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Mutagenesis of the Catalytic Triad of Tissue Transglutaminase Abrogates Coeliac Disease Serum IgA Autoantibody Binding

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Background and aims: Tissue transglutaminase (tTG) is an autoantigen in coeliac disease and the related disorder, dermatitis herpetiformis. The detection of autoantibodies directed against tTG is a highly specific marker of coeliac disease; however, it is unclear if there is a role for these autoantibodies in the disease process. The aim of this study was to investigate whether the catalytic triad of tTG is targeted by coeliac disease autoantibodies.

Methods: A full-length wild-type recombinant tTG and a novel site-directed mutagenic variant lacking the catalytic triad were produced in Escherichia coli. Serum samples from 61 biopsy-proven coeliac disease and 10 dermatitis herpetiformis patients were tested for their recognition of both antigens in enzyme-linked immunosorbent assay.

Results: Although IgA autoantibodies from sera of patients with coeliac disease and dermatitis herpetiformis bound wild-type tTG well, a dramatic decrease in binding to the mutant tTG was observed with a mean reduction of 79% in coeliac disease and 58% in dermatitis herpetiformis samples. IgG anti-tTG antibodies did not show a similar pattern of reduction, with no overall difference in recognition of the wild-type or mutant tTGs.

Conclusions: These results suggest that the IgA anti-tTG response in coeliac disease and dermatitis herpetiformis is focused on the region of tTG responsible for its transamidation and deamidation reactions, whereas the IgG response may target other regions of the enzyme.

Materials and methods

Serum samples

Seventy-six serum samples from 61 patients (46 females, 15 males; age 15 to 76 years, median = 50) with coeliac disease were used to characterise anti-tTG binding. Diagnosis was based upon histology of duodenal biopsy as well as positivity for anti-tTG and anti-endomysial antibodies. Sera from 10 dermatitis herpetiformis patients (3 females, 7 males; age 12 to 64 years, median = 53) were also included in the study. Forty-nine sera (29 females, 20 males; age 17 to 84, median = 53) from individuals with normal intestinal biopsy and negative anti-tTG serology as determined using the Celikey® ELISA

Abbreviations: tTG, tissue transglutaminase; IPTG, Isopropyl β-D-1-thiogalactopyranoside; rpm, revolutions per minute; PBS, phosphate buffered saline
Bacteria were grown in nutrient broth to OD600

The GeneReadir 4200 system (LiCor).

The substituted nucleotides are underlined.

The standard curve was generated using bovine serum albumin (BSA) ranging from 0.1 to 10 ng/ml.

Statistics

The Mann–Whitney U test and Wilcoxon ranked sums test were used to compare reactivity to wild-type and mutant tTG.

Western blotting

SDS–PAGE was performed according to standard methods. Protein was blotted onto polyvinylidene fluoride membranes (Sigma-Aldrich) using a semi-dry apparatus (Apollo). The membrane was blocked at 4°C overnight with 5% non-fat dried milk including 0.5% Tween 20 (Sigma-Aldrich). Primary antibodies used were CUB7402/TG100, a monoclonal mouse anti-tTG antibody (Labvision); GST01, a mouse anti-GST antibody (Labvision); and polyclonal rabbit anti-tTG (Roboscreen). All primary antibodies were diluted 1:1000 in 5% non-fat dried milk including 0.5% Tween. After incubation for 2 h at room temperature and thorough washing with PBS containing 0.1% Tween, the membranes were incubated with 1:1000 dilution of either rabbit anti-mouse conjugated to horseradish peroxidase (HRP) or swine anti-rabbit conjugated to HRP (Dako). Blots were visualised using chromogenic substrate 3,3′-diaminobenzidine (Sigma-Aldrich) and hydrogen peroxide (Sigma-Aldrich).

**Table 1** Primers used in site-directed mutagenesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→ 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C277A-F</td>
<td>GTCAGGATGGCAGGCTGGGCGATTGCAGGCCG</td>
</tr>
<tr>
<td>C277A-R</td>
<td>GGCGCAAAGGCTAAAGAGAGGGGAA</td>
</tr>
<tr>
<td>H358A-F</td>
<td>CGGAGGATGGCCGAGGAGCTGGGAGG</td>
</tr>
<tr>
<td>H358A-R</td>
<td>CAGCAGTCCAGCCAGCAACAGC</td>
</tr>
<tr>
<td>D358A-F</td>
<td>GCAGGCTGCGCCCTGACCCCG</td>
</tr>
<tr>
<td>D358A-R</td>
<td>GGCGGCTGGGGGCGAGGCCG</td>
</tr>
</tbody>
</table>

The substituted nucleotides are underlined.

F, forward primer

R, reverse primer
Ethical approval

Ethical approval for this study was obtained from the joint ethics committee of St James’s and Tallaght hospitals.

RESULTS

Cloning strategy, sequencing and mutagenesis

Recombinant tTG was expressed as a glutathione-S-transferase (GST) fusion protein. The solubility-enhancing properties of the GST tag have been reported to increase yields of difficult proteins. DNA sequencing confirmed the sequence identity, which was homologous with the nucleotide sequence previously characterized by Gentile (1991). The Quickchange system (Stratagene) was used for site-directed mutagenesis on the three amino acids of the catalytic triad of tTG (Cys33, His34, and Asp3).

Protein production and quantification

Conditions for protein production were optimised at 1 mM IPTG overnight at room temperature. Cells were lysed using Celllytic (Sigma-Aldrich), a detergent-based reagent that releases 95% of soluble proteins from *Escherichia coli*. The purified proteins were examined by SDS–PAGE and shown to be highly pure (fig 1). Since protein yields were consistently low, NanoOrange™ (Molecular Probes), a highly sensitive protein quantification reagent, was used to determine tTG concentrations. Wild-type tTG had an average yield of 1.15 mg/l of bacteria whereas mutant protein preparations yielded, on average, 50% less enzyme then wild-type tTG preparations (data not shown).

Wild-type and mutant tTG characterisation

Reactivity of commercially available antibodies to wild-type and mutant tTG was examined by ELISA (fig 2a). To confirm that equivalent amounts of both fusion proteins were binding ELISA plates, a commercial mouse anti-GST antibody was included in all ELISAs and was found to bind equally to wells coated with both wild-type and mutant tTG. To confirm conformational integrity of both wild-type and mutant tTGs, commercially available antibodies directed against tTG were included in ELISAs. A mixture of two mouse monoclonal antibodies directed against tTG (CUB7402 + TG100 Neomarkers) bound mutant tTG with slightly decreased affinity but this finding did not reach statistical significance (Mann–Whitney U test). Rabbit polyclonal anti-tTG (Roboscreen) bound both mutant and wild-type tTGs with equal affinity. All three commercial antibodies reacted with wild-type and mutant tTGs in Western blots (fig 2b).

Wild-type and mutant tTG recognition by coeliac sera

Coeliac disease serum IgA reactivity to wild-type tTG correlated well with IgA anti-tTG results from the Celkey™ tTG ELISA system (r = 0.804). However, coeliac disease serum IgA reactivity to mutant tTG was dramatically reduced compared with wild-type tTG (fig 3a). In fact, although 88.16% of coeliac disease sera were found to be positive in the wild-type antigen assay, only 18.42% of sera exceeded the cut-off point when tested for IgA reactivity to the mutant tTG. There was, on average, a 79% reduction in IgA class autoantibody binding to mutant tTG compared with wild-type tTG.

In IgG ELISAs, sera were used at a higher dilution than in IgA assays and, as a result, AU values were lower. Coeliac disease serum IgG bound wild-type tTG at significantly higher levels than control sera (p < 0.005, Student’s t-test). Interestingly, IgG reactivity did not mirror the pattern observed in IgA assays (fig 3b) as there was no significant difference between coeliac disease IgG recognition of wild-type or mutant tTG (Wilcoxon signed ranks test).

Wild-type and mutant tTG recognition by dermatitis herpetiformis sera

The IgA wild-type tTG ELISA successfully identified 6 of 10 dermatitis herpetiformis sera as anti-tTG positive (fig 3c). In a similar pattern to the results with sera of patients with coeliac disease, dermatitis herpetiformis serum IgA showed a dramatically reduced binding to mutant tTG. All samples tested for IgA binding to mutant tTG bound the antigen at a level below the threshold for positivity, with a mean percentage reduction in binding of 58% compared with wild-type tTG. Once again, IgG responses did not mirror IgA results. IgG antibodies showed no difference in binding to wild-type or mutant tTGs (fig 3d).

DISCUSSION

Recent discoveries in the field of coeliac disease have assigned an important role to tTG in the deamidation of gliadin molecules resulting in strengthening of interactions between DQ2 and gliadin. In the light of these discoveries, a role for the autoimmune response directed against tTG in the pathogenesis of coeliac disease seems more plausible. A previous study has suggested that blockade of tTG by autoantibodies could be responsible for inhibition of epithelial cell differentiation and contribute to the mucosal lesion observed in coeliac disease. In a recent study by Sblattero et al. (2002), truncated tTG fragments were applied as antigen to ELISAs with coeliac disease sera to map immunodominant epitopes. Their results suggested that an important conformation-dependent epitope was to be found within the region spanning amino acids 140 to 376. Interestingly, this region encompasses the three amino acids of the catalytic triad of tTG (Cys33, His34, and Asp3). Since this region of tTG is only revealed upon Ca²⁺ activation and is a site of tTG–gliadin interaction, it represents a valid target for autoantibody binding studies.

In this study, a full-length recombinant tTG and a mutant variant lacking the catalytic triad were produced to investigate autoantibody binding to this region of tTG while attempting to maintain overall protein conformation. This is the first time site-directed mutagenesis has been used to investigate tTG epitopes. These results demonstrate that the IgA anti-tTG response in coeliac disease sera is specifically targeted towards the region of the enzyme responsible for its transamidation and deamidation reactions. In fact, all coeliac disease sera showed a profound reduction in IgA binding to mutant tTG compared with wild-type tTG, with 79% of sera reacting positively to the wild-type tTG having no reactivity to the mutant tTG. This effect was not limited to antigen coated on ELISA plates because pre-incubation of coeliac disease sera with wild-type tTG in solution caused almost complete depletion of IgA anti-tTG antibodies whereas pre-incubation of coeliac disease sera with mutant tTG had little effect on anti-tTG levels (data not shown).

In similar experiments investigating IgG anti-tTG antibodies, there was no significant difference in reactivity to wild-type or mutant tTGs. Although in the assay conditions employed, IgG anti-tTG levels did not exceed the cut-off levels for positivity, IgG anti-tTG binding was still significantly higher in coeliac disease than control sera (p < 0.005, Student’s t-test). The IgG anti-tTG ELISA has been shown to be less sensitive than the IgA assay, with sensitivities as low as 13% being reported previously.

Although mutagenesis could have affected the folding of the mutant tTG and hence, affected autoantibody binding to the mutant tTG, there are three reasons this does not seem to be
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Furthermore, because substrate is in excess in these antibody–antigen interactions are non-covalent and reversible even in the presence of autoantibodies is not surprising because the enzyme is capable of continuing its cross-linking function to some extent from intestinal libraries but not from peripheral blood gliadin, IgA antibodies directed against tTG can be isolated from intestinal libraries but not from peripheral blood. Marzari and colleagues (2001) demonstrated that unlike those against gliadin, IgA antibodies directed against tTG can be isolated from intestinal libraries but not from peripheral blood libraries. These results suggest that the anti-tTG response is generated at a local level whereas anti-gliadin responses are systemic.

Several studies have shown some level of inhibition of tTG by coeliac disease autoantibodies. These studies did not preincubate tTG with antibodies at 37°C, possible because of the instability of tTG preparations. The observation that tTG is capable of continuing its cross-linking function to some extent even in the presence of autoantibodies is not surprising because antibody–antigen interactions are non-covalent and reversible. Furthermore, because substrate is in excess in these artificial systems and the enzyme-substrate affinity is highly evolved, substrate is likely to compete well with autoantibodies for interaction with the catalytic triad.

In experiments investigating IgA anti-tTG antibodies from patients with dermatitis herpetiformis, 6 out of 10 samples were found to react to wild-type tTG. This is in keeping with reports that a lower proportion of sera from patients with dermatitis herpetiformis show positive anti-tTG serology compared with coeliac disease sera. IgA and IgG class anti-tTG autoantibodies from dermatitis herpetiformis patients reacted to wild-type and mutant tTGS in a similar fashion to those from coeliac disease sera. Epidermal transglutaminase has recently been identified as the predominant autoantigen in dermatitis herpetiformis. It has been suggested that dermatitis herpetiformis patients may produce populations of antibodies that react with tTG only, epidermal transglutaminase only, and cross-reactive antibodies that target epitopes common to both enzymes. Results from this study suggest that IgA anti-tTG antibodies in dermatitis herpetiformis are very similar to those found in coeliac disease. It is possible that the autoimmune response in this disorder is initiated in the gut against tTG with subsequent intermolecular epitope spreading to epidermal transglutaminase.

Whether the potential inhibition of tTG activity by autoantibodies has any significance in vivo is yet to be determined. However, the blockade of enzyme activity by autoantibodies is not unprecedented. In Wegener’s granulomatosis, inhibition of proteinase 3 by autoantibodies has been described, whereas in autoimmune atrophic gastritis the inhibition of the H+K+-ATPase proton pump by parietal cell autoantibodies has been well characterised. It is worth noting that although antibody-mediated enzyme inhibition is thought to play a role in both of these conditions, total blockade of enzyme activity is not reported. The highly specific nature of IgA anti-tTG targeting could have implications for the activity of tTG at a local level by two possible mechanisms. First, tTG has been shown to have a role in the wound healing process, and its blockade by IgA anti-tTG could interfere with coeliac lesion repair. A second possibility is the prevention of villous crypt-cell differentiation: tTG is involved in the activation of TGF-β, a cytokine required for maturation of crypt cells into enterocytes. It has been shown that IgA from patients with coeliac disease can block the differentiation of T84 intestinal crypt cells in vitro. Given the prevalence of IgA deficiency in coeliac disease patients, it seems unlikely that these autoantibodies are involved in coeliac disease aetiology but perhaps contribute to failure to repair the gut lesion.

The discovery that tTG may have a pivotal role in coeliac disease pathogenesis has led to speculation about a role for the
anti-tTG autoantibody component of this multifactorial disease. In this study, evidence for the highly specific targeting of the active site of tTG by IgA autoantibodies has been discovered, whereas IgG autoantibodies seem unaffected by mutagenesis of this catalytic triad. These findings suggest that anti-tTG autoantibodies of mucosal origin could, at least partly, inhibit tTG activity. These results help further dissect the role of the autoantibody response against tTG in coeliac disease. Future work could involve the investigation of the inhibitory capacity of coeliac disease IgA anti-tTG on the cross-linking function of tTG.

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Competing interest: none declared

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Figure 3 Coeliac disease serum IgA (n = 76) (a) and IgG (n = 64) (b) were analysed for their ability to bind wild-type and mutant tTGs. Dermatitis herpetiformis serum IgA (c) and IgG (d) were also analysed. Cut-off points are represented by bold lines.
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