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Tissue Transglutaminase Antibodies in Individuals with Celiac Disease Bind to Thyroid Follicles and Extracellular Matrix and May Contribute to Thyroid Dysfunction

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Background: Individuals with active celiac disease (CD+) have an increased incidence of thyroid dysfunction, which improves on a gluten-free diet (CD−). We investigated whether tissue transglutaminase-2 IgA antibodies (anti-TGase II) present in sera of patients with celiac disease react with thyroid tissue and possibly contribute to thyroid disease.

Methods: Serum from 40 active celiac patients taken before a gluten-free diet (CD+), 46 patients on a gluten-free diet (CD−), 40 normal controls (NC), and 25 with Crohn's disease (CROHN) was used. All sera were screened for antithyroperoxidase antibodies (TPO-AB) and thyroglobulin antibodies (TG-AB), and indirect immunofluorescence (IIF) was performed on primate thyroid tissue sections using TPO-AB– and TG-AB–negative sera.

Results: IIF with thyroid seronegative, anti-TGase II–positive CD+ sera ($n = 23$) demonstrated staining of thyroid follicular cells and extracellular matrix, in an identical pattern with monoclonal anti-human TGase II antibody. Evidence of TGase II as the antigen in thyroid tissue was supported by elimination of the IIF pattern when sera were depleted of anti-TGase II by pretreatment with human recombinant TGase II. No staining of thyroid tissue was observed when sera from CD+ patients that were negative for TGase II antibodies, or sera from NC subjects were used. Thyroid antibodies were found in 43% of CD+ patients, significantly higher than NC and CROHN patients ($p < 0.0001$). In addition, a positive correlation was observed between anti-TGase II and TPO-AB titers ($p = 0.0001$; $r = 0.63$).

Conclusions: Anti-TGase II antibodies bind to TGase II in thyroid tissue, and titers correlate with TPO antibody titers. These findings suggest that anti-TGase II antibodies could contribute to the development of thyroid disease in celiac disease.

Introduction

Celiac disease is an autoimmune enteropathy that results in damage to the small intestinal mucosa. It is triggered by gluten ingestion in genetically susceptible individuals (1). Recent studies have emphasized that most patients present with a silent, nondiarrheal form of the disease (2). Autoimmune diseases are often prominent and are increased up to 10-fold in patients with celiac disease compared to the general population (3–5). An association between duration of gluten ingestion and autoimmune diseases has been reported in individuals with celiac disease (6).

Tissue transglutaminase (TGase) is an ubiquitous enzyme presents in all tissues, with both intra- and extracellular localization (7–10). Eight TGase enzymes involved in various functions such as blood clotting, apoptosis, keratinocyte differentiation and maturation, matrix stabilization, and transmembrane signaling have been identified in mammals (11,12). Although transglutaminases are involved in multiple cellular functions, all studied mammalian transglutaminases catalyze calcium-dependent acyl transfer cross-linking reactions and the deamidation of glutamine residues (13). The enzyme (TGase II) is considered integral to the development of celiac disease (14–16). It is responsible for deamidation of gliadin

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that has entered the lamina propria of the small intestinal mucosa, probably during infections (17). Deamidated gliadin, with its altered structure and charge, becomes more immunogenic, interacting with HLA DQ2 or DQ8 on antigen-presenting cells. This results in an inflammatory cascade initiated by the liberation of proinflammatory cytokines, and villous atrophy results. During this inflammatory process, antibodies to TGase II (anti-TGase II), as well as antibodies to other tissue components such as actin, are produced (18). Anti-TGase II antibodies can be detected in the small intestine early in the disease process (19) and later in the serum (20).

While little is currently known concerning the mechanism of extraintestinal disease manifestations in celiac disease, circulating antibodies to TGase II have been proposed to play a role. Dermatitis herpetiformis, the dermatological manifestation of celiac disease, arises due to the generation of TGase antibodies in the intestine that react with epidermal TG (21). Antibodies to TGase II also bind to other extraintestinal tissues, such as liver (22), brain (23), and heart (24). Serum IgA antibodies from celiac disease patients have also been shown to bind extracellular TGase II in the liver, lymph node, and muscle, indicating that this autoantigen is widely accessible to circulating antibodies derived from the intestine (22).

The frequency of celiac disease in patients with autoimmune thyroid disease, Hashimoto's thyroiditis, and Graves' disease is 2–3%, approximately 5–10 times the general population (25,26). Conversely, 4–10% of patients with celiac disease or dermatitis herpetiformis suffer from thyroid disease (27–30).

We hypothesized that anti-TGase II antibody in the sera of celiac disease patients may bind to the TGase II present in the thyroid gland and could potentially contribute to the development of thyroid disease.

Materials and Methods

Study population

We used frozen serum samples (stored at -20°C) that were obtained after informed consent from patients with celiac disease evaluated at the Celiac Disease Center at Columbia University. All samples were analyzed in a blinded manner with respect to diagnosis. Forty consecutive adult patients with biopsy-confirmed active celiac disease contributed sera, referred to as CD+ sera. CD− sera were obtained from 46 patients with celiac disease who were on a gluten-free diet. Normal control (NC) sera came from 40 healthy adults, and Crohn's sera were used as a disease control (CROHN), $n = 26$. There was no age difference among CD+, CD−, and NC groups; however, the CROHN group was significantly older than the CD+ group (50.2 ± 3.3 vs. 41.7 ± 2.4, $p = 0.03$).

Screening of study population sera for anti-TGase II IgA and antithyroglobulin/thyroperoxidase antibodies

The study population was screened for TGase II IgA using the ELISA kit (IMMCO Diagnostics, Buffalo, NY). Screening for antithyroid antibodies was performed using thyroglobulin (TG-AB) and thyroperoxidase (TPO-AB) ELISA kits (The Binding site, Birmingham, UK). Sera found to be positive for either of the antithyroid antibodies were excluded from immunofluorescence analysis.

Indirect immunofluorescence assay

Glass slides with sections of monkey thyroid (The Binding Site) were incubated in a moist chamber for 30 minutes at room temperature, with sera diluted 1:20 in phosphate-buffered saline (PBS), pH 7.2, washed two times for 10 minutes in PBS, incubated for 30 minutes in a moist chamber with fluorescein isothiocyanate (FITC) sheep anti-human IgA (The Binding Site), washed again, and mounted with cover slips. The slides were analyzed using a Nikon fluorescence microscope. Both anti-TGase II–positive and –negative sera were included in every batch of tests. Because TGase is conserved across vertebrates (31), primate tissue was used as an alternative to human tissue to avoid autofluorescence due to the presence of IgA in human tissues (32) (high nonspecific background staining was noted when anti-human IgA FITC secondary antibody was used alone or in combination with PBS or nonceliac disease sera on human thyroid tissue). Monoclonal cytokeratin antibody was used at 1:100 (Immunotech, Marseille, France) to stain the epithelial cells, with a secondary goat anti-mouse Texas red IgG (Jackson Immunoresearch, West Grove, PA) antibody (1:100) as the secondary. Nuclear staining was done with DAPI (chromogen-blue) obtained from Invitrogen (Carlsbad, CA). Monoclonal transglutaminase II (Ab-1) antibody was obtained from Neomarkers (Freemont, CA).

Test for inhibition of indirect immunofluorescence staining using human recombinant tissue transglutaminase to sequester TGase antibodies

Sera, negative for thyroid antibodies, from celiac disease patients, diluted 1:10 in PBS, were preincubated overnight at room temperature with 1, 5, 10, and 20 μg of human recombinant TGase II (Lab vision, Freemont, CA). Anti-TGase II–positive pretreated and untreated sera, as well as bovine serum albumin–treated sera, were processed for indirect immunofluorescence (IIF) as described above.

Test for inhibition of IIF staining subsequent to removing TGase II antibodies using beads conjugated with TGase II

A protein isolation column was packed with 1 mL TGase II–conjugated beads and equilibrated with 10 mL binding buffer (Pierce, Rockford, IL), and thyroid seronegative celiac disease sera diluted 1:1 with binding buffer were applied to the column. The column was washed with 10 mL binding buffer and eluted with 1 mL of elution buffer (Pierce). Both the flow through and eluate were neutralized with the Tris Base (pH 9.0) and processed for IIF as described above. Both flow through and eluate were retested for TGase II IgA antibody titers using the ELISA kit (IMMCO Diagnostics). Flow through was found to be negative for TGase II antibodies.

Exclusion of antigenic mimicry

To exclude the possibility of reactivity between IgA antigliadin antibodies with thyroid tissue at a site similar to where TGase II antibodies react, IIF of thyroid tissue was performed with CD+ serum that was seronegative for anti-TPO and anti-TG antibodies both with and without pervious incubation of the serum with 1, 5, 10, and 20 μg of gliadin.

Table 1. Serologic Characteristics of the Different Patient Groups

	ACD	*GFD*	*NC*	*CROHN*	p-*Value*
Mean age (SE)	41.7 (2.4)	46.2 (2.4)	39.1 (2.4)	50.2 (3.3)	<0.0001
TPO-AB+ (%)	15 (37.5)	2 (4.4)	0	0	<0.0001[a]
TG-AB+ (%)	19 (47.5)	18 (39.1)	7 (18)	1 (4)	0.0004
Anti-TGase II+ (%)	40 (100)	5 (11.1)	0	0	<0.0001[a]

$p = 0.03$ for the difference in age between ACD and DC group. $p = 0.0052$ for ATG comparison between ACD and NC, and $p = 0.0002$ for ATG comparison between ACD and DC.

[a]*p*-Value denotes the comparison between ACD and GFD.

ACD, active celiac disease; GFD, gluten-free diet; NC, normal control; DC, disease control; TPO-AB, antithyroperoxidase antibody; TG-AB, antithyroglobulin antibody; anti-TGase II, antitissue transglutaminase antibody; SE, standard error.

Statistical analysis

The nonparametric Spearman correlation analysis was applied to the data for the TGase II and TPO correlation analysis. Statistical analysis was performed using SAS software (version 9.1; SAS Institute, Cary, NC). Discrete variables were analyzed by the Pearson's χ^2-test and continuous variables by the Student's *t*-test or generalized regression models. For all analyses, significance was determined at $p < 0.05$ (two tailed).

Results

Frequency of anti-TGase II, TPO-AB, and TG-AB positivity in the study groups

Among the CD+ groups, 47.5% were positive for anti-TG antibodies, 37.5% were positive for TPO antibodies, and 35% were positive for both. The frequency of positive thyroid antibodies was 50% and 41% in the CD+ and CD− groups, respectively (Table 1). The percentage of positive thyroid serology in celiac disease was significantly different from NC and disease control groups ($p = 0.0001$). The frequency of anti-TGase II in the groups is also shown in Table 1.

Celiac disease sera react with primate thyroid tissue

Sera from 23 of 40 CD patients who had TGase II IgA antibodies but lacked thyroid antibodies were used for IIF staining. None of the sera from CD patients lacking anti-TGase II IgA or sera from NC and CROHN patients showed any immunofluorescence staining of the thyroid (Fig. 1A); however, all 23 serum samples from CD patients with elevated anti-TGase II IgA demonstrated immunofluorescence staining of thyroid follicular epithelium (Fig. 1B). Immunofluorescence was observed to decrease with serial dilution and was absent below a TGase II IgA titer level of 20 EU/ml.

Absorption of anti-tTG–rich sera with recombinant tTG abolishes immunofluorescence staining of thyroid tissue

Pretreatment of celiac disease sera with human recombinant TGase II abolished the immunofluorescence staining of primate thyroid tissue (Fig. 1C), whereas the simultaneous processing of the same samples, not preincubated with TGase II, showed the characteristic IIF staining pattern. Preincubation of untreated celiac disease patients' sera with gliadin did not block the appearance of the fluorescent reaction on thyroid sections. When the TGase II beads were used to remove the TGase II antibodies from the celiac disease patient sera, we observed a complete abolition of immunofluorescence (Fig. 1D, F), while the same patient sera demonstrated the typical pattern of immunofluorescence (Fig. 1E, G). These data demonstrate that the IIF pattern seen with celiac disease sera is in fact due to IgA antibodies to tissue transglutaminase.

To further explore the tissue antigen responsible for anti-TGase II IgA binding, we used a commercially available mouse monoclonal anti-human TGase II-2 antibody (Fig. 2A) and observed a similar pattern of immunofluorescence as in the celiac disease sera (Fig. 2B). An overlapping pattern of staining was observed with an anti-human TGase II-2 antibody and celiac disease sera (Fig. 2C). This is highlighted in Figure 2 showing thyroid follicles (60× magnification) stained with TGase II (Fig. 2A, inset) and TGase II IgA (Fig. 2B, inset). The merged image (Fig. 2C, inset) demonstrates that celiac disease–associated TGase II IgA antibodies were indeed binding in a similar distribution like TGase II.

TGase II IgA staining is observed in thyroid follicle epithelium and extracellular matrix

To confirm the location of the TGase II IgA binding, we used an antibody directed against cytokeratin. The immunofluorescence staining (FITC) of the patient sera with the TGase II IgA antibodies is shown in Figure 2D, while staining of the cuboidal epithelium of thyroid follicles is seen with a cytokeratin antibody (red) in Figure 2E. Figure 2F shows nuclear staining with DAPI (blue) overlayed with cytokeratin antibody staining (Texas-red) and anti-TGase II IgA from celiac disease sera (FITC), demonstrating that the TGase II IgA staining was centered in the thyroid follicular epithelial cells. In addition, when the tissue was examined at higher magnification, we observed the presence of extracellular TGase II IgA binding of the extracellular matrix in the interfollicular areas, shown in Figure 2G and H, where the arrow indicates the presence of TGase II IgA staining in the interfollicular area not colocalizing with the cytokeratin (Texas-red) and DAPI (blue) staining. The restricted pattern of staining seen only in the follicular epithelium (cytoplasmic and membranous) and stroma or extracellular matrix in the interfollicular areas argues against artifactual TGase II staining due to cellular disruption as a consequence of tissue sectioning.

Correlation of anti-TGase and antithyroid antibody titers

We next determined whether there was a correlation between the titers of anti-TGase II IgA and the presence of

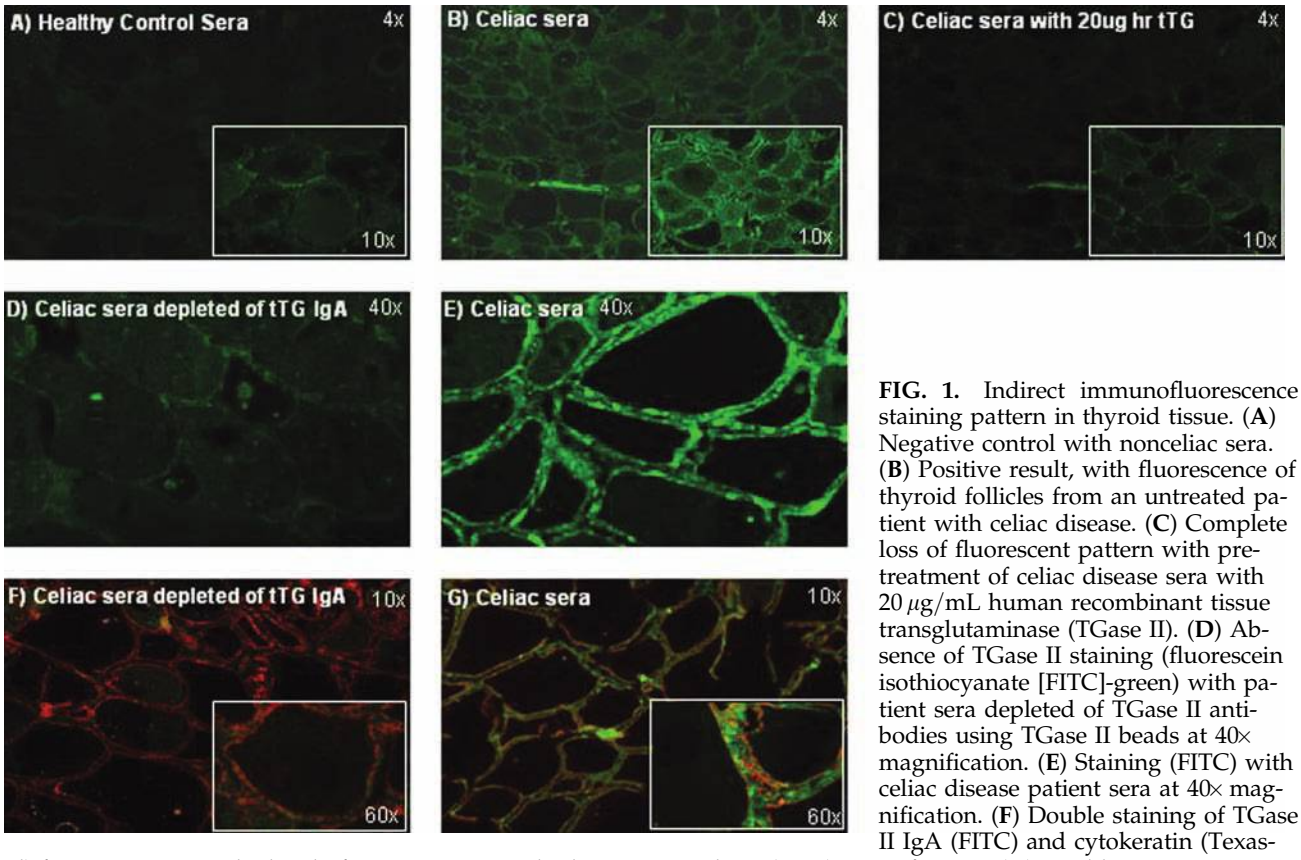

FIG. 1. Indirect immunofluorescence staining pattern in thyroid tissue. (**A**) Negative control with nonceliac sera. (**B**) Positive result, with fluorescence of thyroid follicles from an untreated patient with celiac disease. (**C**) Complete loss of fluorescent pattern with pretreatment of celiac disease sera with 20 μg/mL human recombinant tissue transglutaminase (TGase II). (**D**) Absence of TGase II staining (fluorescein isothiocyanate [FITC]-green) with patient sera depleted of TGase II antibodies using TGase II beads at 40× magnification. (**E**) Staining (FITC) with celiac disease patient sera at 40× magnification. (**F**) Double staining of TGase II IgA (FITC) and cytokeratin (Texas-red) from patient sera depleted of TGase II IgA antibodies at 10× and 60× (inset) magnification. (**G**) Double immunostaining with patient sera TGase II IgA (FITC) and cytokeratin (Texas-red) at 10× and 60× (inset) magnification.

antithyroid antibodies. A positive correlation was observed between TGase II IgA and anti-TPO antibody titers ($r = 0.63$, $p < 0.0001$) for patients with active celiac disease, as shown in Figure 3. No significant correlation was observed between the anti-TGase II and anti-TG antibody titers (Table 1).

Clinical profile of patients with abnormal thyrotropin/free thyroxine and thyroid serologies

We assessed the number of patients with thyroid disease in the active celiac disease and NC groups. Among the CD+ group, eight patients (20%) had thyroid disease. Autoimmune thyroid disease had been diagnosed in four. We also detected four additional patients with thyroid hormone abnormalities. One had hyperthyroidism (seropositive for antithyroid antibodies). The remaining three celiac disease patients were seronegative for thyroid antibodies and had mild hypothyroidism. Three individuals in the NC population (7.5%) were also found to have abnormal thyroid function, two indicative of subclinical hypothyroidism and one of subclinical hyperthyroidism. None of the CROHN group had evidence of thyroid disease.

Discussion

We explored the role of anti-TGase II IgA antibodies in thyroid autoimmunity in patients with celiac disease. Tissue transglutaminase is found in thyroid tissue, in both the cytosol and in the follicle lumen where it has been demonstrated to cross-link TG (8). In our study, we demonstrate that the anti-TGase II IgA–positive serum from patients with celiac disease binds to thyroid follicular cells as well as extracellularly in the interfollicular space. The pattern of immunofluorescence seen with active celiac disease patient sera was not observed with the sera of TGase II-IgA–negative patients on a gluten-free diet or in the control populations. TGase II was the target antigen for the TGase II IgA antibodies as evidenced by the abolition of immunofluorescence in anti-TGase II–positive sera preincubated with human recombinant TGase II or when sera were devoid of TGase II antibodies using TGase II bound to sepharose beads. Colocalization of the immunofluorescence pattern of mouse monoclonal anti-human TGase II IgG antibody and patient sera confirmed TGase II as the antigen.

Several studies have shown the binding of celiac TGase II IgA antibodies to extraintestinal tissue, such as liver (22), brain (23), and heart (24), all organs that manifest extraintestinal conditions associated with celiac disease. Sategna-Guidetti *et al.* have shown the binding of anti-TGase II IgA from celiac patient sera to monkey cardiomyocytes (24), which was abrogated by treating the sera with recombinant TGase II, indicating a possible mechanism to explain the development of the celiac disease–associated autoimmune cardiomyopathy (33). This cardiomyopathy improves in patients with celiac disease on a gluten-free diet (34). In addition, selective cellular dysfunction as an extraintestinal manifestation of celiac disease was reported in a case with idiopathic congestive cardiomyopathy associated with celiac disease (35).

Neurological problems are common in celiac disease, especially cerebellar ataxia (gluten ataxia) and neuropathy, and

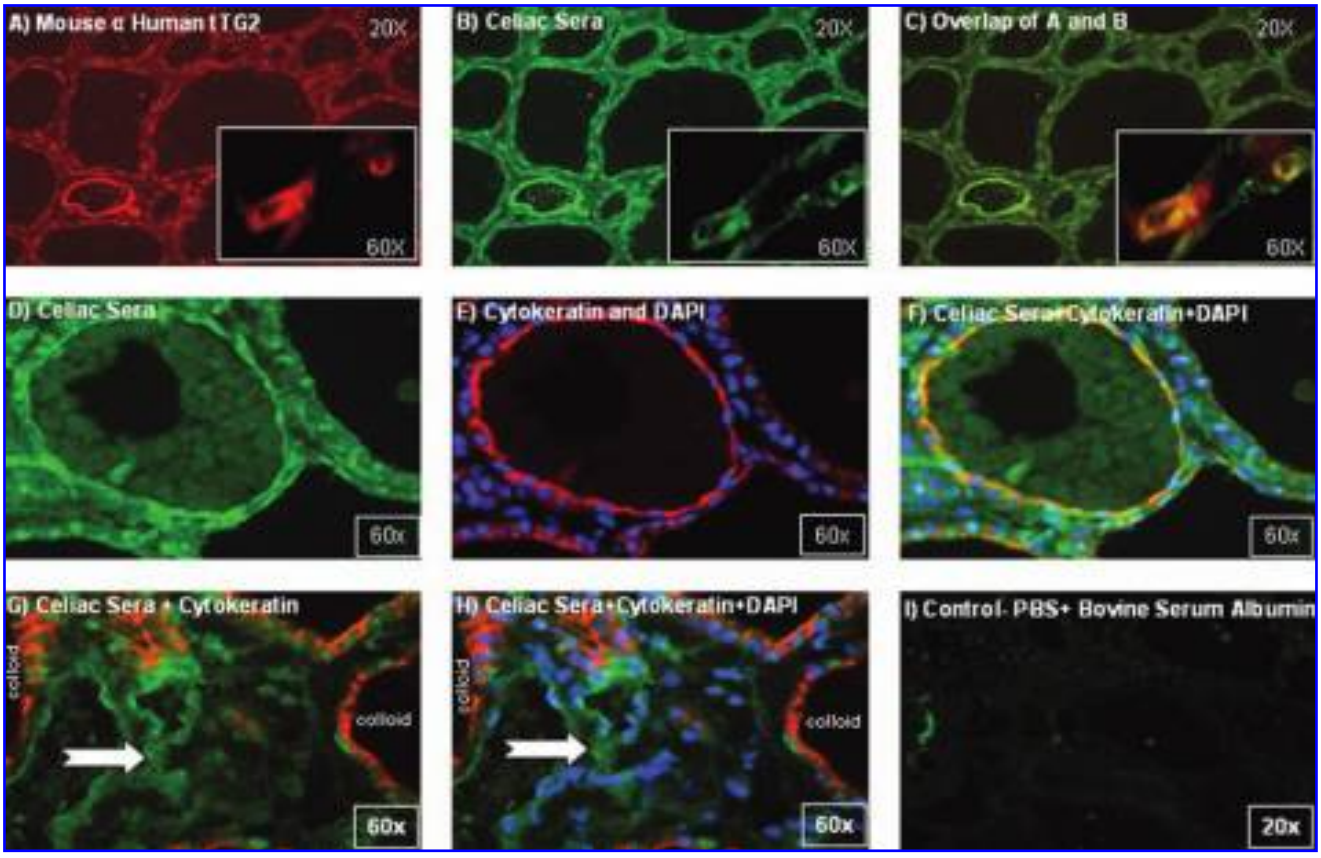

FIG. 2. Indirect immunofluorescence staining pattern of thyroid tissue. (**A**) Thyroid tissue with antibody to transglutaminase-2 (red). (**B**) Thyroid tissue with patient sera TG2 IgA antibodies (fluorescein isothiocyanate [FITC]-green). (**C**) TG2 IgA colocalizes with transglutaminase-2 as indicated by the complete overlap of (**A**) and (**B**) in yellow. (**D**) Typical staining pattern of thyroid follicular cells at 60× magnification. (**E**) Cytokeratin (red) and DAPI nuclear stain. (**F**) An merged image of TG2 IgA (green), cytokeratin (red), and DAPI (blue) staining, demonstrating the presence of TG2-2 in the follicular epithelial cells. (**G**) Cytokeratin (red) staining of a follicular cell (arrow) at 60× along with patient TG2 IgA staining (FITC-green) of the same cell (60× magnification) in the interfollicular area. (**H**) Presence of TG2 in the extracellular matrix in interfollicular areas as demonstrated by the arrow with an overlay of the cytokeratin (red), TG2 IgA (green), and nuclear stain DAPI (blue). (**I**) No staining with bovine serum albumin in phosphate-buffered saline (PBS).

are generally considered to be immune mediated (36). Anti-IgA TGase II antibody deposition has been demonstrated in the intestine of patients with the so called gluten ataxia, without enteropathy, as well as around vessels in the brain of a patient with gluten ataxia, most pronounced in the cerebellum, pons, and medulla (23). This indicates a probable role of anti-TGase II antibodies in the development of this syndrome.

Another study by Bai and coworkers showed that the TGase II IgA antibodies from patients with celiac disease react with fetal rat tibia and also demonstrated an association between circulating anti-TGase II antibodies and the development of osteoporosis (37). They proposed autoimmune mechanisms via TGase II antibodies to explain the development of osteoporosis in these patients. TGase II is important in bone formation (37). The finding that only about 25% of patients with celiac disease have evidence of malabsorption and secondary hyperparathyroidism to explain their osteoporosis (38,39); other factors such as autoimmunity may play a role in the development of bone disease in these patients.

These studies demonstrate that the circulating antibodies of celiac disease interact with the ubiquitous TGase II in different

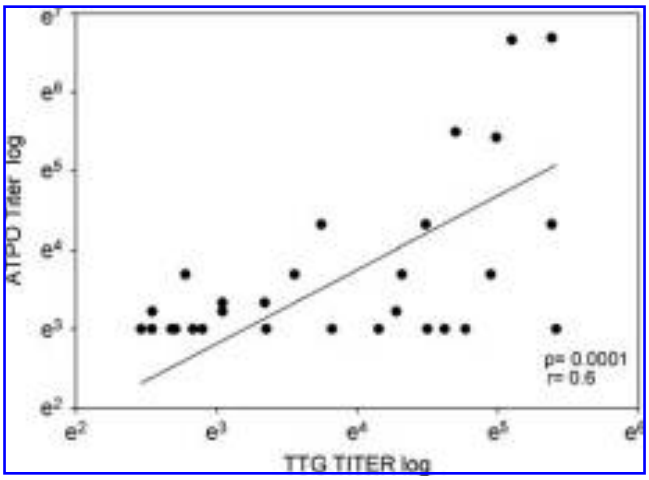

FIG. 3. A positive correlation was observed between the TGase II titers (*x*-axis) and antithyroperoxidase titers (*y*-axis) in active celiac disease patients ($p = 0.0001$, $r = 0.6$). TGase II, anti-tissue transglutaminase antibody; TPO-AB, anti-thyroperoxidase antibody.

organs. One possibility is that the aberrant activation of TGase II activity in tissues, due to antibody binding, contributes to a variety of extraintestinal manifestations, probably by forming inappropriate protein aggregates that may trigger inflammation (40) or apoptosis or both in the end organs. Celiac antibodies have been shown to inhibit the enzymatic activity of TGase II, and may interfere with its normal physiological functions (41) and might influence other cellular functions (42). Although the binding of TGase II antibodies has been demonstrated in numerous extraintestinal organs, barring some inflammation (43), no specific abnormalities have been seen on conventional histology (22).

To observe the significance of the binding of TGase II antibodies to thyroid tissue, we assessed thyroid autoimmunity in patients with celiac disease. Collectively, the frequency of thyroid antibodies in the celiac disease group was significantly different from the control groups and was higher than that shown in prospective studies of autoimmune thyroid disease (14–30.3%) (28,44). A higher age of thyroid seropositive celiac disease patients was observed that confirmed published reports (45). This may represent an isolated effect of age on the development of thyroid autoimmunity, or be a reflection of a long duration of undiagnosed celiac disease. Hence, we went on to explore whether the presence of antithyroid antibodies was related to the presence of anti-TGase II antibodies. We observed a positive correlation between anti-TGase II IgA and anti-TPO antibody titers, suggesting a relationship between celiac disease and the existence of organ-specific antibodies against thyroid tissue. Because an older age at diagnosis of celiac disease may indirectly reflect the duration of gluten exposure, our findings are supportive of the presumption that prolonged duration of gluten exposure in unrecognized patients with celiac disease might predispose to autoimmune diseases such as type 1 diabetes mellitus (46), as well as autoimmune thyroiditis (6).

Our study demonstrates that thyroid TGase II is found within the follicular epithelial cells and also observed extracellularly in the interfollicular area. Although our study does not demonstrate evidence of thyrocyte damage with anti-TGase II IgA binding, it suggests the possibility that TGase II IgA deposition may predict impending thyroid dysfunction as has been shown for jejunal mucosa by Maki and coworkers (22). Additional evidence of thyroid antibody involvement comes from studies that show that patients with celiac disease are more prone to developing atrophic thyroiditis because a hypoechoic pattern is common in thyroid antibody–positive subjects with celiac disease (47,48). A role for celiac disease as a factor in the development of autoimmune thyroid disease, induced via anti-TGase II mechanisms, is supported by the observation that patients with autoimmune thyroid disease and celiac disease show improvement of thyroid disease on a strict gluten-free diet (44,49).

In conclusion, TGase II− is ubiquitous and found within cytoplasm, cell nuclei (7), and extracellular matrix (8,9), and on cellular surfaces (10). It is conceivable that in celiac disease, a systemic autoimmune reaction occurs in some individuals. This has been suggested for cardiac (24), bone (37), and neurological tissues (23). Our studies suggest that this applies to thyroid tissue as well. Although serum autoantibodies to thyroid- and celiac-specific antigens generally do not reflect clinical autoimmune disease (50), other influences such as cellular immunity (51) and genetic and environmental factors may contribute to the development of overt thyroid disease in this population. We did not address the role of IgG antibodies that may also have a role in autoimmunity. However, our study supports a role for anti-TGase II antibodies as a contributing factor in the development of thyroid autoimmunity. This, together with epidemiological data demonstrating a strong association of celiac disease and autoimmune thyroid disease (52), suggests that those with celiac disease should be screened for autoimmune thyroid disease.

Disclosure Statement

No competing financial interests exist.

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