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Skin autoreactivity in Hashimoto's thyroiditis patients without urticaria: Autologous serum skin test positivity correlation with thyroid antibodies, sonographical volume and grading

Recent studies have shown an association between anti-thyroid antibodies and autologous serum skin test (ASST) positive urticaria patients. However, a connection between thyroid and this reliable skin test for mast cell autoreactivity, ASST, has not been reported yet. We investigated ASST in patients with Hashimoto's thyroiditis (HT) without urticaria and compared the results with laboratory and sonographical findings of HT. 154 HT patients, 100 healthy volunteers without HT as a first control group and 46 patients with multinodular goitre but without autoimmune thyroid disease as a second control group underwent testing with ASST. ASST was applied to these groups according to two criteria, first as ASST(new): autologous serum red wheal response 1.5 mm bigger than negative control; second as ASST(old): serum red wheal response 5 mm bigger than negative control accepted as positive. Free triiodothyronine (fT3), free thyroxine (fT4), thyroid-stimulating hormone (TSH), thyroid peroxidase antibody (anti-TPO) and thyroglobulin antibody (anti-Tg) levels were measured. ASST(old), ASST(new) scored positive in 51.3-60.4% of HT patients, with statistically significant differences. Thyroid volume grades were inversely proportional with ASST(old) and (new) positivity. Moderate (+) titers of anti-Tg in ASST(old) and (new) (+) cases were significantly higher than the same titers of anti-Tg in ASST(old) and (new) (-) cases. The prevalence of ASST positivity in HT patients was not affected by the following factors: gender, age at screening, laboratory measurements of thyroid function tests, anti-TPO antibodies and thyroid ultrasound (US) echogenicity. Positivity of ASST in HT has shown that there is a skin mast cell autoreactivity in HT patients independent of autoreactive chronic urticaria (ACU).

Key words: autologous serum skin test, Hashimoto's thyroiditis, autoimmune thyroid disease, thyroid autoantigen, thyroid ultrasound, Fc receptor pathways

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The positive relationship between ASST(+) autoreactive chronic urticaria (ACU) patients and autoimmune thyroid diseases (ATD) with thyroid autoantigen positivity is well known [1, 2]. In CU patients, these autoantigens, anti-TPO and anti-Tg, have been detected to be more common, with a ratio of 5-34% compared with normal populations (5-10%) [3, 4]. Autoreactivity related with ASST positivity has been searched for in association with autoimmune diseases, drug allergy and hypersensitivity. But the effect of ATD on mast cell autoreactivity has not yet been investigated. Inflammatory connections between autoantigen overload of thyroid autoantigens [5, 6], toll-like receptors (TLR), Fc receptor pathways and mast cells remind us that IgE functional autoantibodies and/or histamine releasing IgG autoantibo-

dies can occur at any stage of the pathogenesis of ATD [5, 7-12]. Current studies of autoimmunity have pointed out the controversial issue of whether overload of autoantigens in HT patients makes people candidates for urticaria, or whether there is coexistence of urticaria with autoimmune thyroiditis [5].

The aim of this study was to detect the effects of thyroid gland mass and thyroid hormone levels on mast cell autoreactivity and at which stage this sensitization was effective in HT patients without any urticarial symptoms and history of urticaria. We used ASST, which is the best *in vivo* method for detecting the autoreactivity of mast cells in blood via the Fc receptor pathways. We compared the two forms of ASST results with thyroid hormone levels and ultrasonographic thyroid patterns in both HT and non-ATD patients.

Subjects and methods

Study population

Subjects were recruited from endocrinology and internal medicine clinics at the Istanbul Medeniyet University Goztepe Training and Research Hospital. This prospective study included 154 HT patients. A diagnosis of HT was made in the presence of elevated anti-TPO, anti-Tg and ultrasound patterns suggestive of HT. There were two control groups; the first control group included 100 healthy volunteers without HT, classified as the “healthy control group”, and whose anti-TPO and anti-Tg levels were normal. The second control group included multinodular goitre (MNG) without ATD, the patients were assessed by sonography and their anti-TPO and anti-Tg levels were normal. These 46 patients with MNG were classified as the “patient control group”. Exclusion criteria from the study included previous symptoms and diagnosis of any clinical form of urticaria, autoimmune diseases, allergic or non-allergic respiratory diseases (asthma, allergic rhinitis, nasal polyposis), pregnancy, smoking, NSAID hypersensitivity, drug allergy, intake of antihistaminic treatment, antithyroid drugs, immunosuppressive treatments, high serum immunoglobulin levels, personal history of stool test positivity and laboratory data. All groups were classified according to gender and age range. There were three age ranges were 17-30, 31-50 and 51-70.

Laboratory measurements

All individuals underwent an extensive laboratory work-up that included complete blood count, biochemistry profile, fT3, fT4, TSH, anti-TPO and anti-Tg levels before the intradermal tests. 113 of 154 patients who accepted radiological evaluation in the thyroiditis study group and all of the 46 patients in patient control group were assessed sonographically for disease activity one day before intradermal tests by radiologist (OT) (Ultrasound System GE LOGIQ 3, GE Medical Systems, Solingen, Germany, with GE 7.5L75G Ultrasound Transducer, 7.5-MHz linear array probe). The following US characteristics were examined: 1) thyroid gland volume as low, normal, high (normal values: 17.5 ± 3.2 mL for women and 19.6 ± 4.7 mL for men) [13]; 2) echogenicity of the solid portion (grade 1=normal: similar to submandibular gland, hyperechoic to neck muscles, grade 2: hypoechoic to submandibular gland and hyperechoic to neck muscles, grade 3: iso-/hypoechoic to neck muscles) [14].

Patient and control group thyroid profiles were evaluated with thyroid function tests and thyroid autoantibody levels by an endocrinologist (GT). The thyroid functions of all patients were grouped into four groups: euthyroid, subclinical hypothyroid, overtly hypothyroid, hyperthyroid. Anti-TPO and anti-Tg levels grouped in three groups by titrations: for anti-TPO levels 34-100 IU/ml was accepted as low (+), 100-1,000 IU/mL moderate (+), >1,000 IU/mL high (+) titers and for anti-Tg 115-200 IU/mL was accepted as low (+), 200-1,000 IU/mL moderate (+), >1,000 IU/mL high (+) titers. Normal ranges of thyroid hormones were 0-34 IU/mL for anti-TPO, 0-115 IU/mL for anti-Tg, 0.27-4.2 IU/ml for TSH, 2-4.4 nq/mL for fT3, 0.8-1.76 ng/mL for fT4. Levels of fT3, fT4, TSH, anti-TPO and anti-Tg

were measured with electrochemiluminescence immunoassay method by using Rosh firms' Elecsis 170 device.

Intradermal tests

We performed intradermal tests, ASST, with both autologous serum, normal saline (negative control) and histamine (positive control). Venous bloods of patients both from the study group and control groups were taken in sterile tubes which did not contain coagulation activator factors. Samples were kept at room temperature for 30 mn and centrifuged with 500 g for 15 mn; 3 cm apart, three injections of 0.05 mL autologous serum, normal saline and histamine were given intradermally to the anterior of the left forearm and the wheal and erythema reaction was awaited. Two ASST criteria were used: new and old. According to ASST(new) criteria [15], an autologous serum red wheal response 1.5 mm bigger than the negative control was accepted as positive, after waiting 30 mn and according to ASST(old) criteria [16], a serum red wheal response 5 mm bigger than the negative control was accepted as positive after waiting for both 30 and 60 mn. None of the patients was taking systemic steroids, immunosuppressive or antihistaminic treatment during the tests. The patients and controls gave informed consent and the study was approved by the local Ethics committee.

Statistics

Descriptive statistical methods (mean, standard deviation), student T test, Chi-squared test (χ^2) and Spearman rank correlation analysis were used for interpretation of the results, comparison of groups, comparing quantitative data and correlation of groups, respectively. The results were evaluated within p values of 0.05 and a confidence interval of 95%. NCSS 2007&PASS and 2008 Statistical Software (Utah, USA) programme were used for statistical analysis.

Results

Study group

There were no differences between ages and genders of HT patients and control groups ($p > 0.05$) (table 1). Thyroid US was performed in 113 of 154 HT patients and thyroid volumes of 90 patients (81.1%) were found low, of 16 patients (57.1%) were found normal size and of 7 patients (35%) were found high. Thyroid volumes of HT patients were significantly lower than thyroid volumes in the patient control group ($p < 0.01$) (table 2). According to thyroid US echogenicities, HT patients in the thyroiditis study group and MNG patients in the patient control group were determined as grade 1 at 38.9% and 65.2%, as grade 2 at 45.1% and 30.4%, as grade 3 at 15.9% and 4.3%, respectively.

ASST test

ASST(old) were positive in 79 (51.3%) of the thyroiditis study group and were statistically significant compared to healthy (15%) and patient (15.2%) control groups ($p < 0.01$) (table 1).

Table 1. Clinical characteristics and ASST results of the patients and controls.

		Healthy control group (n=100)	Patient control group (n=46)	Thyroiditis study group (n=154)	(Student T)
Age(17-70)		40.22±14.25	41.65±14.17	40.65±13.44	<i>p=0.884</i>
Gender	Female	88(88.0%)	41(89.1%)	143(92.9%)	<i>p=0.398</i>
(Chi-square)	Male	12(12.0%)	5(10.9%)	11 (7.1%)	
ASST(old)	(-)	85 (%85.0)	39(%84.8)	75 (%48.7)	(Chi-square) <i>p=0.001**</i>
	(+)	15 (%15.0)	7 (%15.2)	79 (%51.3)	
ASST(new)	(-)	77 (%77.0)	33 (%71.7)	61 (%39.6)	(Chi-square) <i>p=0.001**</i>
	(+)	23 (%23.0)	13 (%28.3)	93 (%60.4)	

ASST(new) were positive in 93 (60.4%) of the thyroiditis study group and were statistically significant compared to the healthy control group (23%) and the patient control group (28.2%) ($p<0.01$) (table 1). The patient control group and study groups' ASST(old) and ASST(new) results were not related to thyroid functions and thyroid US grades ($p>0.05$). Ratios of ASST(old) and ASST(new) cases, regarding thyroid functions, were respectively as follows, with decreasing incidence: euthyroid, subclinical hypothyroid, overtly hypothyroid and hyperthyroid (table 2).

In the thyroiditis study group, while thyroid volumes were low in 53 of 62 (85.5%) HT patients with ASST(old)(+), the thyroid volumes of 2 (3.2%) and 7 (11.3%) patients were elevated and normal, respectively. In ASST(old)(-) cases, only the thyroid volumes of 37 (72.5%) HT patients were low and there was a negative correlation between thyroid volumes and ASST positivity ($\uparrow$ ASST($+$) $\rightarrow$ thyroid volume $\downarrow$), although it was not statistically significant ($p>0.05$). In the patient control group, there was no correlation among ASST(old)/ASST(new) results and

Table 2. The association between thyroid functions, volumes and the results of ASST (old)/(new).

Thyroid functions		ASST (old)		ASST(new)	
		(-)	(+)	(-)	(+)
		n (%)	n (%)	n (%)	n (%)
Patient control group (n=46)	Hypothyroidy	1 (2.6%)	0	1 (3%)	0
	Subclinical Hypothyroidy	6(15.4%)	0	4 (12.1%)	2 (15.4%)
	Euthyroidy	29(74.3%)	7 (100%)	25 (75.8%)	11 (84.6%)
	Hyperthyroidy	3 (7.7%)	0	3 (9.1%)	0
	P	0.549		0.059	
Thyroiditis study group (n=154)	Hypothyroidy	14 (14.7%)	6 (7.6%)	10 (16.4%)	10 (10.8%)
	Subclinical Hypothyroidy	25 (26.3%)	32 (40.5%)	22 (36.1%)	35 (37.6%)
	Euthyroidy	31 (32.7%)	35 (44.3%)	27 (44.3%)	39 (41.9%)
	Hyperthyroidy	25 (26.3%)	6 (7.6%)	2 (3.3%)	9 (9.7%)
	P chi-square	0.627		0.379	
Thyroid Volume Grade		ASST(old)		ASST(new)	
		(-)	(++)	(-)	(+)
		n (%)	n (%)	n (%)	n (%)
Patient control group	Normal	9(23.1%)	3 (42.9%)	7 (21.2%)	5 (38.5%)
	Low	19 (48.7%)	2 (%8.6%)	18 (54.5%)	3 (23.1%)
	High	11 (28.2%)	2 (28.6%)	8 (24.2%)	5 (38.5%)
	P	0.344		0.128	
Thyroiditis study group (n=113)* *USG performed	Normal	9 (17.7%)	7 (11.3%)	9 (20.5%)	7 (10.1%)
	Low	37 (72.5%)	53 (85.5%)	30 (68.2%)	60 (87%)
	High	5 (9.8%)	2 (3.2%)	5 (11.4%)	2 (2.9%)
	p chi-square	0.153		0.043*	

thyroid volumes. In the thyroiditis study group, in contrast to the patient control group, ASST(new)(+) patient thyroid volumes were significantly lower than those of the ASST(new)(-) patients ($p<0.05$). In the thyroiditis study group, there was no statistically significant difference between either ASST(old) and anti-TPO titers or ASST(new) and anti-TPO titers ($p>0.05$). However, moderate titers of anti-Tg in ASST(old)(+) cases, moderate and high titers of anti-Tg in ASST(new)(+) cases were significantly higher than the same titers of ASST(old) and (new) (-) cases. ($p<0.05$) (table 3). The prevalence of ASST positivity in HT patients was not affected by the following factors: gender, age at screening, laboratory measurements of thyroid function tests, anti-TPO antibodies and thyroid echogenicity.

Discussion

In this study we found that in HT patients, ASST results were distinctly positive and this positivity was inversely proportional with thyroid gland mass. Besides, the relationship between ASST, thyroid hormone levels and the sonographic grading of thyroiditis were investigated but we did not observe any relationship among them. This is the first study to show skin mast cell autoreactivity with ASST positivity in autoimmune thyroiditis patients without urticaria. O'Donnell *et al.* demonstrated a relationship between anti-TPO and ASST(+), as they reported ASST positivity in 82% urticaria patients with thyroid autoantibody [7]. Positive reactions to intradermal injections of autologous serum, which show functional IgG autoantibodies against the α subunit of high affinity IgE receptors (Fc ϵ RI α) and IgE itself *in vivo*, simply and quickly, is seen in approximately 60% of CU [17]. Autoreactivity related to ASST positivity has been searched for in association with nasal polyposis (55%) [18], autoimmune diseases (lupus erythematosus, scleroderma) (30-40%) [19], seasonal allergic rhinitis (29.8%) [20], intrinsic asthma (45%) [21], multiple intolerance to NSAID (30%) [22], multiple drug allergy syndrome (94%) [23], allergic rhinitis (29%) [24], non-

allergic asthma and rhinitis (20%), allergic rhinitis/allergic bronchial asthma (17.5%) [25]. In our study, we detected statistically significant positivity of both old and new criteria of ASST in HT patients without urticaria, 51.3% and 60.4%, respectively, compared with the positivities of ASST in two control groups (table 1). We aimed to increase the scientific value of our study by planning to work with two control groups, healthy volunteers and non-ATD patients. We also used two ASST criterias; 1) ASST (new) which was formed from the 2009 consensus report (the latest published criteria) [14], 2) The method of Grattan *et al.* (the first published criteria) [15] to reduce false positivity of ASST. Unfortunately, we could not afford basophil histamine release tests, to show correlation with ASST, and this is the weak point of our study. The ratios of ASST positivity in ATD overlapped interestingly with ASST ratios of autoimmune urticaria in the literature [17]. In ATD (including HT and Graves' diseases) autoantibodies are against TSH receptors, TPO and Tg [9-12]. Tg might be involved in the pathogenesis through initiating a T cell immune response to develop ATD, that then spreads to involve other autoantigens like TPO. Tg also reflects thyroid cell mass [11, 26, 27]. TPO is a sequestered antigen but appears in the case of thyroid tissue damage. Antibodies to thyroid peroxidase arise spontaneously with age and appear after thyroglobulin antibodies [9, 26, 28]. It is also known that the extracellular domain of TPO has a similarity of around 45% with myeloperoxidases from eosinophils [5, 9]. In recent years, studies on mast cell biology and Fc receptor associations have demonstrated that there is a hidden autoreactivity in many systems of our body. The thyroid is one of the important organs that shares autoimmune process with the other systems [12, 26]. Mozena *et al.* reported the lack of a role for cross-reacting anti-thyroid antibodies in urticaria in a study on 20 patients, as in older studies planned with small patient groups. These subsequent studies, using classic ELISA or RAST, were unable to show IgE autoantibodies to thyroid antigens in patients with chronic spontaneous urticaria and thyroid autoimmunity [29, 30], probably due to interfering IgG autoantibodies [31] and the limited sensitivity of these assays. Altrichter *et al.* demonstrated the relationship between thyroid, skin and thyroid

Table 3. Anti-TPO/anti-Tg titres correlation with ASST results.

Titers		ASST (old)		ASST(new)	
		(-) (n=75)	(+) (n=79)	(-) (n=61)	(+) (n=93)
		n (%)	n (%)	n (%)	n (%)
Anti TPO	Negative (n=20)	10(13.3%)	10 (12.7%)	9 (14.8%)	11 (11.8%)
	Low(+) (n=26)	13 (17.3%)	13 (16.5%)	12 (19.7%)	14 (15.1%)
	Moderate(+) (n=108)	52 (69.3%)	56 (70.9%)	40 (65.6%)	68 (73.1%)
	<i>P chi-square</i>	0.686		0.604	
Anti Tg	Negative(n=51)	24 (32%)	27 (34.2%)	20 (32.8%)	31 (33.3%)
	Low(+) (n=17)	13 (17.3%)	4 (5.1%)	12 (19.7%)	5 (5.4%)
	Moderate(+) (n=70)	31 (41.3%)	39 (49.4%)	25 (41.0%)	45 (48.4%)
	High(+) (n=16)	7 (9.3%)	9 (11.4%)	4 (6.6%)	12 (12.9%)
	<i>P chi-square</i>	0.020		0.034*	

autoantibodies which had been unproven for so many years. Additionally, they determined that mast cell autoreactivity could be present with systemic involvement in IgE anti-TPO (+) patients, as we pointed out in our study, autoantigen overload of thyroiditis resulted in ASST positivity. In their study, they were able to detect IgE specific for TPO by two methods: classical ELISA and the new human-IgE-capturing-enzyme immunoassay. They demonstrated that the IgE autoantibodies detected in their study were directed against an extracutaneous antigen (=autoallergen), ie TPO, which could be released from the thyroid into the circulation. IgE-anti-TPO is, therefore, likely to be bound to mast cells, basophils and FcεRI-expressing cells throughout the body [5]. This may explain the ASST positivity we found in HT patients.

In our study, the diagnosis of HT was made according to textbook data: Elevated thyroid autoantibody levels (anti-TPO and/or anti-Tg), heterogeneous and (other known) parenchymal alterations in thyroid tissue on sonographic examination [11, 14]. Graves' diseases and Graves' disease + HT were excluded clinically or sonographically by an endocrinologist. HT was mirrored by a hypoechoic ultrasound pattern. Thyroid US measurements were reported to have equal sensitivities and specificities with anti-TPO [14]. Because of that, thyroid US was chosen to confirm whether the severity of thyroid inflammation was related with the positivity of ASST in varying titers of anti-TPO (+) HT patients.

We found no differences between the positivity and negativity of ASST in HT patients with respect to US echogenicity grades. In our study, while 85.5% of ASST(old)(+) HT patients' thyroid volumes were low, in ASST(old)(-) cases, 72.5% of HT patients' thyroid volumes were low and there was a negative correlation with ASST positivity and thyroid volume. In contrast, ASST(new)(+) HT patients' thyroid volumes were significantly lower than those of ASST(new)(-) patients in the thyroiditis study group, when we compared them with the patient control group. These findings showed that, during the chronic stage of HT, thyroid volume gets smaller and, conversely, ASST positivity increases.

We found no differences between the (+)/(-) results of ASST(old) and ASST(new) with respect to thyroid function groups comparing the thyroiditis study group and the control group patients. Many of the HT patients with positive ASST(new) and ASST(old) were euthyroid (table 2). Besides other findings, in ASST(new)(+) cases, especially moderate and high (+) titers of anti-Tg were significantly higher but there was no relation between anti-TPO titers and ASST. This finding was not compatible with our knowledge of anti-TPO and mast cell relation studies in the literature. We found that moderate anti-Tg titers correlated with ASST(old) and (new). Tg and thyroid volumes were inversely proportional in our patients (table 3). In a previous study, Buchanan *et al.* demonstrated an intradermal skin test with an extract of human thyroid gland. Moreover, in this study, Tg antibodies were demonstrated by the passive cutaneous anaphylaxis technique [32]. The anti-Tg and thyroid volume relationships to each other and to ASST that we observed in our study may be due to inflammation seen with ASST(+) ATD and the presence of circulating TPO, IgE-anti-TPO, basophils and eosinophils [5-7].

Pathophysiologically, disorders of cellular immunity exist in HT initially because of a genetic defect of T suppressor

cells, CD4⁺CD25⁺-regulatory T cells (CD25⁺Treg) [33]. As shown by *in vivo* studies, CD25⁺ Treg cells cause suppression of switching IgG class to IgE anti-TPO of B cells but in HT this suppression of switching is not seen. IgE production increases. IL4, in the beginning, and the other ILs are secreted from Th2 cells [33-36]. Moreover, IL-4 increases the number of FcεRI receptors [37, 38]. In summary, FcεRI expression and the switching of IgG anti-TPO to IgE anti-TPO are increased by the reduction of CD25⁺Treg levels [35-38]. In brief, TPO and Tg are higher allergenic proteins that start the circle of thyroid-skin mast cell-humoral/cellular immunity with an excessive autoantigen load, resulting in ASST(+). Maurer *et al.* demonstrated the "Ag overload" to be the main disorder in the pathogenesis of autoreactive urticaria and they defined better results with omalizumab in urticaria through the presence of anti-TPO positivity [39]. Altrichter *et al.* reported in their study that IgG-anti-TPO-positive patients were more likely to express potentially urticaria-inducing (ASST positivity inducing) than IgE-anti-TPO patients [5]. We would like to point out that ASST helps us to show the excessive autoantigen overload that affects mast cells. Our results on ASST(+) autoreactivity in HT patients were not surprising as we previously knew that TPO binds to C4 and leads to complement, mast cell activation and mast cell stimulation threshold decreases when an inflammatory process takes place in the thyroid, and IgE-anti-TPO comes later by circulation [5, 36, 40, 41].

Our study supports the study of Altrichter *et al.* on the thyroid-Fc receptor-mast cell circle, which demonstrates that the autoantigen=autoallergen excess which plays an active role in the pathogenesis of urticaria is formed by thyroid antigens, TPO, IgE-anti-TPO [5, 39]. Our results show that ASST is an *in vivo* marker of autoimmunity that could point out the overload of functionally active autoantibodies like IgG anti-TPO (correlated with IgE-anti-TPO), anti-Tg, especially binding to FcεRIα antibodies [5]. Our results determine that ASST is a test peculiar to the mast cell-related autoimmune disease complex, rather than to ACU diagnosis.

In conclusion we have demonstrated the link between the thyroid gland and skin mast cells through ASST and sonographical findings of thyroid. We believe that ASST will be a widely used skin test to understand potential upregulation of autoreactivity of other systemic diseases related with mast cells. ■

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