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Quantification of human tissue transglutaminase by a luminescence sandwich enzyme-linked immunosorbent assay

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ABSTRACT

Tissue transglutaminase (tTG) is a calcium-dependent enzyme that catalyzes crosslinking of peptidic glutamine residues with primary amines via isopeptide bonds and hydrolysis of ATP or GTP. The enzyme exerts a variety of functions at the cellular and tissue levels that may be disturbed in disease. Its role in pathoprocesses is poorly understood. For investigation of the involvement of tTG in disease, sensitive and specific assays should be available. We have developed the first sandwich enzyme-linked immunosorbent assay (ELISA) based on two monoclonal antibodies (mabs) against human tTG. tTG is captured by mab 3C10 and detected by biotinylated mab 10F3. After incubation with peroxidase-conjugated streptavidin, bound tTG is visualized by peroxidase reaction applying a luminescence substrate. The detection limit was 40 pg/ml. The assay was highly reproducible. Recovery of spiked tTG in crude samples was greater than 92%. The enzyme could be detected in cellular lysates and tissue homogenates of humans. The effect of typical effectors (retinoic acid and interferon-γ) on tTG expression could be demonstrated. A low signal was also obtained in mice samples, suggesting cross-reactivity of the mabs with murine tTG. The new sandwich ELISA may be successfully applied for investigation of physiological functions of tTG and of disorders associated with inadequate tTG expression.

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Transglutaminases (TGs,[1] EC 2.3.2.13) are a family of enzymes involved in calcium-dependent posttranslational modifications of proteins [1,2]. TGs catalyze the irreversible crosslinkage of peptide chains under formation of γ-glutamyl-ε-lysine isopeptide bonds between γ-carboxyamide groups of polypeptide-bound glutamines and ε-amino groups of polypeptide-bound lysine residues or polyamines [3,4]. In the absence of suitable amine substrate or under low pH, TGs catalyze deamidation of the glutamine residue [5].

Up to now, nine TGs were identified in mammals and humans: TG1 to TG7, factor XIIIA, and the enzymatically inactive erythrocyte band 4.2 [6,7]. Tissue transglutaminase (tTG), also known as type 2 TG, is the most diverse and ubiquitous member of the TG family [8]. The complete gene is composed of 13 exons and 12 introns, and it encodes a protein with four domains consisting of 687 amino acids that has a molecular weight of 78 kDa [9,10]. In addition to transamidation and deamidation activity, tTG binds and hydrolyzes GTP and ATP, leading to inhibition of the transamidation activity [11,12]. The enzyme tTG executes a variety of physiological functions (e.g., cell adhesion, migration, and proliferation) and is implicated in cell-to-matrix interaction, formation of the extracellular matrix, maintenance of barrier function in epithelia [13–15], wound healing [16], and apoptosis [17].

In celiac disease, tTG is the antigen recognized by autoantibodies [18], with autoimmunity induced by crosslinking of wheat gliadin peptides to tTG and possibly to other proteins. Deamidation of glutamine residues in gliadins converts wheat gliadin into peptides highly stimulatory for lymphocytes of celiac patients [19,20].

Besides celiac disease, there are many indications for participation of tTG in development of certain types of cancer [9,21] and in neurodegenerative disorders, including Alzheimer's, Huntington's, and Parkinson's diseases [10,22,23]. With respect to Alzheimer's disease, tTG is known to aggregate amyloid-β peptides and tau protein in vitro via crosslinking [24,25]. Furthermore, transamidation activity, messenger RNA (mRNA) levels of tTG, and isopeptide crosslinks are elevated in brains [26–28], and tTG protein is increased in cerebrospinal fluid of Alzheimer patients [29].

For a more detailed investigation of pathological processes, sensitive techniques for quantification of tTG protein should be

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[1] *Abbreviations used:* TG, transglutaminase; tTG, tissue transglutaminase; mRNA, messenger RNA; ELISA, enzyme-linked immunosorbent assay; mab, monoclonal antibody; rhu-tTG, recombinant hu-tTG; TG1, recombinant human keratinocyte transglutaminase; TG3, recombinant human epidermal transglutaminase, TG6, recombinant human neuronal transglutaminase; FXIIIA, recombinant human factor XIII subunit A; biotin–cadaverine, 5-[([*N*-(biotinoyl)amino]hexanoyl)amino]pentylamine, trifluoroacetic acid salt; HRP, horseradish peroxidase; IgG, immunoglobulin G; IFN-γ, interferon-γ; RT, room temperature; PBS, phosphate-buffered saline; RBC, red blood cell; hu-tTG, human tTG; BSA, bovine serum albumin; gp-tTG, guinea pig tTG; SD, standard deviation; TNF-α, tumor necrosis factor-α.

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available. The most frequently used methods for quantification of tTG are activity assays. Most of these activity assays are not specific for tTG but rather detect other TGs as well and have a low sensitivity due to high background [30,31]. In addition, recovery of active tTG is dependent on protein concentration in samples because most proteins are substrates for tTG [32,33].

Despite the increasing number of assays for measuring activity of tTG and of other TGs, until now reports on assays for quantification of tTG have been rare. These assays mainly used a combination of mono- and polyclonal antibodies against tTG that was not of human origin [34].

Here we describe the development of a novel highly sensitive and specific luminescence sandwich enzyme-linked immunosorbent assay (ELISA) for quantification of human tTG based on two monoclonal antibodies (mabs) raised against the human tTG. The assay can be applied for measurement in cell lysates and tissue homogenates.

Materials and methods

Chemicals

Recombinant human tTG (rhu-tTG), recombinant human keratinocyte transglutaminase (TG1), recombinant human epidermal transglutaminase (TG3), recombinant human neuronal transglutaminase (TG6), and recombinant human factor XIII subunit A (FXIIIA) were obtained from Zedira (Darmstadt, Germany). Mouse anti-guinea pig transglutaminase II mabs (TG100 and CUB7402) and 5-[([*N*-(biotinoyl)amino]hexanoyl)amino]pentylamine, trifluoroacetic acid salt (biotin–cadaverine), were obtained from CovalAb (Villeurbanne, France). EZ-Link Sulfo-NHS-Biotin, BCA Protein Assay Kit, and SuperSignal ELISA Femto Maximum Sensitivity Substrate were purchased from Thermo Scientific (Fremont, CA, USA). Biotin- and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (H+L) and HRP-conjugated anti-rabbit IgG (H+L) were purchased from Dianova (Hamburg, Germany). Goat anti-transglutaminase type II was supplied by Millipore (Schwalbach, Germany). Maxisorb immuno 96-well flat-bottom plates (clear and white) were supplied by Nunc (Roskilde, Denmark). Streptavidin/HRP and human recombinant interferon-γ (IFN-γ) were obtained from Roche (Mannheim, Germany). Polyclonal rabbit anti-human tTG (pR08), TMB Staining Kit, and tissue homogenization tubes were obtained from AJ Roboscreen (Leipzig, Germany). CrossDown buffer and retinoic acid were purchased from AppliChem (Darmstadt, Germany). Protease inhibitor cocktail was purchased from Sigma–Aldrich (St. Louis, MO, USA). Culture medium and supplements were supplied by Invitrogen/Gibco (Paisley, UK). Polyacrylamide Tris–glycine gels (SERVA*Gel*) and nitrocellulose membranes were supplied by SERVA (Heidelberg, Germany).

Cells and tissues

HepG2 and PU5-1.8 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37 °C under humidified 5% CO_2. H-358, HL-60, and THP-1 were maintained in RPMI-1640, and MeWo and HeLa cells were cultured in Eagle's minimum essential medium (EMEM) and SH-SY5Y in a 1:1 mixture of EMEM and Ham's F12 nutrient mixture supplemented as above and cultured at 37 °C under humidified 5% CO_2. Medium was refreshed after 2 days. For induction of tTG expression, SH-SY5Y cells were treated in the first experiment with 10, 25, 50, and 100 U/ml IFN-γ in culture medium for 24 h. In the second experiment, SH-SY5Y cells were precultivated for 24 h in medium supplemented with 10 μM retinoic acid, and after removing the retinoic acid-containing medium, cells were cultivated with the already mentioned IFN-γ concentrations for a further 24 h. For cell extraction, the trypsinated cells were harvested by centrifugation at 300*g* for 10 min at room temperature (RT) and washed two times in phosphate-buffered saline (PBS). The cell pellets were frozen at −80 °C. After thawing, the lysed cells were resuspended in ice-cold homogenization buffer (50 mM Tris, 150 mM NaCl, and 2 mM ethylenediaminetetraacetic acid [EDTA], pH 7.5) supplemented with a protease inhibitor cocktail, and the resulting suspension was centrifuged at 16,000*g* for 12 min at 4 °C.

Erythrocytes were separated from buffy coats of five blood donors using separation solution LSM 1077 made with Ficoll density gradient medium from PAA Laboratories (Pasching, Austria) according to the manufacturer's instructions. The red blood cell (RBC)-containing pellet was washed twice with PBS before freezing at −80 °C. Lysis of isolated RBCs was arranged as described above.

Post-mortem tissue from cerebral cortex of three human donors without neurodegenerative disease background was provided by the brain bank of the Interdisciplinary Centre for Clinical Research at the University of Leipzig. All procedures of acquisition of patients' personal data, autopsy, and handling of autopsy material were approved by the ethical committee of the University of Leipzig (no. 063/2000). Mouse liver and cerebral cortex were removed from naive BALB/c mice, which were perfused with 50 ml of PBS before removal of tissue samples. Tissues were stored at −80 °C until homogenization. Before measurement, tissue samples were thawed and washed twice in ice-cold PBS. Samples were solubilized in homogenization tubes containing ice-cold homogenization buffer in a Precellys 24 homogenizer (PeqLab, Erlangen, Germany) at 5500 rpm for 45 s. Homogenates were collected with syringes and centrifuged at 16,000*g* for 12 min and 4 °C.

Protein concentration of supernatants was determined by using a BCA Protein Assay Kit following the manufacturer's instructions. The samples were stored at −80 °C until examination.

Monoclonal antibodies

Generation and characterization of mabs 10F3 and 3C10 to human tTG were described in a previous report [35]. Isotype of mabs was determined by applying the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche).

For construction of a sandwich ELISA, the 10F3 detection mab was labeled with biotin using EZ-Link Sulfo-NHS-Biotin following the manufacturer's instructions. Efficiency of conjugation was tested in an ELISA procedure using microtiter plates coated overnight at 4–8 °C with 2 μg/ml rhu-tTG and blocked 1 h at RT with 2% Tween in PBS. The biotin-conjugated mab 10F3 was diluted from 500 to 1.9 ng/ml and incubated in washing buffer 1 (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) for 1 h at RT. Subsequently, streptavidin/HRP (1:20,000) was added and incubated for 1 h at RT. After final washing, 100 μl of a TMB staining solution was added to the wells and incubated for 10 min at RT in the dark. The reaction was stopped with 150 μl of 1 N H_2SO_4, and the absorbance was determined at 450 nm (with reference to 620 nm) using a microplate reader.

Colorimetric ELISA with mabs against hu-tTG

This ELISA applies 2 mabs against human tTG (hu-tTG). Wells of clear Maxisorb plates were coated with 100 μl of mab 3C10 (3 μg/ml) and blocked for 1 h at RT with blocking buffer 1 (3% bovine serum albumin [BSA] in washing buffer 1). rhu-tTG was diluted in sample buffer (PBS with 0.05% Tween 20 and 3% BSA, pH 7.5). Samples were incubated at 4 °C for 16 h. After washing, the wells were incubated with 100 μl of 2 μg/ml biotin-conjugated mab 10F3 in

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