

RESEARCH ARTICLE

Serum levels of transforming growth factor- β 1 (TGF- β 1) in patients with systemic lupus erythematosus and Hashimoto's thyroiditis

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ABSTRACT. Background: Transforming growth factor- β 1 (TGF- β 1) exerts broad anti-inflammatory and immunosuppressive effects and plays a key role in self-tolerance. Complete knockout of TGF- β 1 in mice results in autoimmunity and multi-organ inflammatory syndrome. The aim of the present study was to determine TGF- β 1 serum levels in healthy individuals and in patients with typical systemic or organ-specific autoimmune disorders such as systemic lupus erythematosus (SLE) and Hashimoto's thyroiditis (HT) in an attempt to elucidate the importance of TGF- β 1 in human autoimmunity. **Patients and methods:** Serum concentrations of TGF- β 1 were determined using an enzyme-linked immunosorbent assay in a group of 53 patients with SLE (87% women) and 123 with HT (95% women). Results were compared with those from 66 healthy controls (HC; 80% women). **Results:** Significantly lower levels of serum TGF- β 1 were found in patients with SLE and HT than those found in HC (mean \pm SD: SLE: 8.7 ± 2.5 ng/mL; HT: 18.74 ± 8.2 ng/mL; HC: 33.01 ± 2.48 ng/mL; SLE versus HC: $p < 0.001$; HT versus HC: $p < 0.001$). Also, serum levels of TGF- β 1 were significantly lower in patients with SLE compared to patients with HT ($p < 0.001$). The serum levels TGF- β 1 were significantly higher in men than in women in the HC group (63.4 ± 28.1 ng/mL versus 26.6 ± 17.5 ng/mL, $P < 0.001$), but were similar for men and women in both patients groups ($p > 0.05$). **Conclusions:** Our data demonstrate that altered TGF- β 1 levels are associated with the presence of autoimmune disorders, and that TGF- β 1 concentrations seem to be more profoundly depressed in systemic autoimmune diseases than in autoimmune thyroid disorders. Autoimmunity may have been triggered as a result of a decreased immunosuppressive effect induced by depressed TGF- β 1 levels in patients with SLE and Hashimoto's thyroiditis.

Key words: autoimmunity, Hashimoto's thyroiditis, systemic lupus erythematosus, transforming growth factor- β 1

Autoimmune diseases (AID) encompass a large variety of organ-specific and systemic disorders with complex etiologies. A typical systemic inflammatory autoimmune disease is systemic lupus erythematosus (SLE), which is characterized by various immunological abnormalities, including dysregulated polyclonal activation of both T and B lymphocytes and subsequent production of large quantities of autoreactive antibodies and the formation of immune complexes causing tissue and organ damage [1]. The most common organ-specific autoimmune disorder is chronic, autoimmune thyroiditis or Hashimoto's thyroiditis (HT), considered the prototype of organ-specific AID. The disease is characterized by diffuse lymphocytic infiltration of the thyroid gland, the presence of anti-thyroglobulin antibodies (anti-Tg), and/or anti-thyroid peroxidase antibodies (anti-TPO), in addition to abnormalities of thyroid function [2].

Recently, experimental studies have demonstrated an association between transforming growth factor- β (TGF- β) and the development of autoimmunity [3]. TGF- β 1 belongs to a large family of multifunctional proteins, secreted by a variety of cell types that act as signal molecules in controlling a great number of biological processes. It is a highly pleiotropic cytokine with an important role in maintaining immune homeostasis [4]. TGF- β 1 has pronounced anti-inflammatory and immunosuppressive functions [5], the latter being brought about by controlling the activation, proliferation, differentiation and survival of all effector immune cells [5, 6].

The great importance of this cytokine in the control of autoimmunity is clearly demonstrated in studies with complete knockout of TGF- β 1 in mice, or genetic manipulation of its receptors in T cells. TGF- β 1-deficient mice and those with impaired TGF- β 1 signaling in T

cells, develop an autoimmune syndrome with multiple organ involvement and death [7-10]. This syndrome resembles SLE and Sjogren's syndrome in humans [7] and is characterized by a multifocal inflammatory process affecting the heart, brain, lungs, skeletal muscle, liver, stomach, pancreas, salivary glands and other organs, lymphoproliferation, spontaneous activation of autoreactive T lymphocytes, and production of autoantibodies [8, 10]. Also, the protective effects of TGF- β against autoimmunity have been established in animal models with colitis, autoimmune diabetes, collagen-induced arthritis, and autoimmune thyroiditis [11].

The aim of the present study was to determine the serum levels of TGF- β 1 in healthy individuals and in patients with typical systemic and organ-specific autoimmune disorders such as SLE and HT, in attempt to elucidate the importance of TGF- β 1 in human autoimmunity. We also examined the possible contribution of some endogenous factors, such as sex and age on the production of TGF- β 1 in healthy individuals and in patients with autoimmune diseases.

MATERIALS AND METHODS

Study subjects

Fifty three patients with SLE were recruited from the St. Ivan Rilski University Hospital (Sofia). The patients satisfied at least four of the 1982 American College of Rheumatology (ACR) criteria for SLE [12]. A total of 123 HT outpatients from the Department of Internal Medicine, Stara Zagora University Hospital (Bulgaria) were also enrolled in the study. In all patients, diagnosis had been established by an enlarged thyroid gland, increased TPO-Abs and typical hypoechogenicity of the thyroid in high-resolution sonography. In negative TPO-Abs patients, fine needle aspiration biopsy was performed and typical cytological features of autoimmune thyroiditis were found. Serum levels of TSH and free thyroxin (fT4) were estimated. Comparisons were made with 66 healthy controls, recruited consecutively from participants in a health check-up program during the study period, without matching to the patients.

At the time of sampling, neither patients nor control subjects had any clinical signs or symptoms of intercurrent illness.

Informed consent was obtained from all participants in the study in accordance with the ethical guidelines of the Helsinki Declaration.

Blood samples

Blood samples in the study were collected in the morning, between 8.00 and 10.00 h, and stored on ice until processed. Processing was completed within two h of collection and serum samples were frozen at -70° C until analysis. Serum samples from cases and controls were analyzed together in the same batch.

Measurement of serum TGF β 1 concentration

The serum concentrations of activated TGF- β 1 protein were measured using a quantitative sandwich ELISA technique according to the manufacturer's instructions (Qantakine®, R&D systems, Abingdon, UK). Before

assay, the latent TGF- β 1 contained in patients' serum was activated to the immunoreactive form using acid activation and neutralization. The results were calculated by reference to the standard curve and expressed as nanograms/picograms per mL (ng/mL). The minimum detectable TGF- β 1 level ranged from 1.7 -15.4 pg/mL.

Statistical analysis

All data for this study were analyzed using SPSS V.16.0. Intergroup comparisons were performed using the *t* test for continuous measures that were normally distributed, The Mann-Whitney *U* test was used for continuous measures that were not normally distributed, and the χ^2 test for the categorical measures. Spearman's correlation coefficients were calculated to investigate univariate associations between serum levels of TGF- β 1 and age. Independent associations between the variables of interest were investigated using the general linear model (GLM). GLM is relatively robust against violation of multivariate normality, so that variables were not transformed before entry. The main explanatory variables age, sex and presence or absence of illness were used as continuous variables or as categorical variables upon categorization. Since serum levels of TGF- β 1 are dependent on sex, age and presence or absence of SLE or HT, interactions of sex, age and the presence of each of the two diseases with respect to explaining serum concentrations of TGF were investigated. Results were expressed as regression coefficients, and estimated marginal means (an estimated marginal mean is the model estimator of the mean value of the dependent variable after adjustment for the covariates). A 2-tailed *P* value of <0.05 was considered significant.

RESULTS

Study subjects

The group of the patients with SLE consisted of seven (13%) males and 46 (87%) females from 16 to 70 years old with a mean (\pm SD) age of 39.42 ± 13.2 years. The group of HT patients consisted of six (5%) males and 117 (95%) females from 18 to 73 years old with a mean (\pm SD) age of 47.5 ± 13.1 years. The sex and age distribution of the healthy controls (HC) was: 13 (19.7%) males and 53 (80.3%) females; mean (\pm SD) age, 51.8 ± 16.4 years, range 19-83 years. The proportion of males in both patient groups was lower than that in the HC group, but a significant difference was found only between HT cases and healthy controls with regard to gender distribution ($\chi^2 = 10.43$, *p* = 0.001). SLE patients were significantly younger than healthy controls (*p* < 0.001), as the proportion of individuals under 45 years old was significantly higher among patients (64.2%) compared to controls (34.8%) ($\chi^2 = 10.11$, *p* = 0.001). The demographic characteristics of the cases and control subjects are summarized in *table 1*.

Serum level of TGF- β 1 in study patients and controls

Serum TGF- β 1 levels ranged from 2.47 to 21.56 ng/mL for SLE cases, with a mean (\pm SD) of $8.88 (\pm 3.8)$ ng/mL and a median of 9.03 ng/mL, from 3.91 to 45.28 ng/mL for HT cases, with a mean of $18.74 (\pm 8.18)$ ng/mL and a median of 18.47 ng/mL and from 5.28 to 107.36 ng/mL for

Table 1
Demographic characteristics of study patients and controls.

Characteristics	HT patients (n = 123)	SLE patients (n = 53)	Healthy controls (n = 66)	Intergroup differences	
				HT versus HC	SLE versus HC
Gender					
Male (%)	6 (4.9)	7 (13.2)	13 (19.7)	$P = 0.001$	$P = 0.347$
Female (%)	117 (95.1)	46 (86.8)	53 (80.3)		
Age (years)					
Mean \pm SD	47.5 \pm 13.8	39.4 \pm 13.2	51.8 \pm 16.4	$P = 0.11$	$P < 0.001$
Median (range)	49.0 (18-73)	38.0 (16-70)	53.0 (19-83)	$P = 0.648$	$P = 0.001$
<45 (%)	47 (38.2)	34 (64.2)	23 (34.8)		
≥ 45 (%)	76 (61.8)	19 (35.8)	43 (65.2)		

SLE, systemic lupus erythematosus; HT, Hashimoto's thyroiditis; SD, standard deviation.

Table 2
Serum levels of TGF- β 1 in both patient groups and healthy controls included in the study.

Serum level of TGF- β 1	Value ng/mL (mean \pm SD)	Significance
SLE		
total (n = 53)	8.88 \pm 3.79	SLE versus HC $p < 0.001$
male (n = 7)	8.57 \pm 2.72	SLE versus HT $p < 0.001$
female (n = 46)	8.93 \pm 3.95	
	male versus female $p = 0.82$	
HT		
total (n = 123)	18.74 \pm 8.18	HT versus HC $p < 0.001$
male (n = 6)	19.27 \pm 10.08	
female (n = 117)	18.71 \pm 8.12	
	male versus female $p = 0.89$	
Healthy controls		
total (n = 66)	32.99 \pm 24.84	
male (n = 13)	60.98 \pm 31.32	
female (n = 53)	26.12 \pm 17.34	
	male versus female $p < 0.001$	

SLE, systemic lupus erythematosus; HT, Hashimoto's thyroiditis; TGF- β 1, transforming growth factor; SD, standard deviation.

controls, with a mean of 32.89 (\pm 24.84) ng/mL and a median of 24.85 ng/mL. TGF- β 1 serum concentrations were significantly lower in SLE patients (n = 53) and HT patients (n = 123) than in the HC group (n = 66) (Mann-Whitney U test, $p < 0.001$). Also, TGF- β 1 serum levels were significantly lower in patients with SLE compared to patients with HT (Mann-Whitney U test, $p < 0.001$). Table 2 presents the serum concentrations of TGF in patients and healthy controls included in the study.

We also examined the possible influence of gender and age on the production of TGF- β 1 in healthy individuals and in patients with either of these two autoimmune diseases. The levels of serum TGF- β 1 were significantly higher (Mann-Whitney U test, $p < 0.001$) in men (n = 13, mean \pm SD, 60.98 \pm 31.32 ng/mL) than in women in the HC group (n = 53, mean \pm SD, 26.12 \pm 17.34 ng/mL, $P < 0.001$), but were similar between the sexes in the SLE group (t test, $p = 0.82$) and the HT group (t test, $p = 0.89$) (table 2). Also, the difference in serum TGF- β 1 levels between men from the SLE group and the control group was more profound (Mann-Whitney U test, $p < 0.001$) than that between healthy men and male patients with HT (Mann-Whitney U test, $p = 0.017$).

There was a positive, moderate correlation between TGF- β 1 serum levels and age ($r = 0.41$, $p = 0.001$) as TGF- β 1 serum levels among healthy controls were lower

in individuals under 45 years (n = 23, mean \pm SD, 21.3 \pm 10.4 ng/mL), and higher in those aged over 45 (n = 43, mean \pm SD, 39.2 \pm 27.9 ng/mL, $P < 0.001$). (figure 1). There was no association between serum TGF- β 1 and age in cases with autoimmune disease as shown in figure 1.

To further investigate the relationship between serum concentrations of TGF- β 1 with the presence of autoimmune disease (SLE/HT) while adjusting for sex and other potential confounders, multivariate analysis was performed using GLM with serum levels of TGF as dependent variable and sex, age and presence of the disease as covariates. Table 3 shows the results of this analysis.

The biological factors of gender and age, independently of the presence of AID, affect the serum levels of TGF, with highly significant parameters estimated. The level of TGF- β 1 is positively associated with sex and age, and negatively associated with the presence of SLE/HT. Age has a weaker statistically significant influence over serum concentrations of TGF- β 1. Most strong is the impact of gender on the levels of TGF- β 1, following that of the presence of autoimmune disease. The regression coefficients describe the independent relationship between the explanatory variable (e.g., the presence of SLE/HT), and dependent variable (e.g., serum levels of TGF- β 1): on average, as compared with healthy controls,

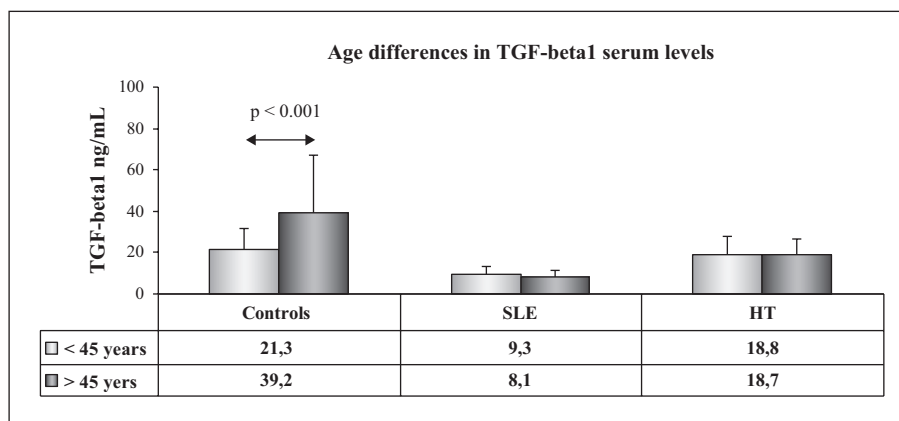


Figure 1

Mean (\pm SD) serum level of TGF- β 1 in both patients' groups and healthy controls according to the age of individuals.

Table 3

Multivariate relationship between sex, age and presence of autoimmune disease (SLE/HT) with serum levels of TGF- β 1 separately, established by the general linear model in the 176 patients included in the study.

Dependent variable		
Independent variable	TGF	
	Parameter estimate (SE)	P
Age (years)	0.13 (0.057)	<0.05
Sex (male)	33.21 (38.82)	<0.001
SLE	-15.88(25.51)	<0.001
HT	-7.28 (20.39)	<0.001

SLE, systemic lupus erythematosus; HT, Hashimoto's thyroiditis; TGF- β 1, transforming growth factor; SE, standard error.

a patient with HT has a lower serum concentration of TGF- β 1, *i.e.* 7.28 ng/mL, and one with SLE also has a reduced serum TGF- β 1 *i.e.* 15.88 ng/mL. The influence of gender is demonstrated in the following results, under substantially equivalent conditions (other conditions being equal), men have higher serum concentrations of TGF compared with women (33.21 ng/mL). Interactions between presence of disease (HT/SLE) and gender were tested and were estimated as significant, while interaction between absence of the two diseases and sex was excluded from the model. *Figure 2* shows the relationship between serum concentrations of TGF and disease status after adjustment for sex.

DISCUSSION

In the present study, we compared serum levels of TGF- β 1 in healthy individuals with those in patients with diseases that are representative of systemic or organ-specific autoimmune disorders namely SLE and HT. We observed a large interindividual variation in serum TGF- β 1 levels among healthy subjects as well as in patients tested. Our results showed lower levels of activated TGF- β 1 in lupus patients and in patients with HT compared with healthy individuals. Also, in patients with SLE, TGF- β 1 levels were lower than those of patients with HT, suggesting that the immune dysregulation is more profound in systemic than in organ-specific AID.

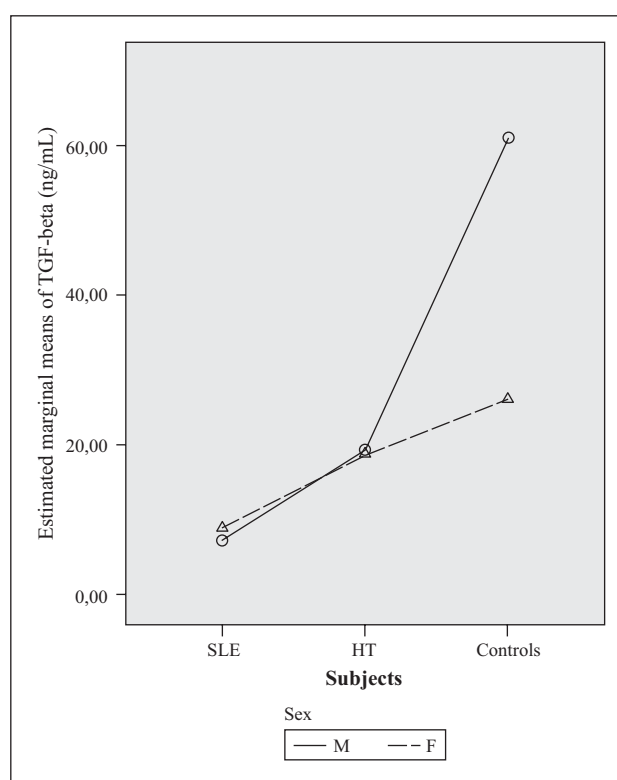


Figure 2

Estimated marginal means of TGF- β 1 serum levels in dependence of disease status (SLE/HT/Controls) and sex.

It has been reported that decreased serum levels of TGF- β 1 in patients with systemic lupus are the most pronounced and constant abnormality for the cytokine levels in these patients [13, 14]. Lower serum levels of TGF- β 1 are associated with susceptibility to SLE, as well as with disease activity and the development of organ damage [15, 16]. Oshuka *et al.* reported decreased levels of total and active isotype of TGF- β 1 in patients with SLE compared with healthy controls [17]. In a parallel study, the same authors reported that the main source of TGF- β 1 (both latent complex, and as an active form) are NK cells, and the level of induced NK cell production of TGF- β 1 is also reduced in patients with SLE [18]. Decreased production of TGF- β 1 in SLE lymphocytes could be explained by a reduced secretion of the precursor molecule or inhibition mechanisms for the conversion of the latent complex to the

active form. Another possibility is related to increased production of IL-10, which downregulates TGF- β 1 [19]. We may also discuss the role of genetic factors on the expression of TGF- β 1. In our association study, it was found that the promoter polymorphism C-509T in the TGF- β 1 gene influences the genetic predisposition to SLE and clinical manifestations of the disease in the Bulgarian population, which defines it as one of the genetic factors contributing to the clinical diversity of this disease [20].

Experimental models in lupus-prone mice and non-autoimmune "wild" type mice have clearly established the key role of this cytokine in the pathogenesis of systemic lupus. In "wild type" mice, disruption of B cell tolerance in response to autoantigen stimulation is transient, and restoration of immunological tolerance to own-antigens correlated with the appearance of TGF- β 1-producing T cells [5]. In lupus-prone mice, decreased production of TGF- β 1 and/or blocking its activity by monoclonal antibodies, leads to immune dysregulation and production of autoantibodies forming immune complexes that induce immune-mediated inflammation in target organs [21]. This triggers the local production of anti-inflammatory cytokines such as TGF- β 1, which stimulates the healing process, but also has a profibrotic activity. Its increased expression in tissues can lead to overproduction and deposition of collagen and matrix proteins, causing progressive tissue fibrosis and organ dysfunction [21].

Few studies have investigated serum TGF- β 1 levels in autoimmune thyroiditis [22, 23]. Our data are similar to those reported by Akinci *et al.* [23]. The authors observed lower TGF- β 1 levels in HT patients compared to controls, and suggest that altered TGF- β 1 levels are associated with the presence of Hashimoto's thyroiditis, not with the treatment of thyroid dysfunction. The immunosuppressive role of TGF- β 1 in thyroiditis development was demonstrated in experimental autoimmune thyroiditis [24]. TGF- β 1 acts to suppress inflammation and is important for protecting against thyroiditis mediated by the transfer of CD4⁺ T cells [25]. In the early stage of the autoimmune process, TGF- β 1 plays an inhibitory role, whereas it may trigger the development of fibrosis during the late stage of the disease as was shown in an experimental granulomatous thyroiditis model [26, 27].

The strengths of our study include a comprehensive approach toward investigating the relationship between serum concentrations of TGF- β 1 and the presence of AID by performing multivariate analysis. We used the GLM with serum levels of TGF- β 1 as the dependent variable and sex, age, and the presence of disease as covariates and observed significant, positive associations of serum TGF- β 1 with sex and age, and a negative association with the presence of AID. Based on the results of the GLM, it can be argued that age has the least impact on serum TGF- β 1. Gender has the strongest influence on serum TGF- β 1, followed by the presence of disease. Also, there is a significant shared effect of gender and presence of disease on TGF- β 1 serum concentrations. Higher levels of TGF- β 1 were found in men and older individuals among the healthy subjects. In patients with SLE or HT, such a correlation between serum levels of TGF- β 1 and gender or age was not observed. These data allow us to suppose that low levels of serum TGF- β 1 may predispose to the onset

of autoimmunity in younger individuals, and that high levels of serum TGF- β 1 may protect against autoimmunity in men.

One limitation of our study is that circulating TGF- β 1 was only measured at one time point. It is unclear how well a single measure may reflect long-term levels. Furthermore, we did not take into account the influence of disease activity and clinical manifestations in patients with SLE or the functional status of the thyroid gland in patients with HT. Thus, inter-individual variation may have reduced our ability to detect a difference in serum levels by case-control status, and it is possible that one serum measure may not be enough to show association with disease. Despite these limitations, significant differences observed between median values TGF-beta1 levels in healthy individuals and those with AID, confirm the importance of low serum levels of this cytokine to the pathogenesis of SLE and Hashimoto's thyroiditis.

In conclusion, altered TGF-beta1 levels are associated with the presence of autoimmune disorders, and TGF-beta1 concentrations seem to be more profoundly depressed in systemic autoimmune diseases than in autoimmune thyroid disorders. Autoimmunity may have been triggered as a result of a decreased immunosuppressive effect induced by depressed TGF-beta1 levels in patients with SLE and Hashimoto's thyroiditis.

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