SERUM LEVELS OF INTERLEUKIN - 23 AND INTERLEUKIN - 17 IN HASHIMOTO'S THYROIDITIS

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Abstract

Context. Overproduction of proinflammatory cytokines plays a significant role in the pathogenesis of Hashimoto's thyroiditis (HT). Recent studies revealed a prominent role of newly discovered Th17 subset in the induction of autoimmune disorders and that the signaling induced by IL-23 on Th17 cells is crucial to obtain a pathogenic and sustained phenotype. The objective of this study was to provide the involvement of interleukin IL-23/IL-17 axis in pathologic processes.

Design. Serum levels of IL-23 and IL-17 in controls and HT patients were studied in different stages of disease activity.

Subjects and methods. We investigated 93 patients with HT: 33 patients with newly diagnosed euthyroid HT (Group I), 11 patients with newly diagnosed hypothyroid HT (Group II), and 49 subjects treated with Levothyroxine (Group III). Thirty healthy subjects were included as controls. Concentrations of IL-23 and IL-17 in the serum samples of patients and controls were evaluated by enzyme-linked immunosorbent assay.

Results. Serum level of IL-23 was significantly higher in all HT patients ($p < 0.0001$) as well as in subgroups of patients in comparison with controls ($p < 0.01$). Serum concentrations of IL-17 were statistically increased in the group of HT patients ($p = 0.014$); the differences in IL-17 levels between groups I and III in comparison to healthy controls were also significant, but not for group II.

Conclusions. Our results highlight the involvement of the IL-23/IL-17 axis in the development of HT and its severity. Moreover, upregulated secretion of IL-23 could be a biomarker for progression and monitoring of HT.

Key words: Hashimoto's thyroiditis, Interleukin-23, Interleukin-17, ultrasonography, fibrosis.

areas of the world, however it is known that many patients are in euthyroid state for years. Microscopically, there is a diffuse process consisting of a combination of epithelial cell destruction, lymphoid cellular infiltration and fibrosis (1). Long time T helper (Th) type 1 (Th1) and Th type 2 (Th2) cell imbalance has been related to the pathogenesis of HT with prevalence of Th1 lymphocytes and there are a lot of studies to support this theory (2, 3). Nowadays, data have shown that HT patients have increased proportions of circulating Th17, both in the peripheral blood and thyroid gland (4-6). Th17 cells are a newly discovered lineage of effector CD4 T cells, which are considered developmentally and functionally distinct from Th1 and Th2 cells. Work of Gaffen *et al.* (7) has shown that the signalling induced by interleukin (IL) - 23 on Th17 cells is crucial to obtain a pathogenic and sustained phenotype. Interleukin-23 is a heterodimeric cytokine (p40/p19) consisting of a p19 subunit and the p40 subunit of IL-12p70. Despite advances in understanding the Th17 cell activities, we still lack clear evidences of IL-23-dependent mechanisms that coordinate the differentiation of pathogenic Th17 cells. Studies concerning the impact of IL-23 and IL-17 in the development of HT are still limited and contradictory (4, 6, 8). Authors of some papers in recent years attempted to find a link between the activation of this axis and the onset of fibrosis in the thyroid gland (6, 9).

The aim of this study was to provide the involvement of IL-23/IL-17 axis in pathologic processes in HT by measuring IL-23 and IL-17 serum levels in different stages of disease activity and comparing them with healthy controls.

MATERIAL AND METHODS

Subjects

We investigated 93 consecutive out-patients (85 women and 8 men, aged 47.6 ± 13.6 years) from the

INTRODUCTION

Hashimoto's thyroiditis (HT) is the most common cause of hypothyroidism in iodine-sufficient

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Department of Endocrinology, Stara Zagora, University Hospital (Bulgaria), with HT. After iodination of table salt, Stara Zagora has been one of the sufficient iodine supply regions of Bulgaria for a long time.

In all patients diagnosis had been made by clinical examination of the thyroid gland, elevated anti - thyroid peroxidase autoantibodies (antiTPO Abs) and/or typical sonographic characteristics of the thyroid in high-resolution sonography. In negative antiTPO Abs patients fine needle aspiration biopsy (FNAB) was performed and typical cytological features of autoimmune thyroiditis were found. With FNAB it was also excluded the presence of thyroid carcinoma in the study population. Thirty healthy subjects age and sex - matched (27 women and 3 men, aged 43.4 ± 10.9 years) were included as controls.

Exclusion criteria for both groups were acute or chronic systemic disease (diabetes mellitus, malignancy, cardiac or any autoimmune disease, renal or liver failure, pituitary or hypothalamic disease) or smoking, alcohol consumption as well as taking medication that may interfere with the immune system or thyroid function as antioxidants, vitamins, corticosteroids, amiodarone or nonsteroidal anti-inflammatory drugs. Pregnant women and those in a period of lactation were not included.

Patients were divided into three subgroups according to the thyroid function: group I (n=33) involved subjects with normal thyroid function - thyroid stimulating hormone (TSH) and free thyroxine (fT4) within the normal range; group II (n=11) included patients with hypothyroidism (high levels of TSH and low or normal serum levels of fT4); group III (n=49) involved subjects with hypothyroidism treated with Levothyroxine (LT4) in a dosage to maintain TSH and fT4 within the normal range. Patients were examined at least 6 months after taking LT4, while a median period of treatment was 3.6 years. Informed consent was obtained in all subjects and the study was approved by the local ethics committee. The study was conducted according to the declaration of Helsinki.

Laboratory evaluation

Fasting samples of venous blood were collected in the morning between 8.00 h. and 10.00 h. Serum samples were routinely collected and stored frozen at -20°C until assayed. At the time of sampling, neither of the patients and control subjects had clinical signs or symptoms of intercurrent illness. FT4 was measured by competitive immunoassay on the Roche Elecsys 2010 (electrochemiluminescence

immunoassay – ECLIA). TSH was measured by a third-generation two-site (sandwich) ECLIA on the Roche Elecsys 2010. The reference range was 11.5– 22.7 pmol/L for FT4 and 0.35–5.5 $\mu\text{IU/mL}$ for TSH. AntiTPO Abs were measured by ELISA, using commercially available kits (The Binding Site Ltd, Birmingham, UK); serum antiTPOAbs levels greater than 150 U/mL were considered as positive.

Cytokine Determination

Quantitative determination of IL-23 and IL-17 were performed by commercially available ELISA kits (Novex®, Invitrogen Corporation, Frederick, USA; BioSource, Nivelles, Belgium; for IL-23 and IL-17, respectively) according to the manufacture's instructions. The minimum detectable concentration of IL-23 ELISA kit was less than 20 pg/mL, and of IL-17 ELISA kit was less than 2.0 pg/mL. Serum samples of cases and controls were analyzed together in the same analytic batch.

Thyroid Ultrasonography

Thyroid ultrasonography was performed as part of clinical examination of patients, using a high-resolution ultrasound (US) with Doppler US device (Aloka Prosound SSD-3500SX, Aloka, Japan). 6-13 MHz linear transducer was used for the examinations. Patients were examined in supine position, with the neck slightly hyperextended. In the US fibrous septa were presented as linear hyperechoic white lines in thyroid parenchyma that in some cases may give the parenchyma “the pseudo-lobular” appearance with pseudonodules. For each patient included in the study, we measured thyroid volume by US (expressed in mL), calculated using the ellipsoid formula (longitudinal diameter X transverse diameter X anteroposterior diameter X p/6) applied to each lobe (10). Thyroid sonography was performed by the same endocrinologist (JG), with experience in US, before all laboratory evaluations.

Statistical Analysis

Normally distributed data were reported as mean $\pm$ SD. Data that did not show a Gaussian distribution were expressed as median (range, minimum – maximum or mean $\pm$ SE). The categorical variables were represented as number and percentage. Differences between two unpaired groups were analyzed by the Mann-Whitney U test and comparison of cytokine concentrations between HT subgroups of patients was done by Kruskal-Wallis test. Correlation

between two variables was evaluated with Pearson's correlation analysis. The level of statistical significance was set at $P < 0.05$. SPSS version 13.0 program was used for statistical analysis.

RESULTS

The baseline characteristics of control group and HT subgroups are summarized in Table 1. The presence of fibrous septa was found in the three subgroups of HT patients without statistical difference between them ($\chi^2 = 1.7$, $p = 0.428$).

IL-23 levels (mean $\pm$ SE) were significantly higher in HT patients than in healthy controls (38.70 ± 3.1 pg/mL vs. 14.76 ± 2.2 pg/mL, $p < 0.0001$). The serum levels of IL-23 in controls and subgroups

of patients are shown in Figure 1. All subgroups of patients had significantly increased IL-23 serum levels according to their stage of HT when compared to controls, but without statistical significance between subgroups of HT patients ($p = 0.944$).

The serum levels of IL-17 (mean $\pm$ SE) were significantly higher in HT patients compared to healthy controls (6.26 ± 0.9 pg/mL vs. 2.11 ± 0.2 pg/mL, $p = 0.014$). When the HT patients were subdivided into 3 groups, the differences in IL-17 levels between subgroups I and III in comparison to healthy controls were also significant ($p = 0.047$ and $p = 0.002$, respectively). Serum levels of IL-17 in group II were lower compared to other patient's groups without statistical significance compared to controls ($p = 0.571$) as seen on Figure 2. No significant difference was

Table 1. Baseline characteristics of healthy controls and Hashimoto's thyroiditis (HT) patients (group I – patients in euthyroid stage; group II – patients with hypothyroidism; group III – patients treated with Levothyroxine)

Variables	Controls n=30	Group I n=33	Group II n=11	Group III n=49	p
TSH (mIU/L)	1.8 (0.9 – 2.8)	3.2 (2.4 – 4.6)	21.9 (10.4 – 65.9)	2.9 (1.7 – 7.2)	<0.0001 a,b,c,d,e,f
ft4 (pmol/L)	15.8 ± 2.6	14.6 ± 4.4	10.3 ± 6.2	17.1 ± 5.0	<0.05 b,d,e,f
antiTPO Abs	9.2 (2.5 – 47)	397 (1.8 – 1301)	738 (57 – 1439)	510 (2.1 – 2700)	<0.001 a,b,c
Thyroid volume (mL)	10.0 ± 3.3	14.7 ± 8.3	14.1 ± 13.2	15.9 ± 12.9	<0.05 a,b, c
Fibrous septae (%)	0	13/33 39.4 %	5/11 45.5 %	14/49 28.6 %	ns

Data are presented as mean $\pm$ SD or median (range), when they were not normally distributed.

a.control vs. euthyroid, b.control vs. hypothyroid, c.control vs. treated with levothyroxine, d.euthyroid vs. hypothyroid, e.euthyroid vs. treated with Levothyroxine, f.hypothyroid vs. treated with Levothyroxine. Abbreviations: TSH – thyroid stimulating hormone; ft4 – free thyroxine; antiTPO Abs – anti – thyroid peroxidase autoantibodies

Figure 1. Serum levels of interleukin (IL)-23 (pg/mL) in Hashimoto's thyroiditis (HT) patients stratified according to the thyroid function and controls (C). Results are presented as mean values with standard errors of the means ($\pm$ SE). * $p < 0.01$; ** $p < 0.001$. Abbreviations: eu – patients in euthyroid stage; hipo – patients with hypothyroidism; treatment – patients treated with Levothyroxine.

Figure 2. Serum levels of interleukin (IL)-17 (pg/mL) in Hashimoto's thyroiditis (HT) patients stratified according to the thyroid function and controls (C). Results are presented as mean values with standard errors of the means ($\pm$ SE). * $p < 0.05$; ** $p < 0.01$. Abbreviations: eu – patients in euthyroid stage; hipo – patients with hypothyroidism; treatment – patients treated with Levothyroxine.

observed regarding IL-17 serum levels between the patient's subgroups ($p=0.178$).

The IL-17 and IL-23 levels correlated positively in the group of euthyroid patients ($r = 0.484$; $p = 0.004$), but not in other two groups. Thyroid anti-TPO autoantibody levels were not correlated with either IL-17 or IL-23 levels ($r < 0.2$; $p > 0.05$). There were no correlation between the volume of the thyroid gland and serum levels of IL-17 ($p=0.54$) or IL-23 ($p=0.184$). Also, the presence of fibrosis was not related to either IL-17 ($p=0.861$) or IL-23 ($p=0.7$) serum levels when the cytokine concentrations were compared in patients with and without fibrous septae.

DISCUSSION

The main objective of our investigation is to provide data for involvement of IL-23/IL-17 axis in the development of HT by measuring IL-23 and IL-17 serum levels in different stages of disease activity. Horie *et al.* in 2009 were the first that reported on the possible role of Th17 for the induction of an autoimmune thyroiditis on animal model (11). Subsequently, Figueroa-Vega *et al.* detected that HT patients have an increased levels of Th17 cells in both peripheral blood and thyroid tissue as well as an enhanced *in vitro* differentiation of their T cells into Th17 lymphocytes, induced by IL-6/IL-23 (4).

In the current study, we have demonstrated clear differences in the serum level of pro-inflammatory cytokine IL-23 and IL-17 in HT patients compared to controls. Our results demonstrated increased serum levels of IL-23 in all HT patients compared to controls as well in subgroups without functional state to influence the results. Our findings in euthyroid stage of HT consists of data from studies of Ruggeri *et al.* and Liu *et al.* where they found increased serum levels of IL-23 in untreated HT patients (12, 13). In these studies most of patients were euthyroid and they did not receive any treatment. The authors concluded that their findings concerning IL-23 could reflect an early step in the natural course of HT. Bossowski *et al.* (14) described increased serum levels of IL-23 and IL-17 in untreated HT children and, during therapy with LT4, they normalized IL-17, while IL-23 remained unchanged.

The greatest advantage of our research is that we evaluated at the same time, separately, patients in euthyroid as well as in hypothyroid stages of Hashimoto's disease, but also patients treated with LT4. To our knowledge we are the first investigators to evaluate the serum levels of IL-23 and IL-17 in

three functionally different groups of HT patients. We found a significant increase of serum levels of IL-23 in all groups of HT patients, which may indicate the role of IL-23 not only for the initiation of pathogenic processes, but also for the maintenance of autoimmune inflammation. Moreover, in our previous study we established a significantly enhanced IL-12p40 serum quantity both in euthyroid and hypothyroid stages as well as a significant higher frequency of IL-12p40 genotype (3'UTR AA genotype in IL12B gene), which is responsible for enhanced cytokine production (15, 16). In addition, IL-23 induces the production of a proinflammatory cytokine, IL-17, from activated T cells, such as Th17 (17). Exposure to IL-23 is essential to maintain activity of inflammatory Th17 cells. The established correlation between IL-17 and IL-23 for euthyroid patients confirms the positive relationship between these two cytokines and their involvement in disease onset.

IL-17 is the main effector cytokine, primarily produced and secreted by Th17 cells that promotes end-stage inflammation (17, 18). Our results demonstrated significant differences in the serum levels of IL-17 between healthy controls and all HT patients. We observed increased serum levels in euthyroid stage (newly diagnosed) compared to controls. We found lower serum levels in hypothyroid HT patients, which increased during therapy with LT4, suggesting that serum IL-17 levels could be influenced by hypothyroidism. Similar findings concerning the serum levels of IL-17 in hypothyroid stage were found in the study of Konca Degertekin *et al.* (9). Decreased levels of IL-17 in hypothyroidism may be related to depression of humoral and cell-mediated immunity in this functional state (19). We assume that these changes in serum levels of IL-17 were developed on the basis of general immune disorders existing in Th17 pathway in HT. The distinctive feature of our results is that the long-lasting treatments of hypothyroid patients with LT4 lead to IL-17 increase. We suggest that this increase of IL-17 is related to the persistence and progress of immunopathology realized by a shift from Th1 to Th17 than to a direct drug effect.

Some of authors in these studies tried to link the activation of Th17 pathway with the presence of fibrosis of the thyroid gland. Modern concepts of autoimmune thyroiditis differ significantly from the definition given by Dr. Hashimoto (1). Based on our knowledge, we believe that at the time of the discovery of the disease, functionally a patient may be from euthyroid stage to severe hypothyroid; the volume of the

thyroid gland may vary considerably between patients. By ultrasonography, there were described different degrees of hypoechogenicity and pseudonodules in the thyroid gland parenchyma, which is a manifestation of inflammatory cell infiltration and fibrosis observed in all stages of the disease (20).

In addition, we analyzed the size of the thyroid gland by ultrasonography and the presence of fibrous septae in the three patient's groups. We detected the presence of fibrosis in 34% of the patients, regardless of the stage of the disease. These ultrasound findings also support the hypothesis about the role of IL-17 axis for the beginning and maintenance of fibrotic processes in the thyroid, manifested to varying degrees in each patient. Our results support the role of Th17 axis in the pathogenesis of the disease. We suppose that it is the leading role in the pathogenesis of the chronic inflammatory phenomenon tissue damage and fibrosis seen in HT.

In the large number of patients with autoimmune thyroiditis, the treatment with thyroid hormones restores functional disorders and reduces thyroid volume. Some of them remain with firm thyroid glands and local compression, despite taking LT4. With the current treatment with thyroid hormones or corticosteroids these symptoms do not respond. Treatment with the drugs blocking axis of IL-23/IL-17 could be a new therapeutic perspective, which would improve the quality of life of this group of patients.

However, there are some discrepancies between studies concerning the change of serum levels of discussed IL-23 and IL-17 cytokines in various stages of TH activity. This may be due to the different sensitivity of detection of cytokines, the number of patients included in the studies, the age of patients. Moreover, some studies simultaneously analyzed patients in hypothyroid and euthyroid stage of disease; the duration of disease before diagnosis also may differ in patient's groups and influence the results. However, all researches unequivocally prove the activation of IL-17 axis in patients with Hashimoto's thyroiditis. There is an increasing interest in whether blocking IL-17 axis and mostly IL-23 will give new perspectives on the treatment in some patients with Hashimoto's thyroiditis, but, new studies need to be carried out in the future.

There are some limitations of our study. First we measured serum levels and we were not able to measure functional expression of IL-17A receptors by thyrocytes. We believe that serum cytokine's levels

may not totally represent the process in the thyroid gland. Second, we identified with ultrasonography the presence of fibrous septa in thyroid parenchyma, but with this method we were unable to quantify the severity of fibrosis. Based on our data the link between thyroid fibrosis and IL-17 axis is rather hypothetical, but it matched perfectly data with histologic descriptions of the group of Li *et al.* (6).

In conclusion, our results demonstrate the involvement of IL-23/IL-17 axis in the development of HT in all stages of the disease. Moreover, our data show that the significance of IL-23 is more pronounced than IL-17 in HT development and severity. Increased secretion of circulating IL-23 could be a biomarker for progression and monitoring of HT.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

This work was supported by Grants: no. 14/2013 and 1/2016 from the Fund for Scientific and Mobile project from Faculty of Medicine at the Trakia University, Stara Zagora, Bulgaria.

Ethics

The authorization was given by the Ethics Review Board of the Faculty of Medicine, Trakia University, Stara Zagora.

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