Chapter

2

Validated sandwich ELISA for the quantification of tissue Transglutaminase in tissue homogenates and cell lysates of multiple species

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Abstract

Tissue Transglutaminase (tTG) is a calcium dependent enzyme that displays diverse functions in various physiological processes. In addition to these physiological functions, there is strong evidence for the implication of tTG in a number of pathologies, including celiac disease, cancer and neurodegeneration. To explore the expression and function of tTG during (patho) physiological conditions, it is of utmost importance to have an assay that specifically measures tTG protein levels in various species and matrices. Therefore, we have developed a sensitive sandwich ELISA to measure tTG protein levels in tissue homogenates and cell lysates of human, rat and mouse origin. The ELISA uses commercially available antibodies, and human recombinant tTG as the standard protein. The limit of detection is 100 pg/ml; the coefficients of intra- and inter-assay variation range from 2.4% to 6.6% and from 12.7% to 15.1%, respectively. Clear detectable levels of tTG protein were measured in human and rat liver and cerebral cortex, as well as in brain-derived neuronal and glial cells. tTG levels in mouse tissues were much lower than observed in human and rat tissues. No cross-reactivity against keratinocyte TG (TG1), epidermal TG (TG3) or blood coagulation factor XIII was observed. The tTG specific sandwich ELISA presented in this paper is a sensitive and reliable tool to accurately measure tTG protein levels in different matrices (cell/tissue) of rat, mouse and human origin. It provides a better alternative for the widely used transglutaminase activity assay with respect to sensitivity and specificity, and may serve as a valuable tool to investigate protein expression levels as part of the approach to unravel the contribution of tTG to health and disease.

Keywords: tTG, human, mouse, rat, brain, liver

Introduction

Transglutaminases (TGs; EC 2.3.2.13) are calcium dependent enzymes that catalyze the covalent crosslinking of proteins by epsilon-gamma glutamyl lysine isopeptide bonds. So far, nine distinct TG isoenzymes have been identified at the genomic level, of which six have been isolated and characterized at the protein level.
Of these, TG1, TG3 are located in e.g. epidermal cells, keratinocytes and platelets, and factor XIII in monocytes, and macrophages. Tissue Transglutaminase (tTG) is a more ubiquitously expressed transglutaminase which can be found in most mammalian tissues. Although its molecular mechanism of action has not been completely resolved, tTG plays an important role in numerous (patho)physiological processes including apoptosis, cell adhesion and migration, cell differentiation, signal transduction (GTPase activity) and stabilization of the extracellular matrix.[106] It can contribute to pathology such as promoting cell attachment, invasion and survival of cancer cells and thus play a role in the development of a metastatic phenotype of the cells.[232] Furthermore, tTG functions as an important auto-antigen in intestinal celiac disease.[90] With regard to extracellular matrix deposition, tTG promotes the assembly of fibronection by acting as a co-receptor for the $\alpha 5\beta 1$ integrin, thereby playing a role in e.g. wound healing,[9] and also promoting adhesion and migration of cells under both physiological and pathological conditions,[22] tTG has been found to play an important role in the central nervous system which is focus of our own research activities. Well defined is the contribution of tTG to the aggregation of huntingtin in Huntington's disease.[86, 401] Furthermore, recent studies indicate that tTG is implicated in the crosslinking of beta amyloid and alpha synuclein in Alzheimer's disease and Parkinson's disease, respectively.[16, 70, 182, 276] Indeed, tTG is expressed abundantly in the human brain, localizing predominantly in neurons, and tTG expression is increased in affected neurons in both Alzheimer and Huntington's disease.[175, 211] To study the expression of tTG under various (patho)physiological conditions as an initial step to unwind the specific role of tTG locally, including the central nervous system, it is of utmost importance to have a reliable method available that quantitatively measures in a sensitive way the amount of tTG in a variety of samples, ranging from cells to animal and human post-mortem material. For that purpose, the detection of tTG from multiple species is a prerequisite. In this article we describe the development and validation of a novel sandwich type of ELISA, using commercially available antibodies, for the sensitive quantification of tTG in cells and tissues of human, rat and mouse origin.

Materials and methods

Materials

Maxisorp immuno 96-well flatbottom plates, 6-well plates and 10 cm culture dishes were obtained from Nunc, Roskilde, Denmark, mouse anti guinea pig transglutaminase II Ab-2 (clone TG100) from Labvision corporation, Fremont CA, USA, goat anti-guinea pig transglutaminase type II from Upstate, Charlottesville VA, USA, biotinylated goat anti mouse IgG from Jackson ImmunoResearch Europe Ltd., Suffolk, UK and streptavidin poly-HRP from Sanquin, Amsterdam, NL, recombinant human tissue Transglutaminase (His6-rhtTG), recombinant human keratinocyte transglutaminase (His6-rhTG1), recombinant

human epidermal transglutaminase (His6-rhTG3) and coagulation factor XIII from human plasma were purchased at N-Zyme Biotec GmbH, Darmstadt, Germany.
Retinoic acid, 0-phenylenediamine (OPD), bovine serum albumin (BSA), aprotinin, pepstatin A, leupeptin and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich, St. Louis MO, USA Lipopolysaccharide (E. coli O55:B5) was from Difco Laboratories, Detroit MI, USA, and all culture media and supplements from Invitrogen/ Gibco, Paisley, UK.

Experimental materials
Human SH-SY5Y neuroblastoma cells (ATCC #CRL-2266) were maintained in a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's F12 Nutrient Mixture, containing 10% fetal bovine serum (FBS), 2.5 mM L-glutamine, 1 mM sodium pyruvate and 1:100 nonessential amino acids. The cells were plated in 10 cm culture dishes in medium supplemented with 10% FBS. After 24 hours the culture medium was removed and replaced by medium containing 3% FBS and 20 μM retinoic acid (RA). Six days after RA administration the cells were harvested and subsequently lysed.
Primary co-cultures of rat astroglial and microglial cells were prepared as described.[206] The cells were plated in 6-well plates and treated with 100 ng/ml bacterial lipopolysaccharide (LPS) for 24 hours and subsequently lysed.
Post-mortem cortical white matter of a human subject without neurodegenerative disease was obtained with informed consent and distributed through the Dutch Brain Bank. Rat liver and cerebral cortex were taken from naive Dark Agouti rats. Mouse liver and cerebral cortex were extracted from naive DBA/2J mice. All animal experiments were performed with permission of the local animal ethical committee. All tissue was stored at -80^{0}C until homogenizing.

Sample preparation
Tissue homogenates were prepared in an ice-cold buffer containing 50 mM Tris and 150 mM NaCl (pH 8.0) supplemented with 1 mM EDTA, 0.1 mM PMSF, 1 μg/ml of aprotinin, leupeptin and pepstatin, using a Heidolph Diax 900 homogenizer, followed by centrifugation at 16,000 g for 15 minutes at 4^{0}C.
Cultured cells were lysed in buffer as described above, followed by sonification (Branson sonifier, Danbury, CT, USA) and centrifugation at 16,000 g for 15 minutes at 4^{0}C. The protein content in the supernatants was measured using the BCA Protein Assay kit as described by the manufacturer's instruction (Pierce, Perbio Science, Etten-Leur, NL). Samples were stored at -80^{0}C until assayed.

Selection of tTG antibody couple
To select a tTG antibody couple that gave optimal signal-to-noise ratio in a sandwich-type of ELISA, a panel of monoclonal and polyclonal antibodies, all of which are commercially available (see Table 1), were subjected to a checkerboard titration, using recombinant human tTG as the antigen (data not shown). After selection of the goat polyclonal anti-tTG antibody from Upstate as the capture and the mouse monoclonal anti-tTG (TG100) antibody from Labvision as the detecting antibody, conditions (e.g. dilutions/ concentrations) for the ELISA were optimized in subsequent experiments.

Table 1: Antibodies used in set-up of tTG sandwich ELISA

Raised in	Type	Immunogen	Description	Company
Mouse	Monoclonal	Purified guinea pig liver tTG	Transglutaminase II Ab-2 (clone TG100) (Epitope: aa 447-538)	Labvision (Fremont, CA, USA)
Mouse	Monoclonal	Purified guinea pig liver tTG	Transglutaminase II Ab-3 (clones CUB 7402 + TG100) (Epitope resp.: aa 447-478 & aa 447-538)	Labvision (Fremont, CA, USA)
Rabbit	Polyclonal	Recombinant full length human tTG	Transglutaminase II Ab-4	Labvision (Fremont, CA, USA)
Rabbit	Polyclonal	Recombinant full length human tTG	Anti-Transglutaminase 2 (ab2972)	Abcam (Cambridge, UK)
Rabbit	Polyclonal	Peptide corresponding to aa 676-687 of human tTG	Tissue Transglutaminase (Ab-1)	Calbiochem (San Diego CA, USA)
Goat	Polyclonal	Purified guinea pig liver tTG	Anti-Transglutaminase Type II	Upstate (Charlottesville VA, USA)

To select an antibody couple that gave optimal signal-to-noise ratio in a sandwich-type of ELISA a panel of commercially available monoclonal and polyclonal antibodies were subjected to a checkerboard titration, using recombinant human tTG as the antigen. Based on this criterion the goat polyclonal from Upstate was chosen as the capture antibody, and the mouse monoclonal AB-2 from Labvision was chosen as the detecting antibody, after which the ELISA protocol was further optimized and validated.

Procedure

Microplate wells were coated for 4 hours at room temperature (RT) with 100 μl of goat anti transglutaminase type II antibody (Upstate) diluted 1:1,000 in 50 mM carbonate buffer, pH 9.6. Plates were rinsed once with wash/dilution buffer (10 mM phosphate buffer pH 7.4 containing 0.5 M NaCl and 0.1% Tween-20) and blocked for 1 hour at RT with 200 μl of 0.5% BSA in wash/dilution buffer.

To prepare an ELISA standard curve, a serial dilution of recombinant human tTG was made in a range of 0.1-20 ng/ml. 100 μl of recombinant tTG or protein samples (ranging from 0.3 μg/ml to 1 mg/ml total protein), diluted in wash/dilution buffer, was added to the wells and incubated overnight at 4^0C. After washing in wash/dilution buffer, 100 μl of 1 μg/ml mouse anti transglutaminase type II antibody (Ab-2, clone TG100, Labvision) diluted in wash/dilution buffer was added for 1 hour at RT. Subsequently, the wells were incubated with 100 μl of 1 μg/ml biotinylated goat anti mouse IgG (H+L) (Jackson) diluted in wash/dilution buffer followed by an incubation with 100 μl of 0.1 μg/ml streptavidin poly-HRP (Sanquin) diluted in wash/dilution buffer, each for 1 hour at RT. After final washes, 100 μl of a 0.5 mg/ml 0-Phenylenediamine (OPD) solution in 100 mM citric acid buffer (pH 5.0) containing 0.01% hydrogen peroxide was added as a chromogen, and incubated at RT for 15 minutes. The reaction was stopped by the addition of 100 μl of 1 N H_2SO_4 and absorbance values were determined at 490 nm using a microplate reader (SPECTRAmax 250, Molecular Devices). All incubations were performed with gentle shaking on a laboratory orbital shaker.

Specificity
Non-specific signals were evaluated by omitting the capture antibody used in the procedure. To evaluate cross-reactivity of the used tTG antibodies with other members of the transglutaminase family, serial dilutions of recombinant human TG1, recombinant human TG3 and human plasma derived coagulation factor XIII were prepared in a range of 1 to 1,000 ng/ml and subjected to the fore mentioned ELISA protocol.

Evaluation of accuracy
To assess the variation of the ELISA, three dilutions of a rat liver homogenate with a known tTG concentration were prepared. These dilutions were chosen in a way that the complete measuring range of the ELISA was covered; i.e. high, medium and low concentration.
To determine the intra-assay variation, each of the three diluted liver samples were measured in 10-fold within one ELISA. The inter-assay variation was determined by measuring each of the three diluted liver samples in 4-fold on 5 different days.
To study the recovery of tTG in the ELISA, rat and human cerebral cortical homogenates containing 5 or 10 μg/ml of protein, respectively, were spiked with 0.2, 1 or 5 ng/ml of human recombinant tTG.

Transglutaminase activity assay
The transamidation activity of transglutaminase was measured in lysates of SH-SY5Y cells, primary rat glial cells, and mouse and rat liver homogenates using the TG Covtest (Covalab, France)[323] according to the instructions of the manufacturer.

Calculations and statistical analysis
Unless otherwise stated, all standards and samples were measured in duplicate in each ELISA. Sample concentrations were calculated by regression analysis for the standard curve. Four parameter logistic curve fitting and data analysis were performed using SoftMax Pro software of Molecular Devices (Sunnyville, CA, USA).

Results

Assay range, limit of detection, and accuracy of the ELISA
A typical standard curve for human recombinant tTG is shown in Fig. 1. The limit of detection (LOD) of the ELISA, defined as mean plus 3 times the SD of the background optical density, was 100 pg/ml. Within the range of 300 pg/ml to 10 ng/ml tTG can be reliably measured. The intra- and interassay coefficients of variation (%CV) ranged from 2.4% to 6.6% and from 12.7% to 15.1%, respectively (Table 2).
Recovery experiments were performed by spiking homogenates of human and rat cerebral cortex with 0.2, 1 or 5 ng/ml of recombinant tTG. TTG levels of the spike alone (x), of the homogenate alone (y), and of the homogenate plus spike (z) were measured and compared. The mean recovery of spiked recombinant human tTG (i.e.(z-y)/x) in homogenates of human and rat cerebral cortex was 85% and 100%, respectively (Table 3).

Table 2: Intra- and inter-assay variation

	tTG (ng/ml)			
	n	mean	SD	% CV
Intra-assay variation	10	6.20	0.41	6.6
	10	1.38	0.03	2.4
	10	0.38	0.02	4.4
Inter-assay variation	5	5.12	0.66	12.7
	5	1.32	0.20	15.1
	5	0.31	0.04	12.8

The intra-assay variation of the tTG ELISA was determined by performing ten replications of three dilutions of a homogenate of rat liver. The inter-assay variation was determined by measuring four replications of each of the dilutions on five different days. CV: coëfficient of variation.

Table 3: Recovery of recombinant human tTG

	tTG in homogenate (ng/ml)	rh tTG spike (ng/ml)	tTG in homogenate + spike (ng/ml)	Spike Recovery (%)
Rat cerebral cortex (10 μg/ml)	0.33	0.21	0.52	90.5
	0.33	1.05	1.44	105.7
	0.33	5.08	5.69	105.5
Human cerebral cortex (5 μg/ml)	2.46	0.21	2.66	95.5
	2.46	1.05	3.32	81.9
	2.46	5.08	6.41	77.8

To determine the recovery of the tTG ELISA, homogenates of human and rat cerebral cortex were spiked with known amounts of rh tTG. tTG levels of the spike alone, of the homogenate minus spike, and of the homogenate plus spike were measured and compared, and subsequent recovery of the spiked tTG was determined.

Specificity

No signal above background was found when the capture antibody was omitted. A very low level of tTG could be detected when normal goat serum was used as capture antibody. This was maximal 6% of the tTG levels measured using the Upstate antibody as capture antibody.

Despite the high degree of sequence similarity of transglutaminases, the cross-reactivity of the tTG antibodies used in the ELISA with other members of the transglutaminase family (i.e. TG1, TG3 and factor XIII) was below 0.01%, as the highest concentration of TG1, TG3 and factor XIII used (i.e. 1,000 ng/ml) was not detectable in the tTG ELISA with a lower detection limit of 100 pg/ml.

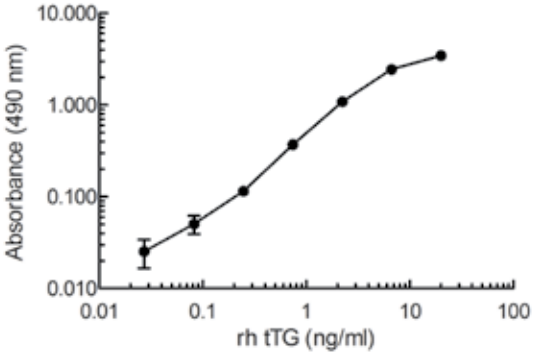

Figure 1: Standard curve of tTG ELISA using recombinant human tTG as a standard. The limit of detection (LOD) of the ELISA, defined as mean plus 3 times the standard deviation of the background optical density, was 0.1 ng/ml. Within the range of 300 pg/ml to 10 ng/ml the standard curve was linear. The results are expressed as mean ± standard deviation.

Measurement of tTG levels in samples

To assess the applicability of this ELISA to measurement of tTG levels in biological samples a number of tissue homogenates and cell lysates of rat, mouse and human origin in which (elevated) tTG levels were expected to be found were subjected to the ELISA protocol. TTG was measured in retinoic acid treated neuroblastoma cells, untreated and LPS treated primary glial cells, human cerebral cortical tissue, rat and mouse cerebral cortex and liver. Dilutional linearity of the different samples was determined and showed that, within a sample-specific range, tTG protein levels could be measured without interference of the amount of matrix components (Fig. 2). Furthermore, parallelism of the sample dilution curves to the human recombinant tTG standard curve is shown, indicating the absence of interfering sample-specific matrix components, and a prerequisite for accurate quantification. Subsequently, the amount of tTG protein in the different types of samples was determined by averaging measured tTG protein levels from 2-5 sample dilutions (Table 4).

Table 4: tTG protein levels in cell lysates and tissue homogenates; TG activity levels in cell lysates

Species	Type	tTG concentration (ng/mg protein)	TG activity (μUnits/mg protein)
Human	SH-SY5Y cells	144.85	202
Human	SH-SY5Y cells (RA treated)	774.63	6599
Human	Cerebral cortex	99.77	ND
Rat	Glial cells	0.77	75
Rat	Glial cells (LPS treated)	45.70	856
Rat	Liver	162.17	5247
Rat	Cerebral cortex	72.24	ND
Mouse	Liver	1.12	3620
Mouse	Cerebral cortex	nd	ND

Values are means of duplicate measurements of 2 to 5 dilutions from within the measuring range and corrected for the total amount of protein. nd: not detectable, ND: not done, RA: retinoic acid, LPS: lipopolysaccharide.

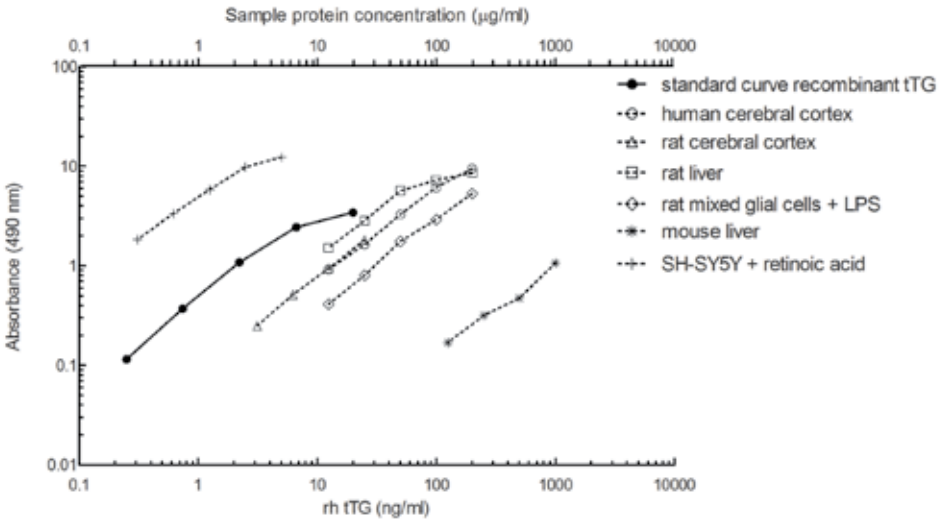

Figure 2: Comparison of standard curve of recombinant human tTG with dilution curves of various samples. Samples and standard protein were diluted in PBS (pH 7.4) containing 0.1% Tween-20.

Treatment of the human neuroblastoma cell line with retinoic acid leads to increased tTG expression and differentiation towards a neuronal phenotype.[96, 341, 377] In our experimental set-up, treatment of SH-SY5Y cells with retinoic acid for 6 days resulted in 775 ng tTG per mg of protein which is about five times more compared to untreated SH-SY5Y cells (145 ng/mg). Similarly, transglutaminase (TG) activity is dramatically enhanced in retinoic acid treated SH-SY5Y cells compared to untreated SH-SY5Y cells.

Glial cells play a prominent role during inflammatory responses in the central nervous system. Neuroinflammation includes activation of astrocytes and microglial cells, and their subsequent production of inflammatory mediators. Bacterial LPS is known to activate glial cells[206, 207] and has been used to evaluate the effect on tTG production. Treatment of co-cultures of rat astrocytes and microglial cells with LPS resulted in elevated tTG protein levels, ranging from 0.8 ng/mg in untreated glial cells to 45.7 ng/mg after LPS stimulation for 24 hours. Also TG activity is upregulated in LPS-treated glial cells compared to untreated glial cells.

tTG protein levels in homogenates of rat and human cerebral cortex were clearly detectable and found to be 72.2 and 99.8 ng/mg respectively. In contrast, the tTG protein level in mouse cerebral cortex was below the limit of detection. (i.e. below 1 ng/mg). In addition, the observed tTG level in mouse liver was significantly lower than that measured in rat liver (1.1 vs 162.2 ng/mg respectively). TG activity was clearly present in both liver samples, but was higher in rat than mouse liver.

Discussion

When trying to identify the role that tTG plays under various (patho)physiological conditions it is initially of importance to be able to reliably measure quantitative amounts of tTG protein in tissue homogenates. In the present study we describe for the first time that detectable levels of specific tTG protein can be measured accurately in liver and/or brain material from human, rat and mouse origin. In addition, tTG protein levels were greatly enhanced in retinoic acid treated neuroblastoma cells and LPS treated glial cells, and in a similar fashion as transglutaminase activity was elevated. To develop the described sandwich type of tTG ELISA, various commercially available antibodies were tested both as capture and detecting antibody to measure human recombinant tTG. One combination of antibodies, a polyclonal goat anti transglutaminase type II antibody from Upstate as capture and the monoclonal mouse anti transglutaminase type II antibody (TG100) from Labvision as detecting antibody, resulted in the best signal-to-noise ratio and a detection range between 0.3 and 10 ng/ml tTG. Based on the tTG sequences the antibodies used are directed to, the assay does not discriminate between full-length tTG and described truncated tTG isoforms.[17, 255] The validity of the assay was verified by precision (intra- and inter-assay) and recovery tests. In our hands, intra- and inter-assay variations as well as recovery of recombinant tTG in various matrices were within an acceptable range defined for immunoassays.[88] Although the transglutaminase family members display a highly conserved amino acid sequence, arranged in four known/characterized conserved domains, the ELISA shows no cross-reactivity with TG1, TG3 and factor XIII, which are three of the other most abundantly expressed transglutaminases. This is of importance to address expression and functional questions with regard to specific TG family members. In this respect, our developed tTG specific ELISA shows an elementary advantage over the TG activity assays that are widely used. These assays measure the transamidating activity of transglutaminase.[78, 145, 171, 345] With minor variations, these methods are based on the transglutaminase facilitated incorporation of [3H]-putrescine or 5-(biotinamido) pentylamine into immobilized casein or N,N'-dimethylcasein. The major drawback of these activity measurements is that they are not specific for the detection of tTG per se. As shown by our own TG activity measurements in neuronal and glial cell lysates, and liver homogenates, the change in TG activity after the *in vitro* cell treatments does not correspond directly to the change in tTG protein levels. Involvement of other potential transglutaminases (e.g. TG1 or TG3) in regulating TG activity within the cells cannot be excluded. Furthermore, the sensitivity of this type of assay is often low compared to immunological detection assays. Indeed, sensitive single or sandwich type of ELISAs to detect tTG have been described.[5, 22, 44, 105] However, these in-house assays were not validated. Moreover, neither of the ELISAs have been applied to measure tTG in tissue homogenates, nor mentioned to be suitable for the measurement of tTG in samples of species other than human. This is a prerequisite to study tTG in cellular processes or animal models of disease. In that respect, tTG protein levels in human neuronal cells that were stimulated to differentiate using retinoic acid were found to be dramatically increased. This is in accordance with observations that elevated tTG plays an important role in retinoic acid induced neuronal outgrowth.[377] Also in primary cultured mixed glial cells elevated tTG protein concentrations were detected after treatment of the cells with the

inflammatory stimulus LPS. Previous studies already indicated that the pro-inflammatory cytokines interleukin-1β and tumor necrosis factor-α increased tTG mRNA and protein in astrocytes.[255] Furthermore, tTG immunoreactivity is clearly present in microglial cells under inflammatory conditions, such as a primate model for NeuroAIDS,[316] and in multinucleated giant cells in humans suffering from HIV-associated encephalomyelitis.[266] Although the precise role of tTG during (neuro) inflammation is unclear, it could contribute to cellular adhesion and migration processes, as shown for other cell types.[9, 22, 252, 368] Additionally, we demonstrated that tTG can be accurately measured in tissue homogenates of human, rat and mouse origin. Particularly liver is known to express high levels of tTG mRNA and TG activity.[71, 114] With our ELISA we observed a rather high amount of tTG protein in rat liver, which is much lower in mouse liver. Also TG activity is lower in mouse than rat liver. This could be due to the presence of an inactive form of tTG in mouse liver as shown in tumor tissue.[193] The same phenomenon was found in rat and human versus mouse cerebral cortical tissue in which no tTG protein could be detected using the ELISA, whereas similar amounts were measured in rat and human cortex. Although we cannot exclude the possibility that mouse tTG is less well detected by our ELISA, the low expression of tTG in mouse compared to human brain and liver has been described before by others.[19] Typically, no tTG protein was observed in mouse brain using immunohistochemical and immunoblotting techniques. Moreover, tTG mRNA and transglutaminase transamidating activity was approximately 70% lower in mouse than in human forebrain. In addition, transglutaminase activity and tTG mRNA in the mouse forebrain was approximately 10% that of the mouse liver.

In conclusion, the specific tTG sandwich ELISA presented provides a sensitive and reliable tool to accurately measure tTG protein levels in different matrices (cell/tissue) of rat, mouse and human origin. The readily detectable (elevated) levels of tTG protein measured in tissue or cells support the validity of the ELISA as a valuable tool to investigate the protein expression levels as part of the approach to unravel the contribution of tTG to health and disease.

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