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ORIGINAL ARTICLE

Autoantibodies from patients with celiac disease inhibit transglutaminase 2 binding to heparin/heparan sulfate and interfere with intestinal epithelial cell adhesion

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Abstract Autoantibodies from patients with celiac disease (CD) can influence transglutaminase 2 (TG2) activity and its cellular functions, but the exact mechanisms have remained unknown. Our objective was to study whether autoantibodies could modulate TG2 binding to heparin/heparan sulfate (HS) and intestinal epithelial cell attachment to fibronectin-TG2 matrix. Anti-TG2 antibodies were purified by TG2 affinity chromatography from sera of patients with active CD. Serum and antibody effects on TG2 binding to heparin/HS, on transamidase activity of TG2, as well as on Caco-2 cell attachment to fibronectin-TG2 matrix were assessed using microplate assays. Both sera and purified anti-TG2 antibodies from CD patients with high anti-TG2 IgA levels reduced TG2 binding to heparin/HS as compared with those with low anti-TG2 IgA or controls. There was a negative correlation between anti-TG2 IgA levels and TG2 binding to heparin/HS. Treatment of fibronectin-TG2 coated wells with CD patients' sera or purified anti-TG2 antibodies reduced attachment of Caco-2 cells onto the plate as compared with the control samples. The effect of CD patients' antibodies on Caco-2 cell attachment to fibronectin-TG2 matrix occurred independently of the inhibition of cell adhesion by Arg-Gly-Asp sequence containing peptides. Anti-TG2 autoantibodies had no effect on transamidase activity of TG2 in vitro. We suggest that modulation of adhesion function of TG2 by autoantibodies from patients with CD could be related to the inhibition of TG2 binding to HS residues of cell surface proteoglycans and could have possible implications for CD pathogenesis.

Keywords Transglutaminase 2 · Autoantibodies · Heparin/heparan sulfate · Adhesion · Caco-2 cells

Abbreviations

ANOVA	Analysis of variance
AP	Alkaline phosphatase
BSA	Bovine serum albumin
CD	Celiac disease
DGR	Asp-Gly-Arg
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FN	Fibronectin
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
RGD	Arg-Gly-Asp
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TG2	Transglutaminase 2

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Introduction

Transglutaminase 2 (TG2) or tissue transglutaminase is a multifunctional protein involved in the cellular processes

of apoptosis, proliferation, adhesion, and extracellular matrix (ECM) modification (Lorand and Graham 2003; Iismaa et al. 2009; Mehta et al. 2010). TG2 localizes in the cytoplasm and on the cell membrane of various cell types but can also be found in the ECM. As the main biochemical activity, TG2 mediates Ca^{2+}-dependent transamidation reaction between protein glutamine and lysine residues resulting in isopeptide bond formation. In addition, TG2 may deamidate glutamine residues to glutamate in the absence of suitable amine donors. Two types of conformational structures for TG2 have been described: an "open" conformation with transamidation activity (Pinkas et al. 2007) and a "closed", compact conformation with bound GTP/GDP and GTPase activity (Liu et al. 2002). Localization of TG2, binding to cofactors and other proteins has importance in realizing its biological function (Iismaa et al. 2009; Mehta et al. 2010).

The role of TG2 in cell interactions with ECM is primarily related to its high-affinity binding to fibronectin (FN), a multifunctional protein (Hang et al. 2005; Zemskov et al. 2006). TG2 can act as integrin co-receptor for binding FN, and complex formation between TG2, FN and integrin $\beta_1/\beta_3/\beta_5$ molecules mediates cell adhesion and spreading (Akimov et al. 2000). The amino acid sequence Arg-Gly-Asp (RGD) within the type III domain of FN is the most important adhesion motif for binding cell surface integrins (Ruoslahti 1996). Recent studies have shown the relevance of TG2 interactions with heparin/HS residues in RGD-independent cell adhesion, and syndecan 4 has been suggested as the main proteoglycan receptor for TG2 (Verderio et al. 2003; Telci et al. 2008; Scarpellini et al. 2009).

Celiac disease (CD) is a chronic immune-mediated disease of the small intestine triggered by wheat gluten or related proteins of the rye and barley. In immunopathogenesis of CD deamidation of gluten peptides by TG2 amplifies T cell response towards gluten (Jabri and Sollid 2009). Anti-TG2 antibodies appear in patients' sera and compose deposits in the small intestinal mucosa (Dieterich et al. 1997; Korponay-Szabo et al. 2004). Serum anti-TG2 IgA has been employed as a sensitive and specific serologic marker for CD, occurring in over 90% of patients (Lewis and Scott 2006). The role of anti-TG2 antibodies in CD has been previously investigated along with the effects of autoantibodies on enzymatic activity and cellular functions of TG2 (Caputo et al. 2009; Lindfors et al. 2009). Autoantibodies from CD patients have been shown to inhibit, enhance, or have no effect on transamidating activity of TG2 in different studies (Esposito et al. 2002; Dieterich et al. 2003; Kiraly et al. 2006; Byrne et al. 2010). In various cell-based experimental approaches, impact of celiac patients' IgA or anti-TG antibodies on cell cycle (Halttunen and Maki 1999; Barone et al. 2007), endocytosis (Caputo et al. 2010), angiogenesis (Myrsky et al. 2008), intestinal and endothelial permeability (Zanoni et al. 2006; Myrsky et al. 2009) have been demonstrated, while the exact mechanisms of antibody action remain still unknown.

Our aim was to study whether anti-TG2 antibodies from patients with CD could affect TG2 interactions with heparin/HS and the related cell adhesion function of TG2. To address the objective of our study, TG2 binding to heparin/HS and Caco-2 cell attachment to FN-TG2 coated surface were assessed in the presence of anti-TG2 antibodies.

Materials and methods

Antibodies, proteins, peptides

Mouse monoclonal anti-TG2 antibodies (clone CUB7402) and rabbit polyclonal anti-TG2 antibodies were obtained from Thermo Fisher Scientific (Fremont, USA), mouse and rabbit normal IgG were from Santa Cruz Biotechnology (Santa Cruz, USA) and Chemicon International (Temecula, USA), respectively. Human plasma fibronectin was from Yo Poteins AB (Huddinge, Sweden). Integrin binding RGD peptide (G1269; sequence GRGDSPK) and a control peptide without effect on integrin function (S3771; sequence SDGRG), were from Sigma-Aldrich (St. Louis, USA).

Human sera, TG2 and affinity purification of antibodies

Serum samples were obtained from 27 children and adolescents with active CD (median age 8.0 years, range 1–21 years) who had intestinal villous atrophy on histological examination and elevated serum anti-TG2 IgA values (except one sera with only anti-TG2 IgG) (Teesalu et al. 2009). Two subgroups were defined based on anti-TG2 IgA level: CD1 group (anti-TG2 IgA $\geq$100 AU; $n = 13$) and CD2 group (anti-TG2 IgA <100 AU; $n = 13$). Control sera ($n = 13$, median age 13.0 years, range 1–22 years), negative for anti-TG2, were from patients with gastrointestinal complaints but with normal intestinal histology. The study was approved by Review Committee on Human Research of the University of Tartu. Full written consent was obtained from all patients and/or their parents where appropriate.

Affinity purification of anti-TG2 antibodies from 13 sera of CD patients was performed and 6 control sera were processed in the same way. Human recombinant TG2 containing C-terminal 6× His-tag was expressed in *E. coli* and purified over 90% homogeneity by two-step chromatography as described earlier (Teesalu et al. 2009). One milligram of TG2 protein was coupled to 1 ml of *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

in 2-ml centrifuge columns (Pierce Biotechnology, Rockford, USA) according to manufacturer's instructions. Human serum (0.5 ml) was diluted 1:4 in PBS, passed through 0.45 μm filter, and applied to TG2-Sepharose for 30 min at room temperature. After washing the column four times with 3 ml PBS, bound proteins were eluted with 2 ml of 0.1 M glycine, pH 2.5, and pH neutralized by adding 0.5 ml of 1 M Tris–HCl, pH 8.0. Antibody samples were dialyzed against PBS using Slide-A-Lyzer® Dialysis Cassettes (Pierce), concentrated by centrifugal ultrafiltration (iCON Concentrator; Pierce Biotechnology) up to initial volume, and stored at −20°C. The TG2-Sepharose column was regenerated with 6 M guanidinium hydrochloride after each purification cycle to remove any bound proteins. Protein concentration in antibody samples was determined by Bradford method using bovine γ-globulin as standard, and anti-TG2 (IgA, IgG) levels were assessed by Enzyme linked immunosorbent assay (ELISA) and expressed as log units per μg of protein (Log U/μg). To test possible competing serum autoantibody effect on anti-TG2 mouse monoclonal antibody (CUB 7402) binding, ELISA was performed as described earlier (Teesalu et al. 2009). TG2-coated wells were first incubated with sera diluted 1:100, washed, and then incubated with CUB 7402 at 0.1 μg/ml followed by alkaline phosphatase (AP)-conjugated rabbit anti-mouse antibodies (Dako, Glostrup, Denmark).

Western blotting

Affinity-purified antibody samples were resolved by SDS-PAGE on 10% polyacrylamide gels (0.5 μg protein per lane) and transferred to nitrocellulose membranes. Western blotting with antibodies was performed as previously described (Teesalu et al. 2001). To determine the presence of antibody isotypes in samples, immunoblots were probed with AP-conjugated goat anti-human IgA, IgG or IgM antibodies (Invitrogen Corporation, Carlsbad, USA), diluted 1:10,000. FN was detected using rabbit anti-human fibronectin antibodies (1:5,000) followed by 1:1,000 diluted AP-conjugated swine anti-rabbit immunoglobulin antibodies (Dako, Glostrup, Denmark).

Heparin/heparan sulfate binding assay

A method of protein binding to noncovalently immobilized heparin was used (Mahoney et al. 2004). Wells of BD Heparin Binding Plate (BD Biosciences, Bedford, USA) were coated either with 100 μl of 4–6 kDa heparin (Fluka 51549; Sigma-Aldrich), heparan sulfate (H9902; Sigma-Aldrich), both from porcine intestinal mucosa, or bovine hyaluronic acid (H7630; Sigma-Aldrich) at 10 μg/ml in PBS overnight at 20°C. After washing three times with assay buffer (SAB-6: 50 mM Na-acetate, 100 mM NaCl, 2 mM EDTA, 0.2% (v/v) Tween 20, pH 6.0), wells were incubated with blocking buffer (2% BSA in SAB-6) for 1 h at 37°C. Then the wells were coated with 100 μl of TG2 at 2.5 μg/ml in blocking buffer for 1 h at 37°C. For binding inhibition studies, TG2 was first incubated either with heparin (0.25–64 μg/ml), human sera (dilution 1:100) or affinity-purified anti-TG2 antibodies (20 μg/ml) in blocking buffer for 15 min at 20°C, and then applied to the wells for 1 h at 37°C. The wells were washed with SAB-6 three times, and incubated with mouse monoclonal anti-TG2 antibodies and subsequently with AP-conjugated rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark) in blocking buffer for 30 min at room temperature. After washing step, wells were covered with 100 μl of substrate 4-nitrophenyl phosphate (1 g/l) in 1 M diethanolamine, 0.5 mM $MgCl_2$ (pH 9.8) for 30 min and reaction stopped by adding 50 μl 0.1 M EDTA. The absorbances were read at 405/492 nm and the binding of TG2 in inhibition experiments was expressed as percentages of results obtained without antibodies.

Cell attachment assay

Cells of the human colon adenocarcinoma cell line Caco-2 (American Type Culture Collection, Rockville, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 2 mM L-glutamine, nonessential amino acids, penicillin/streptomycin at indicated concentrations, and 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria). Cells were maintained at 37°C in 5% CO_2, medium changed in 3 days, and cells passaged or used in experiment when reaching 80–90% confluence.

Cell attachment assay was based on previously described method (Balklava et al. 2002). In particular, 96-well cell culture plates (BD Biosciences, Bedford, USA) were coated with human fibronectin in PBS (5 μg/ml) overnight at 4°C and washed two times with PBS. Plate wells were then incubated with 10 μg/ml human TG2 in PBS for 1 h at room temperature followed by washing step and blocking with 1% BSA for 1 h. Dilutions of human sera (1:50) or affinity-purified antibodies at 20 μg/ml were applied to the wells for 1 h and then washed four times with PBS thereafter. Cultured Caco-2 cells were detached using Trypsin–EDTA and reconstituted in serum-free DMEM at 4×10^5 cells/ml, incubated for 15 min at room temperature either with RGD or DGR control peptides (20 μg/ml), and finally 100 μl of suspension was seeded on FN or FN-TG2 coated wells. Cells were allowed to attach for 90 min at 37°C and then washed gently two times with PBS. Then cells were fixed and stained with 100 μl of 0.5% crystal violet in 70% (v/v) ethanol for a few minutes followed by washing with 350 μl PBS four times and stain solubilised in 100 μl of 30% (v/v) acetic acid. The absorbance was

read at 540 nm by spectrophotometer, and results were expressed as percentage normalized to the cell attachment with the DGR control peptide on FN-TG2.

Fluorescence staining of cells

8-well chamber glass slides (Nalge Nunc International, Rochester, USA) were coated with FN in PBS (10 μg/ml) overnight and air-dried. Incubation of FN-coated surface with TG2 and treatment with series of human serum dilutions were performed as described in cell attachment assay. Detached Caco-2 cells were pretreated with RGD or DGR control peptides (50 μg/ml) in serum-free DMEM for 15 min and 1×10^4 cells were seeded onto FN or FN-TG2 coated wells. After 2 h incubation at 37°C, wells were washed two times with PBS and attached cells fixed with 3.7% paraformaldehyde solution in PBS for 15 min. Cells were permeabilized with 0.2% Triton X-100 in PBS containing 1% normal goat serum (Invitrogen, Camarillo, USA) and thereafter incubated with 1:100 diluted Alexa Fluor 594 phalloidin (Invitrogen Molecular Probes, Eugene, USA) in PBS and 1% goat serum for 1 h. After washing the slides four times with PBS, coverslips were mounted and slides examined under the fluorescence microscope Olympus IX70 (Olympus Corporation, Tokyo, Japan).

TG2 transamidation assay

Transamidation assay of FN bound TG2 was based on the method by Kiraly et al. (2006) and described in more detail elsewhere (Uibo et al. 2011). Briefly, 96-well microtiter plates were coated with human FN at 5 μg/ml and the human recombinant TG2 at 1 μg/ml for a 30 min at 20°C was added. Then wells were incubated with human affinity-purified anti-TG2 antibodies (10 μg/ml) or CUB 7402 monoclonal antibodies (5 μg/ml) for a 30 min at 20°C. After washing step wells were incubated with 0.5 mM 5-(biotinamido)-pentylamine (Pierce Biotechnology, Rockford, USA) for 15 min at 37°C in the reaction buffer (100 mM Tris–HCl pH 8.5, 5 mM $CaCl_2$, 10 mM DTT). Following incubation with AP-conjugated streptavidin (Pierce Biotechnology) the absorbance was read at 405/492 nm and the results were expressed as percentages of OD values without addition of antibodies.

Statistical analysis

Statistical analyses were performed using MedCalc software (Version 11.5.1; Mariakerke, Belgium). One-way analysis of variance (ANOVA) was used for comparison of study groups with Student–Newman–Keuls test for pairwise comparisons. Pearson's correlation coefficient was calculated where appropriate. $p < 0.05$ were considered significant. Average values ± standard deviations (SD) of parameters were presented in the text and figures.

Results

Affinity purification of anti-TG2 antibodies

Affinity purification of antibodies from CD patients' sera was performed on TG2-Sepharose column and all the eluted antibody fractions were tested for the presence of anti-TG2 IgA, IgG and IgM antibodies by quantitative ELISA. Average recovery of specific anti-TG2 IgA was 35 ± 17% as compared with that in sera and no anti-TG2 antibodies were found in control samples. Western blot analysis of affinity-purified anti-TG2 samples from CD patients and affinity column control samples from normal sera revealed the presence of nonspecific IgG and IgM as well as fibronectin among most samples tested (Fig. 1). In further studies control samples were used as antibody controls for affinity-purified anti-TG2 antibodies.

Autoantibodies from CD patients inhibit TG2 binding to heparin/HS

Concentration-dependent binding of human recombinant TG2 to immobilized heparin on heparin binding plate was

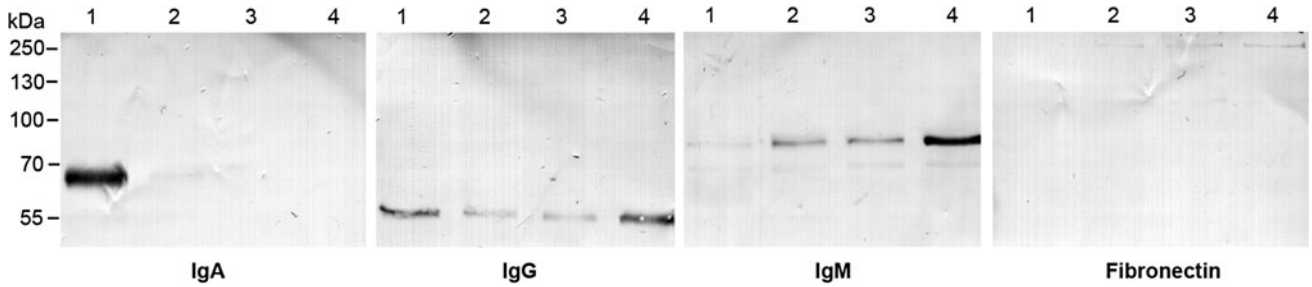

Fig. 1 Western blot analysis of affinity-purified anti-TG2 samples from sera of CD patients by using human immunoglobulin isotype-specific antibodies and anti-fibronectin antibodies. The presence of IgA class antibodies can be seen only in CD samples (*lines 1*, *2*), while nonspecific IgG and IgM as well as fibronectin can also be detected in control samples from normal sera (*lines 3*, *4*)

observed with saturation over 10 μg/ml (Fig. 2a). Less TG2 was bound to hyaluronic acid, a negatively charged glycosaminoglycan with no sulfate groups. The specific interaction between TG2 and heparin was also demonstrated by competitive inhibition of TG2 using increasing concentrations of soluble heparin (Fig. 2b). To rule out the possibility that human serum anti-TG2 antibodies could reduce mouse monoclonal anti-TG2 antibody (CUB 7402) binding by competing for the same epitope, sera were tested for ability to inhibit CUB 7402 reactivity to TG2 in ELISA. No effect of the CD and control group sera on CUB 7402 binding was found (Fig. 2c). By testing serum effect on TG2 binding to immobilized heparin, CD1 sera decreased TG2 binding (23 ± 13% of TG2 without sera) compared with CD2 or control sera (46 ± 17 and 51 ± 18%, respectively, $p < 0.001$; Fig. 3a). There was also a strong negative correlation between anti-TG2 IgA levels and TG2 binding to heparin among CD sera ($r = -0.72$, $p = 0.0003$; Fig. 3b). Similar results were obtained using HS instead of heparin in TG2-binding inhibition experiments (data not shown).

Similar to results obtained with sera, affinity-purified anti-TG2 antibodies demonstrated inhibition of TG2-binding to heparin as revealed by different TG2-binding values between CD1 samples (20 ± 8%), CD2 samples (50 ± 18%), and controls (73 ± 25%, $p < 0.001$; Fig. 3c). There was also high correlation between anti-TG2 IgA level and ability of samples to inhibit TG2 binding to heparin ($r = -0.78$, $p = 0.003$; Fig. 3d). Dose-dependent effect of selected affinity-purified antibody samples (containing IgA/IgG or IgG anti-TG2) on TG2 interactions with heparin and HS is shown in Fig. 4. Rabbit polyclonal anti-TG2 antibodies and control samples had no effect on TG2 binding to heparin/HS.

Effect of anti-TG2 autoantibodies on Caco-2 cell attachment to FN-TG2 matrix

Concentration-dependent inhibition of Caco-2 cell binding to FN-coated plastic was observed using RGD peptide in the range of 10–100 μg/ml to the level 20% of the initial values and no significant effect was observed with RGD control peptide (DGR) in the same concentration range (data not shown). Antibody effects on cell attachment to FN-TG2 were studied in the presence of RGD peptide at 20 μg/ml and normalized to the level obtained with DGR control peptide at the same concentration. Caco-2 cell attachment to FN-TG2 was less inhibited by RGD peptide compared with the FN-coated surface (Fig. 5a). Treatment of FN-TG2 with mouse monoclonal anti-TG2 antibodies (CUB 7402; 10 μg/ml) or rabbit polyclonal anti-TG2 antibodies (20 μg/ml) had no significant effect on cell attachment as compared with the normal IgG controls

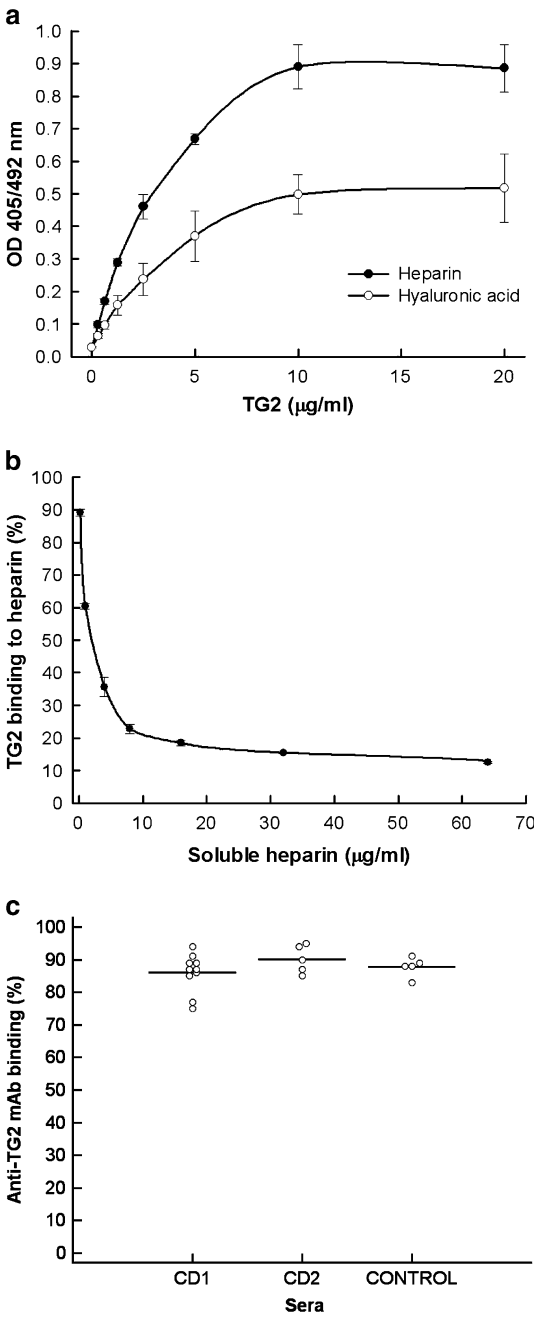

Fig. 2 Specific binding of TG2 to immobilized heparin on heparin-binding plate and specificity of anti-TG2 monoclonal antibodies (CUB 7402). **a** Concentration-dependent binding of TG2 to heparin or hyaluronic acid-coated plate. **b** Competitive inhibition of TG2 (2.5 μg/ml)-binding to immobilized heparin by increasing concentrations of soluble heparin. **c** Preincubation of TG2-coated wells with anti-TG2 containing human sera (CD1, CD2; $n = 15$) or control sera ($n = 5$) had no significant effect on mouse monoclonal anti-TG2 antibody (CUB 7402) binding in ELISA. Mean values ± SD of two experiments, both done in duplicates, are shown

(Fig. 5a). Treatment of FN-TG2 matrix with CD patients' sera resulted in lower Caco-2 cell attachment compared with the control sera both in the presence of DGR or RGD

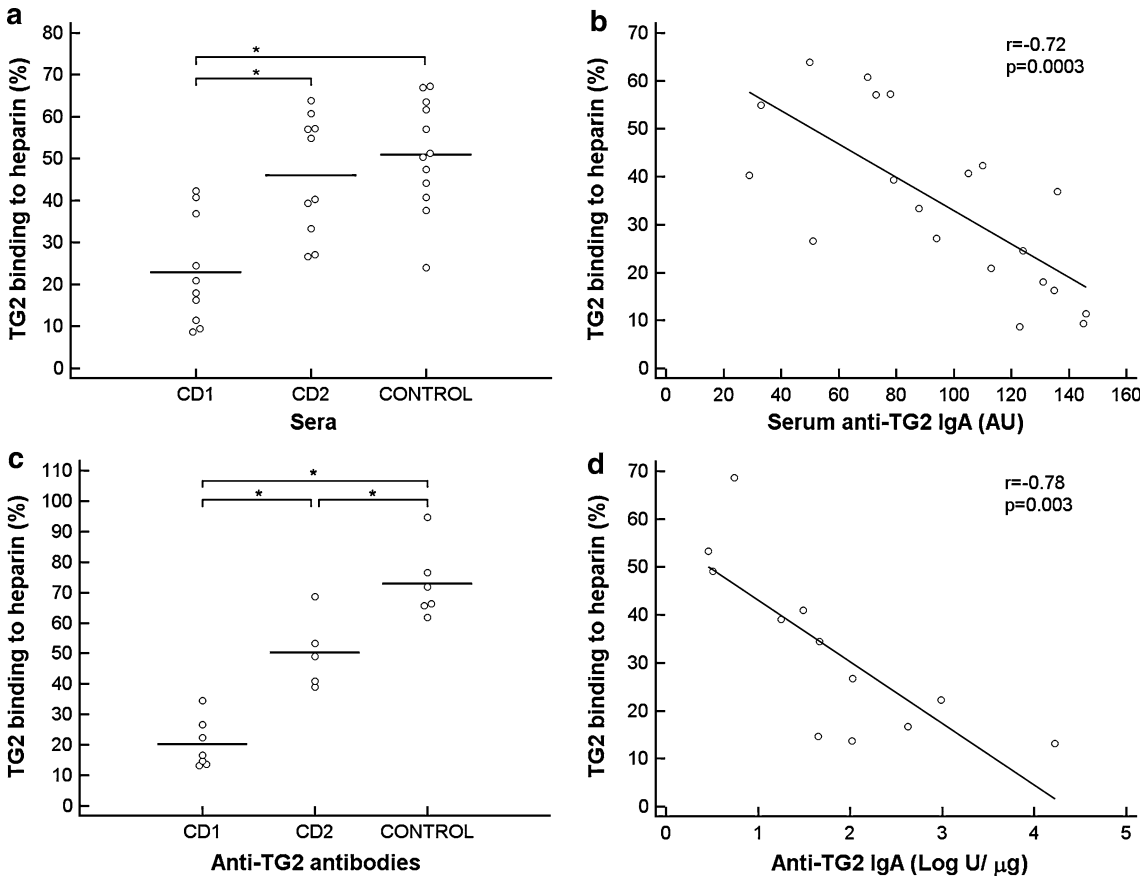

Fig. 3 The effect of human sera and affinity-purified anti-TG2 antibodies on TG2 binding to immobilized heparin. **a** Sera from CD patients with high anti-TG2 IgA levels (CD1, $n = 10$) decreased TG2 binding to heparin compared with those with low antibody levels (CD2, $n = 10$) and controls ($n = 12$). **b** Negative correlation between anti-TG2 IgA values and inhibition of TG2 binding to heparin among CD serum samples. **c** Affinity-purified anti-TG2 antibodies of CD1 ($n = 7$) and CD2 ($n = 5$) groups inhibited significantly TG2 binding to heparin as compared with controls ($n = 6$) and **(d)** inhibition was inversely correlated with anti-TG2 IgA levels (log units per μg of protein). *Dots* represent average of two separate experiments. *$p < 0.05$

peptide (101 ± 13 vs. 129 ± 14% and 66 ± 18 vs. 97 ± 14%, respectively, $p < 0.05$; Fig. 5b, d). The effect of human affinity-purified anti-TG2 antibodies was also studied either by treating FN-TG2 surface with antibodies or adding antibodies to the cell suspension in the attachment assay. As seen in Fig. 5c, incubation of FN-TG2 surface with CD1 antibody samples decreased Caco-2 attachment compared with the controls (65 ± 14 vs. 86 ± 22%, respectively, $p < 0.05$). Negative correlation existed between Caco-2 cell attachment to FN-TG2 and anti-TG IgA values in affinity-purified antibody samples from patients with CD, although statistically not significant ($r = -0.49$, $p = 0.10$). There were no difference between celiac (CD1, $n = 5$) and control samples ($n = 6$) when antibodies were added to the cell medium in the attachment assay (49 ± 6 vs. 53 ± 14%, respectively; $p = 0.31$).

To investigate whether the cell attachment reducing effect of celiac patients' anti-TG2 antibodies could be related to change in transamidation activity of TG2 bound to FN, the impact of affinity-purified antibodies on enzymatic activity of TG2 was studied. No effect of affinity-purified anti-TG2 antibodies ($n = 17$) on transamidation activity of TG2 could be seen in vitro (TG2 activity 93 ± 5% for CD1 and 94 ± 5% for controls) with no correlation between TG2 transamidation and anti-TG2 IgA levels in CD samples ($r = 0.04$, $p = 0.87$). As a positive control, mouse monoclonal CUB 7402 antibody inhibited TG2 activity down to the level of 13 ± 2%.

Discussion

The main objective of our study was to investigate whether autoantibodies against TG2, arising in patients with CD, could influence TG2 as an adhesion molecule in the context of interactions between TG2, FN, and heparin/HS. Using human sera and affinity-purified anti-TG2 antibodies we demonstrated inhibiting effect of CD patients'

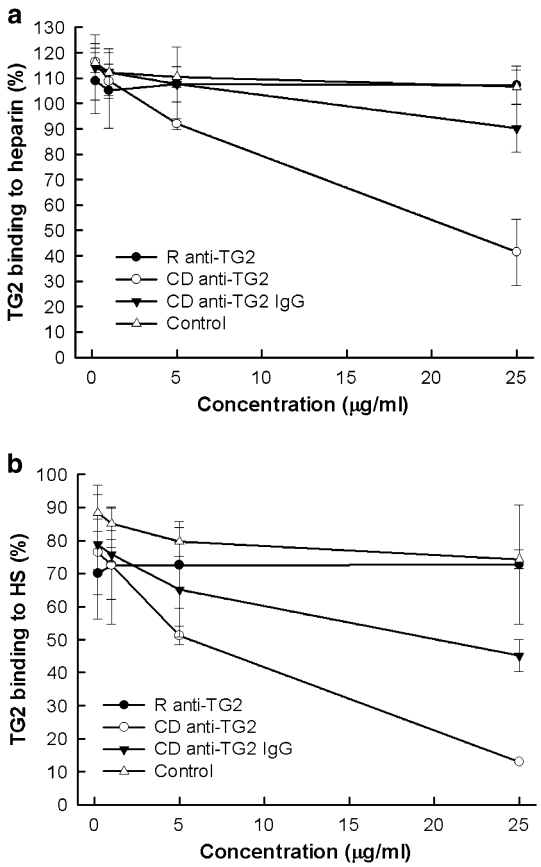

Fig. 4 Affinity-purified anti-TG2 antibodies from CD patients' sera inhibited concentration-dependently TG2 binding to heparin **(a)** and heparan sulfate **(b)**. Purified anti-TG2 antibodies from CD patient's sera with high anti-TG2 IgA/IgG (CD anti-TG2) or with anti-TG2 IgG only (CD anti-TG IgG) decreased TG2 binding to heparin and HS. Rabbit polyclonal anti-TG2 antibodies and normal serum proteins from affinity column eluate represented controls. Mean values ± SD of two experiments are shown

autoantibodies on TG2 binding to heparin/HS as well as on RGD-independent attachment of Caco-2 cells to FN-TG2 matrix.

Heparan sulfate proteoglycans (HSPG) are glycoproteins composed of core protein and one or more linear polysaccharide chains, consisting of sulphated L-iduronic/D-glucuronic acid and glucosamine units. Being involved in processes of cellular communication, HS chains of proteoglycans bind a variety of protein ligands like ECM proteins, growth factors, chemokines, and cytokines (Bishop et al. 2007). By structure, HS is very close to heparin, which is widely used as a model to study protein-HS interactions. The electrostatic interaction between negatively charged residues of heparin/HS and positively charged basic amino acids arginine and lysine in polypeptide chains forms the basis for the protein and heparin/HS binding. High-affinity binding of guinea pig TG2 to heparin/HS has been demonstrated in the study by Scarpellini et al. (2009), but there is no experimental data about heparin-binding sites in TG2. The inhibitory effect of anti-TG2 antibodies from patients with CD on TG2 binding to heparin/HS could be attributed to antibody blocking of TG2 regions responsible for interactions with HS.

The interaction of FN bound TG2 with cell surface HS residues has been related to the cell adhesion pathway which is complementary to direct integrin-mediated, RGD sequence-dependent adhesion process (Verderio et al. 2003; Telci et al. 2008). Similarly, using Caco-2 cell line as a model for intestinal epithelial cells, we found that cell adhesion to FN-TG2 matrix was less inhibited by RGD peptide than to FN alone. Syndecan-4 has been demonstrated as the candidate HSPG for binding TG2 (Scarpellini et al. 2009). Moreover, a model has been proposed in which the FN-TG2 matrix promotes cell adhesion via interactions with syndecan 4 and integrins leading to the activation of protein kinase C-α and focal adhesion kinase (Telci et al. 2008; Verderio et al. 2009). As we could demonstrate the inhibition of TG2 binding to heparin/HS by CD patients' anti-TG2 antibodies, we asked whether antibodies can affect the RGD-independent cell adhesion pathway as well. In support of this view, treatment of FN-TG2 surface with CD patients' sera or purified anti-TG2 antibodies revealed lower cell attachment than using control samples. Our results suggest that the effect seen by anti-TG2 autoantibodies from CD patients on epithelial cell attachment in the assay is related to targeting external TG2 bound to FN matrix, rather than TG2 expressed on the cell surface. Extracellular TG2 is most probably the primary target of CD patients' autoantibodies also in vivo as revealed by finding of deposits of anti-TG IgA in the small intestinal lesion of CD, colocalizing with TG2 and partly with FN network (Korponay-Szabo et al. 2004). In line with these findings, our previous study showed that using of FN as a linker molecule in anti-TG2 ELISA significantly improved the detection of anti-TG2 antibodies in sera from children with CD (Teesalu et al. 2009).

While TG2 retains its enzymatic activity when bound to FN (Kiraly et al. 2006), we tested whether anti-TG2 autoantibodies can modify transamidase activity of TG2 and thereby mediate their effect on cell attachment. No CD patients' anti-TG2 effect could be seen on transamidase activity of TG2 in our assay. Although protein cross-linking function of TG2 plays a role in cell-ECM interactions (Zemskov et al. 2006), the adhesion-promoting effect of TG2 has been shown to be independent of TG2 transamidase activity (Akimov et al. 2000; Verderio et al. 2003). Various effects of CD patients' IgA and anti-TG2 antibodies on transamidase activity of TG2 have been reported: reaching from inhibition (Esposito et al. 2002; Dieterich et al. 2003; Byrne et al. 2010) to enhancement of enzyme activity (Kiraly et al. 2006; Myrsky et al. 2009). Such variability in results can be accounted for differences in

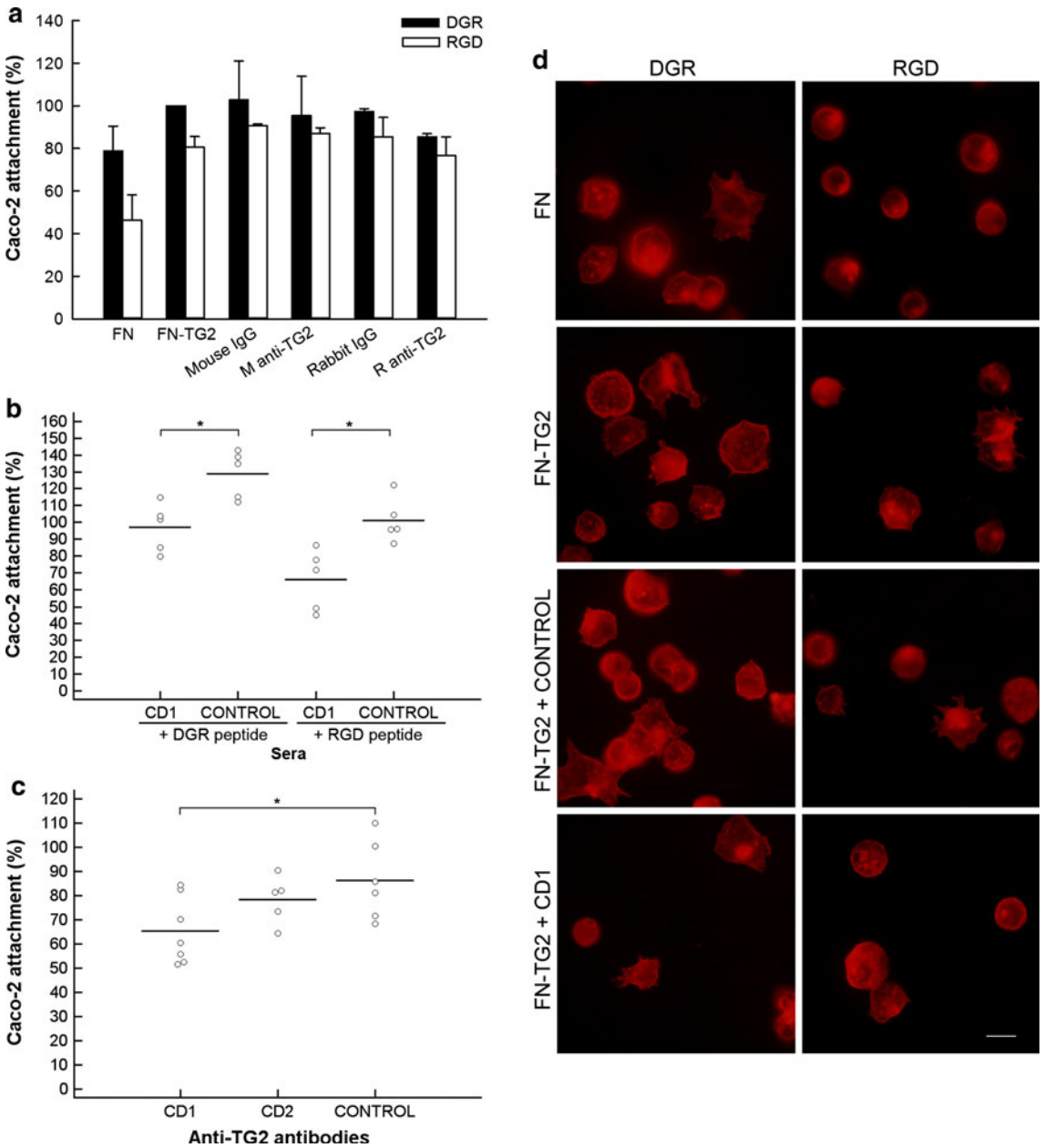

Fig. 5 Caco-2 cell attachment to FN-TG2 coated surface treated with sera or anti-TG2 antibodies from CD patients in the presence of RGD peptide. **a** Caco-2 cell attachment to FN-TG2 was less inhibited by RGD peptide at 20 μg/ml compared with only FN-coated wells in cell attachment assay. Treatment of FN-TG2 coated wells with mouse monoclonal anti-TG2 antibodies (M anti-TG2; 10 μg/ml) or rabbit polyclonal antibodies (R anti-TG2; 20 μg/ml) had no significant effect on cell attachment as compared with normal IgG controls either in the presence of DGR (control) or RGD peptide. **b** Treatment of FN-TG2 wells with CD patients' sera containing high anti-TG2 (CD1, $n = 5$) reduced Caco-2 cell attachment compared with the control sera ($n = 5$) both in the presence of DGR or RGD peptide. **c** Attachment of Caco-2 cells to FN-TG2 was lower when wells were treated with purified anti-TG2 from high-level anti-TG2 sera (CD1, $n = 7$) compared with the control samples ($n = 6$). **d** Fluorescence images of Caco-2 cell attachment to FN-TG2 coated surface treated with sera from CD patient or control, in the presence of RGD or DGR peptides (50 μg/ml). Actin filaments were stained with Alexa Fluor 594 phalloidin. *Bar*, 20 μm. Mean values of two (**a**, **b**) or three (**c**) experiments are shown. $*p < 0.05$

both antibody samples as well as in methods of antibody purification and testing of TG2 activity. In previous studies the IgA or anti-TG2 from CD patients have been shown to inhibit intestinal epithelial cell differentiation (Halttunen and Maki 1999) and endocytosis (Caputo et al. 2010), promote cell proliferation (Barone et al. 2007), disturb angiogenesis (Myrsky et al. 2008), and increase intestinal and endothelial cell permeability (Zanoni et al. 2006; Myrsky et al. 2009). These findings have been explained either by anti-TG2 antibody effects on transamidase

activity (Myrsky et al. 2009) or nonenzymatic functions of TG2 (Barone et al. 2007).

The question remains, as to what extent the proposed inhibitory mechanism of CD autoantibodies on TG2 and heparin/HS interaction and on cell attachment in vitro could have implications for immunopathogenesis and tissue remodeling of intestinal lesion in CD. It has been suggested that TG2-related cell adhesion plays a particular role for cell survival in the inflammatory environment where RGD-dependent adhesion is impaired (Verderio et al. 2003). In this context, binding of anti-TG2 antibodies to extracellular TG2 and hindering its interactions with cell HSPGs can lead to loosening of adhesion contacts between epithelial cells and basement membrane in small intestinal mucosa of CD patients. Indeed, blistering of epithelial cells in small intestinal lesion of CD patients has been previously reported (Kainulainen et al. 2002). The finding that IgG purified from CD patients' sera impaired invasiveness of trophoblastic cells (Di Simone et al. 2010) also indirectly supports the role of anti-TG2 antibodies in modifying cellular adhesion.

In conclusion, we have described the ability of autoantibodies from patients with CD to inhibit TG2 binding to heparin/HS as well as the RGD-independent intestinal epithelial cell attachment to the FN-TG2 matrix. Further studies are required in order to clarify the possible role of TG2-mediated cell adhesion pathway and anti-TG2 antibodies in the pathogenesis of celiac disease.

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