Role of tissue (type 2) transglutaminase in the pathogenesis and diagnosis of coeliac disease and dermatitis herpetiformis

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INTRODUCTION

Coeliac disease (CD; also known as nontropical sprue, gluten-sensitive enteropathy or coeliac sprue) is a common chronic small bowel mucosal disorder associated with a persistent intolerance to gluten as well as concomitant immune and autoimmune phenomena in genetically susceptible individuals. In a few individuals (approx. 1% of CD patients), CD is associated with dermatitis herpetiformis (DH), a bullous skin disease characterised by polymorphic eruptions with typical predilection sites and underlying granular IgA deposits in the papillary dermis. These deposits are most likely immune complexes with epidermal (type 3) transglutaminase. As CD and DH share a similar jejunal pathology, genetic background, pathomechanism, as well as common diagnostic analysis and dietary possibilities for therapy, in this work, I will use the term ‘gluten-sensitive disease’ (GSD) for both of these conditions.

The typical clinical presentations of CD are colourful including chronic diarrhoea, vomiting, abdominal pain, distension and bloating, failure to thrive, fatigue, short stature, loss of weight, iron deficiency anaemia, osteoporosis, habitual abortions, amenorrhoea, infertility, ataxia, and mood disturbances. DH presents with very itching skin symptoms, but gastrointestinal problems are mild or completely absent. CD shows in the majority of cases only atypical symptoms or is absolutely symptomfree. In these patients, the enteropathy is beleived to be localised mosaically and segmentally, thus the small bowel may compensate for the loss of resorption surface. In addition, CD can associate with certain autoimmune diseases (mainly type I diabetes mellitus, autoimmune thyreoiditis, Sjögren’s syndrome) and malignancies (mainly gastrointestinal lymphoma). These indirect consequences are the most important complications of unrecognised CD.

According to current criteria, the diagnosis of CD should always be confirmed by jejunal biopsy, that of DH by skin biopsy (using immunohistochemistry). However, serological examina-
tions are also helpful in the diagnosis because they offer a much less invasive, quicker, and cheaper alternative. In addition, they are more suitable for follow-up of patients on a gluten-free diet and for screening of asymptomatic individuals. These tests detect mainly IgA antibodies because IgG antibodies are usually neither specific, nor sensitive enough, with the exception of a few patients with IgA deficiency. In the practice, anti-gliadin, anti-reticulin, anti-umbilical cord vein and fibroblast, and anti-endomysial antibodies are determined.

In sera from 80-90% of untreated patients with GSD, anti-gliadin antibodies of both IgA and IgG type can occur (gliadins are gluten proteins in wheat). The anti-gliadin antibodies of IgA type are somewhat more specific but less sensitive than those of IgG type; consequently, both types are to be examined. Their sensitivity and specificity is higher in children than adults, this is why this test is most frequently used by paediatric gastroenterologists.

In addition, disease specific IgA antibodies against reticulin, endomysium and jejunum are also present in CD and DH. The most frequently used ‘gold standard’ is the endomysium antibody (EMA) test having a perfect specificity (practically 100%) and a high sensitivity (95% in CD and 70% in DH). A few years ago, an ubiquitous, both intra- and extracellular, multifunctional enzyme, tissue (type 2) transglutaminase (TGC), was identified to be the autoantigen of EMAs in GSD. It is still (July, 2002) unknown, however, why and how GSD can develop.

TGC is a Ca\(^{2+}\)-dependent enzyme primarily catalysing the formation of isopeptide bonds between glutamine and lysine residues within or between certain proteins and polypeptides. In addition, it can cross-link small primary amines to the glutamine residues. Moreover, in the absence of amine donor, it is also able to deamidate changing positively charged glutamines into negatively charged glutamic acids which may affect antigenic features of the substrate. TGC can be found in almost every tissue in both intra-
and extracellular localisation. Its physiological role has not yet been clarified in every detail. TGc has been implicated in several physiological processes as diverse as adrenergic receptor signal transduction, apoptosis, cell adhesion, stabilisation of extracellular matrix in development and wound healing, receptor-mediated endocytosis, cellular adhesion, cornified envelope formation in the keratinocytes, and apoptosis. However, its functions are most likely not vital, because gene disruption of TGc did not lead to major alterations in the mouse development and life cycle.

Although the EMA test is the best of serological investigations, a demand for alternative test methods exists because it is performed on expensive oesophagus sections from endangered primates, is laborious and time consuming, needs skilled personal for evaluation, and is subjective in borderline cases. The identification of TGc as autoantigen of EMAs allowed the use of anti-TGc antibodies in the diagnosis of GSD.
AIMS

In 1997-98, Dieterich et al. and Sulkanen et al. developed and optimised an ELISA for the serological diagnosis of gluten-sensitive enteropathy. This test gave high sensitivity and specificity (above 90%), although it was based upon guinea pig TGc (the sequence identity between guinea pig and human TGcs is 82.8%). Thus the aim of these studies was to confirm the significance of this finding for diagnostic, screening, and follow-up purposes in GSD, and further studies were planned to optimise the assay and especially to detect potential disadvantages.

Three aims were intended to achieve within the framework of this project:
1. The original ELISA test for diagnosis of GSD based on the guinea pig TGc was established in our laboratory for investigations of the nature of autoantibodies directed against TGc (effect of a GFD, association with the EMA signal). This study will be designated as ‘guinea pig TGc ELISA study’.
2. The question was raised whether the performance of the test could be further improved by using the human TGc as antigen, as patient sera not recognised by the guinea pig TGc ELISA may have antibodies directed against epitopes of human TGc not conserved in the guinea pig enzyme (‘human TGc ELISA study’).
3. The specificity of the TGc ELISAs was intended to be further determined using serum samples from patients having a wide spectrum of different diseases (‘autoimmune study’).
PATIENTS AND METHODS

Patients

The patients had been diagnosed at the Gastroenterological Departments of Clinic of Internal Medicine II or Clinic of Pediatrics II and the Department of Dermato-Venereology of the Semmelweis University. The patients had the following diagnoses (patient number ranges in the different projects are shown in parentheses): CD on a gluten-free diet (16-72) or untreated (37-39), DH on a gluten-free diet (16-16) or untreated (30-56), healthy controls (20-84), and patients with other, unrelated disorders (27-484). The latter ones were either diseases important in the differential diagnosis (gastrointestinal illnesses, autoimmune bullous skin diseases), or disorders which might be associated with production of anti-TGc autoantibodies (e.g. systemic autoimmune diseases, patients with non-autoimmune diseases involving organs with enhanced apoptosis and cell lysis and/or putative secondary autoimmune processes). We examined patients with the following diagnoses (number of patients in parenthesis): pemphigus vulgaris (48), bullous pemphigoid (32); ulcerative colitis (20); Goodpasture’s syndrome (20); Crohn disease (31); Wegener’s granulomatosis (20); rheumatoid arthritis (41); progressive systemic sclerosis (30); antiphospholipid syndrome (13); psoriatic arthritis (11); hepatitis C (100); malignant tumours (11). In addition, several other diseases were tested (58).

Recombinant production of TGc

An episomal eukaryotic expression vector containing the puromycin resistance gene, produced from pCEP4 (Invitrogen), was modified to introduce a sequence encoding the Strep II tag (Institut für Bioanalytik, Germany) followed by a stop codon into the multiple cloning site. We received the full-length human TGc cDNA as a kind gift from Dr. Daniel Aeschlimann. This cDNA was inserted into the vector so that it began with a Kozak’s translation initiation sequence, and the stop codon was removed from
its C-terminal end so that the tag can be attached. The correct insertion and sequence of the full construct was verified by cycle sequencing. Human embryonic kidney cells (293-EBNA; Invitrogen) were transfected episomally with this construct and selected by puromycin.

After harvesting, cells were homogenised in sucrose solution, and after two centrifugation steps, TGc was purified with Strep-Tactin affinity chromatography using phenyl-methyl-sulfonyl-fluoride as protease inhibitor. The purification was controlled by SDS-PAGE and immunoblotting with monoclonal antibodies against TGc. The protein concentration was measured using the bicinchoninic acid protein assay reagent (Pierce) according to the protocol provided by the supplier, with bovine serum albumin as the calibrator. TGc activity was measured by incorporation of [1,4-³H]putrescine with inclusion of a buffer containing dithiothreitol to reduce any oxidised sulfhydryl groups important for catalytic activity.

**ELISA**

The ELISA method was similar to the calcium-activated test described previously by Dieterich et al. Nunc MaxiSorp 96 well microtitre plates were coated with 1 μg guinea pig TGc (Sigma) or human recombinant TGc in 100 μl of 50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl₂ per well at 4 °C at least 9 h. No blocking was used because 1 μg of TGc was able to saturate the binding sites of the adhesion surface of ELISA wells. After each step, the wells were washed with 50 mM Tris-HCl containing 10 mM EDTA and 0.1% Tween 20 (TET). Sera were diluted to various concentrations with TET, and incubated on the plates for 1.5 h at room temperature. Bound IgA was detected by peroxidase-conjugated Ab against human IgA (Dako), diluted 1:4000 in TET and incubated for 1 h at room temperature. The colour was developed by the addition of 100 μl of 60 μg/ml 3,3’,5,5’-tetramethylbenzidine substrate in 100 mM sodium acetate, pH
6.0, containing 0.015% $\text{H}_2\text{O}_2$ for 5 min at room temperature. The reaction was stopped by adding 100 µl of 20% $\text{H}_2\text{SO}_4$. The absorbance was read in an ELISA reader at 450 nm.

The amount of protein and the serum concentrations used in the test were optimised. All serum samples were examined in triplicates, and triplicates of a negative and a positive reference serum as well as a buffer blank were included in each assay. The antibody concentrations were expressed in arbitrary units, i.e., as percentages of the positive reference serum.

**Other biochemical methods**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, polymerase chain reaction (PCR), automated cycle sequencing, digestion with restriction endonucleases, and mass spectrometry were performed using standard techniques.

**Routine diagnostic methods**

Total serum IgA measurement, gliadin ELISA, EMA test and other histological and immunohistochemical methods were performed using standard techniques of clinical diagnostics.
RESULTS

The guinea pig TGc ELISA study

The guinea pig TGc ELISA could successfully differentiate between controls and patients with GSD. When comparing with the controls, the difference was significant for both CD and DH. There was also a significant difference between the two groups of GSD patients: CD patients had significantly higher anti-TGc antibody activity than DH patients.

The guinea pig TGc ELISA gave a specificity and a sensitivity of 98.6% and 92.5%, respectively. The coincidence of the results with the gold standard monkey oesophagus EMA test was 95.8%, but the coincidence with the clinical diagnoses was 97.1%. Examination of patients on a gluten-free diet showed that the titre of IgA antibodies against TGc slowly declines during a few weeks or months, and finally they disappear. Similarly, the EMA test also becomes negative. In this respect, the guinea pig TGc ELISA was less sensitive than the EMA test, because almost 10% of EMA-positive patients on a gluten-free diet were negative for guinea pig TGc ELISA whereas the opposite situation was found only in 1%.

The human TGc ELISA study

The human TGc was expressed and purified as a pure enzyme when visualised by Coomassie-stained SDS-PAGE. The activity and molecular mass of the enzyme were as expected. Like the guinea pig TGc, the human TGc was also used in its Ca$^{2+}$-activated form for ELISA because of higher sensitivity.

As expected from the previous study, the human TGc ELISA could also differentiate between controls and patients with gluten sensitivity, the difference being significant for both CD and DH. In contrast, however, there was no significant difference between the anti-TGc antibody activity of CD and DH patients. Upon comparison of untreated GSD patients with controls, the specificity and sensitivity of the ELISAs were high (human TGc: 98.1%
and 98.2%, respectively; guinea pig TGc using the same set of sera as in the human TGc-based assay: 96.2% and 92.7%, respectively). Although the human TGc ELISA seems to be better, the difference between the two tests was not significant, most likely because of the relatively small number of patients studied (n=108). The coincidence with the clinical diagnosis was also somewhat better (human TGc ELISA: 98.1%; guinea pig TGc ELISA: 94.4%), just like when the coincidence with the EMA test was calculated (96.4% and 91.1% for the human and guinea pig TGc ELISA, respectively). Upon examination of patients on a gluten-free diet, the human TGc ELISA was even more sensitive than the EMA test. Both the human and the guinea pig TGc ELISAs showed false positivity only in patients with Crohn disease.

The autoimmune study

However, later it turned out that a considerable number of sera from patients with diverse autoimmune diseases show positive reaction in the TGc ELISAs without evidence of a GSD. The following diseases showed significantly raised IgA antibody titres against TGc: antiphospholipid syndrome, ulcerative colitis, Crohn disease, Goodpasture syndrome, Wegener’s granulomatosis, rheumatoid arthritis, SLE, progressive systemic sclerosis, hepatitis C. Overall approximately 50% of the patients with autoimmune diseases and 25% of the others showed (generally weak) false-positive reaction in the human TGc ELISA and somewhat less in the guinea pig TGc ELISA. The titres of anti-TGc antibodies correlated with the total serum IgA and anti-gliadin antibody levels, although the scatter was high. Interestingly, however, no false-positive patient was EMA-positive.
CONCLUSIONS AND PRACTICAL USE OF RESULTS

In the series of experiments described above I investigated the suitability of guinea pig and human TGc ELISAs for the clinical diagnosis of GSD. The experiments supported previous observations that TGc is the main autoantigen of circulating, disease-specific IgA antibodies in GSD. When the experiments were started, it was still not known that the antibodies against endomysium, reticulin, umbilical cord cells and jejunum are identical and directed against TGc in these tissues. This means the TGc ELISA measures the same parameter detected by the indirect immunofluorescence, but the ELISA is simpler, quantitative, cheaper, it can be automated, and there is no need for highly skilled personal and oesophagus from endangered primates.

It was shown that both the guinea pig and the human TGc ELISA were suitable for simple and non-invasive diagnosis, screening, and follow-up of GSD. The human TGc ELISA was found to be somewhat superior to the guinea pig TGc ELISA and as specific and sensitive as the EMA test, without having its practical disadvantages.

However, in particular in active AI diseases, the TGc ELISA is less specific than the EMA test. No false-positive serum from AI patients was false-positive in the EMA test. Although CD has been described to associate with various AI disorders, it is highly unlikely that almost half of the patients with non-GSD AI disease, particularly in the absence of EMA immunoreactivity, would have GSD. The frequent TGc ELISA positivity suggested either an aspecific detection of IgA antibodies in our assays or the presence of circulating IgA antibodies directed against TGc epitopes hidden in the endomysium. Hence, though the TGc ELISA positivity is a good marker of GSD, it should not alone be taken as the basis for a diagnosis of GSD, in particular in AI diseases, and positive results in symptomless individuals should always be verified by EMA test before submitting them for jejunal biopsy.
The median serum IgA antibody level against TGc was significantly higher in patients with untreated CD than with untreated DH, results in agreement with those of other authors. This finding is supported by the observation that the EMA test is often false-negative in untreated DH patients who usually have no or very mild enteropathy.

The TGc ELISA already showed the effect of a GFD when the EMA test had not yet been turned negative. The apparent advantage of the quantitative TGc ELISA against the semiquantitative EMA test is that the former allows the control of the compliance to the GFD more effectively.

Thus one can conclude that the human TGc-based ELISA should be the method of first choice for simple and non-invasive serological diagnosis, screening, and follow-up of GSD. Positive results in symptomless individuals should always be verified by EMA test before submitting them for jejunal biopsy, while negative results ought to be controlled if the clinical symptoms and signs are suspicious of GSD. The role of TGc in GSD (and possibly in other diseases) remains elusive, further studies are needed to understand whether IgA antibodies against TGc have pathological consequences or are only markers of gluten consumption.

The ‘Expression of the human tissue transglutaminase in human embryonic kidney cells and purification via StrepTactin affinity chromatography’ was patented by the European Patent Office (No. 99111975.1.). The human TGc ELISA presented in this work is currently (July, 2002) produced in Germany on an industrial scale and it is commercially available.
OWN PUBLICATIONS FORMING THE BASIS OF THIS WORK


ABSTRACTS FORMING THE BASIS OF THIS WORK


* In 2000, our research group won the scientific award of the German-Hungarian Dermatological Society with this article.
PUBLICATIONS ON THE TG RESEARCH BEING IN INDIRECT CONNECTION WITH THIS WORK

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