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Review Article

Tissue Transglutaminase, Protein Cross-linking and Alzheimer's Disease: Review and Views

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Abstract: Extensive protein cross-linking and aggregation are some of the most common molecular events in the pathogenesis of Alzheimer's disease (AD). Both β-amyloid (Aβ) plaques and neurofibrillary tangles, which are extracellular and intracellular proteinaceous aggregates, respectively, contribute to neuronal death and progressive cognitive decline. Although protein cross-linking has been recognized and extensively studied for many years, the underlying mechanisms are largely unknown. Recent data indicates that tissue transglutaminase (tTG), which catalyzes the cross-linking of a wide spectrum of proteins including Aβ, tau, α-synuclein and neurofilament proteins, may be involved in protein aggregation in AD. Many AD risk factors, such as trauma, inflammation, ischemia and stress, up-regulate tTG protein and activity levels. In this review, we summarize the evidence that tTG plays a role in AD, especially in cross-linking of Aβ, tau, α-synuclein and neurofilament proteins. An experimentally testable hypothesis is that tTG may play a central role in AD pathogenesis and that it provides a conceptual link between sporadic and familial AD through a shared pathogenic pathway.
Key Words: Tissue transglutaminase (tTG, TG2), Alzheimer's disease, β-amyloid (Aβ), tau, α-synuclein, neurofilament proteins, protein cross-linking.

Introduction

Alzheimer's disease (AD) affects millions of people worldwide with, unfortunately, ever increasing incidence. Currently there is no cure for this devastating disease, and even symptomatic relief remains modestly effective. Underlying the behavioral and cognitive decline of AD is the progressive neuronal dysfunction and ultimately cell death by processes that are not fully understood.

Grossly, the brain of AD usually shows atrophy with reduced volume and weight due to extensive loss of neurons in the neocortex. Histologically, the most remarkable and consistent morphological features are the neuritic senile plaques and neurofibrillary tangles (NFTs) [1]. The major proteinaceous component of the plaques is the extensively cross-linked β-amyloid (Aβ) with non-amyloid components comprising the core of the plaques [2, 3]. Mature NFTs are composed of aggregates of hyperphosphorylated tau [4, 5] and many other proteins, such as ubiquitin [6-8] and neurofilaments [9-11]. The mechanism underlying the extensive protein cross-linking in AD is still unknown, but tissue transglutaminase (tTG) has been implicated in this process [12, 13]. In this review, we will focus on the potential biological significance of tTG in the pathogenesis of AD.

Tissue Transglutaminase

Tissue transglutaminase (also known as TG2, EC 2.3.2.13) is a member of the Ca^{++}-dependent transglutaminase (TG) family that catalyzes protein cross-linking [12, 14, 15] (**Figure 1**). The γ-glutamyl-ε-lysine isopeptide bond formed by the action of these enzymes produces highly insoluble protein complexes that are extremely stable, showing resistance to 2% SDS and 8M urea or enzymatic degradation [14, 16]. These protein scaffolds may stabilize the structural integrity of the dying cells before their clearance by phagocytosis, thus preventing the nonspecific

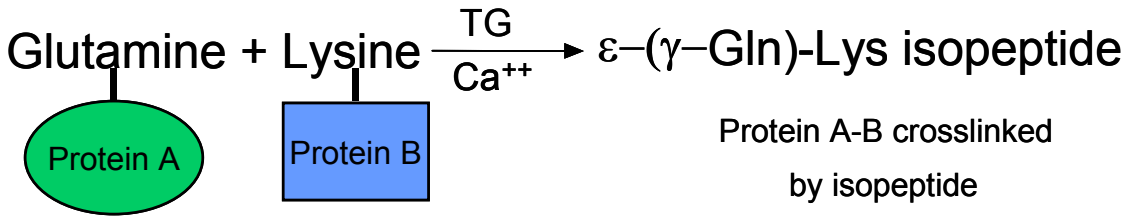

Figure 1 Simplified scheme of tTG-catalyzed isopeptide formation between glutamine and lysin in a calcium-dependent manner. Glutamyl residue in one protein molecule serves as acyl donor or amine acceptor, and lysyl residue in another protein serves acyl acceptor or amine donor. With calcium, tTG catalyzes a covalent cross-linking between the proteins by forming γ-glutamyl-ε-lysine isopeptide bond. Modified from Greenberg CS *et al* [12].

release of harmful intracellular components such as lysosomal enzymes, nucleic acid, and the resulting inflammatory responses.

Nine different TGs have been identified in mammals and human [17-19] including TG C [20, 21], K [22], E [23], P [24], X [18], factor XIII [14, 17] and Band 4.2 protein [25, 26]. These enzymes are subject to various post-translational modifications such as phosphorylation, fatty acylation and proteolytic cleavage which regulate the activity and subcellular distribution of the enzyme under different biological conditions [27, 28]. The tTG gene encodes a monomeric protein composed of 685-691 amino acids in human and other vertebrates [29-33] with a calculated molecular weight of about 80 kDa, although a shorter form of tTG might also exist [34]. The human tTG gene has been mapped to chromosome 20 and includes 13 exons and 12 introns [35, 36]. General features of members of the TG family and detailed biochemistry of tTG have been summarized in several recent reviews [37, 38].

The x-ray crystal structure of human tTG complexed with GDP at 2.8-Å resolution showed that the monomer has four distinct domains that are quite similar to Factor XIII [39-41]. These include an N-terminal β-sandwich domain, a transamidation catalytic core, and two C-terminal barrels (**Figure 2**). These features suggest a structural basis for the negative regulation of transamidation activity by the bound nucleotide, and positive regulation of transamidation by Ca^{++} [41]. With truncated tTG-GST fusion protein, it was found that the N-terminal β-sandwich domain and the catalytic domain are required for tTG enzymatic activity, while the C-terminal barrels are not [42].

Tissue TG is particularly interesting due to its wide spread expression in many tissues including brain. It is expressed in both central and peripheral nervous systems [43-47]. In brains, tTG is localized mostly in the cytoplasmic compartment of neurons [43, 48, 49], although it can also be found in nuclei and extracellular matrix [19]. Growing data suggests that tTG is involved not only in some physiological processes such as differentiation and apoptosis but also in multiple pathological processes such as wound healing and neurodegenerative diseases by producing protein conjugates [50-58]. Among all members of the TG family, tTG is one of the most extensively studied and has been implicated in multiple human diseases including AD [59].

Many AD Risk Factors Induce Expression of tTG

Since the majority of cases of AD are sporadic without a clear genetic cause, and an even a large percentage of familial cases cannot be explained by the overproduction of Aβ, multiple factors, especially environmental factors are likely involved in the pathogenesis of AD. In fact, traumatic brain injury [60, 61], aging [62-64], inflammation [65, 66], ischemic damage (infarcts and ischemia) [67-71] and brain stress [72-75] have all be shown to increase the risk of AD. Many of them overly induce tTG expression and/or activity.

Tissue TG is Increased in Brain after Trauma

For many years, traumatic brain injury (TBI) has been associated with enhanced AD risk [76-78]. Epidemiological evidence and retrospective clinical studies implicated TBI as a common preceding event prior to AD [79,

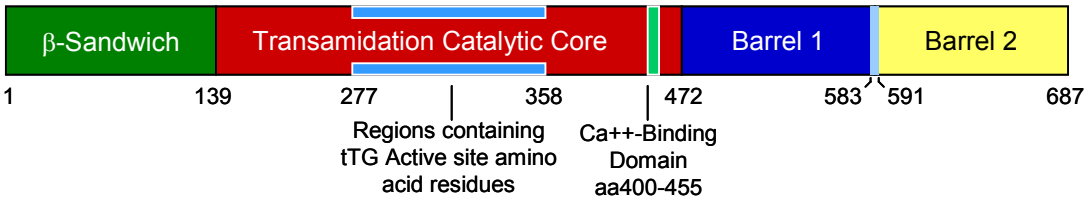

Figure 2 Schematic representation of the structural domains of transglutaminase, amino acid residue distribution region of the catalytic core and Ca^{++}-binding domain. The scheme was drawn based on the data from Liu S. *et al* [42] with reference to [37, 38].

80], especially in those without ApoE4, a known genetic risk factor for AD [81-83]. Dementia pugilistica (DP) is a progressive memory disorder that occurs after repeated head trauma in professional boxers. It is characterized by NFTs that are composed of hyperphosphorylated tau protein indistinguishable from NFTs in AD brains. Animal studies have shown that TBI induces cognitive impairment [84-86] and at an ultrastructural level increases deposition of Aβ [87]. Abnormal tau proteins isolated from DP brains were indistinguishable from the six abnormally phosphorylated brain tau isoforms in AD brains [88]. These data supports the notion that TBI increases susceptibility to AD [89, 90]. There are a variety of other similarities between TBI and AD including highly aggregated Aβ that is typically resistant to proteolytic degradation [91]. These aggregated Aβ species found in AD [92] can also be found in various TBI animal models [87, 93, 94]. In a study on human subjects, significantly more Aβ immunoreactive neurons were observed after head injury than controls [95]. The levels of Aβ was increased in the cerebrospinal fluid of patients after severe brain injury and remained elevated for some time after the initial event [78]. Extensively aggregated and phosphorylated tau is detected in rat brain after traumatic injury. In this model, normal-looking neurons in the telencephalon and brainstem were immunoreactive for phosphorylated tau six months after injury. Cortical neuronal counts gradually decreased, with up to 42% decrease at 6 months after injury [94]. These data suggests that recurrent TBI may cause DP through pathological mechanisms similar to those seen in AD. A single TBI may increase susceptibility to sporadic AD decades after the event.

Tissue TG is usually up-regulated in injured tissues, which suggests that it plays a role in wound repair [96-98]. In model of spinal cord ischemia, overall TG activity increased transiently and then declined to control levels after one week [99]. After injury of superior cervical ganglion or vagus nerve, TG activity was also increased [100, 101]. Recent studies have shown that both tTG mRNA and protein are up-regulated after TBI [102]. While increased tTG synthesis and activation under such circumstances is part of the normal protective cellular response contributing to tissue homeostasis by stabilization of the extracellular matrix and cellular integrity, pathologic protein cross-linking may also occur as seen in AD.

Tissue TG is Up-Regulated in Brain with Ischemia, Inflammation and Other Cell Stresses

In addition to TBI, other AD risk factors such as ischemia, inflammation and cell stress [64, 65, 68, 72, 73, 103-105] induce tTG expression or activity. Focal brain infarct elicits inflammation in the lesion and the surrounding brain tissues with a rapid up-regulation of pro-inflammatory cytokines such as TN F-α and IL-1β [106]. Both TNF-α and IL-1β can induce tTG expression in cultured cells [107]. After global cerebral ischemia in gerbils, tTG activity was followed by incorporation of [^{3}H]-putrescine into dimethyl-casein throughout the 48 hours of reperfusion following a 3 minute occlusion. In experimental animals, significant increases were found in the ischemic hippocampus at 24 hours of reperfusion, while minor changes were observed in the cortex. Both RT-PCR and western-blot demonstrated a substantial up-regulation of tTG in the ischemic hippocampus, suggesting that tTG is part of the tissue stress response after global brain ischemia/reperfusion [108]. Increased expression of tTG at both mRNA and protein levels was also seen following middle cerebral

artery occlusion in rats [109]. Tissue TG mRNA level peaked on day 5 after injury in the ipsilateral cortex. However, in the ipsilateral hippocampus, tTG induction peaked 1 day after injury and to a lesser extent than observed in the ipsilateral cortex. Western blot analysis demonstrated that tTG protein expression progressively increased from day 1 to day 7 after ischemia, with greater expression in cortex than hippocampus. These results demonstrate that tTG mRNA and protein expression increases significantly after ischemic injury. The temporal profile of tTG induction after ischemia was similar to that observed in TBI animal model [102], suggesting a similar role of tTG in both pathological conditions [109].

Tissue TG can also be induced by cerebral inflammation [106] and brain stress induced by glutamate excitotoxicity, calcium influx, oxidative stress, inflammatory cytokines and UV exposure [110]. These data suggests that Aβ can also induce tTG expression, possibly through effects on cellular redox status or calcium flux. There are indications that activated tTG redistributed to the plasma membrane [110]. At this location, tTG may play an active role in excitotoxic neuronal cell death, a likely component of acute central nerve system (CNS) injury and chronic CNS neurodegenerative disease [111, 112].

Tissue TG Catalyzes the Cross-Linking of Critical Proteins of AD Pathology

The most characteristic pathological structures of AD pathology are senile plaques and NFTs [1] (**Figure 3A** and **B**). The major components in senile plaques are Aβ1-40 and Aβ1-42. Some senile plaques have a condensed core that contains truncated α-synuclein fragments [2, 113]. Small amounts of neurofilaments can also be found in plaques [114]. The dominant component of NFTs is the hyperphosphorylated tau, a microtubule binding protein [115-117]. Recently, α-synuclein had also been found in NFTs [118-120]. So far, all of those major components found in senile plaques and NFTs have been shown to be substrates of tTG.

The first suggestion that tTG may play a role in AD was made by Selkoe and colleagues [121] when they showed tTG can covalently cross-link neurofilament proteins into insoluble polymers *in vitro* by forming γ-glutamyl-ε-lysine intermolecular bridges. Later studies indicated that tTG can catalyze cross-linking of Aβ [122], amyloid precursor protein (APP) [123-127], tau [128-135] and α-synuclein [134, 136, 137] in addition to neurofilament proteins.

Tissue TG Cross-Links Aβ and APP

Several years after a potential link between tTG and AD was suggested [121], Ikura and colleagues reported that tTG could cross-link synthetic Aβ1-28 *in vitro* exclusively through Lys16 [123]. This finding was quickly extended to the Aβ1-42 [124] and APP [138] by independent groups. Using the incorporation of site-specific probes followed by enzymatic digestion and sequencing of tracer-containing fractions, Lys16, Lys28 and Gln15 in Aβ were all susceptible to cross-linking by tTG [139]. Aβ cross-linking catalyzed by tTG could be inhibited by specific inhibitors (e.g., dansyl-cadaverine and spermine) and non-steroidal anti-inflammatory drugs (e.g., indomethacin, meclofenamic acid, diflunisal and salicylic acid) [140]. Immunochemical demonstration of tTG in amyloid plaques in AD brains suggests a role in plaque formation by cross-linking Aβ or other components [141]. The *in vivo* data demonstrating a direct link between tTG and cross-linking of Aβ are still missing.

Tissue TG Cross-Links tau

Tau protein is an excellent substrate of TG and tTG both *in vitro* and *in vivo* [126]. Dudek and colleagues showed that in the presence of TG tau formed macromolecular complexes that were insoluble in ionic detergent, β-mercaptoethanol, guanidine-HCl and urea. Furthermore, they demonstrated that the filamentous tau aggregates had increased immunoreactivity to the monoclonal antibody Alz-50 [126]. To determine which domains of tau were modified by tTG, [^{3}H]-putrescine-labeled tau was digested with chymotrypsin. Mass spectrometric analysis demonstrated that tau was modified at only one or a few discrete sites, primarily in the carboxyl half of the molecule. Thus, cross-linking is selective for only a subset of the many glutamine residues in tau. Furthermore, a tau deletion construct (T264) containing a portion of the microtubule binding domain, which is normally a substrate of TG, cannot be cross-linked. This provides evidence that the cross-linking may be conformation-dependent [142].

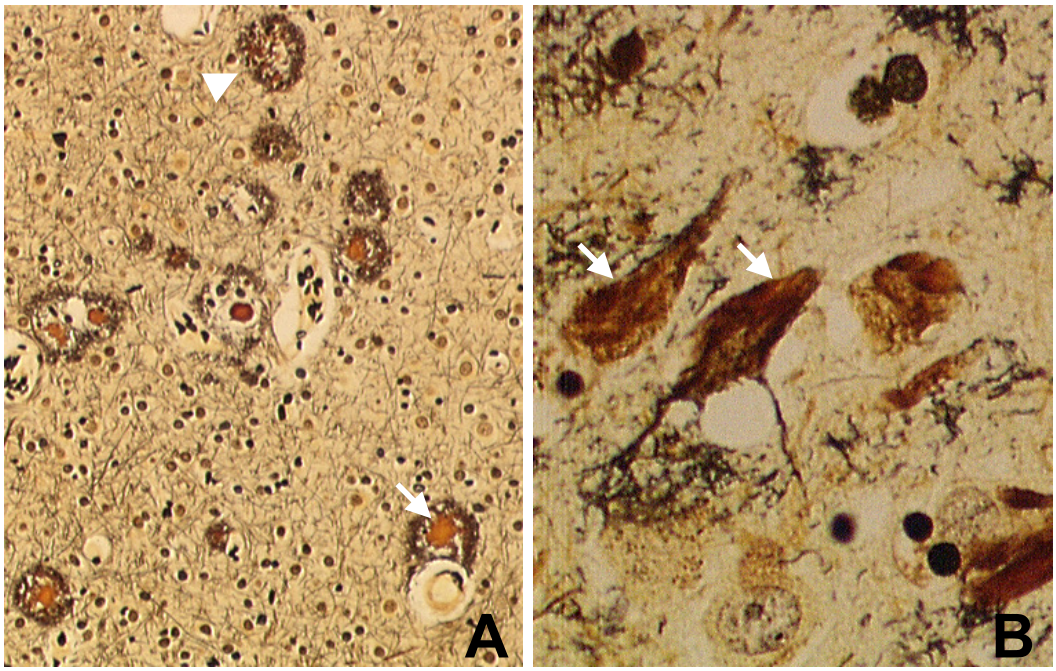

Figure 3 Characteristic structures of AD brain. (**A**) Senile plaque (arrowhead) and senile plaque with a condensed core (arrow) and (**B**) neurofibrillary tangles (arrows) (silver staining x 400).

Similar observations were reported by another group who used purified tTG from guinea pig liver to cross-link recombinant human tau protein [143]. Cross-linking site analysis of human tau (tau23 and tau40) showed that eight glutamines can function as amine acceptor residues, with two major sites at Gln351 and Gln424. In addition, 10 lysine residues were identified as amine donors, most of which are clustered adjacent to the microtubule binding repeats of tau in regions known to be solvent accessible in filamentous tau [144]. When over-expressed in cultured SH-SY5Y cells, tTG was co-immunoprecipitated with tau [145]. Recently, tau protein cross-linking catalyzed by TG was further confirmed in P301L tau transgenic mice [135]. Studies on human specimens indicate that tTG may be involved in cross-linking of tau pathology seen in AD brains. A study performed on frozen prefrontal cortex of 9 AD and 9 age- and postmortem interval-matched controls showed that total TG activity was significantly higher in AD compared to controls. Tissue TG protein levels determined by quantitative immuno-blotting were elevated approximately 3-fold in AD compared to controls. Interestingly, there were no significant differences in TG activity or tTG protein levels in the cerebellum from the same panel of samples between control and AD cases [146]. Furthermore, the level of isopeptide bonds, the catalytic product of tTG, was increased in AD brains compared to controls [147, 148]. In one study, a statistically significant (45%) elevation in ε-(γ-glutamyl)-lysine cross-links was found in AD when compared to control cortex [147]. Using single- and double-label immunofluorescence confocal microscopy and immunoaffinity purification and immunoblotting, another study found isopeptide bonds in NFTs and paired helical filament tau early in AD. The number of neurons that are immunoreactive with the antibody against ε-(γ-glutamyl)-lysine bonds was significantly higher in AD cortex compared with age-matched controls and schizophrenics [148].

TG activity, including tTG, may also play a role in NFTs seen in progressive supranuclear palsy (PSP) brains [149]. We double-stained AD brains with anti-tau and anti-isopeptide antibodies which showed that tau and isopeptide co-localized in many, although not all, tangle-bearing neurons (**Figure 4A**, Wang DS *et al*: unpublished data). Some amyloid plaques also showed colocalization of isopeptide and Aβ, but the intensities of isopeptide immunostaining were relatively weaker (**Figure 4B**). This may reflect further protein degradation in plaques, epitope masking or partially stronger immunostaining

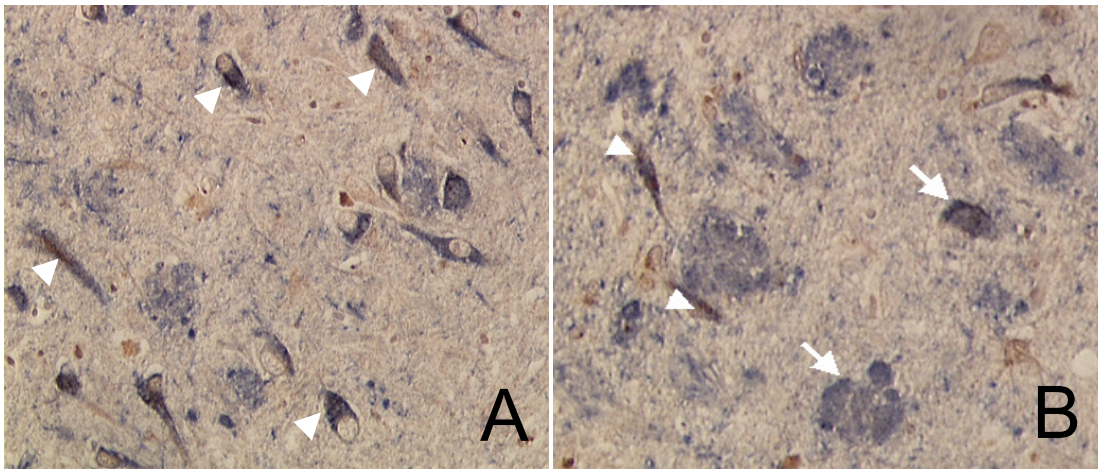

Figure 4 A. Isopeptides and tau protein co-localized in neurofibrillary tangles. The section was stained with mouse monoclonal anti-isopeptide antibody manually first with HRP-DAB. Then the section was treated with DAKO double staining kit followed by CP13 antibody with AP-NACP for color-development. Brown is isopeptide and blue is tau. Arrows indicate tangle-bearing neurons stained by both anti-isopeptide and anti-tau antibodies. **B**. Isopeptide and Aβ double-staining show colocalization of both proteins in the plaques (arrows) and NFTs (arrow head). The intensities of isopeptide in plaques are relatively weaker that are potentially due to further protein degradation in this type of lesions during the relative lengthy morphogenesis compared to structures like NFT. Magnification: 200 x.

of very abundant Aβ. Together, the available data suggests that TG, especially tTG, could be a contributing factor in NFT formation.

Tissue TG Cross-Links α-Synuclein

Alpha-Synuclein, an intracellular neuronal protein and a synaptic marker, is also a common substrate of tTG. A 35-residue peptide derived from α-synuclein is a major, non-Aβ component (NAC) of plaques [3, 150, 151]. It is usually localized to the condensed core of the amyloid plaques [152]. TG catalyzes the formation of covalently linked NAC polymers *in vitro* as well as polymers with Aβ. The tTG-reactive amino acid residues in NAC are Gln79 and Lys80. Lys80 is localized in a consensus motif Lys-Thr-Lys-Glu-Gly-Val, which is conserved in the synuclein gene family [134]. Purified tTG catalyzed α-synuclein cross-linking, leading to the formation of high molecular weight aggregates *in vitro*. Over-expression of tTG resulted in formation of detergent-insoluble α-synuclein aggregates in cellular models. Immunohistochemical studies on postmortem brain tissue confirmed the presence of TG-catalyzed ε-(γ-glutamyl)-lysine isopeptide in the halo of Lewy bodies in Parkinson's disease and dementia with Lewy bodies, co-localizing with α-synuclein [136]. Furthermore, both tTG protein and isopeptide coimmunoprecipitated with α-synuclein in extracts of PD substantia nigra. The isopeptide was detected in both α-synuclein monomer and its higher molecular weight oligomers, indicating this modification was early in Lewy body formation [137]. Interestingly, we and others found α-synuclein and tau co-exist in many NFTs or Lewy body-like structures, usually with an α-synuclein core surrounded by tau-positive periphery [119, 120, 153]. This indicates α-synuclein cross-linking and aggregation may be an initial event which precedes tau aggregation in the morpho-genesis of Lewy bodies and NFTs [120].

Tissue TG and Isopeptide are Increased in AD Compared to the Age-Matched Controls

Recently we showed that tTG and tTG activity are elevated in AD brains compared to controls [37, 48, 154]. As discussed above, iso-peptides have been found in plaques [141] and tangles [148]. Our recent study showed that levels of tTG, tTG activity and isopeptide immunoreactivity in brain homogenates correlate inversely with neuropsychological test scores reflective of overall cognitive function (Wang DS *et al*: manuscript in preparation). In these brains, tTG and tTG activity were increased compared to age-matched normal controls. Isopeptide levels showed a more robust inverse correlation with clinical cognitive measures than tTG or tTG

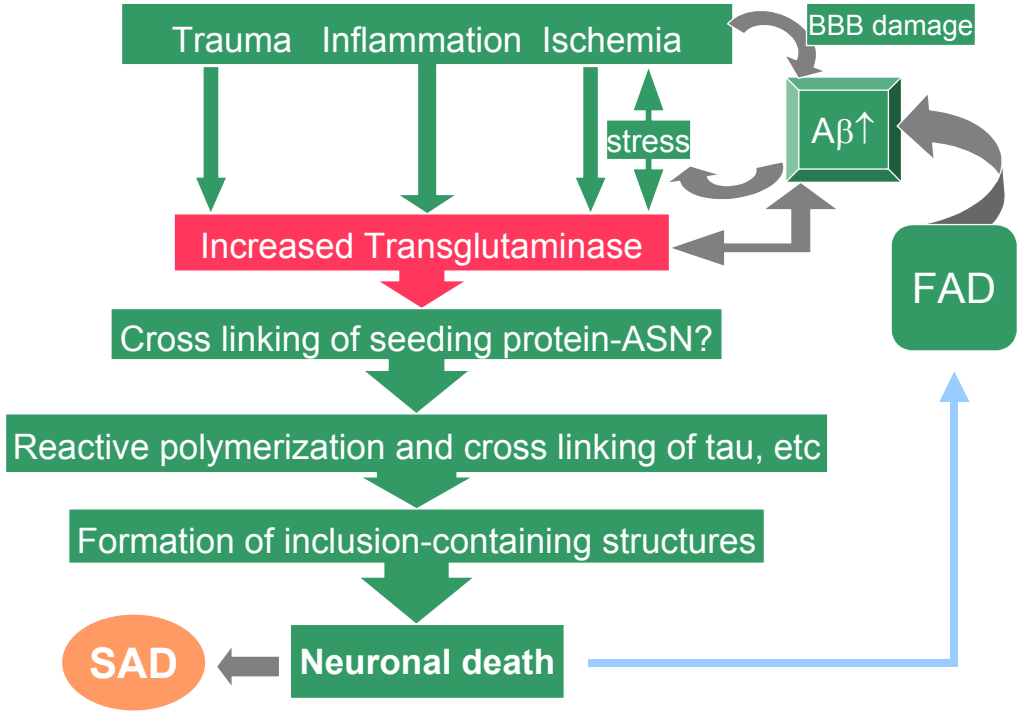

Figure 5 Hypothetical mechanisms for the role of tTG in AD pathogenesis. Increased brain tTG induced by multiple factors such as trauma, inflammation and ischemic injury will cross-link protein like α-synuclein (ASN), Aβ and tau. In sporadic AD, increased Aβ due to trauma, inflammation and ischemia will further increase tTG levels. Aggregated Aβ may serve as a long-term chronic stimulant for the tTG and keep the pathogenic process going even after the initial factors no longer present. In familial AD (FAD), excessive production Aβ may be sufficient to increase tTG and initiate AD pathogenesis, with or without additional factors seen in sporadic AD. FAD: familial Alzheimer's disease; SAD: sporadic Alzheimer's disease.

activity. This suggests that although tTG and its activity are increased during AD pathogenesis, this increase may be limited or may reach a steady-state level, but that isopeptide immunoreactivity, the product of tTG activity, may continue to increase and accumulate during the disease process. The results suggest that accumulation of cross-linked protein gradually results in neuronal dysfunction and cognitive decline.

Although insoluble and 70% formic acid-extractable isopeptide correlated with both neuropathological and neuropsycho-logical data, total isopeptide levels in crude homogenate only showed significant correlation with some neuropsychological, but not neuropathologic measures (Wang DS *et al*: unpublished data). It is likely that formic acid-extractable isopeptides are derived from insoluble end-stage structures such as neuritic plaques and NFTs. Thus, one would expect a robust correlation with neuropathological measures (e.g., plaque and tangles counts) as well as clinical cognitive data. On the other hand, total isopeptide immunoreactivity includes isopeptides from other proteins that may not be present in insoluble lesions. It is tempting to speculate that these soluble isopeptide-containing proteins may contribute to neuronal dysfunction independent of plaques and tangles.

Hypothetical Role of tTG in AD Pathogenesis

Based on the above studies, we propose a hypothetical mechanism for the role of tTG in AD pathogenesis (**Figure 5**). Increased brain tTG induced by environmental factors such as brain trauma, inflammation and ischemic injury will lead to cross-linked proteins, such as α-synuclein [119, 120, 155], Aβ [124, 125] and tau [147, 148]. Increased production of Aβ due to trauma, inflammation and ischemia in sporadic AD or overproduction of Aβ in familial AD due to mutations in presenilin or APP will increase the stress in brain and further up-regulate tTG, causing a feed-forward

response. Aggregated Aβ may serve as a long-term chronic stimulant for tTG and perpetuate the pathogenic process [157]. During this chronic process, neuronal cells are gradually lost, which leads to progressive cognitive decline. Reversal or attenuation of this protein cross-linking and aggregation may help slow cognitive decline and neurodegeneration in AD. Future research is needed to establish the sequence of events after initiating factors are no longer present. In familial AD, the stress due to the excessive production of Aβ alone may be sufficient to increase brain tTG and initiate AD pathogenesis, with or without additional factors needed to initiate the pathogenic cascade in sporadic AD.

Summary

Extensive protein cross-linking and aggregation involving a variety of proteins are commonly occurring molecular processes during the pathogenesis of AD [156]. The initiating factors are likely to be environmental insults (e.g., trauma, inflammation or ischemic damage) that lead to increased tTG activity and increased cross-linking for tau, Aβ and other molecules, which leads to functional impairment, structural lesions characteristic of AD (e.g., plaques and tangles) and eventually neuronal death. If the relationship between increased tTG and deleterious cross-linking of proteins such as α-synuclein, tau and Aβ are critical to AD pathogenesis, therapeutic measures should be developed to manipulate tTG protein and activity levels.

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