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Transglutaminase 1 and its regulator Tazarotene-induced gene 3 localize to neuronal tau inclusions in tauopathies

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Abstract

Alzheimer's disease (AD), progressive supranuclear palsy (PSP), frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) and Pick's disease (PiD) are commonly known as tauopathies. Neurodegeneration observed in these diseases is linked to neuronal fibrillary hyperphosphorylated tau protein inclusions. Transglutaminases (TGs) are inducible enzymes, capable of modifying conformational and/or structural properties of proteins, by inducing molecular cross-links. Both transglutaminase 1 (TG1) and transglutaminase 2 (TG2) are abundantly expressed in brain, and are associated with fibrillary hyperphosphorylated tau protein inclusions in neurons of AD, so-called neurofibrillary tangles (NFTs). However, other data obtained by our group suggested that tau pathology in the brain may be primarily related to TG1 and not to TG2 activity. To obtain more information on this issue, we set out to investigate association of TG1, TG2 and TG-catalyzed cross-links with fibrillary hyperphosphorylated tau inclusions in tauopathies other than AD, using immunohistochemistry. We found strong TG1 and TG-catalyzed cross-link staining in neuronal tau inclusions characteristic of PSP, FTDP-17 with mutations in the tau gene (FTDP-17T) and PiD brain, whereas, in contrast to AD, TG2 was only rarely observed in these inclusions. Furthermore, using a biochemical approach, we demonstrated that tau is a substrate for TG1-mediated cross-linking. Interestingly, we found colocalisation of the TG1 activator, Tazarotene-induced gene 3 (TIG3), in the neuronal tau inclusions of PSP, FTDP-17T and PiD, but not in NFTs of AD cases, indicating that these tau containing protein

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Statement of author contribution

MMM and MJ conceived and carried out experiments. JB and JGJMB carried out experiments. BD conceived experiments. AJMR and RLE analyzed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

aggregates are not identical. We conclude that TG1-catalyzed cross-linking, regulated by TIG3, might play an important role in the formation of neuronal tau inclusions in PSP, FTDP-17T and PiD brain.

Keywords

transglutaminase 1; Tazarotene-induced gene 3; transglutaminase 2; tauopathies; neurodegeneration; neurofibrillary tangles

Introduction

Alzheimer's Disease (AD), progressive supranuclear palsy (PSP), frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) and Pick's disease (PiD) are neurodegenerative diseases collectively known as tauopathies. These neurodegenerative diseases are neuropathologically characterized by the presence of intracellular fibrillary inclusions composed of hyperphosphorylated forms of the microtubule-associated protein tau [1,2], and are thought to play a central role in neuronal dysfunction and neurodegeneration [3]. In AD, tau inclusions are found in neurons, so-called neurofibrillary tangles (NFTs), which are composed of paired-helical filaments (PHF) of tau [4]. In FTDP-17 with mutations in the tau gene (FTDP-17T), the tau protein aggregates into twisted ribbon filaments, PHF and straight filaments in both neurons and glial cells, whereas tau-containing argyrophilic spherical filaments, known as Pick Bodies (PBs), in nerve cell bodies and glial cells are characteristic of PiD [5]. In PSP, both NFTs and hyperphosphorylated tau-containing glial cells are observed [5]. Unfortunately, however, mechanisms underlying tau aggregation, tau-mediated cytotoxicity and neuronal dysfunction observed in tauopathies are not well-understood.

The tau protein is a microtubule-binding protein, involved in microtubule stabilization. As a result of alternative splicing of the tau gene mRNA, multiple tau isoforms are formed [6]. These isoforms also differ by the presence of either 3 (3R) or 4 (4R) tandem repeats of 31 or 32 amino acids, located in the carboxy terminus of the protein, which constitute microtubule-binding domains [7]. For reasons not well-understood, the tau protein is hyperphosphorylated in neurons and glial cells of most tauopathies, which results in abnormal self-polymerization and aggregation of tau, leading to disturbances in microtubule dynamics [8,9]. In this manner, hyperphosphorylated tau aggregation leads to destabilization and disruption of cytoskeleton integrity and thus, cell function and viability [10]. Although the mechanisms underlying tau aggregation within brain cells remain largely unknown, besides phosphorylation, several other post-translational modifications of the tau protein, in particular glycation and transglutaminase-catalyzed cross-linking [11-14], are suggested to play an important role in the aggregation process.

The transglutaminase (TG) protein family (EC 2.3.2.13) consists of nine members, amongst which transglutaminase 1 (TG1), and transglutaminase 2 (TG2) are arguably the best characterized and known to be expressed in the human brain [15]. TG1 is most known for its essential role in the keratinocyte differentiation in the epidermis [16], whereas TG2 plays an important role in a broad range of processes underlying development, cell differentiation and

apoptosis [17]. TGs are calcium-dependent enzymes that catalyze several posttranslational modifications, including the formation of (γ -glutamyl)polyamine bonds and the formation of molecular cross-links [18]. TGs induce such cross-links by catalyzing an acyl transfer reaction between the γ -carboxamide group of a polypeptide-bound glutamine and the ϵ -amino group of a polypeptide bound lysine residue to form a covalent, highly protease-resistant, ϵ -(γ -glutamyl)lysine isopeptide bond [18,19].

Evidence is mounting that TG-catalyzed cross-linking plays an important role in the formation of cytotoxic protein aggregates that are characteristic of neurodegenerative diseases [18,20], Parkinson's disease [21,22] and AD [11]. Our group recently demonstrated association of TG2 and TG-catalyzed cross-links not only with NFTs, but also with senile plaques and cerebral amyloid angiopathy (CAA) in AD [23]. This is in line with earlier findings in human brain using primarily biochemical approaches [11,12]. In fact, both amyloid-beta, present in plaques and CAA, and tau are TG2 substrates, and TG2 affects their aggregation pathway by its cross-linking action [24,25]. Interestingly, however, in our study we also found strong association of TG1 with NFTs in AD [23]. These data suggest that not only TG2 is involved in tau aggregation and possibly NFT formation, but also TG1. This notion is supported by additional data from our group demonstrating immunoreactivity of TG1 and TG-catalyzed cross-links in tau-containing, age-related protein inclusion bodies known as corpora amylacea (CA), in which TG2 was undetectable [26]. Together, our findings hint towards a hitherto unrecognized role for TG1-mediated cross-linking in tau-related brain pathology, in particular NFTs and CA.

Besides a single report on increased levels of TG1 mRNA and protein levels in brain areas affected by tau pathology in AD [15], and our data on TG1 localization in AD and CA [23,26], no information is available about the relationship between the expression of TG1 and tau pathology in classical tauopathies. Investigation of this issue may provide more evidence on the involvement of TG1 versus TG2 in tau aggregation. For this purpose, we investigated the presence of TG1, TG2 and TG-catalyzed cross-links in both control brain and in fibrillary tau inclusions characteristic of PSP, FTDP-17T and PiD using immunohistochemistry and compared this with the staining pattern observed in AD patients. In addition, we investigated whether tau is a substrate for TG1-catalyzed cross-linking using a (biotinamido)pentylamine incorporation assay [27]. Tazarotene-induced gene 3 (TIG3) is a member of the class II tumor suppressors regulating terminal differentiation in human keratinocytes [28]. Recently, it was discovered that TIG3 binds and regulates TG1 activity [29-31]. Therefore, to investigate whether TG1's association with tau pathology forms part of a regulated pathway, we also investigated the association of TIG3 with tau and TG1 immunoreactivity in PSP, FTDP-17T, PiD and AD cases.

Materials and Methods

Brain tissue

Brain areas known to demonstrate tau pathology in each of the tauopathies was obtained by autopsy from 24 patients (14 females, 10 males). Tissue samples of the medial frontal gyrus (MFG) and substantia nigra (SN) of 3 FTDP-17T (P301L) patients (age 54.7 ± 10.3 years (mean $\pm$ standard deviation); post mortem delay 7.6 ± 3.2 hours), MFG tissue of 5 PiD

patients (age 65.6 ± 7.0 years; post mortem delay 6.6 ± 0.6 hours), cerebellum (CER, containing the nucleus dentatus) and thalamus (THA) tissue of 4 PSP patients (age 76.8 ± 5.5 years; post mortem delay 6.7 ± 1.9 hours), temporal neocortex (NEO) of 5 AD patients (age 77.4 ± 9.7 years; post mortem delay 6.8 ± 2.5 hours) and CER, MFG, SN, THA and NEO tissue of 5 control subjects without neurological disease (age 82.2 ± 2.4 years; post mortem delay 6.1 ± 2.1 hours) were rapidly dissected following autopsy and immediately frozen in liquid nitrogen (The Netherlands Brain Bank, Amsterdam, The Netherlands). AD cases demonstrated both tau and A β pathology (NFT Braak grade between 4-6 and all patients demonstrated a C grade for A β pathology). Control cases showed no pathological abnormalities (Braak A β grade 0, Braak NFT grade 0). The samples were stored at -80° until use. The diagnosis of the patients was based on a combination of neuropathological and clinical criteria [32]. Supplementary Table 1 provides an overview of the diagnosis, gender, age and post-mortem delay of the patients used in this study.

Immunohistochemistry

Experiments were performed as described previously [22,23,26]. The primary antibodies were diluted in a Tris buffered saline-Triton (TBS-T)/ bovine serum albumin (BSA) (3%) solution and incubated at 4° overnight. Primary antibodies used in this study are listed in Supplementary Table 2. The secondary antibodies (for monoclonal: Jackson biotin-labeled goat anti-mouse: dilution 1:400, for polyclonal Jackson biotin-labeled donkey anti-goat, 1:400, and Jackson biotin-labeled goat anti-rabbit, dilution 1:400) were also diluted in TBS-T/3% BSA and incubated for 2 hours at room temperature. Subsequently, the sections were incubated with avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA,) and visualized using 3,3'-diaminobenzidine (DAB). Thereafter, sections were counterstained using haematoxylin, dehydrated using ethanol followed by two steps of pure xylene. Finally, coverslips were mounted on the sections using entellan[®]. The specificity of the antibodies directed against TG1 and TG2 in human brains was demonstrated in our previous report [23]. The specificity of the anti-TG-catalyzed cross-link antibody was demonstrated by preadsorption with H-Glu(H-Lys-OH)-OH (Bachem AG, Bubendorf, Switzerland) [23,33,34]. Hyperphosphorylated tau inclusions in astrocytes were identified using a double immunofluorescence staining of the anti-GFAP antibody with the AT8 antibody. CA were identified using serial sections stained with Periodic acid-Schiff, as described previously [26,35].

Double Immunofluorescence

Sections were fixed and stained as described above. Negative controls were incubated in this solution without the primary antibodies. Secondary antibodies used were: donkey anti-goat coupled to Alexa594 (dilution 1:400, Molecular Probes, Eugene, OR, USA), donkey anti-rabbit coupled to Alexa594 (dilution 1:400, Molecular Probes), donkey anti-rabbit coupled to Alexa488 (dilution 1:400, Molecular Probes), and donkey anti-mouse coupled to Alexa488 (dilution 1:400, Molecular Probes). Fluorescence was analyzed using a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Rijswijk, The Netherlands). To exclude false positive fluorescence signals for each channel, a series of images was obtained separately in both channels through a $63 \times$ glycerin lens (zoom factor $4\times$, Z-increment $0.12 \mu\text{m}$, approximately 100 images of 1024×1024 pixels).

In vitro transamidation of 3R and 4R tau by TG1

In vitro TG1 catalyzed transamidation reactions were performed with 2.4 μ M recombinant human TG1 (Zedira Biotech.) and 2.4 μ M recombinant human tau 2N3R or human tau 2N4R (Protein purity >90%, rPeptide, Bogart, GA, USA) in buffer with or without 1 mM 5-(biotinamido)pentylamine (BAP), streptavidin coupled to agarose (Pierce, Rockford IL, USA), 50 mM Tris-Cl, 10 mM CaCl_2 and 5 mM DTT (Promega, Leiden, The Netherlands), pH 8.0, for 1 hour at room temperature, as described previously [27]. The transamidation reaction was started by addition TG1. The reaction was stopped by addition TBS-T, containing 1 mM EDTA (Sigma). Thereafter, the samples were heated for 10 min at 95°C in Laemmli sample buffer containing 10 mM DTT.

SDS-PAGE and immunoblot analysis

Protein fractions obtained from recombinant protein incubations or streptavidin precipitations were subjected to 4-12% gradient BIS/TRIS-poly acrylamide gel electroforese (Invitrogen Gibco, Paisly, UK) and transferred to polyvinylidene fluoride membranes via immunoblotting. After blocking the membranes for 2 hours with TBS-T containing 5% non-fat skimmed milk (ELK)(Campina, Woerden, The Netherlands), membranes were probed with primary antibodies directed against tau (dilution 1:100, monoclonal anti-human tau, Innogenetics, Belgium) in TBST containing 2.5% non-fat skimmed milk. Goat anti-mouse HRP-coupled antibody (DAKO, Glostrup, Denmark) was used as secondary antibody to detect the anti-tau antibody, or a streptavidin poly-HRP (dilution 1:10.000, Sanquin, Amsterdam, The Netherlands) to detect BAP incorporated into proteins. After washing the membranes three times in 10 mM Tris-Cl, 150 mM NaCl, proteins were visualized with the Supersignal West Dura extended duration substrate (Pierce) and imaged using the Chemidoc XRS imaging system (Bio-Rad, Veenendaal, The Netherlands).

Results

Presence of TG1, TG2 and TG-catalyzed cross-links in NFTs in PSP brain

The general staining pattern of TG1, TG2 and TG-catalyzed cross-links observed in the studied brain areas of PSP, FTDP-17T and PiD cases are in line with our findings described in previous reports [22,23,26]. Until now, presence of TG-catalyzed cross-links in fibrillary tau inclusions has only been observed in NFTs in PSP, a typical 4R tauopathy [36]. To determine whether TG1 and/or TG2-mediated cross-linking might be implicated in 4R fibrillary tau inclusion formation in PSP, we investigated the presence of TG1, TG2 and TG-catalyzed cross-links in both THA and CER of PSP cases. The antibody directed against fibrillary tau demonstrated the presence of NFTs and fibrillary tau inclusions in glial cells in both the THA and CER of PSP brains (not shown). Interestingly, immunohistochemical analysis of TG1 in both THA and CER of PSP cases demonstrated the presence of TG1-positive NFT-like neurons (Fig. 1A), whereas only a small minority of these structures showed TG2 immunoreactivity. Double immunofluorescence staining of the anti-TG1 antibody with the anti-tau antibody showed colocalization of TG1 with hyperphosphorylated tau in all observed pretangles, defined as PHF tau-positive neurons without fibrillary structure, and NFTs present in both THA and CER of PSP cases (Fig. 1B-D). TG2 immunoreactivity was very weak or not present at all in hyperphosphorylated tau in NFTs in

PSP brain (Fig. 1E-G). Furthermore, TG-catalyzed cross-link staining was present in all observed NFTs found in the affected brain areas of PSP cases (Fig. 1H-J). However, immunoreactivity of TG1, TG2, and TG-catalyzed cross-links was lacking in hyperphosphorylated fibrillar tau inclusions in GFAP-positive astrocytes, in both PSP and FTDP-17T (not shown). In addition, no colocalisation of TG1, TG2 and TG-catalyzed cross-links was observed with an anti-TDP-43 antibody demonstrating cytosolic TDP43-positive inclusions in the studied tauopathies (not shown).

Presence of TG1, TG2 and TG-catalyzed cross-links in fibrillary tau-containing neurons in FTDP-17T brain

In FTDP-17T (P301L mutation), alike PSP, both tangles and pre-tangles are observed [37]. To study whether TGs are associated with these tau-containing neurons, we first investigated the presence of TG1, TG2 and TG-catalyzed cross-links in fibrillary tau inclusions in both MFG and SN of FTDP-17T cases. Alike the findings in PSP cases, and in contrast to TG2, TG1 immunoreactivity was observed in fibrillary tau containing neurons in both the MFG and SN (Fig. 2A). Double immunofluorescence staining of anti-TG1 antibody with the anti-hyperphosphorylated tau antibody confirmed colocalization of TG1 with hyperphosphorylated tau in all observed fibrillary tau-containing neurons and pre-tangles present in both MFG and SN of FTDP-17T cases (Fig. 2B-D). In contrast, only weak or no TG2 immunoreactivity was detected in fibrillary tau-containing neurons present in these specific FTDP-17T cases (not shown), whereas strong TG-catalyzed cross-link staining was observed in these affected neurons (Fig. 2H-J).

Presence of TG1, TG2 and TG-catalyzed cross-links in neuronal PBs in PiD brain, and both 3R and 4R tau are substrates for TG1-mediated cross-linking

In contrast to PSP, tau inclusions in PiD are predominantly 3R tau-containing inclusions [38]. Similar to the above-described tauopathies, strong TG1-positive neurons containing PBs (demonstrated by AT8 staining) were detected in the MFG of PiD cases (Fig. 3A), whereas only faint TG2 staining was observed in these neurons (not shown). Double immunofluorescence staining of the anti-TG1 antibody with the anti-hyperphosphorylated tau antibody demonstrated presence of TG1 in all observed PBs in PiD cases (Fig. 3B-D). In contrast, weak or no TG2 staining was detected in PBs in the MFG of PiD cases (not shown). Immunoreactivity of the anti-TG-catalyzed cross-link antibody colocalized with PB-containing neurons in MFG of PiD brain (Fig. 3H-J).

Our above-described data suggest that in fibrillary tau inclusions found in tauopathies, tau might be a substrate for TG1-mediated cross-linking. NFTs in AD consist of a mixture of 3R and 4R tau [39], in contrast to the above-described tauopathies [38,39]. We therefore investigated whether both 3R and/or 4R tau are substrates for TG1. Co-incubation of TG1 with either 3R (Fig. 3K) or 4R tau (Fig. 3L) for 1 hour at room temperature resulted in formation of tau multimers (first lane of Fig 3K and L). Co-incubation of TG1 with 3R tau or 4R tau, in combination with an amine donor (BAP) to detect TG-mediated covalent transamidation [27], demonstrated that both 3R tau (Fig. 3K) and 4R tau (Fig. 3L) are substrates of TG1-mediated cross-linking. Incubation of BAP with tau in the absence of TG1 had no effect on tau multimerization, and was not incorporated in tau multimers (Fig

3K, L). Together, these data demonstrate that both 3R and 4R tau are substrates for TG1-mediated cross-linking, and that this interaction leads to tau aggregation.

TIG3 is present in neurons showing tau inclusions in PSP, FTD-17 and PiD cases

TIG3 has recently been identified to bind to TG1, and act as a prime regulator of TG1 action [40]. In order to study whether TG1's presence and possible activation in fibrillary tau-containing cells might form part of a tightly regulated pathway, we analyzed the presence of TIG3 in cells showing tau inclusions in PSP, FTDP-17T and PiD cases. Alike TG1 immunoreactivity, TIG3 staining demonstrated NFTs-like neurons in THA and CER of PSP cases (Fig. 4A). Moreover, double immunofluorescence staining of anti-hyperphosphorylated tau antibody and the anti-TIG3 antibody in PSP cases demonstrated the presence of TIG3 in all observed NFTs and pretangles (Fig. 4B-D). Colocalization of TIG3 with fibrillary tau inclusions in neurons was also observed in FTDP-17T (Fig. 4E-G) and PBs in PiD (Fig. 4H-J). In addition, colocalisation of TIG3 and TG1 was observed in NFTs in PSP (Fig. 4K-M), in PBs in PiD and in fibrillary tau-containing neurons in FTDP-17T (not shown). However, TIG3 antibody immunoreactivity was not observed in tau inclusions in GFAP-positive glial cells, or in TDP-43-positive inclusions (not shown).

TIG3 is present in CA, but absent from NFTs in AD brain

In a previous study of our group, we described the presence of TG1 and TG-catalyzed cross-links in CA in both control and AD cases [26]. In contrast, TG2 immunoreactivity was not observed in these CA [26]. The above-described data suggest that TG1 activation in neuronal fibrillary tau inclusions in tauopathies might be regulated by TIG3. In order to investigate whether TG1-TIG3 association is a more regular phenomenon in TG1-related tau pathology, we studied the presence of TIG3 immunoreactivity in CA in control and PSP cases, and in NFTs in AD. In control (Fig. 5A-C), PSP and AD cases (not shown), TIG3 immunoreactivity was found in all observed CA. Interestingly, however, TIG3 staining was not observed in NFTs in the neocortex of AD cases (Fig. 5D-F).

Discussion

Here we describe the presence of TG1 and its regulator TIG3 in neurons showing fibrillary tau inclusions in PSP, FTDP-17 and PiD cases. In contrast, these neurons demonstrated only faint or no TG2 immunoreactivity in the studied tauopathies. Together with our observation that tau is a substrate for TG1 cross-linking, these data strongly implicate TG1, but not TG2, activity in formation of cross-links in neuronal fibrillary tau inclusions in PSP, PiD and FTDP-17T. In addition, a specific role for TG1 activation in neuronal fibrillary tau formation and stabilisation (see beneath) in PSP, FTDP-17T and PiD is suggested by the absence of TIG3 from NFTs in AD, in which TG2 has been shown to be abundantly present by us and others [11,20,23]. A similar role may be played by TG1 in formation of CA [26], as TIG3 was present in these age and neurodegeneration-related spherical bodies containing polymerized proteins, including tau [26].

Until recently, cross-linking activity observed in aggregated protein inclusions found in the brain during normal aging and in neurodegenerative diseases was almost exclusively linked

to TG2. However, it has been known for some time that, apart from TG2, TG1 is also expressed in the brain [15]. Furthermore, elevated TG1 mRNA levels were observed in AD brain, although the cellular source of this elevation was unclear [15]. Recently, our group demonstrated that in addition to TG2, TG1 is present in NFTs in AD, and that in CA, formed in the normally aging brain but also in AD and PD brain, only TG1 but not TG2 was found [23,26]. Together with the current findings, these data emphasize the importance of TG1 in the formation of protein inclusions in the human brain. However, considering the limitations of the use of human brain material, although only faint or no TG2 staining was found in NFTs of PSP, PiD and FTDP-17T, we cannot exclude a contribution of TG2 to formation of tau inclusions in these tauopathies.

In line with a previous report [36], but in contrast to neurons, we found no colocalisation of TGs or their cross-linking activity with tau inclusions in glial cells in the studied tauopathies. These observations suggest that although both pathologically altered neurons and glial cells contain a mixture of straight filaments and paired helical filament-like structures containing tau protein [41], the processes underlying their formation, the properties of the aggregated tau and/or the cellular response to these tau inclusions differ between cell types. Clearly neuronal survival is more affected by tau pathology than glial survival [42]. Our data implied the activity of TG's, in particular TG1, in this discrepancy between both cell types. First, TG cross-linked tau filaments are more resistant to degradation or proteolytic digestion [12,20], and in this way contribute to elevated cytotoxicity in neurons. Second, intracellular activation of TG activity under cell stress conditions might also result in cytotoxicity by other means, since TG activity is directly associated with regulating cell death and survival [43]. Therefore, TG activity in neurons, associated with tau inclusions, might determine cell fate. This hypothesis remains subject for further studies. Although AD, PSP, PiD and FTDP-17 are all collectively known as tauopathies, the predominant form of tau protein accumulating in the NFTs of these diseases differs. In tau-inclusions of PiD the predominant tau form accumulating is 3R tau, whereas in PSP the 4R tau is the predominant form [38]. In AD, NFTs are composed of a mixture of 3R and 4R tau [39]. In neuronal tau inclusions of PSP, PiD and FTDP-17T, strong TG1 staining was observed, whereas TG2 staining was absent or at the most very faint. In contrast, in NFTs in AD both a strong TG1 and TG2 staining was observed [23]. This suggests that when NFTs are composed of predominantly one tau isoform, TG1 is the most prominently expressed TG, a notion that is strengthened by our observation that the TG1 regulator TIG3 is present in NFTs in PSP, PiD and FTDP-17T, whereas it is not detected in NFTs in AD brain. In an as yet to be defined manner, this may be related to the fact that NFTs in AD can be considered as a secondary pathological event that is preceded by the formation of toxic amyloid-beta species and the deposition of amyloid-beta aggregates in the form of senile plaques [44]. This is in contrast to the fibrillary tau inclusion formation in PSP, PiD and FTDP-17T, in which tau aggregation is the prime pathological lesion.

Although TG1 is observed in NFTs of AD [23], PSP, PiD and FTDP-17T (this study), and in CA [26], it was unknown whether tau is a substrate for TG1-catalyzed cross-linking. So far, it had only been demonstrated that both tau and hyperphosphorylated tau are substrates for TG2 [24], and that TG2 can transform tau into straight helical filaments [11]. Moreover, purified helical filaments from AD and PSP tissue contain TG cross-linked tau protein, and

cross-linking of tau filaments occurs before the presence of immunohistochemically detectable NFTs [11,12,36]. These data indicate that TG-catalyzed cross-linking plays an early role in the tau aggregation pathway. Our results show for the first time that both 3R and 4R tau are substrates for TG1, and that TG1 is indeed capable of cross-linking tau leading to formation of SDS-stable tau polymers. In addition, we observed that both TG1 and its activator TIG3 are present in pretangles, supporting an upstream contribution of TG1 activity in neuronal tau inclusion formation. Although further studies are required to elucidate the effect of TG1-catalyzed cross-linking on tau aggregation and stabilization, our data underscore the notion that TG1 might be the prime TG family member responsible for the formation of TG-catalyzed cross-links found in tau inclusions in classic tauopathies and in CA.

TIG3 is a member of the class II tumor suppressors, initially identified in human keratinocytes where it plays a key role in regulating terminal differentiation [28]. Recently, it was discovered that TIG3 binds and regulates TG1 activity [29-31], and that TIG3 overexpression markedly induces TG1 activity, thereby stimulating keratinocyte death [31]. This is in line with our data, showing that in both pretangles and mature neuronal fibrillary tau inclusions in PSP, FTDP-17T and PiD immunoreactivity of TG1 and TG-catalyzed cross-links colocalizes with TIG3. These data suggest that TIG3 is upregulated and activates TG1 in early stages of fibrillary tau formation, and in this way may be linked to neuronal cell death. A similar mechanism might account for the presence of TG1 and TIG3 in CA, which are stress-related inclusions bodies allegedly formed as a remnant of dead brain cells [45]. As TIG3, perhaps via regulation of TG1 activation, plays a crucial role in determining cell survival and death [40], identification of the mechanisms regulating its expression and function in the brain may offer new targets to counteract neuronal death in classic tauopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Immunoreactivity of TG1, TG2 and TG-catalyzed cross-link antibodies in thalamus of PSP cases. The anti-TG1 antibody demonstrated TG1 immunoreactivity in NFT-like neurons in the thalamus of PSP cases (A, arrow). The anti-tau (AT8) antibody stained NFTs (C, arrow). Double immunofluorescence staining of an anti-tau antibody with an anti-TG1, anti-TG2 or anti-TG-catalyzed cross-link antibody was performed. TG1 staining was present in NFTs (B-D, arrow). In contrast, only weak TG2 immunoreactivity was observed in NFTs (E-G, arrows). TG-catalyzed cross-link immunoreactivity was found in NFTs (H-J, arrow).

Original magnification: A-J $\times$ 400. Abbreviations: TG1 = transglutaminase 1; TG2 = transglutaminase 2; AT8 = anti-hyperphosphorylated tau antibody; cross-links = TG-catalyzed cross-links.

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Figure 2. Immunoreactivity of TG1, TG2 and TG-catalyzed cross-link antibodies in FTDP-17T cases. The anti-TG1 antibody demonstrated fibrillary tau containing neurons in the medial frontal gyrus of FTDP-17T cases (A, arrow). The AT8 antibody stained fibrillary tau containing neurons (C, arrow). Double immunofluorescence staining of an anti-tau antibody with an anti-TG1, anti-TG2 or anti-TG-catalyzed cross-link antibody was performed. TG1 staining was present in neurons showing fibrillary tau inclusions (B-D, arrow). TG-catalyzed cross-link immunoreactivity colocalized with fibrillary tau containing inclusions in neurons (E-G,

arrow). Original magnification: A-G $\times$ 400. Abbreviations: TG1 = transglutaminase 1; AT8 = anti-hyperphosphorylated tau antibody; cross-links = TG-catalyzed cross-links

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Figure 3.

Immunoreactivity of TG1, TG2 and TG-catalyzed cross-link antibodies in PiD cases, and cross-linking of both 3R and 4R tau by TG1. The anti-TG1 antibody demonstrated TG1 immunoreactivity in Pick Bodies (PB)-containing neurons in the medial frontal gyrus of PiD cases (A, arrow). The AT8 antibody stained PBs (C, arrow). Double immunofluorescence staining of an anti-tau antibody with an anti-TG1, anti-TG2 or anti-TG-catalyzed cross-link antibody was performed. TG1 staining was present in PBs (B-D, arrow). Strong TG-catalyzed cross-link immunoreactivity was found in PBs (E-G, arrow). Both 3R tau (H) or

4R tau (I) demonstrate multimerization when co-incubated with TG1 at room temperature for 1 hour, according to the methods described in the materials and methods section. Co-incubation of 3R (H) and 4R (I) tau with TG1, in the presence of BAP (as described in the materials and methods section), demonstrated incorporation of BAP into both low and high molecular weight tau multimers. In contrast, no BAP incorporation was observed in both 3R tau (H) and 4R tau (I) in the absence of TG1. Experiment was repeated 3 times with similar results. Typical experiment is shown. Original magnification: A-G $\times$ 400. Abbreviations: TG1 = transglutaminase 1; AT8 = anti-hyperphosphorylated tau antibody; cross-links = TG-catalyzed cross-links; Av/str = avidine/streptavidine.

Figure 4.

TIG3 staining in PSP, FTDP-17T and PiD cases. The anti-TIG3 immunoreactivity demonstrated NFT-like neurons in the substantia nigra (SN) of PSP cases (A, arrow). The AT8 antibody stained pretangles (B, arrow) and fibrillary tau containing neurons (E, H, arrow). Double immunofluorescence staining of the AT8 antibody with the anti-TIG3 antibody was performed in SN of PSP cases and the medial frontal gyrus of PiD and FTDP-17T cases. TIG3 was found present in pretangles of PSP cases (B-D, arrow), in fibrillary tau containing neurons in FTDP-17T cases (E-G, arrow), and in PBs in PiD cases

(H-J, arrow). Double immunofluorescence staining of the anti-TG1 antibody with the anti-TIG3 antibody was performed in SN of PSP cases. Colocalisation of TG1 staining with TIG3 staining in NFTs in SN of PSP cases was observed (K-M, arrow). Original magnification: A-M $\times$ 400. Abbreviations: TIG3 = Tazarotene-induced gene 3; PSP = progressive supranuclear palsy; TG1 = transglutaminase 1; PiD = Pick's Disease; FTDP-17T = frontotemporal dementia and parkinsonism linked to chromosome 17; AT8 = anti-hyperphosphorylated tau antibody.

Figure 5.

Immunoreactivity of the TIG3 antibody in corpora amylacea (CA) in control brain, and its absence in NFTs in AD brain. The anti-TG-catalyzed cross-link antibody stained CA in neocortex of control cases (A, arrow). Double immunofluorescence staining of the anti-TG-catalyzed cross-linking antibody with anti-TIG3 antibody showed colocalization in CA (A-C, arrow). The AT8 antibody stained NFTs in the neocortex of AD cases (D, arrow). Double immunofluorescence staining of the AT8 antibody with the TIG3 antibody demonstrated no colocalization of TIG3 with hyperphosphorylated tau in NFTs of AD cases (D-F, arrow). Original magnification: A-F $\times 400$. Abbreviations: TIG3 = Tazarotene-induced gene 3; AT8 = anti-hyperphosphorylated tau antibody; cross-links = TG-catalyzed cross-links, AD = Alzheimer's disease.