LETTER TO THE EDITOR

A SURVEY OF AUTOANTIBODIES TO SELF ANTIGENS IN GRAVES' DISEASE PATIENTS WITH THYROID-ASSOCIATED OPHTHALMOPATHY

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Thyroid Associated Ophthalmopathy (TAO) is a complex pathology to treat and a considerable threat for Graves' Disease (GD) patients. Thus, there is great interest to find tools able to predict the onset and prognosis of TAO. Chronic inflamed tissues are characterized by tissue damage and recruitment of cells from the bloodstream, events that can lead to self-antigen exposure and induce autoimmune phenomena. In this study, we determined whether the occurrence of antibody anti-extracellular matrix (ECM) molecules [Collagen CI, CIII, CIV, CV, laminin (LM) and fibronectin, (FN)], anti-smooth muscle (ASM), anti-nuclear antigen (ANA), anti-ribonucleoprotein (RNP) and anti-thyroid-stimulating hormone (TSH) receptor (TRAbs) were associated with TAO in GD patients. We analyzed serum of 50 patients affected by GD, 24 of whom were affected by TAO, and 40 healthy donors (HD). The occurrence of TRAbs or ANA, anti-SM and anti-RNP antibodies did not allow to discriminate TAO+ from TAO- GD patients. Among the 24 TAO+ and 26 TAO- patients, 15/24 and 17/26 displayed TRAbs, respectively. None of the GD patients displayed anti-RNP antibodies, while 20/24 TAO+ and 17/26 TAO- patients were found to be positive for ANA and 3/24 TAO+ and 4/26 TAO- patients showed anti-SM antibodies. Conversely, when compared with HD control sera, GD sera showed antibodies to all individual ECM molecules. Remarkably, anti-CIII autoantibodies of the IgG isotype were significantly associated with GD TAO+ patients (p=0.045). Indeed, 6 out of 24 GD TAO+ patients scored positive for anti-CIII IgGs as compared to only 1 out of 26 TAO-. Our results suggest the potential involvement of anti-ECM antibodies in GD and a contribution of anti-CIII IgGs in TAO pathogenesis of GD patients.

GD is an autoimmune disease caused by the presence of autoantibodies against the thyroid-stimulating hormone (TSH) receptor (TRAbs) which induces the production of excess thyroid hormone

Key words: Graves' disease, autoantibodies, ophthalmopathy, extracellular matrix

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

(1). Graves-associated ophthalmopathy is currently more accurately defined as TAO (1, 2). TAO is an inflammatory autoimmune disorder which affects the thyroid gland, orbital and periorbital tissues, and rarely the pretibial skin or digits is also associated with hyperthyroidism (1, 2). TAO clinical manifestations differ from simple diplopia to an important proptosis with retrobulbar tissue oedema (1, 2).

To our knowledge no humoral markers are able to associate GD with the appearance and stage of TAO, although different studies have reported that TRAbs are not only associated with the pathogenesis of autoimmune thyroid disease, but are also involved in the pathogenesis of TAO (1-3). It has to be considered that autoantibodies are the feature trait of several autoimmune diseases (4). For example, anti-smooth muscle antibodies (SMA) are found in autoimmune hepatitis (AIH) type 1 and anti-RNP (ribonucleoprotein) antibodies are associated with mixed connective tissue disease and occasionally are present in SLE and less commonly in systemic sclerosis (5). Anti-RNP antibodies detect proteins (70 Kd, A, C) that are associated with U1 RNA and form U1snRNP (6). Antinuclear antibodies (ANA) recognize various nuclear antigens and are helpful in diagnosing systemic rheumatic disease patients (7). A high incidence of autoantibodies to ECM components during inflammation and cancer has also been reported (8-13). Autoantibodies to ECM were found in patients with rheumatoid arthritis, scleroderma, autoimmune thyroiditis etc. (9, 12, 13). Bednarczuk T. et al. previously reported the occurrence of high levels of anti-CIII antibodies in GD patients with TAO (14).

The extracellular matrix (ECM) is a composite and dynamic macromolecular network with both structural and regulatory functions. Indeed, besides playing an essential role in the development and maintenance of tissue architecture, the ECM provides spatially constrained mechanical and biochemical signals that participate in the integrated control of cell proliferation, survival, migration, and differentiation (15). ECM components belong to four major types of macromolecules: collagens, elastin, proteoglycans, and noncollagenous glycoproteins (laminin, fibronectin, tenascin).

In the effort to determine potential markers of TAO, we analyzed the serum of GD patients for the presence of autoantibodies to SM, RNP, NA, TSH receptor and to several ECM proteins including collagen type I, III, IV, V (CI, CIII, CIV, CV), laminin (LM) and fibronectin (FN).

MATERIALS AND METHODS

Patients

Fifty outpatients affected by GD with no history of malignancies and without any corticosteroid therapy for the last 6 months were enrolled. All patients were recruited from the endocrinology outpatient department of the "Tor Vergata" University Hospital, Rome. The patients signed the informed consent approved by the Hospital's Ethics Committee. Patients received medical and blood examination, color doppler ultrasound evaluation of thyroid, ECG, measurement of proptosis (with Hertel exophthalmometer) and evaluation of the ophthalmopathy by the Clinical Activity Score (CAS, 1 to 10) in the day-hospital unit of "Tor Vergata" University Hospital of Rome. A proptosis ³20 mm was considered diagnostic of TAO. Control sera were collected from 44 healthy blood donors [14 women (mean age: 45.5±14) and 30 men (mean age: 49±11)] at the blood transfusion center, University Hospital Policlinico "Tor Vergata".

Determination of anti-TSH receptor, anti-SM, anti-RNP and anti-NA antibodies

Quantification of TRAbs was performed by BRAHMS TRAK human Radioimmunoassay (RIA, BRAHMS Aktiengesellschaft, Germany). Serum levels above 1.5 U/l were scored positive as indicated by the manufacturer.

Quantification of anti-SM and anti-RNP antibodies was performed by ALPHADIA ENA Screen DOT kit Enzyme Immunoassay (Alphadia SA/NV, Wavre, Belgium), according to the manufacturer's recommendation.

Quantification of anti-NA antibodies was performed by using the HEp-2 cell-line as substrate (Immuno Concepts, N.A. Ltd. Sacramento, CA), according to the manufacturer's recommendation.

Determination of anti-ECM antibodies

Purified human CI, CIII, CIV, CV and FN were obtained from Chemicon International (Temecula, CA, USA). Purified LM was obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). The purity of antigens was greater than 95% by SDS-PAGE and Coomassie Blue staining (16). Mouse monoclonal anti-human CI, CIII, CIV and CV antibodies were obtained from Chemicon International. Mouse monoclonal anti-human FN and rabbit polyclonal anti-human LM, as well as peroxidase-conjugated anti-human IgG and IgM or anti-mouse and

-rabbit IgG were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA).

Sera were assayed for the presence of antibodies directed toward native ECM antigens by ELISA as previously described (9-11). Briefly, ECM antigens as well as bovine serum albumin (BSA) were diluted at 1-5 µg/ml. One hundred microliters of each mixture were incubated overnight at 37°C in polyvinylchloride microtiter plates (Dynatech, Chantilly, VA, USA). Antigen-coated wells were then blocked with 5% non-fat dry milk in PBS for 1 h at 37°C and incubated with human sera. Sera were initially assayed at 1:25, 1:50 and 1:100 dilutions. The 1:100 dilution was chosen for further experiments because it was the best serum dilution that lacked background reactivity. Each serum was assayed in duplicate for reactivity to ECM antigens or BSA. Anti-ECM monoclonal and polyclonal antibodies diluted at 1 µg/ml were used for positive controls. After a 4-h incubation at 37°C, the plates were washed 5 times with 1% non-fat dry milk in PBS and goat anti-human IgG, IgM or goat anti-mouse or anti-rabbit peroxidase-conjugated antibodies were added and incubated for 1 hat 37°C. Plates were washed and the wells were layered with a solution containing o-phenylene-diamine dihydrochloride in the presence of H_2O_2. The reaction was blocked with 50 µl of H_2SO_4 4N and the absorbance of the samples was read at 492 nm. Any serum with an O.D. exceeding the mean of control sera O.D. values plus 2SD (standard deviations) was considered positive for the presence of autoantibodies (9-11). Intra-plate and inter-plate variations did not exceed 10-15%.

To determine the specific isotype of serum antibodies separate ELISAs were performed using anti-human IgG, IgM secondary antibodies (17).

Statistical analysis

Statistical associations were considered significant at *p*-values <0.05 using Fisher's exact test two-tailed analysis (18).

RESULTS

Patients

Sera were obtained from 50 GD patients and 44 healthy donors (HD). At the time of the clinical observation, 32/50 GD patients showed acute symptoms of hyperthyroid function and the presence of TRAbs. Conversely 18/50 GD patients were in a remission phase. Among the 32 symptomatic GD patients, 23, 6 and 3 were at the first, second and third manifestation of the disease, respectively. Twenty-four out of 50 patients were affected by TAO, displaying 17 and 7 bilateral or monolateral proptosis, respectively. CAS was used to assess disease activity. A score of 5, 3, 2 and 1 was found in 2, 5, 8 and 9 patients, respectively.

Presence of anti-TRAbs and anti-nuclear antigens (NA), anti-smooth-muscle (SM) and anti-RNP antibodies in GD patients with and without TAO (TAO+ and TAO-)

In order to determine whether the presence of TRAbs and ANA and anti-SM or anti-RNP antibodies was associated with the appearance of TAO in GD patients, we analyzed serum of GD patients for the occurrence of TRAbs, ANA, anti-SM and anti-RNP antibodies. Our results indicated that of the 24 TAO+ and 26 TAO- patients, 15/24 and 17/26 displayed TRAbs, respectively. The occurrence of TRAbs did not allow to discriminate TAO+ from TAO- GD patients (Table I).

Table I. *Occurrence of autoantibodies to NA, SM, RNP and TRAbs in Graves' Disease (GD) patients with and without Thyroid-Associated Ophthalmopathy (TAO+ and TAO-).*

GD Patients	TRA bs[a]	ANA	Anti-SM	Anti-RNP
TAO+ (n=24)	15/24	20/24	3/24	0/24
TAO- (n=26)	17/26	17/26	4/26	0/26

[a]*Autoantibodies were determined by commercial kits according to the manufacturer's recommendation.*

Table II. *Autoantibodies against native collagen (C) types I, III, IV, V, laminin (LM) and fibronectin (FN) in sera of patients with GD versus healthy donors (HD).*

ECM Molecules	HD	GD	p[a]
Collagens + Glycoproteins	7/44	30/50[b]	$p \leq 10^{-4}$
CI	3/44	16/50	p=0.0038
CIII	1/44	17/50	$p \leq 10^{-4}$
CIV	3/44	7/50	p=n.s
CV	2/44	13/50	p=0.0048
LM	2/44	11/50	$p=17x10^{-3}$
FN	3/44	19/50	$p=5x10^{-4}$

[a]GD patients serum reactivity vs HD control serum, two tailed analysis; [b]Number of positive sera with autoantibodies (IgG plus IgM isotypes) to ECM antigens by ELISA. Any serum with an O.D. exceeding the mean of HD sera O.D. values plus 2SD (standard deviations) was considered positive for the presence of autoantibodies.

Table III. *Autoantibodies against native collagen (C) types I, III, IV, V, laminin (LM) and fibronectin (FN) in sera of patients with GD with and without TAO versus healthy donors (HD).*

ECM Molecules	HD	GD TAO+	p[a]	GD TAO-	p[a]
Collagens + Glycoproteins	7/44	16/24[b]	0.0001	14/26	0.0012
CI	3/44	10/24	0.001	6/26	n.s.
CIII	1/44	10/24	0.0001	7/26	0.0032
CIV	3/44	4/24	n.s.	3/26	n.s.
CV	1/44	7/24	0.002	6/26	0.009
LM	2/44	6/24	0.019	5/26	n.s.
FN	3/44	10/24	0.0009	9/26	0.006

[a]TAO+ or TAO- GD patients serum reactivity vs HD control serum, two tailed analysis; [b]Number of positive sera with autoantibodies (IgG plus IgM isotypes) to ECM antigens by ELISA. Any serum with an O.D. exceeding the mean of HD sera O.D. values plus 2SD (standard deviations) was considered positive for the presence of autoantibodies.

Similarly, ANA, anti-SM and anti-RNP antibodies did not appear to be helpful markers for TAO discrimination within GD patients. Indeed, 20/24 TAO+ and 17/26 TAO- patients were found to be positive for ANA, while 3/24 TAO+ and 4/26 TAO- patients showed anti-SM antibodies. In

Table IV. *Frequencies of autoantibodies positivity of IgG or IgM isotypes against native collagen (C) types I, III, IV, V, laminin (LM) and fibronectin (FN) in serum of patients with Graves' Disease (GD) with and without Thyroid-Associated Ophthalmopathy (TAO+ and TAO- and healthy donors (HD).*

ECM Molecules	HD	GD	p[a]	TAO +	TAO–	p[b]
CI						
IgG	1/44	11/50[c]	0.0045	8/24	3/26	n. s.
IgM	1/44	10/50	0.009	5/24	5/26	n. s.
CIII						
IgG	0/44	7/50	0.0135	6/24	1/26	**0.045**
IgM	1/44	10/50	0.009	6/24	4/26	n. s.
CIV						
IgG	1/44	5/50	n. s.	3/24	2/26	n. s.
IgM	2/44	6/50	n. s.	4/24	2/26	n. s.
CV						
IgG	1/44	8/50	0.033	4/24	4/26	n. s.
IgM	1/44	10/50	0.009	6/24	4/26	n. s.
LM						
IgG	1/44	8/50	0.033	4/24	4/26	n. s.
IgM	1/44	5/50	n. s.	3/24	2/26	n. s.
FN						
IgG	1/44	13/50	0.001	6/24	7/26	n. s.
IgM	2/44	12/50	0.009	8/24	4/26	n. s.

[a]GD patients serum reactivity vs HD control serum, two tailed analysis; [b]TAO+ GD patients serum reactivity vs TAO- GD patients serum reactivity, two tailed analysis; [c]Number of positive sera with autoantibodies (IgG or IgM isotype) to ECM antigens by ELISA. Any serum with an O.D. exceeding the mean of HD sera O.D. values plus 2SD (standard deviations) was considered positive for the presence of autoantibodies.

addition, none of the GD patients displayed anti-RNP antibodies (Table I).

Anti-ECM molecule autoantibodies in GD patients with and without TAO

Sera of GD patients and healthy donors (HD, n=40) were assayed for the presence of autoantibodies to CI, CIII, CIV, CV, LM and FN (Table II). Our results demonstrated the occurrence of autoantibodies of the IgG plus IgM isotypes to at least one ECM protein in 30 out of 50 sera from GD patients and in 7 out of 44 control sera (GD *vs* HD, p£10^{-4}) (Table II). Significant serum reactivity of GD patients was directed toward CI (16/50), CIII (17/50), CV (13/50), LM (11/50) and FN (19/50). When comparing the presence of anti-ECM autoantibodies of the IgG plus IgM isotypes in GD patients with (TAO+) or without TAO (TAO-), we observed that 16/24 GD TAO+ (p=0.0001, *vs* HD) and 14/26 GD TAO- (p=0.0012, *vs* HD) sera displayed antibodies to at least one ECM protein (Table III). The optical density values obtained from the binding of autoantibodies of the IgG isotype to ECM molecules both in GD patients and HD are shown in Fig. 1. As shown in Table III, anti-CI

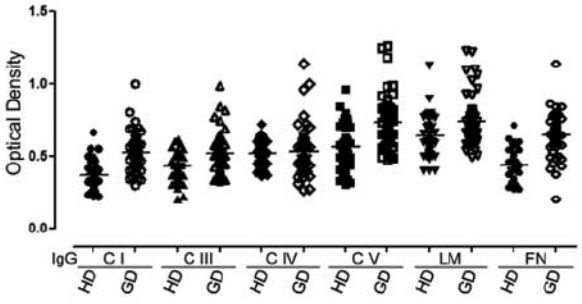

Fig. 1. *Quantitative analysis of anti-ECM autoantibodies in GD patients and healthy donors. The optical density values obtained from the binding of autoantibodies of the IgG isotype to ECM molecules both in GD patients and healthy donors (HD) are shown.*

autoantibodies were found in 3/44 HD, in 10/24 GD TAO+ (p=0.001, *vs* HD) and in 6/26 TAO- sera; anti-CIII antibodies were detected in 1/44 HD, in 10/24 GD TAO+ (p=0.0001, *vs* HD) and in 7/26 GD TAO- (p=0.0032, *vs* HD) sera; anti-CV antibodies were revealed in 1/44 HD, in 7/24 GD TAO+ (p=0.002, *vs* HD) and in 6/26 GD TAO- (p=0.009, *vs* HD) sera; anti-LM immunoglobulins were observed in 2/44 HD sera and in 6/24 GD TAO+ sera (p=0.019, *vs* HD); anti-FN reactivity was found in 3/44 HD sera, in 10/24 GD TAO+ (p=0.0009, *vs* HD) and in 9/26 GD TAO- sera (p=0.006, *vs* HD). The presence of autoantibodies to CIV in GD patients was not significant as compared to HD.

Next, we determined whether the occurrence of TAO was associated with a specific isotype of anti-ECM autoantibodies (Table IV). Overall, when compared with HD sera, GD sera testing positive showed significant anti-ECM antibodies of the IgG or IgM isotype with the exception of the IgM anti-LM antibodies (Table IV).

Remarkably, anti-CIII autoantibodies of the IgG isotype were significantly associated with GD TAO+ patients. Indeed, 6 out of 24 GD TAO+ patients scored positive for anti-CIII IgGs as compared to only 1 out of 26 TAO- (p=0.045) subjects. Thus, our results demonstrated that among the anti-ECM autoantibodies of the IgG isotype those reacting with CIII were able to discriminate TAO+ from TAO- GD patients. It is also of notice that among TAO+ GD patients, four simultaneously displayed autoantibodies to four ECM proteins, as compared to only one TAO- GD patient. It is worth noting that 5 patients (4 with TAO and 1 without TAO) had simultaneous antibodies to at least 4 out of 5 ECM molecules. These patients were all young females and showed an aggressive Basedow's disease (3/5 patients were submitted to surgery for medical failure at first presentation; 4/5 showed levels of anti-TPO at 270-3000 IU/ml; 1/5 had TRAb=400). Three of these patients referred autoimmunity family history and one was affected by other autoimmune diseases [Polyglandular autoimmune syndrome (PGA) type 3].

We did not find any association between the occurrence of anti-ECM autoantibodies and TRAbs or anti-SM, anti-RNP and anti-NA antibodies. In addition, no correlation between biochemical parameters of inflammation (erythrocyte sedimentation rate, C-reactive protein, IL-6, TNFα) and anti-ECM autoantibodies was observed.

DISCUSSION

There is a growing interest in finding biological markers able to predict the onset and prognosis of TAO in GD patients. In this study, we determined whether the occurrence of anti-ECM, anti-SM, anti-RNP, anti-NA (ANA) and anti-TSH receptor (TRAbs) antibodies was associated with TAO in GD patients. Previous studies reported that TRAbs, ANA and anti-SM antibodies occur in autoimmune thyroid diseases and are involved in TAO pathogenesis (1-3, 19). However, in our study we could not associate the presence of these antibodies with the development of TAO in GD patients. Differences in the assay used could account for this discrepancy. Thus, we determined whether the occurrence of anti-ECM autoantibodies was associated with the presence of TAO in GD patients. Here we demonstrate a significant incidence of ECM molecule autoantibodies in GD patients as compared to healthy donors. In addition, anti-CIII autoantibodies of the IgG isotype were associated with TAO in GD patients. The presence of ECM autoantibodies in 7/44 healthy donors is not surprising and has already bee reported (9-11, 14). It has been demonstrated that in chronic inflamed tissues there is a remodelling of the ECM which, in turn, elaborates inflammatory signals (4, 8, 20). These events can lead to the exposition of ECM self-epitopes and induce autoimmune phenomena (4, 8).

The role of ECM proteins as antigenic factors for autoimmunity processes in the pathogenesis of TAO, GD and different autoimmune diseases is still to be fully understood and could probably lead to a re-evaluation of organ and non-organ specific autoimmunity in predisposed patients. Thus, longitudinal studies with a large number of patients and functional studies are needed to confirm a role for autoantibodies to ECM molecules in GD patients with TAO.

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