25 HYDROXYVITAMIN D AND CYTOKINES IN MULTIPLE SCLEROSIS

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25 HYDROXYVITAMIN D И ЦИТОКИНЫ ПРИ РАССЕЯННОМ СКЛЕРОЗЕ

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ABSTRACT

INTRODUCTION: Clinical trials of patients with multiple sclerosis (MS) have produced inconsistent results for the profile of cytokine secretion in serum and cerebrospinal fluid in patients with multiple sclerosis during periods of relapse and remission. Epidemiological and clinical observations data reveal an association of the changes in vitamin D serum concentration with the risk of developing MS. **Aim:** To evaluate changes in serum concentrations of 25(OH)D, IL17, IFN-gamma, TGFβ1, IL4, IL10 in relapse and remission and their correlation with the severity of disability. **PATIENTS AND METHODS:** Fifty-three persons (30 clinically healthy controls and 23 patients with relapsing-remitting multiple sclerosis) living between 41° and 42° northern latitude were registered during the astronomical winter period (October 2012- May 2013).-Patients were diagnosed according to Mc Donald 2010 criteria. The degree of neurological deficit was assessed by EDSS. Serum concentrations of 25(OH)D (nmol/l) and cytokines (pg/ml) were tested by ELISA - once for controls and twice for patients (during relapse and remission). **RESULTS:** In the studied population average levels of 25(OH)D were close to insufficiency, most pronounced in patients in relapse, as differences were not statistically significant. A reverse correlation was found between the levels of 25(OH)D and the deficit in relapse and remission. Concentrations of TGFβ1 significantly increased in remission compared with exacerbation and controls. Serum level of IL4 was significantly lower in relapse compared with controls. In remission there was a marked tendency of increase compared with exacerbation. During clinical improvement IL17 and IFN-gamma tended to decrease compared to the average levels in relapse. In both periods, the average concentrations of IFN-gamma in patients were significantly lower compared with controls. No statistically significant differences were found comparing cytokine changes with those of 25(OH)D and deficit. **CONCLUSION:** Persistent cytokine imbalance in patients compared with controls is a marker for Th1-mediated CNS demyelination. Anti-inflammatory TGFβ1, IL4 are indicators of immune response intensity. The deficit severity does not depend on changes of the tested cytokines, but correlates with 25(OH)D levels during periods of relapse and remission.

Key words: vitamin D, cytokines, multiple sclerosis

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РЕЗЮМЕ

Цель: Оценить изменения в сывороточных концентрациях 25(OH)D, IL17, IFN-gamma, TGFβ1, IL4, IL10 в периоды приступа и ремиссии и их связь с тяжестью инвалидизации. **МАТЕРИАЛ И МЕТОДЫ:** 53 лица (контрольная группа из 30 клинически здоровых лиц и 23 пациента с приступно-ремитентным ходом рассеянного склероза) с местообитанием в координатах 41° - 42° северной широты, зарегистрированы в астрономически зимний период (октябрь 2012 – май 2013г.). Диагноз был поставлен в соответствии с критериями Mc Donald 2010. Степень неврологического дефицита оценена при помощи EDSS. Сывороточные концентрации 25(OH)D (nmol/l) и цитокинов (pg/ml) исследованы при помощи ELI-

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SA – однократно в контрольной группе и двукратно среди пациентов (во время приступа и ремиссии). **Результаты:** У обследованного контингента средние уровни 25(OH)D находятся на пороге недостаточности, сильнее всего выраженной среди больных в состоянии приступа, без наличия статистически значимых различий. Установлена обратная корреляционная зависимость между уровнями дефицита 25(OH)D во время приступа и ремиссии (r приступ = -0.593, $p = 0.03$; r ремиссия = -0.46, $p = 0.024$). Во время ремиссии значительно возрастают концентрации TGF β 1 по сравнению с эксацербацией ($p = 0.0001$) и с контрольной группой ($p = 0.001$). Сывороточный уровень IL4 является сигнификантно более низким во время приступа по сравнению с контрольной группой ($p = 0.049$). Во время ремиссии наблюдается выраженная тенденция повышения по сравнению с эксацербацией ($p = 0.054$). В период клинического улучшения наблюдается тенденция понижения уровней IL17, IFN-gamma по сравнению со средними уровнями в период приступа. Во время обоих периодов средние концентрации IFN-gamma у пациентов являются сигнификантно более низкими по сравнению с контрольной группой ($p = 0.036$, $p = 0.05$). При сопоставлении изменений уровня цитокинов с изменениями уровня дефицита 25(OH)D не установлены статистически значимые взаимосвязи. **Заключение:** Персистирующий цитокиновый дисбаланс у пациентов по сравнению с контрольной группой является маркером Th1, вызванной демиелинизацией в ЦНС. Антивоспалительные цитокины TGF β 1, IL4 являются показателями интенсивности иммунной реакции. Тяжесть дефицита не зависит от изменений исследуемых цитокинов, но коррелирует с уровнями 25(OH)D во время периодов приступа и ремиссии.

Ключевые слова: витамин D, цитокины, рассеянный склероз

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INTRODUCTION

Multiple sclerosis (MS) is an immune disorder with multifactorial etiology, characterized by demyelination, axonal transection and oligodendropathy in the central nervous system (CNS). Data obtained in experimental allergic encephalomyelitis (EAE) support the thesis of CD4+ T cell-mediated destruction of the myelin with imbalance in cytokine secretion from Th1, Th17, Th2 lymphocyte subpopulations. In exacerbation the imbalance in the periphery is characterized by reduction of the regulatory CD4+, CD25+ Foxp3 subpopulation related to the production of the anti-inflammatory cytokines TGF β 1, IL10, inhibited synthesis of IL4 and enhanced secretion of proinflammatory cytokines TNF α , IFN-gamma, IL17.^{1,2} Clinical studies establish inconsistent results for cytokine secretion profile in serum and cerebrospinal fluid of patients during periods of relapse and remission when compared with controls.³⁻⁵ The hypothesis of correlation between the pattern of myelin destruction, clinical manifestations, and potential therapeutic response is a reason to examine the involvement of various factors in the etiology and pathogenesis of MS. Extracorporeal vitamin D-factor prevents the development of EAE if administered before the induction with myelin protein and arrests the progress of the disease during treatment after the first clinical signs.^{6,7} Data from epidemiological and clinical observations suggest a link of the changes in serum concentrations of vitamin D with

the risk of developing MS, and with the phases of relapse and remission.^{8,11} An indicator of vitamin D status in the body is the serum concentration of 25 hydroxyvitamin D /25(OH)D/, which correlates with the biologically active metabolite having properties of the hormone 1,25 dihydroxyvitamin D /1,25(OH)2D/.⁸ Data obtained in vitro support the hypothesis of suppressive effect of 1,25(OH)2D on the synthesis of the pro-inflammatory cytokines IFN-gamma, IL17, IL2, of the induction of anti-inflammatory Th2 mediated secretion of IL4, IL5, IL13, and of the regulatory CD4+, CD25+ Foxp3 subpopulation. Treatment of EAE with 1,25(OH)2D stimulates the synthesis of IL4 and TGF β 1. Conflicting results have been reported on minor changes of Th2-mediated secretion of cytokines in the CNS and peripheral organs of the immune system after treatment with 1,25(OH)2D.^{10,12}

Research on the causal link between the dynamics in serum concentrations of immune parameters, vitamin D levels and clinical indicators of disease activity will clarify aspects of the mechanism of the immune process so that the therapeutic control of disturbed immune regulation is optimized.

AIM

To evaluate changes in serum concentrations of IFN-gamma, IL17, TGF β 1, IL4, IL10, 25(OH)D in relapse and remission phases and their relationship with the degree of neurological deficit.

PATIENTS AND METHODS

DESIGN

The present study is prospective, case-control. Patients were recruited on an outpatient basis at the MS Diagnosis and Treatment Center at St. George University Hospital, Plovdiv, during the winter from October 2012 to May 2013. The research was approved by the Ethics Committee at MU Plovdiv by Protocol № 3/05.07.2012. The design involves a single test of serum IFN-gamma, IL17, TGFβ1, IL4, IL10, 25(OH)D of the clinically healthy controls and two tests of the patients - during periods of relapse and remission (≥ 2 months after a consecutive relapse). Patients with relapses were routinely hospitalized at the Clinic of Neurology, St. George University Hospital, Plovdiv. Treatment was conducted with methylprednisolone (Sopharma) 500 mg, i.v. in the morning, the course dose - 2500 mg.

PATIENTS

Data of 53 individuals were analyzed. Of these, 30 were controls (15 women, 15 men) and 23 patients (15 women, 8 men) with inpatient monitoring during relapse and outpatient monitoring during remission.

Inclusion criteria - Caucasians community dwelling at 41°-42° northern latitude, aged 18-50 years; patients with relapsing-remitting MS, degree of neurological deficit - 1.5-5.0 after EDSS;

Exclusion criteria - primary and secondary progressive course; drug treatment modifying the disease course during the year preceding the date of registration; therapy with vitamin D and drugs affecting the metabolism of vitamin D (oral contraceptives, hormone replacement therapy, anti-epileptic agents, laxatives, thiazide diuretics), accompanying autoimmune diseases, acute and chronic infections, liver, kidney, and neoplastic diseases, hyperparathyroidism, diabetes, hypercalcaemia.

CONTROLS

Inclusion criteria - clinically healthy, age- and sex-matched subjects who were not on oral con-

traceptives, hormone replacement therapy, laxative preparations, or polyvitamins.

METHODS

Clinical methods: Diagnosis was made according to the McDonald criteria (2010). Neurological deficit degree was assessed with the Expanded Disability Status Scale (EDSS, Kurtzke, 1983). Relapse is defined as the onset of new neurological symptoms or worsening of old ones with duration >24 hours, aggravation of EDSS ≥ 0.5 degree in the absence of fever and after a period of 30-day improvement or stable condition after another relapse in succession.

Laboratory methods: Serum concentrations of cytokines IFN-gamma, IL17, TGFβ1, IL4, IL10 and 25-OH Vitamin D were determined by enzyme-linked immunosorbent analysis with original ELISA-kits, eBioscience, Austria, and original ELISA-kits, Immundiagnostik AG, Germany. Each sample was tested in duplicate analyses. Tests were serially read by ELISA-reader (Sirio- microplate reader, SEAC- Italy) at 450 nm (reference - 620 nm).

STATISTICAL ANALYSIS

The number of observed objects was estimated at 5% α error and β error - 20%. Quantitative indicators are presented as mean and standard error $\pm$ SEM/. Comparison of results between groups was performed using the independent sample t test and Mann-Whitney test at a significance level of $p < 0.05$, depending on the results of the Kolmogorov-Smirnov test. Correlation analysis was also applied. For statistical data processing statistical software SPSS 17.0 was used.

RESULTS

The clinical characteristics of the participants is presented in Table 1.

Comparison of mean age in patients and controls found no statistically significant differences ($p > 0.05$, $t = 1.316$). All patients were registered by the end of the first month of a consecutive relapse (between 3rd and 25th day), the highest proportion

Table 1. Clinical characteristics of participants

Groups	Number	Sex		Age	Duration of illness	Age at onset of first signs
		female	male	(mean $\pm$ SEM)	(mean $\pm$ SEM)	(mean $\pm$ SEM)
		(n)	(n)	years	years	years
Patients	23	15	8	35.09 $\pm$ 2.87	7.65 $\pm$ 1.75	27.30 $\pm$ 1.97
Controls	30	15	15	31.17 $\pm$ 1.41	-	-

of registered patients occurring by the end of week 3 (47.83%, $n = 11$). The average degree of neurological deficit in relapse was 2.48 ± 0.19 ; during remission it was 1.72 ± 0.19 . The deficit decrease was statistically significant during remission compared with relapse ($p < 0.001$). Tables 2 and 3 present results of the tested pro- and anti-inflammatory cytokines in patients and controls during periods of relapse and remission. The following analyses were used: IFN γ , IL17 – parametric analysis; for IL4 – parametric analysis, for TGF B1, IL10 – non-parametric Mann-Whitney test.

In remission the mean levels of IFN-gamma and IL17 tended to decrease compared to the levels in relapse. During both periods the mean concentrations of IFN-gamma were significantly lower than those in controls ($p = 0.036$ in relapse, $p = 0.005$ in remission).

During remission mean concentrations of TGF β 1 increase statistically significantly in comparison with exacerbation ($p < 0.0001$) and with controls ($p < 0.001$). The mean concentration of IL4 is significantly lower in relapse than this in controls ($p = 0.049$), but in remission it tends to increase compared with exacerbation period ($p = 0.054$). No statistically significant differences were found

in the dynamics of IL10 in comparisons between patients and controls, relapse and remission. Analysis of mean serum concentrations of 25(OH)D between controls, patients in relapse and those in remission, did not find any statistically significant differences, but the established trends have to be mentioned. The mean levels in healthy subjects and patients were within the insufficiency range for Bulgarian population ($25\text{-}49.99$ nmol/l), most pronounced in patients with relapse ($\bar{x} \pm \text{SEM}$; healthy - 31.46 ± 7.3 ; in relapse - 26.93 ± 7.44 ; in remission - 28.06 ± 7.28).

During remission average levels increase compared to those in relapse, but fail to reach statistical significance. Results are presented in Fig. 1.

Values for EDSS and 25(OH)D levels in relapse and remission are presented in Figure 2. Correlation analysis (Spearman) found a negative correlation between the concentration of 25(OH)D in relapse and remission, and the degree of deficit in both periods ($r_{\text{relapse}} = -0.593$, $p = 0.03$; $r_{\text{remission}} = -0.46$, $p = 0.027$).

DISCUSSION

This study revealed persistent immune disbalance in patients during the two clinical periods in compari-

Table 2. Serum concentrations of tested proinflammatory cytokines

Cytokines	IL17		p_1	p_2	IFN γ		p_1	p_2
	(mean $\pm$ SEM)	pg/ml			(mean $\pm$ SEM)	pg/ml		
Relapse	69.82 $\pm$ 10.88		0.728		2.04 $\pm$ 0.48		0.036*	
Remission	58.50 $\pm$ 11.10		0.073	0.520	1.31 $\pm$ 0.25		0.005*	0.192
Control	63.77 $\pm$ 10.88				4.55 $\pm$ 0.95			

p_1 – comparison with controls;

p_2 – comparison between relapse and remission.

Table 3. Serum concentrations of tested anti-inflammatory cytokines

Cytokines	IL4		p_1	p_2	IL10		p_1	p_2	TGF B1		p_1	p_2
	(mean $\pm$ SEM)	pg/ml			(mean $\pm$ SEM)	pg/ml			(mean $\pm$ SEM)	pg/ml		
Relapse	5.29 $\pm$ 1.00		0.049*		1.68 $\pm$ 1.28		0.986		0.50 $\pm$ 0.04		0.802	
Remission	8.54 $\pm$ 1.31		0.368	0.054	1.36 $\pm$ 0.29		0.169	0.332	0.77 $\pm$ 0.04		0.001*	0.0001*
Controls	10.99 $\pm$ 2.34				1.67 $\pm$ 1.20				0.52 $\pm$ 0.05			

p_1 – comparison with controls;

p_2 – comparison between relapse and remission.

Figure 1. Serum levels of 25(OH)D.

Figure 2. Changes in deficit severity /EDSS/ and serum levels of 25(OH)D.

son with controls. Secretion of cytokine inducers of inflammation - IFN-gamma, IL17 dominates during exacerbation. Various authors find in patients in relapse significantly higher secretion of IFN-gamma from peripheral blood mononuclear cells (PBMC), higher serum levels compared to remission. In brain lesions enhanced production of IFN-gamma, an inducer of oligodendrocyte apoptosis, is detected.^{13,14} We have found statistically significant lower serum levels of IFN-gamma in patients in the two phases compared to controls. We assume that these data are related to the fluctuation of anti-myelin reactivity. N. Hellings et al. find transient increase in IFN-gamma, TNF α , IL6 before exacerbation. In vitro studies confirm these results.⁴ Research on seasonal fluctuations of cytokines reveals significantly higher IFN-gamma levels in healthy individuals during winter than during summer.¹⁵ We assume that the obtained results may reflect such an aspect in the dynamics of cytokine secretion. The identified tendency of decrease in serum concentrations of IL17

during remission compared to relapse is consistent with the results reported by V. Brucklacher-Waldert et al. for a significantly higher proportion of IL17 lymphocytes during exacerbation compared to remission.¹⁶ There has been experimental evidence proving an anti-inflammatory potential of Th17 subpopulation. Results for the increase of mRNA levels in the inflammation areas of the CNS are illustrative for elevated levels of IL17 in the serum and CSF of patients compared with controls.^{5,17,18} No statistically significant difference is found upon comparison of IL17 serum concentrations in patients and controls. Data of Kallaur A. et al. are similar.⁵ We believe that significantly increased levels of TGF β 1 during remission compared to relapse and controls is the result of enhanced secretion of TGF β 1 during this period and is an aspect of immune tolerance disorders in patients. Experimental data confirm the regulatory function of TGF β 1 - retards the development of EAE, reduces the severity of clinical manifestations, suppresses reactivity of proinflammatory cytokines - TNF α , IFN-gamma, IL1, IL2, IL6. Clinical studies establish: negative correlation between serum concentrations of TGF β 1 and magnetic resonance activity; high TGF β 1mRNA levels in patients with low-degree neurological deficit as opposed to those with severe deficiency.¹⁹ Studies on the secretion of IL4 and IL10 during both clinical periods report inconsistent results. We evaluate the tendency of increase in IL4 levels during remission compared with the relapse as an act of anti-inflammatory mediator activity, confirmed by experimental data: elevated levels of IL4 reduce the severity of EAE; antiIL4mAb treatment significantly increased IFN-gamma secreting cells.^{5,20} Similar data have been obtained by D. Franciotta et al. for statistically significant enhanced secretion of IL4 from PBMC during remission.²¹ K. Hohnoki et al. report just the opposite - significantly higher concentrations of IL4 in relapse.¹³ The result for statistically significant lower levels of IL4 during exacerbation compared to controls is a manifestation of immune disbalance in patients. There were no significant differences in the dynamics of IL10 levels. V. Ozenci et al. prove the anti-inflammatory activity of IL10: suppressed expression of class II molecules on Human Leukocyte Antigens (HLA), of adhesion and costimulatory molecules on monocytes, macrophages and dendritic cells. K. Kahl et al. report significantly elevated levels of IL10mRNA in patients in relapse compared with healthy subjects. B. Cannella et al. record exacerbations of EAE after administration of IL10 in doses higher than the physiological doses.²²⁻²⁴

Similar to the results of this study are the data reported by C. Brandão et al.³ No correlation was detected between changes in the deficit and serum concentrations of the cytokines during both clinical periods. We assume that the regulatory cytokine disbalance and EDSS scale reflect with different specificity the intensity of immune inflammation. The degree of neurological deficit is directly related to the loss of axons, but axonal damage have different mechanisms - immune-inflammatory, degenerative, ischemic. In the group under observation unlike other observations no significant differences were found in the dynamics of 25(OH)D levels in both periods and between patients and controls.^{8,9} The recorded significant negative correlation between changes in the deficit during the two phases and serum concentrations of 25(OH)D is established also by other authors.²⁵ Complex analysis of detected tendencies and statistically significant relationships between changes in survey indicators are of interest. During exacerbation - a phase with aggravated neurological deficit, there is dominance of Th1, Th17-mediated secretion of IFN-gamma, IL17 cytokine, 25(OH)D levels border the threshold adopted for deficit (≤ 25 nmol/l). During remission, a phase with reduced degree of neurological deficit, there is dominance of CD4⁺, CD25⁺ Foxp3-mediated synthesis of TGFβ1 and Th2 mediated production of IL4, serum concentrations of 25(OH)D increase. Clinical observations provide evidence for the potential of vitamin D to induce an anti-inflammatory cytokine secretion of IL4, IL5, IL10. J. Smolders et al. detected a positive correlation between serum concentration of 25(OH)D and T regulatory function. Treatment in patients with RRMS, 1000 IU vitamin D, significantly increases TGFβ1 levels in serum compared to untreated.¹⁹ At the current stage no detectable causal relationship is found between changes in cytokines and 25(OH)D levels.

CONCLUSIONS

Persistent cytokine disbalance in patients compared to controls is indicative of Th1-mediated CNS demyelination.

Anti-inflammatory cytokines, TGFβ1, IL4 are indicators of the immune response intensity.

Neurological deficit severity is not related to changes of the studied cytokines, but correlates negatively with changes in 25(OH)D serum levels during periods of relapse and remission.

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