percentage of CD4+CD25+ T cells in the patient group was statistically significantly lower than in the control group ($p=0.018$). The percentage of CD4+FOXP3+ T cells was 2.6 ± 1.8 in the CSU patients and 4.2 ± 2.0 in the control group. The percentage of CD4+FOXP3+ T cell in the patient group was statistically significantly lower than in the control group ($p=0.000$). The percentage of CD4+CD25+FOXP3+ T cells was 1.9 ± 1.6 in the CSU patients and 1.9 ± 1.0 in the control group. The percentage of CD4+CD25+FOXP3+ T cells was found to be similar in both groups ($p=0.192$). The serum 25(OH)Vit D levels were 11.2 ± 2.5 ng/mL in the patient group and 11.7 ± 4.6 ng/mL in the control group. The serum 25(OH)Vit D levels were found statistically similar in the patient and control groups ($p=0.218$). The demographic and clinical characteristics of the patients and control groups were presented in Table 1.

No significant relationship was found between the percentages of CD4+CD25+, CD4+FOXP3+, CD4+CD25+FOXP3+ T cells and duration of disease, UAS7, ASST and serum 25(OH)Vit D levels ($p>0.05$; Table 2).

Table 1. Demographic and clinical characteristics of the patient and control groups

	Patient n=38	Control n=30	P
Gender (Female/male)	29/9	21/9	0.558
Age (year)	44 (17-72) 43.7 ± 15.1	50 (25-66) 46.2 ± 10.1	0.528
Disease duration (month)	12 (2-180) 28.1 ± 38.5	-	-
UAS7	28.7 ± 8.1	-	-
ASST			
Negative	16 (42.1%)	-	-
Positive	22 (57.9%)	-	-
CD4+CD25+ T cells (%)	4.1 ± 2.8	5.4 ± 1.9	0.018
CD4+FOXP3+ T cells (%)	2.6 ± 1.8	4.2 ± 2.0	0.000
CD4+CD25+FOXP3+ T cells (%)	1.9 ± 1.6	1.9 ± 1.0	0.192
25(OH)Vit D ng/mL	11.2 ± 2.5	11.7 ± 4.6	0.218

ASST: Autologous serum skin test, UAS7: Weekly urticaria activity score, 25(OH)Vit D: 25 hydroxy vitamin D

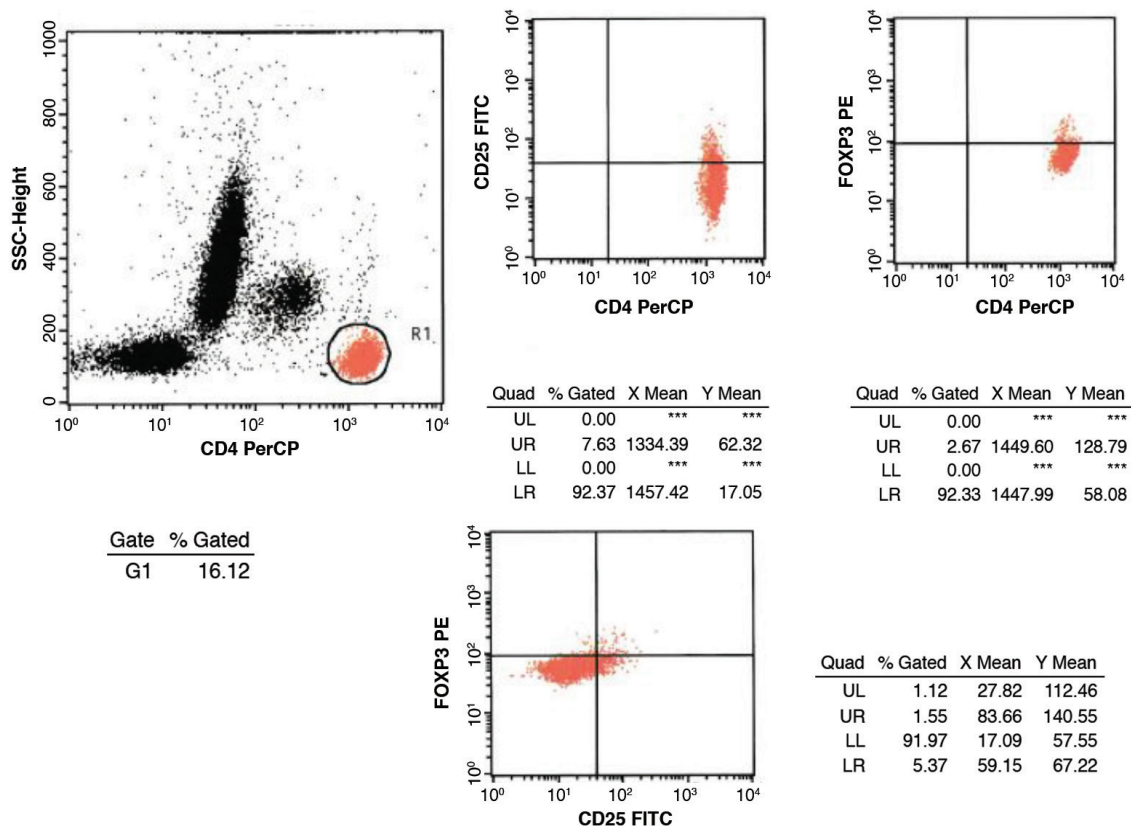


Figure 1. Flow cytometric analysis of T regulatory cells

Table 2. The evaluation of relationship between T regulatory cells subgroups and autologous serum skin test, disease duration, weekly urticaria activity score and 25 hydroxy vitamin D

	CD4 ⁺ CD25 ⁺ (%) r/p	CD4 ⁺ FOXP3 ⁺ (%) r/p	CD4 ⁺ CD25 ⁺ FOXP3 ⁺ (%) r/p
Disease duration (months)	0.184/0.270	0.020/0.544	-0.148/0.375
UAS7	0.087/0.603	0.147/0.380	0.106/0.527
ASST	-0.114/0.495	0.093/0.581	-0.015/0.738
25(OH)Vit D ng/mL	0.068/0.685	0.035/0.833	0.260/0.116

ASST: Autologous serum skin test, UAS7: Weekly urticaria activity score summed over 7 days, 25(OH)Vit D: 25 hydroxy vitamin D

Discussion

Although autoimmunity is thought to play a role in more than one third of the urticaria patients, the immunopathogenesis regarding the disease has not yet been clarified. Treg cells are classified as CD4⁺CD25⁺, CD4⁺FOXP3⁺ and CD4⁺CD25⁺FOXP3⁺, and FOXP3 is essential for Treg cells function (9). Although they play an important role in the development of immunotolerance by suppressing the inflammatory response developing against self-antigens, the exact role of Treg cells' subtypes is not known. Autoimmune disorders develop with a disturbance in the control of self-reactive T lymphocytes (20,21). CD4⁺CD25⁺ Treg cells suppress the functions of various types of hematopoietic cells in the peripheral blood and they are highly specific for the suppression of effector T cells. Treg cells therefore play an important role in immune homeostasis and are protective against autoimmunity development (19,20). Low number of or defective Treg cells have been reported to play a role in the development of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, multiple sclerosis and also myasthenia gravis (19,22).

There are a limited number of publications reporting that Treg cells are low or have defective function in chronic urticaria. Sun et al. (11) classified chronic urticaria as a chronic idiopathic urticaria (CIU) and chronic autoimmune urticaria (CAU) by using basophil histamine release in their study where they evaluated Treg cells levels. They found lower CD4⁺CD25⁺FOXP3⁺ regulatory T cells and FOXP3 levels in CAU patients than in the control and CIU groups, while the FOXP3 expression levels were similar in the CIU patients and the healthy group (11). They suggested that the reduced number of CD4⁺CD25⁺FOXP3⁺ regulatory T cells in CAU patients may result from the migration of these lymphocytes to the inflammation on urticaria plaque (11). On the other hand, they also emphasized that FOXP3 reduction may be related to autoimmunity in chronic urticaria (11). Chen et al. (12) found high level of CD4⁺CD25⁺ T cell percentage and observed a decrease in CD4⁺CD25⁺ and CD4⁺CD25⁻ cytokine release in chronic urticaria patients. They indicated that Treg cells have defective function in chronic urticaria patients (12). Arshi et al. (13) found significantly lower CD4⁺CD25⁺FOXP3⁺ T cell levels in chronic urticaria patients than the control group while the serum interleukin (IL)-10, transforming growth

factor- β , and IL-17 levels were similar in the two groups. These findings suggest that reduced number of Treg cells or its defective functions related to autoimmune nature of CSU.

Similar to the studies in the literature, we found lower percentages of CD4⁺CD25⁺ and CD4⁺FOXP3⁺ Treg cells in CSU patients than the healthy controls, while no difference was found in terms of the percentage of CD4⁺CD25⁺FOXP3⁺ Treg cells.

One of the immunomodulatory effects of vitamin D is to inhibit the T helper (Th)1 and Th17 response and induce the Treg cells response. Low vitamin D levels lead to increase in the pro-inflammatory cytokine levels and imbalance in the Treg cell levels (18,19,23,24). The serum vitamin D levels have been reported to be lower in chronic urticaria patients than in healthy controls (25-28). Besides, it is reported that the replacement vitamin D reduces the symptoms and improve quality of life in patients with chronic urticaria (29). To date, the relationship between vitamin D levels and Treg cells levels have not been reported in chronic urticaria patients. In our study, 25(OH)Vit D levels were found to be similar in both groups and no relationship was found between Treg cell groups and serum 25(OH)Vit D levels. These results indicate that the Treg cells levels decreases independently from the vitamin D levels in CSU patients.

ASST is a clinical test for *in vivo* detection of functional circulating autoantibodies in chronic urticaria. The positivity of ASST level supports immunologic pathogenesis in CSU patients. Although there are publications showing that 25(OH)Vit D levels are lower in ASST-positive urticaria patients, some studies report that 25(OH)Vit D levels have no difference between ASST positive and negative groups (25-28). In our study, no difference was found between Treg cell subtypes and ASST positive and negative patient groups. The studies conducted on this subject have not reported a relationship between disease activity and disease duration and Treg cells. In this study there was no relationship between the percentages of Treg cells and duration of the disease and UAS7. This result indicates that Treg cells have a contribution to disease pathogenesis independent from the disease severity and duration.

Study Limitations

The effect of the treatment on Treg cells levels in urticaria patients was not shown in this study.

Conclusion

The data we have obtained from our study supports that Treg cells are involved in immunopathogenesis in CSU patients. The clarification of the role of Treg cells in disease pathogenesis will be helpful in establishing treatment alternatives in CSU patients.

Ethics

Ethics Committee Approval: This study was approved by Eskişehir Osmangazi University Clinical Research Ethics Committee (approval number: 04-2016).

Informed Consent: It was taken.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: I.B., Concept: I.B., Design: I.B., O.M.A., Z.N.S., Data Collection or Processing: I.B., H.K.E., Analysis or Interpretation: S.Y., Literature Search: I.B., H.K.E., S.Y., O.M.A., Z.N.S., Writing: I.B.

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