RESEARCH ARTICLE

Collagen XVIII: a Novel Heparan Sulfate Proteoglycan Associated with Vascular Amyloid Depositions and Senile Plaques in Alzheimer's Disease Brains

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Heparan sulfate proteoglycans (HSPGs) may play a role in the formation and persistence of senile plaques and neurofibrillary tangles in Alzheimer's disease brains. Recently, it has been demonstrated that the human extracellular matrix-associated molecule collagen XVIII is the first collagen carrying heparan sulfate side-chains. Two variants of collagen XVIII with both different signal peptides and N-terminal domains have been described and are referred to as the short and long form. To investigate the distribution of these variants we performed an immunohistochemical analysis by using specific well-characterized polyclonal antibodies. Anti-long huXVIII, a polyclonal antibody directed against the long variant of collagen XVIII, weakly stained large cortical and leptomeningeal vessels, whereas small cortical vessels remained unstained. Interestingly, all amyloid-laden vessels and classic senile plaques were strongly stained. Anti-all huXVIII, a polyclonal antibody directed against an epitope common to both collagen XVIII variants, intensely stained all types of cerebral blood vessels, cerebral amyloid angiopathy-affected vessels and classic senile plaques. Collagen XVIII expression was absent in neurofibrillary tangles. We conclude that collagen XVIII is a novel heparan sulfate proteoglycan associated with vascular Aβ and classic senile plaques and that at least the long form of collagen XVIII accumulates in amyloid-laden vessels and classic senile plaques.

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder and the most prevalent form of late-life mental failure in humans. Histopathologically, 3 types of lesions characterize AD brains. First, neurofibrillary tangles (NFTs), which are intraneuronal accumulations composed of hyperphosphorylated tau protein (6). Second, senile plaques (SPs), which consist of extracellular deposits of amyloid β protein (Aβ) and finally Aβ deposition in blood vessel walls, often referred to as cerebral amyloid angiopathy (CAA) or congophilic angiopathy (19, 20).

Besides Aβ, SPs and CAA contain several other proteins, the so-called Aβ-associated proteins. Among these are inflammatory proteins (28), apolipoprotein E (ApoE) (11, 18, 24) and heparan sulfate proteoglycans (HSPGs) (14, 22, 23, 26, 27, 29) (for review, see 30). In particular, we demonstrated the expression of the HSPGs agrin, syndecan and glypican in SPs and NFTs. We also demonstrated the differential expression of these HSPGs in amyloid-laden vessels in AD on the one hand and hereditary cerebral hemorrhages with amyloidosis of the Dutch type (HCHWA-D) on the other. Furthermore, the pattern of HSPG deposition in CAA differed from that in SPs, suggesting that specific HSPGs might be involved in the pathogenesis of these vascular lesions (27). In addition, it has been suggested that HSPGs participate in the formation of paired helical filaments (2).

In 1998, Halfter et al characterized chicken collagen XVIII and showed that it is the third HSPG type associated with basement membranes, next to agrin and perlecan, and the first member of the collagen family with heparan sulfate side-chains (4). In addition, Saarela et al demonstrated that human collagen XVIII is also a HSPG (16).

Collagenous proteins are characterized by the presence of at least one triple helical sequence of a repeated Gly-*X*-*Y* motif and contain non-collagenous domains at their N- and C-termini. On basis of its primary structure, collagen XVIII belongs to the non-fibrillar collagens, which are characterized by the presence of one or more interruptions in the collagenous sequence (15). The

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function and significance of collagen XVIII is not well known, yet. However, it has been shown that it may generate a 20 kDa proteolytic fragment called endostatin. This molecule, released from the C-terminus of the α1 collagen (XVIII) chain is capable of inhibiting endothelial proliferation and angiogenesis (13).

Recently, the gene encoding human XVIII collagen has been described, which encodes for either a 1516- or a 1336-residue α1 chain (17). These 2 forms share 301 residues of their non-collagenous-1 (NC-1) sequence, a 688-residue highly interrupted collagenous fragment and a 312-residue C-terminal non-collagenous part, but contain different signal peptides and have different N-terminal noncollagenous NC1 domains. The signal peptide of the short form is connected to 2 amino acids specific to this variant, whereas the signal peptide of the long variant is followed by 192 residues specific to this variant. Either isoform is differentially expressed in tissues. The short form is ubiquitously present in basement membranes of most fetal and adult tissues and particularly in blood vessels and various epithelial structures, whereas the long variant is predominantly observed in hepatic sinusoids, but only in negligible amounts in a number of other tissues (17).

We performed an immunohistochemical analysis to investigate the distribution of the collagen XVIII isoforms in AD brains. Hereto, specific well-characterized polyclonal antibodies, directed against either a common collagen XVIII epitope or specifically against the long form of collagen XVIII, were used for immunostaining in order to investigate the expression pattern of these macromolecules in the AD brain.

Materials and Methods

Autopsy material. Brain tissue from patients with clinically diagnosed and neuropathologically confirmed AD was obtained at autopsy. Post mortem diagnosis of AD was confirmed by a consultant neuropathologist according to the Consortium to Establish a Registry for AD (CERAD) criteria (7). After a rapid autopsy procedure tissue samples from hippocampus and frontal or occipital neocortex were obtained and immediately frozen in liquid nitrogen. Neocortical sections of 11 patients (6 male, 5 female) were selected for the presence of multiple CAA-affected vessels (age 79.8 ± 7.8 years; post mortem delay 3.6 ± 1.0 hours). Additionally, hippocampal and neocortical sections of 5 other AD patients were selected (age 78.5 ± 7.4 years; post mortem delay 3.2 ± 0.9 hours). Three cases without neurological disease (age 83 ± 1.5 years; post mortem delay 3 ± 1 hours) were used as controls.

Antibodies. For more specific details about the production, specificity and characterization of the affinity purified pAbs anti-all huXVIII (QH48.18) and anti-long huXVIII (QH1415.7), see Saarela et al (16). The 6C6 antibody was a kind gift of Dr Schenk (Elan Pharmaceuticals); AT8 was purchased from Innogenetics. All secondary antibodies, the avidin-biotin-peroxidase complex (ABC) and Texas Red-labeled avidin were obtained from Vector (Burlingame, Calif) except the FITC-labeled sheep anti-mouse antibody, which was obtained from ICN Pharmaceuticals (Costa Mesa, Calif).

Immunofluorescence staining. To investigate the distribution of collagen XVIII in AD brains, serial sections (4 μm) were used. For immunohistochemical staining, sections were air-dried, fixed in acetone for 5 minutes, subsequently for 5 minutes in acetone containing 0.15% H_2O_2 to block endogenous peroxidase activity, and preincubated for 30 minutes with 20% normal goat serum. Then, sections were incubated overnight at 4°C with polyclonal antibodies anti-all huXVIII (directed against an epitope shared by the long and short form of collagen XVIII) or anti-long huXVIII (directed against the long form of collagen XVIII) at concentrations of 10 μg/ml and 20 μg/ml, respectively. Subsequently, the sections were incubated with biotin-labeled goat anti-rabbit antibody for one hour at room temperature and with avidin-labeled Texas Red for 45 minutes. Between the incubation steps, sections were thoroughly washed with phosphate buffered saline (PBS). After the last rinse in PBS, sections were incubated overnight at 4°C with either monoclonal anti-Aβ antibody 6C6 (dilution 1:500) for the detection of SPs and CAA, or monoclonal anti-tau antibody AT8 (dilution 1:200) for the detection of NFTs. Then, the sections were incubated with sheep anti-mouse FITC-labeled for one hour at room temperature. Between the incubation steps, sections were thoroughly washed with PBS and finally mounted in Vectashield (Vector, Burlingame, Calif). All antibodies were diluted in PBS containing 0.1% bovine serum albumin, which also served as a negative control.

For the detection of fibrillar Aβ, sections were stained with Thioflavin S (0.0125 % in 40% ethanol and 60% 10 mM PBS) for 3 minutes at room temperature, in the dark. Then, sections were differentiated by washing them twice with 50% ethanol and 50% 0.01 M PBS for 10 minutes before they were finally washed in 0.01 M PBS. Sections were examined using a confocal laser-scanning microscope (Leica, Wetzlar, Germany).

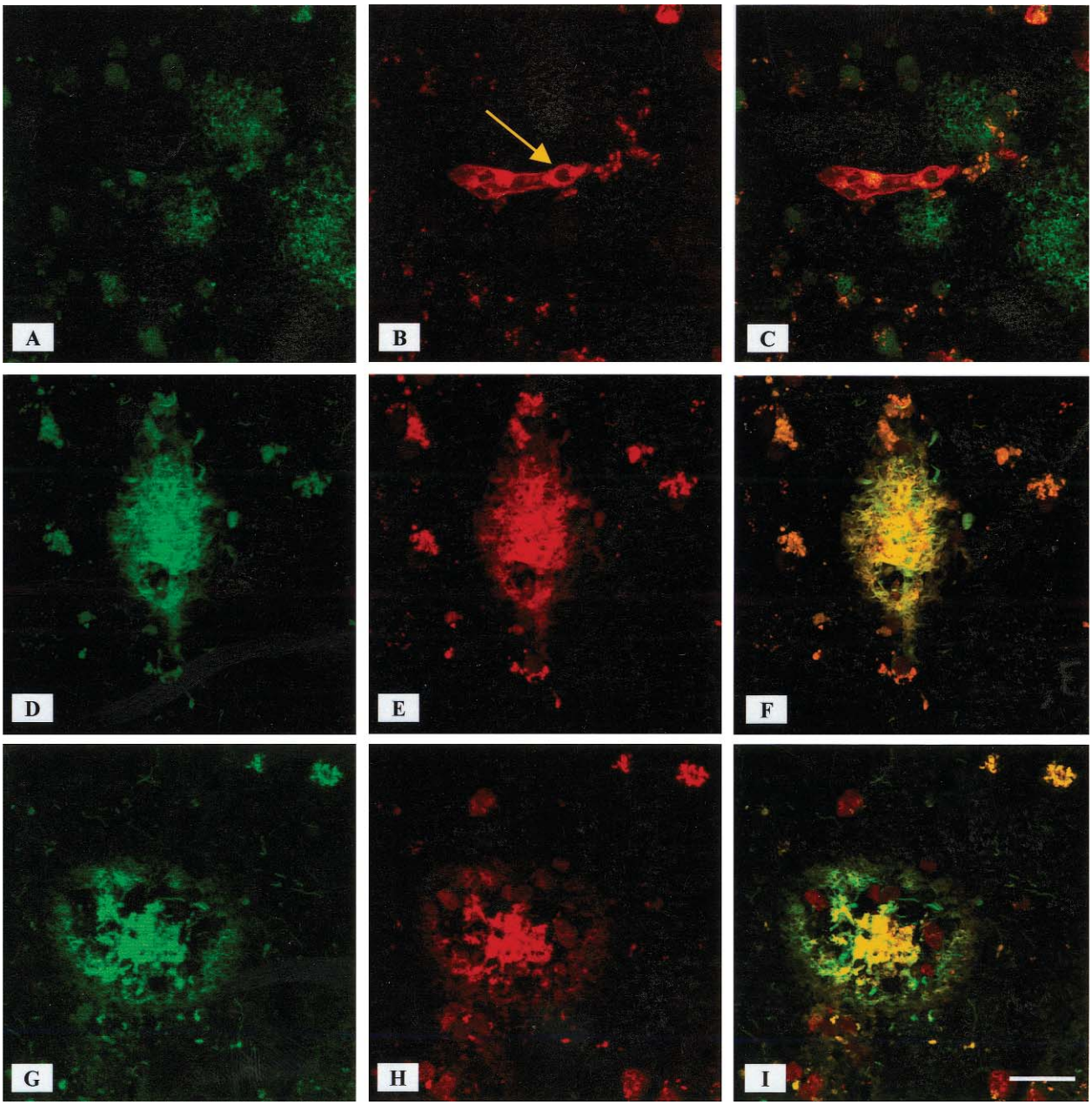

Figure 1. Immunofluorescence staining of senile plaques in sections from AD brains for Aβ (mAb 6C6) (**A**), (Thioflavin-S) (**D, G**), collagen XVIII both isoforms (pAb anti-all huXVIII) (**B, E**) and collagen XVIII long variant (pAb anti-long huXVIII) (H), double immunofluorescence (**C, F, I**). Collagen XVIII was not expressed in diffuse senile plaques (**B, C**), whereas classic senile plaques, in particular the dense cores, were intensely stained by both pAb anti-all huXVIII (**E**) and pAb anti-long huXVIII (**H**). Collagen XVIII staining clearly colocalized with Thioflavin-S staining of classic senile plaques (**F, I**). Collagen XVIII also strongly stained the cerebral (micro)vasculature (**B**; example indicated by arrow). Original magnifications: **A-I** ×630; bar = 50 μm

Results

Aβ and tau in AD brains. Senile plaques and vascular Aβ in AD brains were visualized by using the anti-Aβ monoclonal antibody (mAb) 6C6 and Thioflavin S. By using mAb 6C6 numerous diffuse (Figure 1A) and classic SPs, distributed throughout the grey matter, were observed in the brains of all patients. Thioflavin S strongly stained the dense cores of classic SPs (Figure 1D, G). Severe CAA was found in 7 patients, moderate

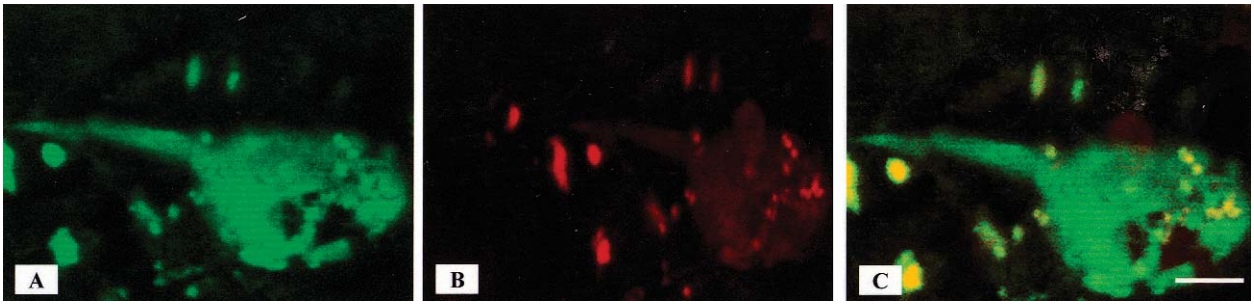

Figure 2. Immunofluorescence staining of neurofibrillary tangles in AD brains by Thioflavin-S (**A**) and anti-collagen XVIII (**B**), double immunofluorescence (**C**). Thioflavin-S strongly stained neurofibrillary tangles (**A**), however no colocalization with collagen XVIII was observed (**B, C**). Original magnifications: **A-C** ×630; bar = 10 μm

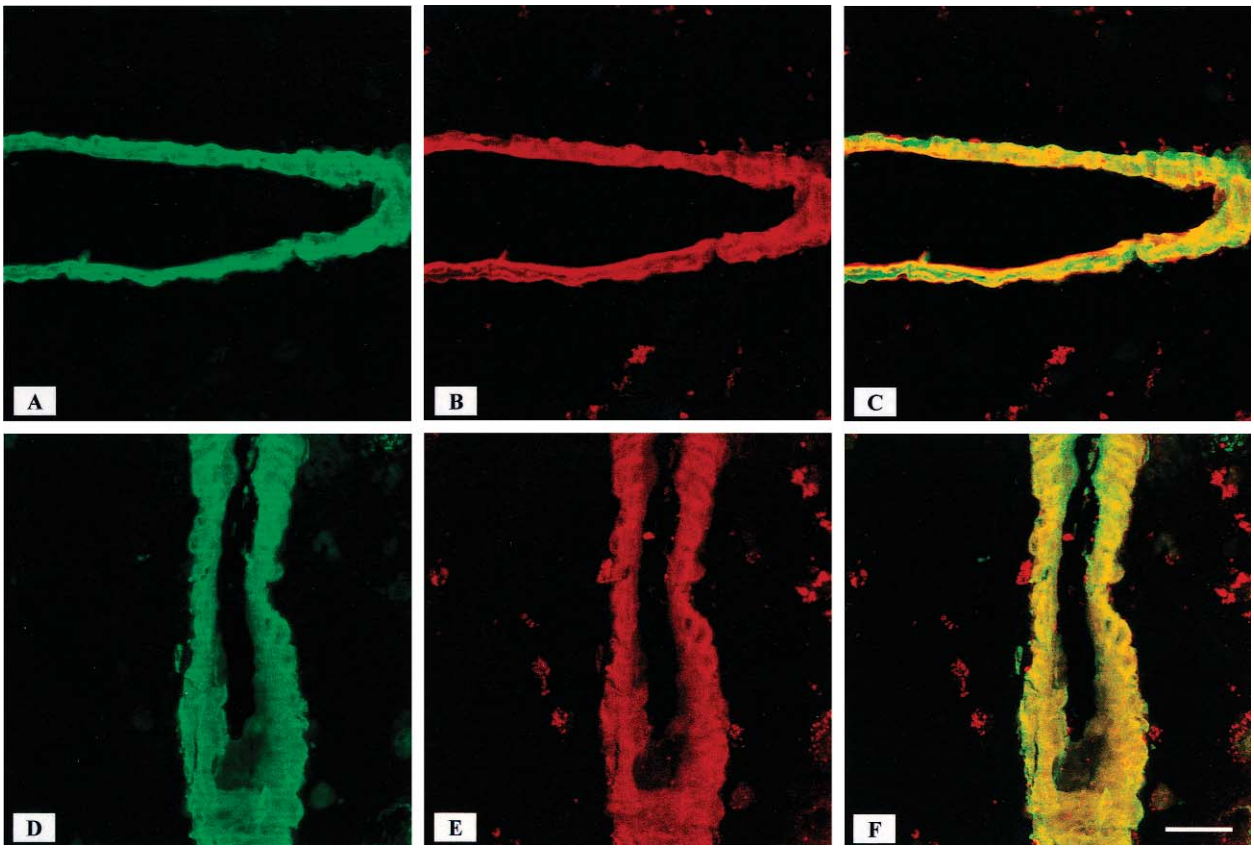

Figure 3. Immunofluorescence staining of cerebral amyloid angiopathy in serial sections from AD brain with Thioflavin-S (**A, D**) and pAb anti-all huXVIII (**B**) and pAb anti-long huXVIII (**E**), double immunofluorescence (**C, F**). Both pAbs anti-all huXVIII and anti-long huXVIII intensely stained amyloid-laden vessels (**B, E**), which was confirmed by colocalization of fibrillar Aβ deposits with collagen XVIII (**C, F**). Original magnifications: **A-F** ×630; bar = 50 μm

CAA in 4 patients. Both cortical and leptomeningeal vessels were affected by deposition of Aβ and strongly stained by both mAb 6C6 and Thioflavin S (Figure 3A, D).

AT8, a mAb directed against hyperphosphorylated tau protein was used to investigate the presence of NFTs in hippocampal and neocortical sections. NFTs and dystrophic neurites were intensely stained by mAb AT8 and observed throughout the hippocampus and frontal neocortex. Furthermore, NFTs and dystrophic neurites were also strongly stained by Thioflavin S (Figure 2A).

Collagen XVIII in AD brains. Anti-all huXVIII pAb intensely stained all types of cerebral blood vessels, including arteries, arterioles, capillaries, venules and veins (Figure 1B and data not shown). This antibody also clearly decorated CAA-affected vessels (Figure 3B) and classic SPs (Figure 1E), whereas expression was neither observed in diffuse SPs (Figure 1B, C) nor in NFTs (Figure 2B, C). Anti-long huXVIII weakly stained large cortical and leptomeningeal vessels, whereas small cortical vessels remained unstained (data not shown). Remarkably, all amyloid-laden vessels (Figure 3B, C, E, F) and classic SPs (Figure 1H, I) were clearly stained by anti-long huXVIII. The long variant of collagen XVIII was consistently absent from diffuse SPs and NFTs (data not shown).

Discussion

It is assumed that heparan sulfate proteoglycans (HSPGs) and in particular the sulfated glycosaminoglycans are involved in several aspects of the pathogenesis of Alzheimer's disease (AD). HSPGs have the ability to enhance Aβ fibrillization as demonstrated, both in vitro (1) and in vivo in a rat model (21), and inhibit proteolytic breakdown of Aβ fibrils by stabilizing these deposits (3). Moreover, immunohistochemical studies revealed that HSPGs colocalize with senile plaques, neurofibrillary tangles and cerebrovascular amyloid deposits (14, 22, 23, 26, 27, 29).

In previous studies we demonstrated that agrin is the major HSPG accumulating in SPs (29), whereas glypican-1 and syndecan-2 are particularly associated with vascular amyloid in AD brains (27). These studies clearly indicated that HSPGs are involved in AD pathology and that the interaction between Aβ and HSPGs might be important for the progression of Aβ depositions in these patients. In this study we demonstrated that, by immunohistochemistry, collagen XVIII is a prominent constituent of basal laminae of cerebral blood vessels and colocalizes with amyloid-laden vessels and classic SPs, but not with diffuse SPs and NFTs. This suggests that collagen XVIII is associated with fibrillar Aβ deposits, but has neither affinity for non-fibrillar Aβ nor for hyperphosphorylated tau protein. These observations are in remarkable contrast to our previous studies, in which we observed that most of the other HSPG subtypes accumulated in diffuse SPs and NFTs to a variable degree (29).

Differences between the expression signals of anti-all huXVIII and anti-long huXVIII in AD brains may reflect expression of the short variant of collagen XVIII. Since antibody anti-long huXVIII does not stain normal cerebral capillaries and only weakly stains larger vessels, the long form of collagen XVIII does not seem to be present in these vessel types, or only in small undetectable amounts. The strong vascular staining with anti-all huXVIII therefore likely predominantly reflects expression of the short form of collagen XVIII. Since both pAbs anti-long huXVIII and anti-all huXVIII similarly stained amyloid-laden vessels and fibrillar SPs, this confirms that at least the long variant accumulated in amyloid-laden vessels and fibrillar SPs. The expression of the short form in CAA and SPs can not be excluded, however. It has been reported that both endothelial cells and pericytes produce collagen XVIII (31), however which specific form is produced remains unclear.

Two possibilities can be proposed for the association between collagen XVIII and CAA. First, accumulation of Aβ in the vascular wall is associated with degeneration of vascular smooth muscle cells, pericytes and endothelial cells, which ultimately leads to vessel wall impairment. As a reaction to this phenomenon the remaining vascular cells might increase their production of extracellular matrix components, eg, collagen IV (5) and collagen XVIII, in an effort to strengthen and stabilize the affected vessel wall. Alternatively, Aβ might trigger vascular, neuronal or glial cells to produce collagen XVIII, which may then become associated with fibrillar Aβ deposits in the vessel wall and brain parenchyma. From this point of view it is remarkable that the long form of collagen XVIII is produced, since this specific variant is normally not, or only weakly, associated with vessels. However, it is been demonstrated that certain pathological conditions result in differential expression of collagen XVIII (8, 9, 10).

The presence of extracellular matrix components in SPs and CAA-affected vessels is of interest since it has been proposed that amyloidogenesis may be facilitated by extracellular matrix components, including basal lamina products (32). It has been reported that extracellular matrix proteins colocalize to SPs and CAA and have the ability to bind Aβ and its precursor with relatively high affinity. In addition, SPs and amyloid-laden vessels of patients with HCHWA-D also contain various extracellular matrix constituents (25). The binding of Aβ to extracellular matrix constituents may structurally influence the amyloidogenic protein ultimately initiating the process of amyloid deposition (12). Furthermore, immunoreactivity of fibronectin, collagen IV and laminin was maintained or even increased in severely CAA-affected vessels, suggesting that these ECM molecules might strengthen the vessel wall (5, 32).

In conclusion, based on the observation that collagen XVIII is associated with CAA and classic SPs, it is suggested that collagen XVIII has a particular affinity for fibrillar Aβ. Collagen XVIII might interact with Aβ either via its core protein or its heparan sulfate side-chains. Especially the long form of collagen XVIII accumulated in amyloid-laden vessels and classic SPs. Since the long variant is normally not, or only weakly, expressed in cerebral vessels, it is possible that Aβ triggers cells in the vessel wall or surrounding brain parenchyma to produce this specific type of collagen XVIII, which then becomes associated with classic SPs and CAA-affected vessels. The exact role of both the long and the short variant of collagen XVIII in the pathogenesis of Alzheimer's disease remains to be elucidated.

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