

Original Article

Serum vitamin D/25(OH)D associated with toll-like receptor (TLR) 2 expression of immune cells in the saliva of Systemic Lupus Erythematosus: a preliminary study

Hendri Susanto^{1,*}, Bagus Soebadi², Diah Savitri Ernawati², Adiastruti Endah Pamardiati², Hening Tuti Hendarti², Iwan Hernawan², Desiana Radithia², Awalia³

¹ Department of Oral Medicine, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia

² Department of Oral Medicine, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia

³ Department of Internal Medicine, Dr. Soetomo Hospital, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

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Abstract – Objective: Vitamin D deficiency may contribute to Systemic Lupus Erythematosus (SLE) development. Vitamin D may involve in pathogen recognition through Toll-like receptor (TLR) 2 in immune cells in saliva. This study aimed to determine the correlation between serum vitamin D/25(OH)D and *TLR2* expression of immune cells in the saliva of SLE. **Materials and methods:** This cross-sectional study conducted at the the SLE patients who met the inclusion and exclusion criteria. Those who had signed informed consent involved to underwent unstimulated saliva collection and blood samples for TLR2 and vitamin D/25(OH)D examination. The correlation between serum vitamin D/25(OH)D concentration and salivary TLR2 expression was analyzed using the correlation test, linear regression with 95% confidence level. **Results:** Thirty SLE patients had a mean serum vitamin D/25(OH)D concentration of 9.98 ± 4.64 ng/ml. The mean of TLR2 expression of CD11b⁺ cells in saliva was $26.03 \pm 20.92\%$. There was a significant positive correlation between serum vitamin D/25(OH)D concentration and TLR 2 expression of CD11b⁺ cells in the saliva. ($r=0.434$; $P < 0.05$). Vitamin D/25(OH)D was the only predictor for TLR 2 expression. **Conclusion:** Serum vitamin D/25(OH)D concentrations associated with TLR2 expression of CD11b⁺ cells in the saliva of SLE.

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic systemic autoimmune disease that has systemic manifestations involving multiple organ systems. The prevalence of SLE is around 20% in the population, depending on the study background and research methods [1]. The prevalence of SLE in Indonesia is not known yet. However, The prevalence of SLE in Asia-Pacific countries was range 4.3–45.3 (per 100.000) [2].

The etiology of SLE may be genetic and environmental factors. The environmental factors that have been identified as SLE etiologies are viral infections (Epstein Barr Virus), smoking, exposure to chemicals (Mercury, Pesticides, cosmetics, asbestos), air pollution, sunlight, drugs (Hydralazine, Methylodopa, Chlorpromazine, Quinidine, Procainamide). Other risk factors for SLE are age and gender [1,3]. Besides, nutritional factors are also involved as the etiology factor for SLE. One of the nutritional factors that may be the etiology factor of SLE is vitamin D deficiency [4].

Studies had shown that a low level of vitamin D concentrations was associated with the development of SLE. It was found that vitamin D concentrations in SLE patients were lower than healthy people (<10 mg/ml), while normal vitamin D concentrations were >30 mg/ml [5]. A cohort study showed that patients with active SLE had lower serum vitamin D/25(OH)D concentrations compared to those with inactive SLE [6]. Low vitamin D concentrations in SLE are associated with high disease activity, whereas an increase in serum vitamin D concentrations correlated with decreased disease activity [7]. Other study showed that vitamin D supplementation in SLE patients who have serum vitamin D/25(OH)D concentrations less than 30 ng/ml and less than 10 ng/ml for approximately 2 years showed an increase of vitamin D concentration and associated with decreased fatigue levels in patients with SLE [8].

The dysregulation of innate immunity in SLE was shown by a report which revealed transmembrane toll-like receptor (TLR) may trigger the formation of autoantibodies and cytokines. Toll-like receptor 2 was thought to be associated with the formation of auto-antibody DNA in SLE [9]. The activation of TLR2 may be involved in the loss of tolerance of CD4⁺T cells in

* Correspondence: drghendri@ugm.ac.id

SLE. However, TLR2 activation also contributes to protection against microbial infection by increasing the function of CD4+T cells to inhibit inflammation and tissue damage [10]. A study has shown that serum TLR2 was lower in SLE than normal healthy people and inversely related to the severity of SLE [11]. The role of TLR2 in the recognition of pathogenic microorganisms also found in oral cavity. Thereby, the low TLR2 expression in oral cavity may increase susceptibility to the development of infectious diseases in the oral cavity of SLE, such as fungal (*Candida spp*) infections [12].

Interestingly, there is a relationship between vitamin D and TLR2 expression. Vitamin D decrease TLR2 expression [13]. A study showed that vitamin D supplementation in vitamin D deficiency conditions increased TLR2 expression in blood mononuclear cells in healthy people [14]. However, another study reported that there was a negative relationship between vitamin D/25(OH)D concentration and TLR2 expression in monocytes in Behcet's Disease. The administration of vitamin D3/1,25(OH)2D3 to monocytes showed a decreased TLR2 expression in Behcet's Disease patients [15]. The difference of the results of those studies indicated that the relationship between vitamin D and TLR2 can be positive or negative and is influenced by systemic conditions [13].

Hence, the decrease of vitamin D in SLE may also impact to the TLR2 expression in immune cells in the oral cavity such as monocytes, neutrophils, dendritic cells, natural (NK) cells, B cells, T cells, T regulatory (T Reg). Those immune cells in saliva may express TLR2 which have essential role to protect oral cavity from infections [16,17]. All those cells originate from the parotid gland, blood, cells and plays an important role in the TLR2-mediated immune response [18]. The fact that vitamin D has an important contribution to the function of immune cells especially in pathogen recognition in the oral cavity, vitamin D may correlate with the TLR 2 expression of immune cells in the saliva in SLE. However, there was not a study that investigates the correlation between vitamin D serum and TLR2 expression in saliva of SLE. Hence, this study aimed to investigate the correlation between serum vitamin D/25(OH)D and the expression of TLR2 in immune cells in saliva of SLE.

Materials and methods

SLE patients recruitment

The subjects of this study were those who had been diagnosed with SLE. Subjects of this current study were SLE patients diagnosed by Rheumatologists based on the American College of Rheumatology criteria (ACRC) 1997. They were informed about the study and were asked to participate. The inclusion criteria were the SLE patients who did not have other diseases, did not smoke and drink alcohol, did not use oral contraceptives. The exclusion criteria were pregnant, consumed steroids including immunosuppressants for more than 6 months, have a history of the allergy reaction to blood sample equipments, and wearing prosthesis. Informed consent was obtained from all patients. Additionally, information about age, gender/sex, ethnicity, marital status, education level,

were obtained by a questionnaire. This study was approved by the Ethical Committee for Research of the Faculty of Medicine of Universitas Gadjah Mada, Yogyakarta, Indonesia.

Blood and saliva collection for vitamin D/25(OH)D and TLR2 expression measurement

All participants underwent a whole unstimulated saliva sample collection using the spitting method in the morning of their regular visit to the Rheumatology clinic. To determine immune cells in saliva, Anti-human CD11bFITC conjugated antibody (Biolegend) was used and the TLR2 expression used Anti-human TLR2PE-conjugated antibody (Santa Cruz) in saliva. Both immune cells (CD11b+ cells) and TLR2 were measured using flow cytometry method according to the previous study [19]. The blood sample was obtained from all subjects by venipuncture taken from the median cubital vein on the patient's upper right limb following saliva collection. An EDTA vacutainer was used to collect a blood sample to determine Vitamin D/25(OH)D using Electro-chemiluminescence immunoassay (ECLIA) methods. The vitamin D/25(OH)D <20 ng/ml categorized vitamin D/25(OH)D deficiency, when vitamin D: 21–29 ng/ml categorized insufficiency, Normal serum vitamin D/25(OH)D >30 ng/ml [20].

Statistical analysis

The demographics of SLE patients were analyzed using descriptive statistics. The Shapiro Wilk test was used to analyze whether the data were normally distributed or not. The correlation between serum vitamin D/25(OH)D and TLR2 expression of immune cells (CD11b+ cells) in saliva analyzed using Pearson correlation test when the data was normally distributed or Spearman correlation test when the data was not normally distributed. Multiple linear regression was used to analyse whether Vitamin D/25(OH)D, age, gender, ethnicity, disease duration, and medications were predictors for TLR2 expression of CD11b+ cells in saliva of SLE patients. Statistical analysis was performed with 95% Confidence Interval (CI) using SPSS version 17.00.

Results

Thirty SLE patients meet the inclusion criteria that participated in this study. Twenty-nine subjects (96%) were women. The mean age of the subject was 27.73 years old with the highest percentage for education level was high school (40%). Seventeen subjects were not married yet and the most subjects ethnicity was Javanese (73.3%). Twenty-eight subjects consumed steroid medication (methylprednisolone) (93%). Forty-three percent of subjects have one-month disease duration and 23% of subjects have 6 months duration of disease (Tab. I).

All subjects have low serum vitamin D/25(OH)D concentration with the mean value of vitamin D/25(OH)D concentration was 9.98 ± 4.64 ng/ml and categorized by vitamin D

deficiency (<20 ng/ml). The mean value of TLR2 expression of immune cells (CD11b+ cells) in the saliva of the subjects was $26.03 \pm 20.92\%$ (Tab. I).

Since the serum vitamin D/25(OH)D concentration and TLR2 expression in CD11b+ cells in the saliva was not normally distributed ($P < 0.05$) according to Shapiro Wilk analysis, the Spearman correlation test was used to analyse the association between serum Vitamin D/25(OH)D concentration and the TLR2 expression of CD11b+ cells in the saliva of SLE. The result of the Spearman correlation test analysis showed that there was a positive significant correlation between serum Vitamin D/25(OH)D concentration and saliva TLR2 expression ($r=0.434$; $P < 0.05$) (Tab. II). Result multiple linear regression showed that serum vitamin D/25(OH)D concentration was the only predictor for TLR2 expression of CD11b+ cells in saliva of SLE patients ($P < 0.05$) (Tab. III).

Discussion

This was the first study that investigated the association between serum vitamin D/25(OH)D concentrations and TLR2 expression of immune cells (CD11b+ cells) in the saliva in Systemic Lupus Erythematosus (SLE) patients. The results of this study showed that the mean age of SLE subjects approximately was 27 years old. It was found also that the majority of subjects were female (96%) with an age range of 12–50 years old. Our results were accordance with the fact that SLE patients are more common in women with a percentage of about 90% and a ratio of women and men is 9–15: 1 [1]. Age may be a risk factor for the development of SLE with the age range of 18–65 years old and the mean age of SLE patients is 30 years old [21].

Our study also showed that the mean value of serum vitamin D/25(OH)D concentrations, was 9.98 ng/ml. All the subjects of current study had low serum vitamin D concentrations, which is the main indicator of body vitamin D status, below 20 ng/ml and categorized as vitamin D deficiency [20]. The result of this study was accordance with other studies which showed SLE patients have low vitamin D concentrations [6,7]. The low level of vitamin D may cause the impairment of the immunomodulatory function of Vitamin D, and may result in the dysregulation of the immune system in SLE [3]. Vitamin D deficiency also has been accepted to be one of the etiology of SLE and contributed to the complications of SLE [3,4]. The function of Vitamin D as an immunomodulator is associated with the presence of Vitamin D receptors (VDRs). VDRs are expressed in cells of the body, including in both innate immune cells, (*i.e.* neutrophils, macrophages) and cellular immune cells such as antigen-presenting cells (APC), dendritic cells, CD4+ and CD8+T cells [22]. We found that immune cells (CD11b+) in saliva of our SLE patients expressed TLR2. TLR2 expression which found in saliva-derived from blood and salivary gland cells, may be stimulated by the invasion of colonies of microorganisms in oral cavity [16,17,23]. This study was similar with other study that showed TLR2 expressed in CD11b+ cells in saliva of newborns [19].

Table I. The characteristic of SLE subjects.

Variable	Value
Number: <i>n</i>	30
Age: mean \pm SD (years)	27.73 \pm 10.70
Gender: <i>n</i> (%)	
Women	29 (96.7)
Education level: <i>n</i> (%)	
Elementary school	7 (23.3)
Junior high school	8 (26.7)
High school	12 (40)
Higher education	2 (6.7)
Occupation: <i>n</i> (%)	
Government employees	2 (6.7)
Student	10 (33.3)
House wife	14 (46.7)
Unemployment	4 (13.3)
Married status: <i>n</i> (%)	
Married	13 (43.3)
Not married yet	17 (56.7)
Ethnicity: <i>n</i> (%)	
Javanese	22 (73.3)
Maduranist	8 (26.7)
Medications: <i>n</i> (%)	
Methylprednisolone	28 (93)
Cyclosporine	16 (53)
Paracetamol	5 (16)
Omeprazole	4 (13)
Azathioprine	2 (6)
Ranitidine	3 (10)
Mycophenolate mofetil	2 (6)
Amlodipine	2 (6)
Folic acid	2 (6)
Disease duration: mean + SD (months)	2.73 \pm 2.05
1 Month: <i>n</i> (%)	13 (43)
2 Month: <i>n</i> (%)	5 (17)
3 Month: <i>n</i> (%)	4 (13)
4 Month: <i>n</i> (%)	1 (3)
5 Month: <i>n</i> (%)	6 (24)
Serum vitamin D/25(OH)D: mean \pm SD (ng/ml)	9.98 \pm 4.64
Saliva TLR2 expression on cd11b: (%)	26.03 \pm 20.92

cd11b: neutrophils in saliva; *n*: number; ng: nanogram; SD: Standard Deviation; TLR2 : Toll-like receptor 2.

The decreased of TLR2 expression may be one of the risk factors for the increased infection in oral cavity in SLE patients such as oral candidiasis [12]. This oral infection which caused by *Candida albicans* may be not only predisposed by low immune cells (*i.e.* neutrophils) migration towards the site of infection in oral mucous and increased neutrophil apoptosis [24], but also a decrease of TLR2 expression in immune cells in oral

Table II. The result of The Spearman correlation test between serum vitamin D/25(OH)D concentration and TLR2 expression of neutrophils in saliva of SLE.

Variable	Correlation coefficient (<i>r</i>)	<i>P</i>
serum Vitamin D/25(OH)D concentration Saliva TLR2 expression	0.434	0.017*

*Significantly different ($P < 0.05$); TLR2: *Toll-like Receptor 2*.

Table III. Multiple linear regression analysis Vitamin D/25(OH)D predictor for TLR2 expression of Neutrophils in the saliva of SLE.

Model predictors	<i>B</i>	<i>P</i> -value of β	R^2	95% confidence interval of β
Model			0.175	
Vitamin D/25(OH)D (ng/ml)	1.886	0.021		−10.178 to 24.573
Constant	7.198	0.403		0.303 to 3.469

P=probability, a *P*-value of ≤ 0.05 was considered statistically significant; β : unstandardized coefficient; Dependent variable: salivary TLR2 expression; Independent variables: age, gender, education level, ethnicity; occupation, medications, disease duration, serum Vitamin D/25(OH)D.

cavity. Hence, it may impair the function of immune cells to recognize pathogenic microorganisms through TLR2 mediated immune response. The mechanism of TLR in infectious diseases was still uncertain, but several studies have shown that TLR stimulation in macrophages will induce CYP27b1 (1 α -hydroxylase) enzyme that catalyzes vitamin D/25(OH)D₃ to calcitriol (1,25(OH)₂D₃) and vitamin D receptor (VDR), thus activates cathelicidin antimicrobial peptides [25]. In the innate immune cells, vitamin D/25(OH)D may induce vitamin D receptor (VDR) through TLR2 stimulation and produce antimicrobial peptide cathelicidin production [26].

The results of this study indicated that vitamin D/25(OH)D has a positive correlation with TLR 2 expression of CD11b+ cells in saliva, which means that low levels of vitamin D/25(OH)D will have low TLR2 expressions. Our result was similar to the results of another study that showed a positive relationship between vitamin D and TLR2 expression in monocytes [14]. Other evidence showed that there is a relationship between reduced cathelicidin (LL37) production by immune cells and low vitamin D concentrations. The cathelicidin production (LL37) is influenced by low vitamin D concentrations depending on vitamin D receptor (VDR) mediated by TLR 2 [27]. Another study had shown that 1,25(OH)D will reduce cytokine production such as TNF α after the TLR2 interact with the

ligands on the cells surfaces. This indicates that TNF α production depends on vitamin D receptor (VDR). Moreover, low TNF α concentration was shown by low TLR 2 expression [28] and TLR activation induces the expression of vitamin D receptor (VDR) and 1- α hydroxylase. Hence, low TLR2 expression will result in low vitamin D receptor (VDR) and 25(OH)D cannot be converted to 1.25(OH)D [26].

The subjects of this study mostly were treated by systemic corticosteroids. Ninety-three percent of subjects consuming methylprednisolone. Methylprednisolone is a moderate potency steroid antiinflammatory drug that commonly used to reduce inflammation in SLE [21,29]. Methylprednisolone has an antagonistic effect on vitamin D. However, the consumption corticosteroid for up to 6 months has no effect on vitamin D status in serum [30]. Since the duration of SLE was not exceed 6 months, hence, the possibility of the influence of corticosteroids (methylprednisolone) on serum vitamin D concentrations may be ruled out. Whereas the effect of other drugs consumed by this study subjects on serum vitamin D concentrations is not known yet. The low level of vitamin D concentrations in this study subject may be probably caused by two factors, a relatively lack of food intake rich in vitamin D sources. Other factor is a lack of synthesis of vitamin D in the skin through exposure to ultraviolet B (UVB) from sunlight which is the main source of vitamin D in the body [31]. However, the etiology factor of SLE is multifactorial, Vitamin D deficiency may be one of the etiology of SLE development in the subjects of this study.

Conclusion

It can be concluded that there was a significant positive correlation between serum vitamin D/25(OH)D concentration and TLR 2 expression of CD11b+ cells in the saliva of SLE. This correlation may explain the essential role of vitamin D in immune response in oral cavity in SLE. Further study needed to investigate the role of vitamin D therapy in SLE may reduce the not only the severity of SLE but also reduce oral infection of SLE.

Conflicts of interests

The authors declare that they have no conflict of interest in this study. Funding has been made available from Universitas Gadjah Mada, Indonesia.

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