Amino Acids (2004) 26: 381–386
DOI 10.1007/s00726-004-0083-7

Amino Acids
Printed in Austria

Transglutaminase 2 in celiac disease: Minireview article

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Received October 10, 2003
Accepted January 15, 2004
Published online April 14, 2004;

Summary. Celiac disease (CD) is an autoimmune pathology of the small intestine triggered, in genetically predisposed patients, by the exposition to gliadin, a flour protein, thus evoking local immune reactions and mucosal atrophy. The discovery that type 2 transglutaminase (TG2) is the main, if not the sole, target of the endomysium CD-specific autoantibodies assigned to this enzyme a master regulator role of CD. Two separated events, both based on the finding that gliadin is able to act as a TG2 substrate, have been described to indicate that TG2 is involved in both the humoral and cellular immune responses. In this paper we review the novel insights on the localization and enzymatic activity of TG2 in the small intestinal mucosa. Moreover, we report on the capability of gliadin and its peptides to act as TG2 substrates.

Keywords: Transglutaminase 2 – Celiac disease – Gluten – Enterocytes

Introduction

Transglutaminase 2 (TG2) (EC 2.3.2.13), the first member of the TG family to be discovered, catalyses the posttranslational modification of proteins. Its calcium-dependent catalytic activity is exhibited toward γ-carboxamide groups of peptide-bound glutamine residues and ε-amino groups of peptide-bound lysines leading to an intra- or interchain isopeptide bond (Folk and Finlayson, 1977). Low molecular weight amines may substitute lysines in transamidating reactions. In the absence of suitable amines, water may act as an acyl acceptor substrate with the consequent deamidation of protein-bound glutamine residues (Folk and Finlayson, 1977; Mycek and Waelsch, 1960). Another intriguing property of TG2 is to possess a site that binds and hydrolyses GTP (Achyuthan and Greenberg, 1987). Such GTPase activity of TG2 is independent of the crosslinking activity, but both activities are regulate, in a reciprocal manner, by the binding of GTP and Ca^{2+} (Achyuthan and Greenberg, 1987; Bergamini, 1988). It is worth of note that the GTP hydrolysis by TG2 is strictly an intracellular function, while the crosslinking reaction could take place both in intracellular and extracellular compartment. In fact, despite the lack of a leader sequence, TG2 appears to be secreted from cells into the extracellular space where it has been implicated in matrix deposition (Aeschlimann and Thomazy, 2000). Moreover, a new potentially important extracellular role of TG2, that does not involve its crosslinking activity, and depends on the close association of the TG2 with the β1 and β3 integrin to the cell surface, has been described. TG2, acting as an integrin-binding co-receptor, could participate in fibronectin assembly as well as in cell adhesion (Gaudry et al., 1999; Akimov and Belkin, 2001; Balklava et al., 2002). Intracellular TG2 is expressed in selected mammalian tissues and seems involved in the regulation of several biological events including cellular proliferation, differentiation, and apoptosis (Aeschlimann and Paulsson, 1994; Griffin et al., 2002). TG2 functions might therefore depend on its peculiar subcellular and cellular localization. In particular, the presence of different accessible proteins able to act as substrates for the enzyme in specific cell type may determine the activity of TG2 and its control. However, in some circumstances, TG2-catalysed post-translational modifications of proteins may generate auto-antibodies, as happens in autoimmune disorders such as celiac disease (CD) (Molberg et al., 2000; Kim et al., 2002).

CD, or gluten-sensitive enteropathy, is a chronic multifactorial disease (Sollid, 2000). It is considered to be the

result of a dysregulated T cell mucosal immune response to wheat gliadin and related proteins from rye and barley. It is strongly associated with the genes encoding for HLA-DQ2 and -DQ8 (Sollid, 2000). Gluten-specific $CD4^+$ intestinal T cells can be isolated from intestinal biopsies of CD patients but neither from healthy controls (Sollid, 2000). Interestingly, the intestinal T cells best recognize gluten peptides in which glutamine are converted to glutamic acid. The deamidation of gliadin may take place in the acidic environment of the stomach; alternatively, TG2 could catalyse *in situ* the deamidation of specific glutamine residues present in immunodominant gluten peptides (Molberg et al., 1998; Molberg et al., 2001).

Since CD patients on a gluten-containing diet have increased levels of serum antibodies not only to gluten but also to TG2 (Dieterich et al., 1997), it has been hypothesized that, apart from the deamidation of gliadin peptide, TG2 can crosslink itself to gliadin thus acting as hapten in the generation of autoantibodies, the carrier being gliadin; in fact it has been suggested that the production of anti-TG2 IgA antibodies is dependent on the help provided by gliadin-specific T cells to normally silent B cells specific for TG2 (Sollid et al., 1997). Recently, Marzari et al. demonstrated that it was possible to isolate anti-TG2 antibodies from all intestinal lymphocyte libraries, but not from those obtained from peripheral lymphocytes (Marzari et al., 2001). This is in contrast to antibodies against gliadin, which could be obtained from all libraries, indicating that while the humoral response against TG2 occurs at the local level, that against gliadin occurs both peripherally and centrally.

Despite significant steps forward have been taken on the understanding of the molecular mechanisms of CD, some crucial questions remain to be answered. For instance, i) where deamidation by TG2 occurs? ii) which is the role of enterocytes, the first barrier against exogenous toxic dietary proteins, in the pathophysiology of CD?

Localization and enzymatic activity of TG2 in the small intestinal mucosa

An immunohistochemical study on the differential expression of TG2 in human cells showed that the small intestine expresses TG2 and that such expression parallels epithelial maturation (Thomazy and Fesus, 1989). More recently, observations have been published on the immunohistochemical localization of the enzyme in normal and CD patients (Molberg et al., 1998; Brusco et al., 1999). In normal subjects, TG2 has been detected in all layers of the small intestinal wall; the enzyme is expressed in the submucosa while only a little amount is located within the epithelium. An increased expression of TG2 in celiac patients was reported in defined areas of the small intestinal mucosa such as enterocytes, both at brush border and cytoplasm levels, as well as in the extra-cellular matrix (Hansson et al., 2002; Esposito et al., 2003).

The presence of TG2 within the extracellular compartment in bioptic fragments from CD patients compared to controls suggests its translocation from the intracellular to the pericellular environment. Since the consequence of the externalisation is an increase in crosslink products in the extracellular space, it has been suggested that changes in both the expression and the location of TG2 is part of a cellular stress response (Wang at al., 1992; Skill et al., 2001; Gross et al., 2003). Furthermore, we demonstrated that in intestinal mucosa of CD patients there is an evident increase of the enzymatic activity compared to control concentrated extra-cellularly in specific areas of the mucosa, particularly in the subepithelial region (Esposito et al., 2003). These findings were obtained by TG assays *in situ* using both 5-(biotinamido)pentylamine, that represents the amino-donor TG2 substrate, and two biotinylated peptides, that represent the glutamine-donor TG2 substrates, as probes for endogenous TG activity. In this way we demonstrated not only that in intestinal mucosa there are accessible proteins able to act as amine-acceptor and glutamine-acceptor substrates, but also that TG2 activity correlates with the pattern of TG2 protein expression. It is interesting to note that, by using the biotinylated glutamine-donor probes, we demonstrated for the first time that in intestinal mucosa there are proteins able to act as glutamine-acceptor substrates (Esposito et al., 2003). These findings are compatible with the hypothesis that TG2 catalyses the formation of gliadin (glutamine-donor)-protein complexes thus generating novel self-antigen responsible of the autoimmune responses.

Gluten peptides as substrates of TG2-deamidating activity

In a pioneering study in 1990, some of us demonstrated the ability of several cereal proteins to act as substrates for TG purified from guinea pig liver (Porta et al., 1990). Among the various dietary proteins tested, wheat glutelins and gliadins, as well as purified A-gliadin, were found to

be the most effective acyl donor substrates for TG. In particular, by performing incubations *in vitro* both in the presence of radiolabeled polyamines and in their absence, these proteins seemed to be able to produce not only γ(glutamyl)polyamine adducts but also polymeric complexes, probably through intermolecular $\varepsilon(\gamma$-glutamyl)lysine crosslinks. In the case of A-gliadin, the single lysil residue occurring in the amino acid sequence (K-186) is supposed to act as acyl acceptor site. It was also reported that the peptic-tryptic fragments of gliadins and prolamines of different origin behaved as TG substrates similarly to native gliadin, mostly in giving rise to large *Mr* polymers.

In the light of the recent acquisitions on CD, it is hypothesized that TG2 might be responsible for the deamidation of specific glutamine residues in gluten derived peptides. Such TG2-catalyzed posttranslational modification generates negatively charged amino acid residues that bind with an increased affinity to the HLA-DQ2 and HLA-DQ8 molecules so potentiating T cells activation (Molberg et al., 1998; Molberg et al., 2001).

Several researches for many years were performed to find which peptide in gluten and the other disease-activating dietary grains activates disease, and more recently which gliadin peptide is selectively used by TG2 as substrate to generate epitopes for recognition by $CD4^+$ in celiac patients. The achievement of this goal was particularly difficult since gliadin for its peculiar amino acid composition, which is characterized by a high content of glutamine (approximately 35 mol%), proline (15 mol%) and hydrophobic amino acids (19 mol%), is an excellent TG2 substrate. It is well known that it has not been possible to derive a consensus sequence around the reactive glutamine residues, however, the spacing between the targeted glutamine and neighbouring proline residues in the sequence plays a dominating role in the specificity of TG2. The enzyme preferred mostly QxP but not QP or QxxP. Therefore, a variety of peptides derived from gliadin containing this specific sequence have been identified as epitopes for gliadin-specific mucosal T-cell clones. In addition, those peptides often differed from patient to patient, and from adult to children (Fleckenstein et al., 2002).

In 2000 two independent studies identified two overlapping TG2-modified α-gliadin peptides as the dominant gliadin T-cell epitopes (Anderson et al., 2000; Arentz-Hansen et al., 2000). The authors reported that TG2 specifically deamidated Q65 in the 57–75 peptide of α-gliadin. With the contribution of a later study (Arentz-Hansen et al., 2002), three overlapping peptides, rich in proline and glutamine (PFPQPQLPY, PQPQLPYPQ, and PYPQPQLPY) were described as the main T-cell epitopes in gliadin. Recently, Shan and colleagues demonstrated that the *in vitro* digestion of recombinant α2 gliadin produced a digestion-resistant 33-mer peptide (residues 57 to 89) containing all three previously described epitopes (Shan et al., 2002). Moreover, the 33-mer product shows the highest specificity toward TG2-deamidation than that reported for any peptide studied thus far, therefore it has been indicated as the primary initiator of the inflammatory response to gluten in CD patients. Interestingly, Molberg et al. described an intestinal T cell response also to TG2-deamidated high molecular weight glutenin proteins (Molberg et al., 2003). Finally, Koning and Vader reported that in children with CD there were multiple gliadin or glutenin peptides that could bind to DQ2 or DQ8 and activate mucosal T-cell populations without to be subjected to TG2 deamidation. On the contrary, in adult patients only one or a few deamidates peptides activate mucosal response. Therefore, they conclude that the deamidation of peptides occurs as disease develops, but may not be required for the initial activation of mucosal T cells in the very early stages of disease (Vader et al., 2002).

TG2 crosslinking activity and autoimmunity

The ability of TG2 to crosslink gliadin peptides to itself and to other protein substrates supports the hypothesis that this event is responsible for the humoral autoimmune response in CD. According to this hypothesis TG2-gliadin complexes bind to TG2-specific B cells, are endocytosed and processed. Gliadin-DQ2 complexes are then presented by the TG2-specific B cells to gliadin-specific T cells which give the necessary help to produce anti-TG2 antibodies (Sollid et al., 1997; Schuppan et al., 1998; Molberg et al., 2000). An immune reaction was observed against the cytoskeleton in both children and adults with CD. In particular, antiactin antibodies are shown to be more strongly associated with more severe degrees of villous atrophy (Clemente et al., 2000). Antibodies against calreticulin, desmin, and a 90 kDa dermal glycoprotein have also been described in CD patients (Maki, 1995; Krupickova et al., 1999; Sanchez et al., 2000; Teesalu et al., 2001). Recently, three new autoantigens as ATP synthase β chain and two variants of enolase α have been identified by mass fingerprinting approach (Stulík et al., 2003). However, it has been demonstrated using TG2 knockout mice that the endomysial binding patterns of celiac serum samples is clearly and exclusively TG2 autoantibody dependent (Korponay-Szabo et al.,

2003). It is tempting to hypothesize that gliadin peptides can trigger a cascade of events leading to the inappropriate presentation of TG2 and crosslinked substrates to the immune system contributing to the immune aspect of CD. This mechanism could in part explain the increased prevalence of concomitant auto-immune diseases, such as collagen diseases, type I diabetes, auto-immune alopecia, hypophysitis and others, in CD patients with prolonged gluten exposure (Collin et al., 2002).

It would be very important to address the question if TG2 autoantibodies can contribute to the intestinal lesion in CD, given the strong disease specific presence of TG2 antibodies and the role played by this enzyme in several crucial biological processes. Increasing evidences suggest that TG2 autoantibodies can affect TG2 function, although this hypothesis seems to be controversial. A study published in 1999 provided an indirect evidence of a blocking function of TG2 autoantibodies. In that study it was demonstrated that the transforming growth factor β (TGF-β) dependent differentiation of T84 crypt epithelial cells can be prevented by the addition of anti-TG2 IgA. The authors suggested that blocking TG2 prevented the generation of the active form of TGF-β, which relies on TG2 for its maturation, and they further speculated that anti-TG2 antibodies may also affect the differentiation of the celiac epithelium (Haltunnen and Maki, 1999).

Recently, we demonstrated that IgA and IgG from serum of celiac patients as well as monoclonal anti-TG2 antibodies obtained from CD patients, display inhibitory effects toward the transamidating activity of human TG2 both *in vitro* and *in situ*. The inhibition of recombinant human TG2 activity was shown in all sera tested, even if the degree of inhibition was variable (Esposito et al., 2002). Such variability was also reported by Kiraly et al. Moreover, they found that some antibodies activated the enzyme also to varying degrees (Kiraly et al., 2002). On the other hand Keaveny et al. did not find any inhibition of TG2 activity by anti-endomysial antibodies (Keaveny et al., 2000). In any case it appears that TG2 activity could be differently modified in the intestine of celiac patients by the antibodies modulating the course of the disease. Furthermore autoantibodies could interfere with some of functions that TG2 exert at the surface of cells and that are independent from the catalytic activity.

What about enterocytes contribution to CD pathogenesis?

The presence of an active TG2 in intestinal mucosa at the level of endothelial, fibroblast, macrophages, mononuclear cells, enterocytes as well as in the extra-cellular matrix poses the question where gluten peptides are specifically modified by TG2. It is tempting to hypothesize that a primary pathogenic event of CD could happen at the level of the enterocytes which are crucial to maintain barrier function between the lumenal milieu and the internal environment.

There are increasing evidences that enterocytes modulate immunological functions of the intestinal mucosa by expressing HLA class II antigens and presenting antigens to T-lymphocytes (Martin-Villa et al., 1997; Hershberg et al., 1997; Biagi et al., 1999; Hershberg and Mayer, 2000). Gliadin could be deamidated by TG2 at the surface or inside the enterocytes and presented by DQ2 molecules triggering the immune response. Moreover, an accumulation of active TG2 as well as the presence of glutamine-donor and lysine-donor TG2 substrates have been demonstrated in the enterocytes of celiac patients compared to controls (Esposito et al., 2003; Farrace et al., 2001). We identified in an enterocyte-like system, by a proteomic approach, more than 25 endogenous proteins, both acyl-acceptor and acyl-donor, that may represent putative substrates of TG2 (Orrù et al., 2003). In relation to their biological significance, the identified proteins fall into four groups. These targets include proteins involved in cytoskeletal network organization, folding of proteins, transport processes. A fourth group finally consists of proteins involved in a miscellaneous of metabolic functions.

It is intriguing to suggest that TG2 could come in contact with gliadin inside enterocytes and modify gliadin peptides by crosslinking to itself or to other acyl-acceptor substrates thus originating neo-antigens recognized by the immune system. This mechanism could explain the existence of auto-antibodies in CD with several distinct specificities.

Conclusion

Experimental evidences suggest that TG2 play a master role in the onset of CD. TG2 transamidating and deamidating activity seem to be greatly responsible for the cellular and humoral immune response, respectively. TG2 expression is ubiquitary increased in CD mucosa and the enzyme is catalitically active. We focus our attention on the possibility that enterocytes, which represent the first barrier for dietary gluten peptides, may represent a primary site where gliadin is modified by TG2 so generating toxic gliadin epitopes and neoantigens that are presented to immunocompetent cells.

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