Chapter 2

Tissue transglutaminase colocalises with extracellular matrix proteins in cerebral amyloid angiopathy

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Abstract

Cerebral amyloid angiopathy (CAA) is a key histopathological hallmark of Alzheimer’s disease (AD) and hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D). CAA is characterised by amyloid-beta (Aβ) depositions and remodelling of the extracellular matrix (ECM) in brain vessels and plays an important role in the development and progression of both AD and HCHWA-D. Tissue transglutaminase (tTG) modulates the ECM by molecular cross-linking of ECM proteins. Here, we investigated the distribution pattern, cellular source and activity of tTG in CAA in control, AD and HCHWA-D cases. We observed increased tTG immunoreactivity and colocalisation with Aβ in the vessel wall in early stage CAA, whereas in later CAA stages, tTG and its cross-links were present in halos enclosing the Aβ deposition. In CAA, tTG and its cross-links at the abluminal side of the vessel were demonstrated to be either of astrocytic origin in parenchymal vessels, of fibroblastic origin in leptomeningeal vessels, and of endothelial origin at the luminal side of the deposited Aβ. Furthermore, the ECM proteins fibronectin and laminin colocalised with the tTG-positive halos surrounding the deposited Aβ in CAA. However, we observed that in situ tTG activity was present throughout the vessel wall in late stage CAA. Together, our data suggest that tTG and its activity might play a differential role in the development and progression of CAA, possibly evolving from direct modulation of Aβ aggregation to cross-linking of ECM proteins resulting in ECM restructuring.

Keywords: Alzheimer’s disease, HCHWA-D, cerebral amyloid angiopathy, tissue transglutaminase, extracellular matrix proteins

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterised by deposition of amyloid-beta (Aβ) peptide aggregates in the brain. In the parenchyma, Aβ aggregates in the form of senile plaques (SPs), whereas Aβ deposition in the vasculature results in cerebral amyloid angiopathy (CAA) [1]. The Aβ protein is a proteolytic cleavage product of the amyloid precursor protein (APP) and is produced in two major forms (Aβ1-40 and Aβ1-42), both capable of interacting with themselves which results in toxic Aβ aggregates [2]. SPs mainly consist of Aβ1-42, whereas Aβ1-40 is the major component of CAA [3]. CAA is present in more than 90% of all AD patients [4] and is also the major pathological hallmark in patients with hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D). HCHWA-D is characterized by the E22Q mutation in the APP gene that results in a highly toxic form of Aβ (i.e. D-Aβ40) which accumulates preferentially in brain vessel walls and leads to severe cerebral haemorrhaging [5]. Importantly, the presence of CAA does correlate with cognitive decline of CAA-affected patients [3, 6]. In general, two types of CAA have been identified: type I CAA includes capillary Aβ depositions in addition to CAA in other cortical and leptomeningeal vessels, whereas in type II CAA, capillary Aβ depositions are not present [7]. CAA is characterized by degeneration of smooth muscle cells, disruption of the basement membrane and remodelling of extracellular matrix (ECM) [3, 8, 9]. Together, these alterations lead to weakening of the vessel wall, impaired vascular functioning and, ultimately, haemorrhages [3, 9]. Despite the strong connection between cognitive decline, reduced functioning of brain vasculature and CAA, the factors and mechanisms underlying accumulation and deposition of Aβ in the vessel wall remain largely unknown.

Tissue transglutaminase (tTG) is a member of the transglutaminase family (TGs, EC 2.3.2.13) and is ubiquitously expressed in the human brain [10, 11]. tTG is a calcium-dependent enzyme involved in covalent posttranslational modifications of proteins such as amine incorporation and molecular cross-linking [12]. The latter is formed by the formation of a γ-glutamyl-ε-lysine bond between a glutamine residue and a lysine residue of a peptide chain, which can be either intra- or intermolecular [12–14]. This cross-linking activity provides tTG with the powerful capacity to stably alter conformation of proteins and induce formation of protein complexes [12]. An important biological role of this cross-linking activity is formation of ECM protein complexes, which results in remodelling of the ECM in response to e.g. cell stress and tissue injury [14–16]. Evidence is mounting that tTG and its transamidation activity play an important role in AD pathogenesis [17]. In AD brain, tTG levels and its cross-links are elevated [18–20] and correlate with the cognitive decline observed in these patients [20, 21]. Furthermore, tTG binds to Aβ, and tTG-mediated cross-linking modulates the Aβ aggregation pathway [22–25]. In fact, a recent study demonstrated that tTG activity is able to induce formation of neurotoxic and protease...
resistant Aβ complexes at low, i.e. nanomolar, concentrations of Aβ [26]. We recently demonstrated for the first time that not only tTG, but also tTG-mediated cross-links are associated with the pathological hallmarks of AD [11]. Interestingly, in this study we found that tTG and its cross-links are associated with both SPs and CAA; however, the distribution pattern of tTG and its activity in both lesions appeared different [11]. In SPs, immunoreactivity of both tTG and its cross-links colocalised with the deposited Aβ, in contrast to CAA where no spatial overlay was observed. Instead, in CAA, tTG and its cross-links were present as a luminal and abluminal halo which enclosed the deposited Aβ in middle-sized parenchymal vessels [11]. Remarkably, the tTG-immunoactive halos that surround the deposited Aβ, resembled that of earlier immunohistochemical stainings of major ECM proteins (i.e. fibronectin [FN] and laminin [LN]), and other known tTG substrates reported by others [27–29]. These results suggest a role for tTG-mediated ECM remodelling in CAA which might be one of the unknown factors that affect Aβ deposition in the vessel wall and CAA development [30].

In order to gain more insight into the role of tTG and its transamidation activity in CAA, we studied the distribution pattern and cellular source of tTG and its cross-links in different brain vessels affected by CAA by use of immunofluorescence on snap-frozen brain tissue of AD, HCHWA-D and control cases. In addition, we investigated colocalisation of tTG and its cross-links with ECM proteins, in particular the tTG substrates fibronectin and laminin [14, 31], in both AD and HCHWA-D brains. Finally, we also investigated the distribution pattern and overlay of in situ TG activity with CAA in control, AD and HCHWA-D cases.

Materials and methods

Brain tissue

Human neocortex tissue samples from 5 AD patients with CAA, including two patients with capillary CAA (capCAA) (age 83.8 ± 11.8 years; post-mortem interval 8.0 ± 2.2 hr) and 7 non-demented control subjects without neurological disease including two controls with CAA (age 81.4 ± 4.7 years; post-mortem interval 6.6 ± 1.5 hr) were obtained from The Netherlands Brain Bank (Amsterdam, The Netherlands). Brain tissue of the neocortex from 5 HCHWA-D patients was obtained (age 55.2 ± 4.0 years; post-mortem interval 4.4 ± 1.1 hr) from Dr. S van Duinen (Department of Pathology, Leiden University Medical Centre, Leiden, The Netherlands). After autopsy, samples were immediately frozen in liquid nitrogen. Table 1 shows an overview of patient details including gender, age, post-mortem interval, diagnosis, cause of death, Braak grading (both for neurofibrillary tangles and SPs) and CAA score. The diagnosis of AD was based on neuropathological and clinical criteria [32, 33]. CAA scoring was performed as described previously [11]: the number of CAA-affected vessels of at least 4 microscopic fields (magnification 4x, 16 mm² per micro-

scopic field) were counted and classified as follows: 0 (-, no CAA), 1-10 (+, sparse CAA), 11-20 (+++, moderate CAA) and >21 (+++, severe CAA).

Double immunofluorescence

Experiments were performed as described previously [11, 34, 35]. Serial sections of temporal neocortex (6 µm) were fixed with acetone (100%) for 10 minutes and blocked with 3% bovine serum albumin (BSA; PAA Laboratories, Pasching, Austria) in Tris buffered saline (TBS) with 0.5% TritonX-100 (TBS-T). Negative controls were incubated in this solution without the primary antibodies. Primary antibodies (Table 2) were diluted in 3% BSA/TBS-T and incubated overnight at 4°C. After washing with TBS, sections were incubated with the appropriate secondary antibody. Secondary antibodies used were donkey anti-mouse, donkey anti-goat and donkey anti-rabbit, all coupled to Alexa 488 or Alexa 594, (dilution 1:400, Invitrogen, Carlsbad, CA, USA). Biotin-labelled donkey anti-mouse-IgM (dilution 1:800, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was used for the tTG crosslink antibody (81D4) and staining was enhanced with the Vectastain avidin-biotin kit (dilution 1:800 in TBS-T, Vector laboratories Inc., Burlingame, CA, USA). As a second enhancement step for the 81D4 antibody, biotinylated tyramide in 0.005% H2O2-TBS was used (generous gift from Dr. I. Huitinga, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands). The conjugate streptavidin Alexa 488 (Invitrogen) was used as a fluorochrome for the 81D4 antibody; streptavidin Alexa 594 was used as a secondary antibody for biotin-labelled mouse anti-human Aβ antibody. In between incubation steps, sections were extensively washed with TBS. Sections were mounted with Vectashield (Vector Laboratories Inc). The specificity of the antibodies directed against tTG in human brain was demonstrated in our previous reports [11, 34]. The specificity of the anti-TG-catalysed cross-link antibody was demonstrated by preadsorption with H-Glu(H-Lys-OH)-OH (Bachem AG, Bubendorf, Switserland) in human brain tissue [11]. To visualise the fluorescence stainings, a Leica TCS SP2 AOBs confocal laser scanning microscope (Leica Microsystems, Rijswijk, The Netherlands) was used. To exclude false positive fluorescence signals for each channel, a series of images was obtained separately in both channels through a 40x lens (zoom factor 1 to 4x, Z-increment 0.15 µm, approximately 20 images of 512×512 pixels).
### Table 1 Patient characteristics

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<th>Patient number</th>
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<th>Age</th>
<th>PMI (hr)</th>
<th>Grade (Braak, NFTs)</th>
<th>Grade (Braak, Aβ)</th>
<th>Grade CAA</th>
<th>Cause of death</th>
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<td>F</td>
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<td>7</td>
<td>1</td>
<td>O</td>
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<td>Myelodysplasia</td>
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<td>F</td>
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<td>1</td>
<td>O</td>
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<td>6</td>
<td>Control/CAA</td>
<td>F</td>
<td>73</td>
<td>8</td>
<td>1</td>
<td>B **</td>
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<td>Palliative sedation</td>
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<tr>
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<td>F</td>
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<td>6</td>
<td>6</td>
<td>C **</td>
<td></td>
<td>Pneumonia</td>
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<tr>
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<td>F</td>
<td>90</td>
<td>3</td>
<td>5</td>
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<td>C ***</td>
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<tr>
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<td>87</td>
<td>6</td>
<td>6</td>
<td>C ***</td>
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<tr>
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<td>53</td>
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<tr>
<td>17</td>
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<td>- ***</td>
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<td>Cerebral hemorrhage</td>
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</table>

**Abbriviations:** AD = Alzheimer’s disease, CAA = cerebral amyloid angiopathy, cap-CAA = capillary CAA, F = Female, M = Male, ND = not determined, NFT = neurofibrillary tangles, PMI = Post Mortem interval (hr = hours). Grading of AD (Braak) and of CAA was performed as described in the materials and methods section.

### In situ TG activity

In situ TG activity detection was performed as described previously [36], with some minor changes. In short, unfixed 6 μm thick tissue sections of neocortex of AD, HCHWA-D and control patients were pre-incubated for 30 minutes at room temperature in a 100 mM Tris-HCl, pH 7.4, 5mM CaCl2, 1 mM dithiothreitol (DTT, Promega, Leiden, The Netherlands) buffer with or without 100 μM of the tTG activity inhibitor Z-DON-Val-Pro-Leu-OMe (Z-006) [37], purchased from Zedira GmbH, Darmstadt, Germany. Then, incubation was continued for 40 minutes at 37°C with the same incubation buffer with or without inhibitor to which 0.05 mM biotinylated 5-(biotinamido)-pentylamine (BAP; Thermo Fisher Scientific, Fremont, CA, USA), a substrate for TGs [38, 39], was added. Thereafter, sections were air dried, fixed for 10 minutes with 100% acetone and subsequently incubated with a primary antibody directed against tTG (Ab1, Neomarkers) in 3% BSA/TBS-T for 1 hour at room temperature followed by 1 hour incubation at room temperature with secondary antibodies donkey anti-rabbit coupled to Alexa 594 to detect tTG (dilution 1:400) and streptavidin coupled to Alexa 488 to detect BAP incorporation (dilution 1:400). Sections were washed with TBS in between and after antibody incubation and mounted with Vectashield. The Leica TCS SP2 AOBS confocal laser scanning microscope was used to visualise the staining as described above.

### Table 2 Primary antibodies

<table>
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<th>Primary antibody</th>
<th>Species raised in</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
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<td>Mouse</td>
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<td>Thermo Fisher Scientific, Fremont, CA, USA</td>
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<tr>
<td></td>
<td>Guinea pig tTG (06471)</td>
<td>Goat</td>
<td>1:4000</td>
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<td>Aβ</td>
<td>Human Aβ biotinylated, clone 4G8</td>
<td>Mouse</td>
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<td>Human Aβ1-16 AB10</td>
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<td></td>
<td>Human Aβ (715800)</td>
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<td>1:100</td>
<td>Invitrogen, Camarillo, CA, USA</td>
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<tr>
<td>tTG cross-link</td>
<td>H-Glu(H-Lys-OH)-OH, 81D4</td>
<td>Mouse</td>
<td>1:100</td>
<td>Covlab, Villeurbanne, France</td>
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<td>Astrocytes</td>
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<td>Rabbit</td>
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<tr>
<td>Endothelial cells</td>
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<td>Rabbit</td>
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<td>Fibroblasts</td>
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</table>

**Abbriviations:** tTG = tissue transglutaminase; Aβ = amyloid-β; GFAP = glial fibrillary acidic protein
Results

Distribution pattern of tTG and its cross-links in CAA of control, AD and HCHWA-D cases

The general staining pattern of tTG and TG-catalysed cross-links observed in the neocortex of control and AD cases are in line with our previous findings [11]. Thus, in vessels of control cases and non-CAA affected vessels in AD and HCHWA-D cases, tTG staining was present in all layers of the vessel wall in both leptomeningeal and parenchymal vessels (Figure 1A-C, arrow), and in capillaries. In addition, weak anti-tTG cross-link immunoreactivity was observed in all layers of the vessel wall in both leptomeningeal and parenchymal vessels, predominantly at the endothelial side of the vessel wall (not shown). In CAA of AD cases, no spatial overlay of anti-tTG immunoreactivity with anti-Aβ staining was observed (Figure 1A-C). However, tTG staining was present as a halo at both the luminal and abluminal side of deposited Aβ, in parenchymal vessels (Figure 1A-C), leptomeningeal vessels (Figure 1D-F) and in capillaries (Figure 1G-I). Similar to tTG immunoreactivity in CAA, tTG cross-link staining was present in an abluminal and/or luminal halo surrounding Aβ deposition in leptomeningeal and parenchymal vessels (Figure 1J-L), but only in an abluminal halo in capillary CAA (Figure 1M-O). In CAA present in non-demented control cases, a similar staining pattern for both tTG and its cross-links was observed (not shown).

Interestingly, in probable CAA precursors, observed in the form of local Aβ deposition in a large parenchymal vessel (Figure 2A-C) and a capillary vessel (Figure 2D-F) of AD cases, anti-tTG immunoreactivity was elevated compared to the unaffected part of the vessel, and, in contrast to the fully developed CAA, spatially colocalised with the deposited Aβ. To investigate whether the appearance of tTG and its cross-links as halos surrounding CAA might be a general phenomenon in CAA, we performed the above-described stainings in HCHWA-D patients. The distribution of tTG (Figure 3) and cross-links (not shown) in CAA in HCHWA-D cases was similar to CAA in AD cases, although tTG staining was more intense as compared with AD.

Colocalisation of tTG with cellular markers in CAA of AD cases

In order to investigate the cellular origin of the observed tTG staining enclosing the deposited Aβ in CAA, double immunofluorescence staining of tTG with cell specific markers (glial fibrillary acidic protein [GFAP] for astrocytes [40], Von Willebrand Factor [VWF] for endothelial cells [41, 42], vimentin for fibroblasts [43] and smooth muscle actin [SMA] for smooth muscle cells [44]) was performed. In control cases, weak tTG staining was observed in astrocytes associated with brain vessels (Figure 4A-C). In CAA of AD cases however, strong anti-tTG immunoreactivity was observed in GFAP-positive astrocytes at the abluminal side of CAA in parenchymal vessels (Figure 4D-F). Especially the astrocytic end feet demonstrated strong tTG staining in CAA (Figure 4D-F). In CAA of

Figure 1 Double immunofluorescence staining of tTG and tTG cross-links in CAA in neocortex of AD cases. The anti-Aβ antibody (715800) stained Aβ deposition in CAA-affected vessels (B, E, H, K, N). Double immunofluorescence staining of the anti-Aβ antibody with either the anti-tTG (Ab1) or anti-TG-catalysed cross-link antibody was performed. In AD cases, tTG staining was observed in control vessels (A, arrow). In CAA however, tTG staining was observed in an abluminal and luminal halo enclosing the Aβ deposition in both parenchymal (A-C) and leptomeningeal (D-F) vessels. In capillary CAA, tTG (Ab1 antibody) was present in an abluminal halo (G-I). Anti-tTG cross-link immunoreactivity was present at the abluminal and luminal side of the Aβ deposition in larger cortical vessels (J-L) and at the abluminal side of capillary CAA (M-O). Scale bars: B, E, H, K, N: 30 μm. Abbreviations: tTG = tissue transglutaminase, Aβ = amyloid-beta, CAA = cerebral amyloid angiopathy.
Figure 2 Double immunofluorescence staining of tTG with Aβ in vessels partly affected by Aβ deposition. In a large parenchymal vessel (A-C) and capillary vessel (D-F), the anti-Aβ antibody (AB10 and 715800, respectively) stained the Aβ deposition (B, E). The anti-tTG antibody (06471 and Ab-1, respectively) showed a strong immunoreactivity in the Aβ-affected part of the vessel compared to the non-affected part of the blood vessel. In addition, tTG staining colocalised with the Aβ staining (A-F, arrows). Scale bars: B: 30 µm, E: 15 µm. Abbreviations: tTG = tissue transglutaminase, Aβ = amyloid-beta.

Figure 3 Double immunofluorescence staining of tTG in CAA in neocortex of HCHWA-D cases
The anti-Aβ antibody (4G8) stained Aβ deposition in CAA (B). Double immunofluorescence staining of the anti-Aβ antibody with the anti-tTG (Ab1) antibody was performed. In CAA, tTG staining was observed in an abluminal and luminal halo enclosing the Aβ deposition (A-C). Scale bar B: 30 µm. Abbreviations: tTG = tissue transglutaminase, Aβ = amyloid-beta.

Figure 4 Double immunofluorescence staining of tTG with cellular markers in CAA in control and AD cases. The anti-GFAP antibody stained astrocytes in parenchymal vessels (B, E), whereas the anti-vimentin antibody stained fibroblasts in the adventitia of the vessel wall of leptomeningeal vessels (H). The anti-VWF antibody stained endothelial cells in blood vessels (K) and the anti-SMA antibody stained smooth muscle cells in the media of the vessel wall (N). Double immunofluorescence staining of the anti-tTG (Ab1) antibody with either the anti-GFAP or anti-VWF antibody and double immunofluorescence of the anti-tTG (06471) antibody with either the anti-vimentin or anti-SMA antibody was performed. In control vessels, sporadic colocalisation of tTG staining with GFAP staining was observed (A-C). In parenchymal CAA vessels, tTG staining in the abluminal halo (D) colocalised with the GFAP staining in astrocytic endfeet (E, F). In leptomeningeal CAA vessels, tTG in the abluminal halo (G) colocalised with the vimentin staining (H, I, arrow). The tTG staining in the luminal halo J) in CAA vessels colocalised with the anti-VWF antibody (K, L). tTG in the abluminal and luminal halo surrounding CAA did not spatially colocalise with SMA staining (M-O). Scale bars B, H, K, N: 30 µm, e: 15 µm. Abbreviations: tTG = tissue transglutaminase, GFAP = glial fibrillary acidic protein, VWF = Von Willebrand Factor, SMA = smooth muscle actin, CAA = cerebral amyloid angiopathy.
leptomeningeal vessels tTG colocalised partly with vimentin, a marker for fibroblasts, in the abluminal halo (Figure 4G-I). Double immunofluorescence staining of tTG immunoreactivity with the anti-VWF antibody in control cases and in non-CAA affected vessels of AD cases, demonstrated tTG staining in all layers of the vessel wall, which at the luminal side colocalised with endothelial cells (not shown). In CAA of AD cases the luminal halo of tTG colocalised with endothelial cells, in both parenchymal (Figure 4J-L) and leptomeningeal vessels (not shown). SMA staining was present in all layers of the vessel wall in control vessels where it colocalised with tTG (not shown). However, although SMA staining was present in all layers of vessels affected by CAA, no colocalisation with tTG was observed (Figure 4M-O).

In HCHWA-D cases, like the AD staining, tTG and its cross-links colocalised with the astrocyte marker GFAP in astrocytic endfeet at the abluminal side of Aβ in parenchymal vessels, and with the endothelial marker VWF at the luminal side of Aβ (not shown).

**Colocalisation of tTG and its cross-links with ECM proteins in CAA of AD cases**

In CAA of AD cases, the staining pattern of these ECM proteins resembles that of tTG and cross-links in CAA, as described in this study. Together, these data suggested that tTG might cross-link the ECM in CAA. We therefore performed double immunofluorescence staining of tTG and cross-links with antibodies directed against the ECM proteins FN and LN which are well-known tTG substrates [14, 31]. In vessels of control cases and non-affected vessels in AD cases, FN and LN were present in all layers of the vessel wall of leptomeningeal and parenchymal blood vessels, and in capillaries (not shown). These data are in line with previous reports [45, 46]. In CAA in AD cases, elevated anti-FN and anti-LN immunoreactivity was found as an abluminal and/or luminal halo enclosing the deposited Aβ (Figure 5B, E). In addition, both the anti-FN and anti-LN immunoreactivity colocalised with tTG (Figure 5A-C, D-F respectively) and tTG cross-link staining (Figure 6A-C, D-F). In CAA of HCHWA-D cases, the distribution of FN and LN staining was similar to the AD cases, showing an abluminal and/or luminal halo that spatially colocalised with the anti-tTG antibody (Figure 5G-I and J-L respectively).

**In situ TG activity in CAA in AD and HCHWA-D cases**

We observed that tTG staining was present in the Aβ part of CAA precursors, whereas in later stages of CAA, tTG was not present in the Aβ deposition. Epitope masking of tTG may have caused this lack of staining. In addition, the conformation of tTG, which is an important property of the enzyme that influences its function [47], may affect the recognition by the antibodies we used. Therefore, to investigate whether tTG is still present and active in the Aβ part of CAA, we investigated the in situ activity of TGs by the incorporation of radiolabelled substrates.
of the TG substrate BAP. Snap frozen tissue sections of the neocortex of AD, HCHWA-D and control cases were incubated with the TG substrate BAP and stained for the presence of tTG and BAP. In vessels of control cases and non-CAA affected vessels in AD and HCHWA-D cases, weak BAP staining was observed in all layers of the vessel wall which colocalised with tTG staining (Figure 7A-C). After co-incubation with the specific tTG inhibitor Z-006, no BAP staining was detectable, in contrast to the tTG staining, which remained unaffected (Figure 7D-F). In CAA in AD and HCHWA-D cases, however, BAP staining was remarkably increased compared to control vessels, and was present in all layers of the vessel wall (Figure 7G). In addition, BAP staining also colocalised with the typical tTG halo enclosing the Aβ part of CAA (Figure 7G-I, arrow). After co-incubation with the specific tTG inhibitor Z-006, no BAP staining was detectable in CAA, whereas tTG staining was still present in the two halos surrounding the deposited Aβ (Figure 7J-L).

Discussion

In this study, we describe for the first time that immunoreactivity of tTG in a vessel wall partly affected by Aβ deposition, which may indicate an early stage, is increased and colocalises with the deposited Aβ. In contrast, in later stages of CAA, both tTG and its cross-links do not colocalise with the deposited Aβ anymore, but are present in an abluminal and luminal halo enclosing the Aβ deposition. We observed that the abluminal halo of parenchymal vessels in CAA are of astrocytic origin and that tTG might derive from fibroblasts in leptomeningeal vessels, whereas tTG in the luminal halo in all vessel types is of endothelial origin. Furthermore, tTG substrates and important ECM components FN and LN colocalise with tTG and its cross-links in the halos that enclose Aβ deposition in CAA. We observed this distribution pattern of tTG and its cross-links in CAA in non-demented controls, AD and HCHWA-D cases, which suggests that this pattern is a general phenomenon of CAA. Surprisingly however, although we did not observe tTG and tTG cross-link staining in the actual Aβ deposition in CAA, we found that tTG is still present and could be activated in situ in the deposited Aβ. Together our data suggest that tTG plays a unique role in CAA development and progression. Initially, tTG levels are elevated in early stages of CAA, and may even precede Aβ deposition, whereas in later stages tTG is likely to be involved in alteration and/or remodelling of the ECM in CAA.

Previously, we noticed an important discrepancy between the distribution pattern of tTG and its activity in SPs and CAA. tTG and its cross-links colocalised with the deposited Aβ in both diffuse and classic SPs, whereas in CAA, tTG and its cross-links did not colocalise with the Aβ aggregate itself [11]. Instead, in CAA, both tTG and its cross-links were present in an abluminal and luminal halo enclosing the Aβ deposition in middle-sized parenchymal vessels with CAA in AD patients [11]. In the present study, we found that apart from middle-sized parenchymal vessels affected by CAA in AD cases, tTG and its cross-links enclose the Aβ deposition in all parenchymal vessels and leptomeningeal vessels, as well as in capillaries affected by CAA. In addition, tTG and its cross-links were present in a similar distribution pattern in CAA in HCHWA-D cases and in CAA-affected vessels of non-demented controls. Together, these findings point out that the presence of tTG and cross-links in halos that enclose Aβ is a common phenomenon found in both type I and II CAA (with or without capillary CAA respectively), in different diseases that are characterised by CAA, and even in CAA in non-demented controls.

Interestingly, in a parenchymal and capillary vessel only partly affected by CAA, which may indicate an early stage of CAA development, tTG was present in the Aβ deposition itself. In later stages of CAA, tTG and its cross-links did not colocalise with Aβ, although tTG could still be activated with our in situ assay in both the Aβ part of CAA and the tTG-immunoreactive halos enclosing the Aβ. However, it should be noted that although it is highly likely that our in situ approach demonstrates tTG activity, this technique might also activate other TGs. However, although both TG1 and TG3 are present in the human brain, there is no association of these TGs with cerebral vessels or CAA [10, 11]. In addition, the potent tTG inhibitor Z-006 that was used in our experiments could inhibit other TGs, yet it has a significantly higher affinity for tTG compared to other TGs [37]. Together we therefore conclude that tTG is present in CAA in early and later stages and that its activity may have a unique role in Aβ deposition and development of CAA.

![Image of double immunofluorescence staining of tTG cross-links with ECM proteins in CAA in AD cases.](image-url)

**Figure 6** Double immunofluorescence staining of tTG cross-links with ECM proteins in CAA in AD cases. Double immunofluorescence staining was performed with either the anti-FN or anti-LN antibody with the anti-tTG cross-link antibody. In CAA vessels of AD cases, FN and LN staining colocalised with the tTG cross-link staining (FN: A-C, LN: D-F) in an abluminal and/or luminal halo enclosing the Aβ deposition. Scale bars B, E: 30 µm. Abbreviations: tTG = tissue transglutaminase, ECM = extracellular matrix, FN = fibronectin, LN = laminin, CAA = cerebral amyloid angiopathy
In situ transglutaminase activity. Double immunofluorescence was performed with the anti-tTG antibody (Ab1) and streptavidin coupled to Alexa 488 directed against the biotin-label of BAP. In non-CAA vessels of AD cases, weak staining of both BAP and tTG was observed in all layers of the vessel wall (A-C). In addition, BAP and tTG staining colocalised in all layers of the vessel wall (A-C). Co-incubation of the specific tTG inhibitor Z-006 (100 µM) with BAP resulted in absence of BAP staining in the control vessel (D). In CAA, strong BAP staining was observed in all layers of the vessel wall (G), whereas no BAP staining was present after co-incubation with Z-006 (100 µM) (J). tTG was again present in an abluminal and luminal halo surrounding the Aβ deposition in CAA (H, K). Colocalisation of BAP with tTG staining was observed in CAA (G-I arrow). Scale bar B, E: 15 µm, H, K: 30 µm. Abbreviations: tTG = tissue transglutaminase, CAA = cerebral amyloid angiopathy, BAP = biotinylated 5-(biotinamido)-pentylamine.

In early stages of CAA development, Aβ accumulates in the media around the vascular smooth muscle cells (SMCs) [3]. We observed tTG immunostaining in the Aβ deposition itself in vessels partly affected by CAA. In this stage of CAA, tTG might originate from SMCs, as these cells are known to produce and secrete tTG [48, 49]. A similar tTG staining was present in capillaries suggesting that pericytes are the cellular source in capillaries. Hence, endothelial cells are also known to produce tTG [50]. Although the cellular origin of tTG in capillaries and larger vessels might differ, tTG staining in the Aβ deposition was increased compared to the non CAA-affected part of the vessel wall. This suggests that either tTG levels are elevated in response to the Aβ deposition in the vessel wall, or that induced tTG levels and activity result in Aβ deposition in the vessel wall. An in vitro study has shown that tTG is upregulated in a monocytic cell line upon treatment with Aβ1-42 [51]. Furthermore, tTG can cross-link Aβ and affect its aggregation pathway [22–26]. Therefore, in vivo tTG expression might be increased in response to Aβ deposition in the vessel wall, which may lead to Aβ cross-linking in the initial stages of CAA. On the contrary, increased levels of tTG may also precede Aβ deposition. tTG is normally present in the vessel wall, where it is important in cross-linking of ECM proteins leading to remodelling of the vessel wall [15]. Levels of tTG in the vasculature increase with age, which leads to enhanced ECM cross-linking and subsequent vascular stiffness [52]. These changes in the vessel wall might influence Aβ deposition. In addition, several proteins that are known to affect the Aβ cascade, in particular heparan sulphate proteoglycans (HSPGs) and small heat shock protein B2 [53, 54], are also substrates for tTG [55, 56]. The fact that these tTG substrates are present in CAA as well suggests that tTG activity might affect Aβ aggregation and deposition, either directly or via cross-linking of Aβ chaperones. However, the role of tTG in initial stages of CAA remains to be investigated, and the use of in vivo models, in particular animal models of CAA, will be instrumental for this purpose.

In late stages of CAA, we found that tTG staining in the abluminal halo was of astrocytic origin in parenchymal vessel and derived from fibroblasts in leptomeningeal vessels, whereas tTG was of endothelial origin in the luminal halo. All these cell types are known to produce tTG [31, 57–59], and tTG is upregulated in response to cell stress and inflammation [60]. Aβ deposition is suggested to precede inflammation [61], thus expression of tTG may be increased especially in the cells surrounding the Aβ deposition in CAA. In parenchymal vessels, fibroblasts may produce tTG as well, although we could not distinguish astrocytes from fibroblasts, as vimentin stains mesenchymal cells including astrocytes and fibroblasts. Taken together, although the tTG in CAA is likely to be derived from astrocytes, fibroblasts and endothelial cells, a role in the production of tTG by smooth muscle cells, as in early stage CAA, cannot be excluded.

The presence and activity of tTG in the abluminal and luminal halo points towards a role in remodelling of the ECM. Altered expression of ECM proteins has already been observed in CAA and the brain microvasculature in AD [8, 27–29, 62, 63]. In our study we observed, in line with previous studies [27–29], altered expression of the ECM proteins FN and LN. Both FN and LN were present in an abluminal and/or luminal halo that surrounded the Aβ deposition. We here found that FN and LN colocalised with tTG and its cross-links in CAA of AD and HCHWA-D patients, suggesting that tTG might cross-link these ECM proteins in CAA. The putative cross-linking of FN and LN by tTG might have detrimental effects, since excess production and cross-linking of ECM proteins in diabetic nephropathy is associated with renal fibrosis and scarring [64]. Furthermore, the formation of cross-linked proteins at the endothelial side of the vessel could impair transport of solutes and
molecules across the blood brain barrier leading to impaired supply of nutrients in the brain thus compromising brain functioning. In addition, tTG-mediated cross-linking of ECM proteins may stiffen the vessel wall, resulting in impaired clearance of Aβ from the brain via the intestinal fluid (ISF) drainage [30, 65, 66]. Furthermore, impaired clearance of Aβ via this route might be not only the underlying cause of Aβ accumulation in the vessel wall leading to CAA, but also contributes to the accumulation of Aβ in the brain parenchyma [66].

On the other hand, the cross-linked ECM proteins might have a protective effect in CAA, because this cross-linked barrier may prevent the weakening of the vessel wall [9] and leakage of the blood-brain barrier, possibly reducing the risk of haemorrhages which can be the result of CAA [3, 9, 67]. In addition, at the abluminal side, the cross-linked ECM proteins may serve as a barrier to prevent spreading of toxic Aβ into the brain parenchyma. Clearly, further investigations are required to investigate these issues.

In conclusion, we found that tTG immunoreactivity is increased in early stages of CAA where it colocalised with Aβ deposition in the vessel wall, whereas tTG and its cross-links in more mature CAA lesions colocalised with ECM proteins in an abluminal and luminal halo enclosing the Aβ deposition. The role of tTG in early stage CAA might therefore be different from end stage CAA, when tTG-mediated ECM remodelling might be more important than its effect on Aβ aggregation per se. However, since we observed an increased ITG transmission activity in the Aβ deposition also in end stage CAA lesions, this may also be the consequence of epitope masking in these densely packed Aβ depositions. Chronological evaluation of CAA lesion development in animal models of CAA will hopefully provide answers to the outstanding questions on the role of ITG in vascular pathology in AD and related disorders.

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Disclosure Statement

The authors declare that they have no conflict of interest.

References


