In vivo targeting of intestinal and extraintestinal transglutaminase 2 by coeliac autoantibodies

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**Background:** IgA class serum autoantibodies against type 2 (tissue) transglutaminase (TG2) bind to both intestinal and extraintestinal normal tissue sections in vitro, eliciting endomysial, reticulin, and jejunal antibody reactions. It is not known whether similar binding also occurs in coeliac patients in vivo, and may thereby contribute to disease manifestations.

**Aims:** To investigate intestinal and extraintestinal coeliac tissues for the presence of in vivo bound TG2 specific IgA and its relation to small intestinal mucosal atrophy.

**Patients:** We investigated jejunal samples with normal villous morphology from 10 patients with developing coeliac disease who subsequently progressed to a flat lesion, from 11 patients with dermatitis herpetiformis, and from 12 non-coeliac controls. Six extrajejunal biopsy samples (liver, lymph node, muscle, appendix), obtained based on independent clinical indications from patients with active coeliac disease, were also studied.

**Methods:** Double colour immunofluorescent studies for in situ IgA, TG2, and laminin were performed. IgA was eluted from tissue sections and tested for TG2 specificity by enzyme linked immunosorbent assay and indirect immunofluorescence.

**Results:** IgA (in one IgA deficient case IgG) deposition on extracellularly located TG2 was detected in jejunal and extrajejunal specimens of all coeliac patients, and also in seven of 11 dermatitis herpetiformis patients, of whom two had no circulating endomysial antibodies. IgA eluted from extraintestinal coeliac tissues was targeted against TG2.

**Conclusions:** Coeliac IgA targets jejunal TG2 early in disease development even when endomysial antibodies are not present in the circulation. Extraintestinal target sites of coeliac IgA further indicate that humoral immunity may have a pathogenetic role.
immunoglobulin deposition occurs in the early stages of disease development when the mucosal morphology is still normal.

MATERIALS AND METHODS

Jejunal biopsy samples

Samples from three sources were investigated. (i) Ten patients (aged 4.4–32 years at presentation, median 7.8) with developing coeliac disease and an initially normal small intestinal villous structure were studied. These patients were prospectively followed up, they consumed normal gluten containing food, and 4–33 months later (mean 9.3) developed overt coeliac disease with a flat mucosa. Initial samples were evaluated at the time of the first biopsy when the final diagnosis was not known. (ii) Eleven patients (aged 3.6–57 years, median 10.4) with untreated dermatitis herpetiformis were studied who had typical IgA deposits in the dermal papillae but normal jejunal mucosal architecture at presentation. (iii) The third group comprised 12 non-coeliac control patients (aged 1–17 years, median 4.6) with a normal small intestinal mucosa. Controls were negative for serum endomysial antibodies while all 10 patients in group (i) and five in group (ii) had circulating endomysial antibodies in serum. Clinical characteristics are presented in table 1.

Biopsies were obtained from the jejunum by Watson capsule and were cut into two pieces. One piece was fixed in formaldehyde for routine histology and the other was snap frozen in liquid nitrogen and stored at −70°C until use.

Other organ samples

A study was made of frozen tissue samples from six additional patients who underwent biopsies from organs other than the small bowel while they had active coeliac disease. All of these biopsies had been performed based on clinical indications independent of the evaluation of coeliac disease (table 2). Samples were traced retrospectively and included two lymph nodes, one skeletal muscle, two liver biopsy samples, and a non-inflamed appendix. As controls, one lymph node, three liver, and six appendix samples from non-coeliac patients were included. These had been removed in unrelated surgical interventions and were histologically normal. All biopsies were performed for diagnostic purposes and informed consent was obtained.

Table 1 Presenting clinical characteristics and symptoms in patients with a normal small intestinal villous architecture

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Sex/age (y)</th>
<th>Symptoms and signs</th>
<th>Removed tissue</th>
<th>Indication for biopsy/removal</th>
<th>Routine histology</th>
<th>Serum EMA titre</th>
<th>Jejunal biopsy histology</th>
<th>Outcome on a gluten free diet</th>
<th>Colocalisation of antibody deposits with TG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/13</td>
<td>Anaemia, iron deficiency, mesenterial lymph node and right testis enlargement</td>
<td>Mesenterial lymph node</td>
<td>Suspicion of malignancy</td>
<td>Normal*</td>
<td>640 (IgA)</td>
<td>SVA</td>
<td>Marked clinical improvement, normal villi</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>F/14</td>
<td>Diarrhoea, weight loss, anaemia, mesenterial lymph node enlargement</td>
<td>Testis biopsy</td>
<td>Suspicion of malignancy</td>
<td>Normal</td>
<td>640 (IgA)</td>
<td>SVA</td>
<td>Symptom free, refused a control jejunal biopsy</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>M/18</td>
<td>Progressive gait disturbance, known but not treated coeliac disease</td>
<td>Appendix</td>
<td>Technical at laparoscopy Suspicion of progressive neuromuscular disease</td>
<td>Normal†</td>
<td>40 (IgA)</td>
<td>PVA</td>
<td>Improved gait, normal villi</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>F/3</td>
<td>Cystic fibrosis, anaemia, steatorrhoea, parvovirus B19 infection, elevated liver transaminase values</td>
<td>Liver biopsy</td>
<td>Acute liver enlargement</td>
<td>Steatosis</td>
<td>320 (IgA)</td>
<td>SVA</td>
<td>Died of progressive Yes liver failure. Near normal villi at autopsy</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F/12</td>
<td>Elevated liver transaminase values, selective IgA deficiency</td>
<td>Liver biopsy</td>
<td>HBsAg positivity</td>
<td>Normal</td>
<td>5120 (IgG)</td>
<td>SVA</td>
<td>Symptom free, control jejunal biopsy not done</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>M/3</td>
<td>Proteinuria, selective IgA deficiency</td>
<td>Kidney biopsy</td>
<td>Proteinuria</td>
<td>Mesangial IgG deposition</td>
<td>1280 (IgG)</td>
<td>SVA</td>
<td>Symptom free, normal villi</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Also with immunohistochemistry for T and B cell markers.
†Also with histochemistry and electron microscopy.

EMA, serum endomysial antibodies; PVA, partial villous atrophy with crypt hyperplasia; SVA, subtotal villous atrophy; TG2, transglutaminase type 2; HBsAg, hepatitis B surface antigen; ND, not done.
Immunofluorescent studies

Unfixed 5 μm thick sections of patient tissues were investigated for deposited immunoglobulins by direct immunofluorescence using fluorescein isothiocyanate labelled rabbit antibodies against human IgA, IgG, and IgM (Dako AS, Glostrup, Denmark) at a dilution of 1:40 in phosphate buffered saline (PBS), pH 7.4. These stainings were also performed as multicolour procedures where patient immunoglobulins were shown in green and extracellular matrix proteins in red using rhodamine conjugated antimouse or antirabbit secondary antibodies, as described elsewhere. The primary antibodies used were monoclonal mouse antibodies against TG2 (CUB7402; NeoMarkers, Fremont, California, USA), and rabbit antibodies against fibronectin (Dako) and laminin (Dako), respectively, diluted 1:200 in PBS. For investigation of the relation of patient immunoglobulins to blood vessels, green was used for detection of patient IgA, red for TG2, and blue for an endothelial marker (rabbit antibodies against von Willebrand factor (Dako) followed by AMCA conjugated secondary antirabbit antibodies (Vector Laboratories, Burlingame, California, USA)).

Elution of IgA and TG2 from tissue sections

Unfixed cryosections were washed twice in PBS to remove blood and related antibodies. A solution of 0.25% chloroacetic

Figure 1 IgA (green) and transglutaminase 2 (TG2, red) in the normal jejunum (A, D, G), in the early developing stage of coeliac disease (B, E, H) and in the coeliac flat lesion (C, F, I). (B) Subepithelial IgA deposits in the villi and around crypts in the mucosa with a normal architecture and (C) in the flat mucosa developed four months later in the same patient. IgA deposits colocalised with TG2 (E, F, H, I), as indicated by the yellow merging of the labels. Both deposited IgA and TG2 were closely related to vessels shown in blue (I, J, arrows). (K) Similar jejunal IgA deposits in a patient with dermatitis herpetiformis and negative serum endomysial antibodies. (L) Crypt basement membrane laminin (red band) with coeliac IgA (green) deposits. There was only partial overlap (yellow) on the extracellular surface of the laminin. Bar = 50 μm.
Acid (Fluka Chemie AG, Buchs, Switzerland) with 0.2 M/l NaCl, pH 2.7, was then used as the eluent and applied to the sections for 20 minutes. The eluates with chloroacetic acid were neutralised with 1 M/l imidazole, washed in PBS, concentrated by centrifugation on 10K membranes (Microsep, Pall Corporation, Ann Harbor, Michigan, USA), and lyophilised. Treated sections were washed twice in PBS and examined by immunofluorescence for the remaining IgA and TG2 in tissues, as described above.

Sections from extraintestinal samples, from six coeliac and three dermatitis herpetiformis jejunum samples, were investigated by immunofluorescence after choroacetic acid treatment. Lyophilised tissue eluates were prepared from a coeliac liver, lymph node, and a non-coeliac liver.

**Detection of coeliac antibodies in serum samples and tissue eluates**

Endomysial antibodies were investigated in serum samples and tissue eluates by an indirect immunofluorescent method on monkey oesophagus, normal human umbilical cord, jejunum, and appendix sections, as described elsewhere. The initial dilution was 1:2.5 in PBS, and samples non-reactive at this dilution were considered negative. Eluates were also tested on TG2 knockout mouse (TG2−/−) jejunum as a substrate, and on TG2−/− jejunum coated with human recombinant TG2 expressed in *Escherichia coli*, as previously described.

Tissue eluates were tested for the presence TG2 and IgA by western blot and for TG2 specific IgA by ELISA using microtitre plates coated with fibronectin (Sigma, St Louis, Missouri, USA) and recombinant TG2, as described by Achyuthan and colleagues. Also, plates coated with rabbit antibodies against mouse IgG1 (ICN, Aurora; dissolved in 0.03 M bicarbonate buffer, pH 9.6) and CUB7402 monoclonal TG2 specific capture antibodies were applied to further verify the presence of both TG2 specific IgA and TG2 in the eluates. Bound IgA was detected with peroxidase conjugated rabbit antibodies against human IgA, as described previously.

**RESULTS**

**In vivo deposited IgA in the coeliac jejunum**

In non-coeliac jejunal biopsy samples, IgA was detected only inside plasma cells and epithelial cells (fig1A). In contrast, clear subepithelial IgA positivity was found along the villous...
In vivo deposited IgA in extrajejunal tissues

To establish whether coeliac IgA can reach TG2 in extraintestinal tissues, we examined by double immunofluorescence two coeliac lymph nodes, two liver samples, one appendix, and one skeletal muscle specimen from coeliac patients (table 2, figs 2, 3). Specimens were normal (cases 1, 2, 3, 5, and 6 in table 2) or showed only non-specific changes (case 4, liver steatosis; table 2) by conventional microscopy. All of these tissues contained abundant TG2 in endomysial localisations or around reticulin fibres both in non-coeliac controls (fig 2A, 3B) and in coeliaics (fig 2G, J; fig 3D, F, H, red). Tissue IgA was not related to endomysial or reticulin structures in non-coeliac lymph node (n = 1), appendix (n = 6), or liver (n = 3) samples and appeared only inside epithelial cells or in the centre of lymphoid follicles (fig 2A, D; fig 3A, B, green). However, in coeliac samples there were heavy IgA deposits on reticulin fibres also (lymph nodes, appendix, liver (fig 2B, E, H; fig 3C)) and on endomysial structures (muscular layer of the appendix, skeletal muscle (fig 2F, K)) which were colocalised with tissue TG2 (fig 2C, I, L; fig 3D).

In vivo deposited IgG in coeliac disease with IgA deficiency

In the case of IgA deficiency (cases 5 and 6 in table 2), IgA deposits were missing (fig 3E, F) but TG2 related IgG deposits were seen in the liver (fig 3G, H) and also on archive electron microscopic kidney pictures of case 6 (not shown).

Investigation of in vivo IgA-TG2 autoantigen binding

Colocalisation of in vivo deposited antibodies with TG2, both in the jejunum and also in extraintestinal tissues, strongly suggested that the specific target for coeliac antibody binding is TG2. To obtain direct evidence, we eluted deposited IgA from extraintestinal tissues for further studies.

We used chloroacetic acid to disrupt TG2 binding to fibronectin and to release the in vivo deposited IgA together with released TG2. Treatment of the sections resulted in disappearance of extracellular IgA deposits in sections from both the extrajejunal and jejunal coeliac samples, while IgA in plasma cells and epithelial cells remained visible on immunofluorescence (fig 4A, B). Likewise, in tissues, TG2 became undetectable by monoclonal TG2 specific autoantibodies after elutions with choroacetic acid.

TG2 specific IgA in coeliac tissue eluates

To ascertain that both TG2 and IgA are found in eluates, western blot and ELISA studies were conducted with concentrated eluates prepared from a coeliac and a non-coeliac control liver, and from a coeliac lymph node. A clear band at 80 kDa, corresponding to the molecular weight of TG2 was, as expected, equally detectable with monoclonal TG2 antibodies both in coeliac and non-coeliac eluates (fig 4C). Human IgA was also detected both in coeliac and non-coeliac eluates (data not shown).

However, when the eluates were tested by ELISA for TG2 specific IgA class antibodies, high optical density values were obtained only with IgA eluted from the coeliac liver and lymph node (0.578 and 2.551, respectively) but not with IgA eluted from the control liver (0.052, representative values from two independent elution experiments). This finding indicates that IgA originating from non-coeliac tissues was not directed against TG2. The result was confirmed in an additional experiment where the eluates were added to ELISA plates coated with TG2 specific monoclonal antibodies and IgA was measured from the bound TG2 human IgA complexes; coeliac liver eluate gave an optical density of 0.493 versus 0.107 obtained with the control liver eluate (fig 4D).
Similarly, IgA eluted from coeliac tissues bound to human recombinant TG2 applied on TG2 knockout mouse tissues (fig 4E, F), and also to the endomysial and reticulin structures of human umbilical cord (fig 4I), monkey oesophagus (fig 4J), and normal appendix (fig 4K) sections. In contrast, IgA eluted from the non-coeliac liver did not bind to any of these structures (fig 4G, H). Furthermore, there was no IgA binding to the TG2 knockout mouse tissues with either the coeliac or non-coeliac eluates.

The experiments above collectively demonstrate that deposited IgA seen in coeliac tissues is targeted against TG2.

**DISCUSSION**

In the present study, we showed for the first time that in vivo targeting of TG2 in coeliac disease occurs in the form of in situ endomysial, reticulin, and jejunal subepithelial autoantibody binding. Patient IgA found deposited on extracellular TG2 in the liver, lymph nodes, and muscles indicates that the coeliac disease autoantigen is widely accessible to the intestinally produced circulating coeliac autoantibodies throughout the body. Based on clinical evidence of the occurrence of extraintestinal manifestations, coeliac disease should be regarded as a systemic disease and not solely involving the intestinal tract. The present primary observations may constitute one important denominator for gluten induced extraintestinal disease manifestations.

This study also showed that coeliac autoantibodies are deposited in the morphologically normal small intestinal mucosa before they can be detected in the circulation, and furthermore that the TG2 specific IgA deposition precedes the formation of the gluten induced flat jejunal lesion. This raises the possibility that autoantibodies against TG2, apart from T lymphocytes, contribute to the pathogenesis of mucosal atrophy in both coeliac disease and dermatitis herpetiformis. Also, in all other organs studied, TG2 was targeted by autoantibodies, as shown by the TG2 specificity of the IgA co-eluted together with TG2 from coeliac tissues. At the same time, conventional elution agents (for example, urea) capable of removing serum endomysial antibodies coated on tissue sections, did not extract any specific IgA from the same tissues (data not shown), which would strongly argue against the possibility that the eluted IgA represents circulating (blood) antibodies.

However, in the gluten dependent skin disease of dermatitis herpetiformis, IgA further targets type 3 transglutaminase in the skin.

The coeliac disease autoantigen, TG2, is a ubiquitous cellular protein which also functions on the cell membrane associated with integrins. In the extracellular space, TG2 is involved in the cell-extracellular matrix interactions and also in the remodelling and stabilisation of the extracellular matrix. As coeliac antibodies have been shown to influence the protein cross linking enzymatic activity of TG2, their presence may interfere with the normal performance of the enzyme in the turnover of the extracellular matrix and cytokines. It may also be hypothesised that binding of autoantibodies to TG2 also disturbs TG2 mediated cellular adhesion. It has been shown that subepithelial blistering leading to loss of surface epithelial cells occurs in the coeliac mucosa, coincident with findings indicating that the most pronounced IgA deposition is characteristically seen subepithelially. None the less, ulceration is not a feature of coeliac disease. Our study also showed that TG2 stands in intimate relation with mucosal blood vessels where deposition of coeliac IgA (fig II) might influence their nutritive and architectural function. There is remodelling of vessels in the...
In conclusion, the widespread in situ endomyosal, reticulin, and jejunal binding of TG2 specific IgA antibodies found in the tissues of our coeliac patients with developing disease or extraintestinal organ manifestations strongly supports the concept that humoral immunity is involved in pathogenesis of coeliac disease.

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