Heparan sulfate proteoglycans and vascular pathology in Alzheimer’s disease
Cover illustration: 3D overview of senile plaques and vascular amyloid (depicted in brown)

Heparan sulfate proteoglycans and vascular pathology in Alzheimer’s disease.
J. van Horssen. Thesis University Medical Center Nijmegen.

The studies presented in this thesis were performed in the Department of Pathology (head Prof. Dr. D.J. Ruiter) at the University Medical Center Nijmegen, Nijmegen, The Netherlands.

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Heparan sulfate proteoglycans and vascular pathology in Alzheimer’s disease

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Jacobus van Horssen
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Promotor: Prof. dr. D.J. Ruiter

Co-promotores: Dr. Ir. M.M. Verbeek
Dr. R.M.W. de Waal

Manuscriptcommissie: Prof. dr. B. Hillen
Prof. dr. G.J.M. Martens
Prof. dr. A.R. Cools

The studies presented in this thesis were performed in the Department of Pathology (head Prof. dr. D.J. Ruiter) at the University Medical Center Nijmegen, Nijmegen, The Netherlands
An ounce of prevention is worth more than a pound of cure
(Benjamin Franklin ± 1800)

Voor mijn ouders
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Chapter 1

General introduction
Alzheimer’s disease

General introduction

The tremendous rise in average life expectancy during the last century can be largely attributed to the reduction in infant mortality, development of antibiotics, balanced nutrition and improved sanitary conditions. This increase in life expectancy has led to a significant increase in incidence of age-related neurodegenerative disorders such as Alzheimer’s disease (AD). Today, AD is the most common form of dementia, a generic name for syndromes characterized by impaired memory function and cognitive declinements. In later stages of AD, patients may develop psychotic symptoms like hallucinations and delusions and finally patients may become mute, incontinent and bedridden and usually die due to infections such as pneumonia.

In 1907 the German neuropsychiatrist Alois Alzheimer described the first case of this multifactorial neurological syndrome. A middle-aged woman had developed memory deficits, behavioral problems and progressive loss of cognitive abilities. After 5 years of institutionalization in a psychiatric hospital she died and histopathological examination of the affected brain was performed. On the basis of this case, the first description of the pathological changes that occur in AD was published. Today, neuroimaging and biochemical analysis of cerebrospinal fluid, combined with neuropsychological examinations and detailed information on the family history, are useful diagnostic tools. However, there are still no definitive tests to diagnose AD during lifetime and postmortem histopathological analysis of the brain is needed to confirm the diagnosis of AD.

Neuropathological features of Alzheimer’s disease

Macroscopically, AD brains are characterized by severe atrophy of selective brain regions, particularly the neocortex, hippocampus, amygdala and entorhinal area. Conversely, several brain areas are usually devoid of AD changes including the cerebellum and striatum. Further analysis reveals widening of the sulci, narrowing of the gyri and in most cases enlargement of the ventricles. Histopathological examination using silver staining techniques demonstrated the presence of a number of characteristic pathological lesions. These hallmarks include senile plaques (SPs), neurofibrillary tangles (NFTs), and cerebral amyloid angiopathy (CAA). This triad is associated with widespread neuronal loss and vascular degeneration.

Senile plaques

Senile plaques are extracellular lesions that contain small polypeptides called amyloid β proteins (Aβ) (Figure 1a). Immunohistochemical analysis, based on the appearance and isoforms of Aβ and the presence and type of neuritic degeneration, revealed several distinct types of SPs (Dickson, 1997). In general, two major subsets of SPs can be distinguished: 1) diffuse senile plaques, which are abundantly present in upper cortical layers, are primarily composed of Aβ1-42 and often lack neuritic changes. However, in the advanced stages of AD a substantial part of the diffuse SPs are associated with paired helical filament (PHF)-type neurites. 2) Classic senile
plaques contain an amyloid core consisting of fibrillar Aβ and are mainly found in the deeper layers of the cortex. Classic SPs contain both fibrillar Aβ1-40 and Aβ1-42 and are surrounded by a wreath of dystrophic PHF-type neurites and dystrophic neurites. Dystrophic neurites contain degenerating synaptic components and enlarged lysosomes. Neuritic classic plaques are also intimately associated with a microglial and astrocytic response. Reactive microglia are usually located within and adjacent to the central amyloid core, whereas astrocytes often ring the outside of the plaque. Sporadically, burned-out plaques can be observed in AD brains, especially in primary cortices and Purkinje cell layer of the vermis. This type of plaque, which is closely related to the classic SPs, consists of an amyloid core but lacks other characteristics of classic SPs. It has been hypothesized that diffuse SPs represent the earliest stage of SP formation and during a protracted process gradually transform into classic neuritic in AD brains (Selkoe, 1991). On the other hand, in transgenic mouse models, both cored SPs and diffuse SPs appear simultaneously suggesting that either plaque type may develop independently, and that diffuse plaques do not necessarily transform into cored plaques (Hsiao et al., 1996).

**Neurofibrillary tangles**

In the human central nervous system the tau gene, located on chromosome 17, contains 15 exons (Andreadis et al., 1992). Alternative splicing of exons 2, 3 and 10 results in six main isoforms (Lee et al., 1988). Tau is a multifunctional neuronal microtubule-associated protein that plays an essential role in the assembly and stabilization of microtubules (Friedhoff et al., 2000). Additionally, tau is involved in facilitating neurite outgrowth (Biernat and Mandelkow, 1999) and signal transduction (Jenkins and Johnson, 1998). In AD brains tau is abnormally phosphorylated by several protein kinases, however the underlying mechanisms of phosphorylation and accumulation of tau in AD remains largely unclear. This hyperphosphorylated form of tau is the major constituent of paired helical filaments (PHF) and neurofibrillary tangles (NFT) found in AD brains (Figure 1b) (Lee et al., 1991; Johnson and Bailey, 2002; Johnson and Hartigan, 1999). Next to AD, a variety of other neurodegenerative disorders, including corticobasal degeneration, progressive supranuclear palsy (Dickson, 1999) and frontotemporal dementia (Hutton et al., 1998) is associated with intraneuronal deposition of hyperphosphorylated tau protein and these are therefore named “tauopathies” (Forman et al., 2000). Transgenic models of tauopathies may serve as experimental systems for elucidating the role of abnormal tau in a variety of neurodegenerative disorders. Additionally, these models may be useful in the development and testing of novel therapies.

**Cerebral amyloid angiopathy**

Cerebral amyloid angiopathy (CAA) is characterized by the deposition of Aβ in the media and adventitia of meningeal and cortical vessels (Figure 1c) and, less frequently, in capillaries and veins. CAA can be encountered in approximately 30% of elderly non-demented individuals and in 80-90% of patients with neuropathologically confirmed AD (Namba et al., 1991; Itoh et al., 1993; Ellis et al., 1996; Itoh and Yamada, 1997; Jellinger, 2002). In the context of AD, the functional impact of CAA on the pathogenesis of AD is relatively unclear and often considered as a coincidental finding, although the vascular damage inflicted by Aβ may lead to severe defects in the vessel wall (Roher et al., 1993). It has been demonstrated that Aβ accumulation
in the vascular media and/or adventitia is associated with degeneration of endothelial cells (Thomas et al., 1997), vascular smooth muscle cells (Davis-Salinas et al., 1995; Van Nostrand et al., 1996b) (Van Nostrand et al., 1996a) and pericytes (Verbeek et al., 1997). Together, these actions may lead to a dysfunction of the blood-brain barrier and finally cause cerebral hemorrhages. In fact, the best-known clinical presentation of CAA is lobar cerebral hemorrhage (Gilbert and Vinters, 1983; Itoh et al., 1993; Yamada, 2000; Kalyan-Raman and Kalyan-Raman, 1984). Vascular amyloid, like classic SPs, is composed of both $\mathrm{A\beta}_{1-42}$ and $\mathrm{A\beta}_{1-40}$, the latter being the most predominant isoform in more advanced stages of AD pathology.

![Figure 1.](image1)

**Figure 1.** Immunohistochemical staining of senile plaques (a), neurofibrillary tangles (b) and cerebral amyloid angiopathy (c).

**Amyloid precursor protein: The origin of amyloid-$\beta$ protein species**

The main component of SPs and amyloid-laden vessels is $\mathrm{A\beta}$ protein, a small polypeptide derived from the much larger amyloid precursor protein (APP). APP belongs to the family of amyloid precursor-like proteins (APLPs) and is a type I transmembrane glycoprotein that is ubiquitously expressed (Kang et al., 1987). Western blot analysis revealed three major isoforms of 695-, 751- and 770-residues. Neuronal cells particularly express the 695-residue isoform, whereas the other two can also be detected in non-neuronal tissue. Next to alternative splicing, the heterogeneity of APP is exemplified by posttranslational modification including the addition of sugars, phosphorylation and sulfation. APP is encoded by a gene on chromosome 21 and contains 18 exons of which exons 16 and 17 contain the $\mathrm{A\beta}$ fragment. Processing of APP in vivo occurs by two major pathways. The first enzyme involved in the proteolytic cleavage of APP was designated as $\alpha$-secretase. Inhibitor studies have shown that $\alpha$-secretase is a zinc-depandant metalloproteinase. Today, several proteins have been identified that have $\alpha$-secretase activity. They are membrane-bound disintegrin and metalloproteinases including ADAM 10, ADAM 17 (also named tumour necrosis factor alpha converting enzyme (TACE) and ADAM 9 (Blobel et al., 1997). $\alpha$-Secretase cleaves APP in the middle of the amyloid peptide region resulting in the release of a large $\mathrm{APP}_{\alpha-\alpha}$ fragment and retention of a C83 residue in the cell membrane. Alternatively, in the amyloidogenic pathway, cleavage of APP at the N-terminus of the amyloid-$\beta$ protein ($\mathrm{A\beta}$) region occurs by $\beta$-secretases. Two enzymes capable of $\beta$-cleavage have been
identified, the so-called β-site APP-cleavage enzymes BACE 1 and BACE 2 (Esler and Wolfe, 2001). BACE 1 is a classic type 1 transmembrane protein of the family of aspartyl proteinases and the key rate-limiting enzyme that initiates the formation of Aβ (Vasar, 2001). Studies in BACE1 knockout mice provide strong evidence that BACE1 is the major β-secretases in brain and its inhibition a target for therapeutic intervention (Cai et al., 2001). Over-expression of BACE1 in cell culture increased the amount of β-secretase cleavage products (Vassar et al, 1999). Moreover, a transgenic mouse line expressing human BACE1 in the brain induces the amyloidogenic processing of APP and elevates the steady-state levels of Aβ1-40/42. In addition, knockout of the BACE1 gene completely impairs the β-secretase cleavage of APP and abolishes the generation of Aβ (Cai et al., 2001). So far, the precise role of BACE2 in APP processing remains relatively unclear. β-Secretases cleave APP between residues Met671 and Asp672 and yield APPs-β and a membrane bound C99 fragment encompassing Aβ. Finally, APP peptides C83 and C99 can be proteolytically cleaved by γ-secretases generating a small peptide designated P3 (non-toxic) and Aβ, respectively. Although the integrity of γ-secretases has not been fully characterized, the presenilins PS1 and PS2 are two candidates for the catalytic components of γ-secretase. PS1 and PS2 are ubiquitously expressed membrane proteins comprising 463 and 448 amino acids, respectively. They share 76% homology and are similar in size and intracellular localization. Both were predominantly found in endoplasmic reticulum and Golgi membranes, however the proteins have also been detected in other intracellular compartments. Presenilin knockout mice have reduced amounts of Aβ1-40/42 in neuronal tissue but larger amounts of γ-secretase substrates C83 and C99 implicating a role in APP processing. PS1/PS2 double-knockout mice have no γ-secretase at all, suggesting that presenilins are required for γ-secretase activity (Wolfe, 2002).

In conclusion, normally APP can undergo at least two post-translational processing pathways resulting in the generation of Aβ in both control and AD patients. This finding is supported by the fact that Aβ peptides can be detected in the brain and cerebrospinal fluid of non-demented individuals. It is conceivable that AD represents an imbalance between Aβ production and clearance leading to the deposition of Aβ in the brain parenchyma and vasculature. Although there has been intensive study on the processing of APP to generate toxic Aβ only recently has attention turned to mechanisms of Aβ degradation and clearance. It has been even suggested that decreased clearance of Aβ from brain and CSF is the main cause of Aβ accumulation in sporadic AD. Since Aβ is continuously produced in the brain, there has to be an efficient clearance mechanism at the blood-brain barrier to prevent its accumulation and subsequent aggregation. Once formed, Aβ can be degraded by several enzymes, including neprilysin (Iwata et al., 2001) and insulin-degrading enzyme (Farris et al., 2003; Kurochkin and Goto, 1994).

**Aβ fibrillogenesis in the context of the amyloid cascade theory**

Amyloid fibrils comprising primarily Aβ1-40 and Aβ1-42 isoforms are a characteristic feature of AD. The “amyloid cascade” theory states that the aggregation of monomeric Aβ forming neurotoxic Aβ fibrils is an early and necessary event in neuronal degeneration in AD (Hardy and Higgins, 1992). A wide range of molecular
and animal studies support the amyloid cascade hypothesis. Additionally, genetic studies support this proposition since mutations leading to early-onset familial AD increase the production of Aβ_{1-42}, which is more prone to form fibrils than Aβ_{1-40}. Still, one of the central key questions is how monomeric soluble Aβ molecules are converted into mature fibrils. Electron microscopy and chromatography techniques have been used to study the initial stages of Aβ fibrillogenesis. Monomeric Aβ is gradually converted into oligomeric structures and pre-fibrillar intermediates, also named protofibrils (Walsh et al., 1999; Walsh et al., 1997; Walsh et al., 1997). Several studies have shown that protofibrils, and not mature amyloid fibrils, may be the key toxic species in AD (Walsh et al., 2002; Bucciantini et al., 2002). These contradictory studies show the need to elucidate the mechanism of Aβ fibrillogenesis in order to identify compounds that alter Aβ assembly. Although the amyloid cascade postulates that Aβ fibrillogenesis is the major culprit in the pathogenesis of AD, alternative theories have been proposed and several research groups have tried to tackle the amyloid cascade hypothesis of Hardy and Higgins. These relatively “new” theories posit that Aβ next to neurotoxic properties also acts as a neurotrophic and neuroprotective agent. Aβ might function as a ligand for neurotoxic compounds (Bishop and Robinson, 2002). After binding, these complexes can be phagocytosed by microglial cells. It has even been hypothesized that Aβ has anti-oxidant properties by binding to metals, thereby protecting neurons against oxidative stress (Kontush, 2001).

**Genetic risk factors for Alzheimer’s disease**

Although most Alzheimer’s disease patients exhibit the first clinical signs during their seventh decade, occasionally the conditions develop at midlife, as with the case Alzheimer described. To date, mutations in three genes are implicated in this rare early onset form of AD, including the PS1 gene, PS2 gene, and the APP gene. Mutations in these genes, however, account for less than 1% of the total number of AD patients. The remaining cases of are probably the result of a complex etiology due to interactions between environmental factors and genetic features of the individual. There are several other genes that are considered susceptibility or risk factors for AD (Rocchi et al., 2003). In this respect, the apolipoprotein E gene is recognized as a major risk factor for sporadic late-onset AD cases.

**Amyloid precursor protein mutations**

Studies on APP started after the observation that patients with Down syndrome developed clinical and neuropathological features of AD (Mann, 1988). These data demonstrated the involvement of chromosome 21 in AD pathology. Several years later, genetic analysis indeed confirmed that specific mutations in the APP gene pathologically affect APP processing by increasing Aβ production, especially its highly amyloidogenic 42 amino acid variant. The mechanism by which APP missense mutations are causative for autosomal dominant early-onset familial AD is not yet clear, but some of these mutations might be responsible for an altered metabolism of APP, since they are located in close proximity to the secretase cleavage sites (figure 2). Mutations at codons 670/671 produce an apparently characteristic AD pathology, whereas those at codon 692 and 693 produce a pathological picture of parenchymal and vascular amyloidosis often leading to intracerebral hemorrhages and the clinical
phenotype of hereditary cerebral hemorrhages with amyloidosis (HCHWA), Flemish and Dutch type, respectively (Cras et al., 1998; Van-Broeckhoven et al., 1990).

![Diagram](image)

**Figure 2.** Overview of several mutations in the APP sequence (bold letters represent identified mutations). Notice that mutations primarily occur before the β-secretase cleavage position and immediately after the α- and γ-secretase cleavage sites. Underlined amino acids represent Aβ_{1-42}.

**Presenilins**

Mutations in the APP sequence are very rare, hence it was expected that several other mutations might exist. In 1995, linkage analysis led to the identification of two other genes named PSEN1 and PSEN2 (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). These genes code for two highly homologous transmembrane proteins called presenilin 1 (PS1) and 2 (PS2). Mutations in PSEN1 and PSEN2 are thought to cause up to 80% of familial early-onset AD cases (Suh and Checler, 2002; De Strooper et al., 1998). After the identification of mutations in the presenilin 1 and 2 genes, molecular and biochemical research demonstrated that presenilins are involved in the proteolytic processing of specific proteins. Each presenilin forms a large multi-protein complex with three other essential compounds, nicastrin, APH-1 and PEN-2 (Suh and Checler, 2002; LaVoie et al., 2003). Gamma-secretase resides within this multisubunit complex. Although the molecular mechanisms by which mutant PS1 exerts its pathogenic effect are not fully understood there is growing evidence that presenilin 1 has γ-secretase activity or at least is a co-factor for γ-secretase, the enzyme involved in transmembrane metabolism of not only APP, but also Notch and at least ten other substrates. To date there are over 120 different mutations, predominantly located in the highly conserved transmembrane region, identified in the PSEN1 gene. Only eight missense mutations are found in the PSEN2 gene (Rocchi et al., 2003; Suh and Checler, 2002). Like the mutations identified within the APP gene, mutations in PS1 and PS2 cause increased generation of Aβ_{1-42} (Rocchi et al., 2003).

**Apolipoprotein E**

Early-onset AD may be explained by single gene defects, however most AD cases are late onset (> 65 years). Although there is no known definite cause for this form of
the disease, there are several known risk factors. Of these, the possession of the apolipoprotein e4 allele is recognized as the most common identifiable genetic risk factor for late-onset AD among most populations (Schmechel et al., 1993). Apolipoprotein E (ApoE) is a plasma glycoprotein involved in cholesterol metabolism and several other pathways (Siest et al., 1995). Three major isoforms of ApoE can be distinguished (ApoE2, ApoE3 and ApoE4), which are encoded by a gene on chromosome 19. Many studies have demonstrated an association between the ApoE e4 variant and late-onset familial and sporadic forms of AD (Strittmatter et al., 1993). Unlike the pathogenic mutations in the amyloid precursor protein or those in the presenilins, ApoE e4 alleles appear to be a risk factor and not an invariant cause of AD, indicating that other environmental or genetic factors, operating in combination with the e4 allele, are necessary to cause AD.

**Therapeutic approaches**

Cholinergic deficits have been closely linked to altered processing of APP and to cognitive impairment (Roberson and Harrell, 1997). To date, the only treatment available for the disease is pharmacotherapy with acetylcholinesterase inhibitors, a palliative strategy aimed at temporary improvement of cognitive function. Cholinergic therapies target late aspects of the disease, but do not stop the progressive mental decline (Lau et al., 2002).

Recent progress in understanding the molecular and cellular pathophysiology of Alzheimer’s disease has suggested possible pharmacological interventions that could modify the development and progress of the disease, such as treatment with secretase inhibitors, non-steroidal anti-inflammatory drugs, transition metal chelators, cholesterol-lowering drugs, Aβ immunization, estrogen replacement therapy and antioxidants (Dominguez and De Strooper, 2002). Inhibitors of tau hyperphosphorylation may also modulate the development and progress of the disease. It can be expected that in the near future this panel of therapeutic approaches will extend.

Both β- and γ-secretase inhibitors decrease Aβ synthesis by modulating the proteolytic processing of APP. A major concern when considering γ-secretase inhibitors for the treatment of AD is their potential side effects. For example, γ-secretase also participates in the cleavage of several membrane proteins like Notch-1, a protein required for transcriptional regulation during development (Kopan and Goate, 2000). Inhibition of γ-secretase in a mouse model led to premature death (Strooper and Annaert, 2001). Among the secretases, BACE is at present the most attractive target for the inhibition of amyloid production. Although a number of β-secretase inhibitors have been shown to be effective in mouse models of AD no effective inhibitors in human brain have been detected or designed yet. Another fundamental question remains: what are the other substrates for BACE *in vivo*? Cholesterol, like β- and γ-secretase inhibitors, affects APP processing. High levels of cholesterol seems to favor processing of APP through the amyloidogenic pathway (Simons et al., 1998; Fassbender et al., 2001), whereas low cholesterol increases the processing of APP by α-secretase (Kojro et al., 2001). Thus, cholesterol-lowering drugs like statins reduce Aβ production (Stuve et al., 2003). Future research is
needed to understand the exact mechanism by which cholesterol modulates secretase activity.

Molecular and cellular research demonstrated that Aβ exerts its toxic effects by several mechanisms including oxidative stress, inflammatory processes and disturbance in calcium homeostasis (Aisen, 2002; Butterfield, 2002). Pharmacological suppression of inflammation and oxidative stress may therefore ameliorate the neuropathology. Epidemiological studies demonstrate that non-steroidal anti-inflammatory drugs offer some protection against the development of AD, but the appropriate dosage and duration of drug administration are still unclear (Etminan et al., 2003). Oxidative stress has been established as an initial event in AD pathology and an attractive therapeutic target since free radical scavengers, like vitamin E, prevent Aβ-induced reactive oxygen species formation (Butterfield, 2002; Perry et al., 2002b; Perry et al., 2002a).

In 1999, Schenk and colleagues showed that immunization against Aβ was effective in a transgenic animal model of AD (Schenk et al., 1999). Active immunization with preaggregated Aβ resulted in a significant reduction of plaque load when compared to control groups. In older animals with established pathology immunization led to a reduced number of amyloid depositions. These positive results prompted researchers to investigate whether the use of antibodies against Aβ alone was sufficient enough to clear amyloid burden in APP transgenic mice. Indeed, administration of antibodies against Aβ caused phagocytic clearance of Aβ by microglial cells (Bard et al., 2000). Even more interesting was the fact that immunization of transgenic mice not only affected the plaque load, but also improved behavioral and cognitive functions (Janus et al., 2000; Morgan et al., 2000). Human phase I trials showed that the vaccine based on Aβ was well tolerated and gave a good immunological response in a subset of individuals. After the completion of phase I, phase IIa trials started to test the efficacy in AD patients with mild to moderate symptoms. However, the phase II study was soon suspended after four patients showed signs of meningencephalitis and, by October 2002, the trial was definitively stopped.

Aforementioned therapeutic strategies focus on the inhibition of amyloid-related pathological processes, however AD brains are also characterized by neurofibrillary degeneration. Phosphorylation of tau protein is regulated by the activity of several phosphatases and it is known that in AD brains their activity is compromised. Hence, modulation of the activities of phosphatases and tau kinases offers promising therapeutic opportunities to inhibit neurofibrillary degeneration (Dalrymple, 2002; Lau et al., 2002). Recently, it was shown that therapeutic concentrations of lithium blocked depositions of Aβ in a transgenic mouse model. Lithium inhibits glycogen synthase kinase-3α (GSK-3α), an enzyme involved in both APP processing and tau phosphorylation. Inhibition of GSK-3α by lithium may therefore not only reduce the formation of senile plaques but also affect neurofibrillary degeneration (Phiel et al., 2003).

The aggregation of Aβ into fibrillar Aβ deposits is thought to be a critical event in AD pathogenesis. Therefore, interference with the fibrillogenesis process may be an important target for AD therapy. Short synthetic peptides, designed as beta-sheet
breakers, have been shown to inhibit fibril formation (Soto et al., 1998; Rijkers et al., 2002). It has been shown that heparan sulfate proteoglycans (HSPGs) and heparan sulfate glycosaminoglycan (HS GAG) side-chains promote Aβ/tau-fibrillization on the one hand and provide resistance against proteolytic breakdown, at the other (van Horssen et al., 2003). Small polysulfated compounds compete with natural HS GAGs and prevent formation of amyloid fibrils and binding of amyloidogenic proteins to the cell surface (Kisilevsky and Szarek, 2002; Kisilevsky et al., 1995; Kisilevsky et al., 2003; McLaurin et al., 1999a) (McLaurin et al., 1999b). Currently, phase II clinical trials with this new class of drugs are underway.

**Outline of the thesis**

**Chapter 2**
This chapter provides general information about the involvement of HSPGs in the pathogenesis of AD. HSPGs are invariably associated with senile plaques and vascular amyloid, but the exact nature of these amyloid-associated HSPGs has remained largely elusive. Today, antibodies directed against specific HSPG subtypes allow us to investigate the distribution pattern of these sulfated macromolecules in vascular amyloid and SPs in AD brains. This expression pattern may reveal which HSPG species are involved in the various stages of SP and CAA formation.

**Chapter 3**
Since it has been suggested that the pathogenesis of senile plaques and CAA differs in several aspects, we compared possible differences between the expression patterns of HSPGs in senile plaques and vascular amyloid. Hereto, we analyzed the distribution of several HSPG species in vascular amyloid and cerebral SPs.

**Chapter 4**
Diffuse plaques in the cerebellum only sporadically transform into fibrillar SPs. Since HSPGs are involved in the conversion of non-fibrillar Aβ into fibrillar Aβ, we decided to study the distribution of HSPGs in AD cerebellum.

**Chapter 5**
Two variants of human collagen XVIII with both different signal peptides and N-terminal domains have been described and are referred to as the short and long form. Using specific anti-collagen XVIII antibodies we studied the distribution of collagen XVIII in AD brains.

**Chapter 6**
Parkinson’s disease (PD) brains are characterized by the presence of Lewy bodies (LBs), intraneuronal inclusion bodies composed of α-synuclein aggregates. Since the fibrillization process of α-synuclein is comparable with the aggregation of Aβ, HSPGs might also be involved in α-synuclein aggregation. Here we describe the distribution of HSPGs and GAGs in PD brains.
Chapter 7
Our immunohistochemical data shows that various types of HSPGs, including agrin, glypican-1, collagen XVIII and several syndecans are associated with AD lesions. However, it is yet unknown if and how excessive deposition of HSPGs in the brains of AD patients is related to the production of Aβ. Here we investigate the cellular origin of these Aβ-associated HSPGs and the influence of DAβ1-40 on HSPG metabolism in human brain pericytes.

Chapter 8
On the basis of the distribution of HSPGs in CAA-affected vessels, we identified a small group of patients with severe CAA associated with secondary vascular changes. These patients were neuropathologically characterized as vascular dementia or AD with a vascular component. This chapter describes the macroscopic and microscopic neuropathological findings of five patients with severe CAA-associated secondary vascular changes in detail.

Chapter 9
In this final chapter we summarize and discuss the results of this thesis and propose future research plans.
Met het oog op de vergrijzing van het huidige docentencorps, zou er meer geïnvesteerd moeten worden in het opleiden van jonge docenten.
Chapter 2

Heparan sulfate proteoglycans in Alzheimer’s disease and amyloid-related disorders

Jack van Horssen\textsuperscript{1}, Pieter Wesseling\textsuperscript{1,2}, Lambert P.W.J. van den Heuvel\textsuperscript{3}, Robert M.W. de Waal\textsuperscript{1}, Marcel M. Verbeek\textsuperscript{1,2}

From the Departments of Pathology\textsuperscript{1}, Neurology\textsuperscript{2}, Pediatrics\textsuperscript{3}, University Medical Center, Nijmegen, The Netherlands

Parts of this chapter have been published in modified form as:

Heparan sulfate proteoglycans

Introduction
Already in the 19th century Virchow described colocalization of carbohydrates with amyloid deposits, but it was not until the 70s that researchers unraveled details of the structure of these molecules. Biochemical analysis revealed that glycosaminoglycans were the basic component of these hydrocarbons. From that time on the relationship between proteoglycans/glycosaminoglycans and amyloid deposits became a focus of interest for several research groups.

Biochemistry and biosynthesis
Proteoglycans (PGs) form a heterogeneous family of complex biological macromolecules composed of linear sulfated polysaccharides covalently attached to a core protein. These long unbranched polysaccharide chains named glycosaminoglycans (GAGs) consist of repeating disaccharide units. On basis of these disaccharide units four major groups of GAGs can be distinguished: chondroitin sulfate (CS), keratan sulfate (KS), dermatan sulfate (DS) and heparan sulfate (HS). This chapter describes the biosynthesis of heparan sulfate proteoglycan (HSGPs), which is the major group of PGs associated with amyloid deposits.

The process of HSPG formation requires several initial steps. The formation of the core protein and assembly of O-linked oligosaccharides occurs in the rough endoplasmic reticulum. Next, the protein backbone is transported to the Golgi apparatus. Here, the biosynthesis of the polysaccharide chain is initiated by the transfer of xylose from uridine-diphosphate-xylose to a hydroxyl group of a specific serine residue on the core protein. Through the stepwise addition of monosaccharide residues by specific glycosyltransferases the tetrasaccharide glucuronic acid-galactose-galactose-xylose is formed. After addition of an N-acetylglucosamine, polymerization is induced by alternated joining of glucuronic acid and N-acetylglucosamine residues via β1,4 and α1,4 linkages, respectively. During this polymerization process a complex system of enzymes subsequently modifies the bare HS chain into an active molecule with a unique sequence. The first enzyme involved in the modification of the polysaccharide chain is the bifunctional glucosyl N-deacetylase/N-sulfotransferase (NDST) that replaces acetyl groups in N-acetylglucosamine by sulfate groups. Epimerases have the ability to convert glucuronic acid residues into iduronic acid. 3’-Phosphoadenosine-5’-phosphosulfate (PAPS) functions as a donor for the sulfate groups, which are attached to specific sites on the GAG side-chain by sulfotransferases, like heparan sulfate glucosaminyl 3-O-sulfotransferase (3-OST) and heparan sulfate glucosaminyl 6-O-sulfotransferase (HS6ST). Various modifications lead to so-called N-acetylated (NA) domains where the saccharide backbones have undergone few modifications and thus mainly consist of D-glucuronic acid–N-acetyl-glucosamine repeats. These largely unmodified regions act as spacers between the highly modified and sulfated domains (NS-domains), which are, because of the sulfate and/or carboxyl groups on most of their sugar residues, highly negatively charged. The large number of modifications leads to structural heterogeneity of HS GAG side-chains (Figure 1). Together, the overall biosynthesis of the GAG side-chain in the Golgi apparatus is a rapid process. After
completing this highly regulated process the various HSPGs are transported in vesicles to the cell membrane and subsequently incorporated in this membrane or released into the extracellular matrix. Alternatively, PGs are internalized and degraded or recycled and transported back to the membrane.

**Figure 1.**
Biosynthesis of heparan sulfate. After the attachment of a xylose to a specific serine residue on the core protein, galactosyltransferases I and II and glucuronic acid transferase I add two galactose residues and a glucuronic acid residue, respectively. After addition of an N-acetylglucosamine, polymerization is induced by specific glycosyltransferases resulting in yet unsulfated HS chain. Finally, enzymatic modifications by NDST, epimerases and sulfotransferases lead to highly modified and sulfated domains (NS), so-called N-acetylated (NA) domains with relatively few modifications and miscellaneous NS/NA regions.
Heparan sulfate proteoglycan subtypes: structures and functions

Heparan sulfate proteoglycans can be divided into two major families including the extracellular matrix-associated HSPGs perlecan, agrin and collagen XVIII, and the cell surface proteoglycans of the syndecan and glypican family (Bernfield et al., 1999) (Figure 2).

**Perlecan**

Perlecan, the largest ECM-associated HSPG, has a 467 kDa core protein composed of five domains and was first isolated from Engelbreth-Holm-Swarm tumor tissue (Hassell et al., 1980). The N-terminal domain contains three functional attachment sites for HS, domain II has four LDL receptor-like repeats, domain III shares similarity with the short arms of laminin chains, domain IV contains Ig-like repeats and domain V shows similarity to the large globular G domain located on the laminin A chain and on EGF motifs (Costell et al., 1997; Groffen et al., 1996; Noonan et al., 1991). Mutations in the mouse perlecan gene result in deterioration of basement membranes (BM) in regions exposed to intense mechanical stress such as the contractile myocardium and the expanding brain ventricles, showing that perlecan is crucial for maintaining BM integrity (Olsen, 1999; Costell et al., 1999). Mutations in the human perlecan gene cause two types of skeletal disorders: the relatively mild Schwartz-Jampel syndrome (SJS), which is defined by myotonia and chondrodysplasia, and the lethal neonatal dyssegmental dysplasia, Silverman-Handmaker type (DDSH). Both diseases show that perlecan plays a cardinal role in neuromuscular function and cartilage formation (Arikawa-Hirasawa et al., 2002; Arikawa-Hirasawa et al., 2001b) (Arikawa-Hirasawa et al., 2001a; Prabhu et al., 1998). Studies of embryogenesis have shown that perlecan expression appears early in tissues of vasculogenesis as the heart and major blood vessels (Handler et al., 1997).

![Figure 2. Schematic representation of the five HSPG subtypes. A total of thirteen distinct genes encode perlecan, agrin, collagen XVIII, four syndecans and six glypicans. The bottom panel provides the functional protein modules and HS attachment sites. Reproduced with permission from American Society for Clinical Investigation.](image-url)
Agrin
Agrin is the major HSPG identified in neuromuscular synapses and was originally identified as an extracellular matrix protein with the ability to promote the aggregation of acetylcholine receptors during synaptogenesis at the neuromuscular junction (Nitkin et al., 1987; Wallace, 1989; Hoch, 1999). The human agrin mRNA molecule encodes for a core protein of 212 kDa containing three HS GAG attachment sites (Tsen et al., 1995). The primary structure of agrin shows a laminin-binding domain, nine follistatin-like protease inhibitor domains, two laminin-like EGF repeats, two serine/threonine-rich domains, a SEA module, named after the three proteins sperm protein, enterokinase and agrin in which it was first recognized, four EGF repeats and three domains sharing homology with globules of laminin α chains (Groffen et al., 1998). Alternative splicing at several sites results in three different isoforms namely x-, y- and z-agrin (O'Toole et al., 1996; Gesemann et al., 1996). In mice the agrin gene encodes for two proteins with different NH₂ termini. These two isoforms, referred to as short and long NH₂ terminal subtypes, have a different tissue distribution and are thought to play distinct roles in the central nervous system (Burgess et al., 2000). Mutant mice lacking specific exons required for clustering of the acetylcholine receptor die perinatally. Additionally, axons of motor neurons do not stop growing, and differentiation of growth cones into presynaptic nerve terminals is disturbed in these animals (Gautam et al., 1996). Besides their role in clustering of acetylcholine receptors, agrin is ubiquitously present in the microvascular network of the brain, suggesting that it may be involved in the selective permeability and maintenance of the blood brain barrier (Donahue et al., 1999). Agrin is also highly expressed in the glomerular basement membrane of the kidney, suggesting that this HSPG contributes to glomerular function (Groffen et al., 1999).

Collagen XVIII
Recently, it was demonstrated that human collagen XVIII was the first member of the collagen family carrying heparan sulfate side-chains (Halfter et al., 1998; Saarela et al., 1998a). Three isoforms of this ECM-associated molecule have been identified with different signal peptides and various N-terminal regions (Oh et al., 1994; Rehn and Pihlajaniemi, 1994; Rehn and Pihlajaniemi, 1995). Human type collagen XVIII cDNA may encode for either 1516- or 1336-residue α1 (XVIII) chains (Saarela et al., 1998b). Collagen XVIII, which is a member of the non-fibrillar collagen family (Prockop and Kivirikko, 1995), became a topic of medical interest when a small 20 kDa C-terminal fragment, referred to as endostatin, turned out to be a potent anti-angiogenic factor with anti-tumor activity (O'Reilly et al., 1997). In the nervous system collagen XVIII is found in the basal lamina of blood vessels and Schwann cells and in the retina (Halfter et al., 1998). Mutations in the human gene encoding for the α1 chain of collagen XVIII were detected in patients with Knobloch syndrome. This autosomal recessive disorder is defined by the occurrence of severe myopia, vitreoretinal degeneration with retinal detachment, macular abnormalities and occipital encephalocele, demonstrating that it plays a pivotal role in the organization of the retina as well as in the closure of the neural tube (Sertie et al., 2000).
Syndecans
The second group of HSPGs species resides in the cell membrane and comprises the syndecans. Syndecan is derived from the Greek word syndein, which means, “to bind together”. The syndecan family includes four different members with highly conserved, relatively short cytoplasmic regions, which are capable of binding a variety of cytosolic regulatory factors (Carey, 1997; Rapraeger, 2001). The transmembrane domains are highly homologous and contain an unusual motif of alanines and/or glycines. At the junction between the ectodomain and the transmembrane region there is a cleavage site for proteases, which may cause shedding of the ectodomains from the cell surface. While syndecans contain several heparan sulfate chains, hybrid forms also carry chondroitin sulfate chains (Bernfield et al., 1992). After heparitinase and chondroitinase treatment the four types migrate in a SDS PAGE system with different sizes between ~35 and 120 kDa. Virtually all cell types express at least one syndecan family member, but most cells and tissues express multiple syndecans (Kim et al., 1994; Zimmermann and David, 1999). Syndecans, as well as glypicans, have been implicated in the regulation of neuritic outgrowth, a crucial step in the development of a functional neuronal network. These actions are mainly regulated by the ability of these HSPGs to bind specific growth factors including fibroblast growth factor (FGF) 1 and -2 and vascular endothelial growth factor (VEGF). This binding, which is predominantly realized through electrostatic interactions between the highly negatively charged sulfate group regions and the basic amino acids arranged on the protein, may function as a reservoir of FGF. On the other hand, FGFs require HSPGs as coreceptors to bind to and activate their cognate cell surface receptors (Halfter et al., 1997; Bovolenta and Fernaud-Espinosa, 2000).

Glypicans
Glypican was first cloned and characterized as a HSPG from human lung fibroblasts (de-Boeck et al., 1987; Anders et al., 1997; David et al., 1990). Currently, the glypicans family consists of six vertebrate, two Drosophila and one C. elegans member (De Cat and David, 2001). Glypicans are covalently linked to the cell surface via a glycosylphosphatidylinositol-anchor and are characterized by an N-terminal signaling sequence, a cysteine-rich globular domain and a hydrophobic C-terminal domain. The average size of the various glypican core proteins is approximately 60 kDa. The multiple GAG chains, which are close to the cell surface, are exclusively of the heparan sulfate type. In general, glypicans are predominantly expressed in the developing nervous system but are also produced by non-neural cells, except glypican-2, which is exclusively expressed in the developing brain. Glypican has been detected in the nuclear compartment, suggesting that they might directly regulate cell activity (Liang et al., 1997). Glypican-1 is strongly expressed in human pancreatic cancer and plays an essential role in responses of pancreatic cancer cells to certain mitogenic stimuli (Kleeff et al., 1998). Mutations in the gene that encodes glypicans results in the Simpson-Golabi-Behmel syndrome (SGBS) (Selleck, 1999; Pilia et al., 1996). This member of the glypican family closely interacts with insulin-like growth factor 2 (IGF2), ultimately leading to this human overgrowth syndrome.

During the last decade, much progress has been made in unraveling the functional roles of proteoglycans, and nowadays HSPGs have been linked to a number of
physiological and pathological processes including neurogenesis, development and regeneration of axonal outgrowth and synaptogenesis (De Cat and David, 2001; David et al., 1993; Bandtlow and Zimmermann, 2000; Yamaguchi, 2001; Small et al., 1996) (Karthikeyan et al., 1994; Lander et al., 1996), glomerular basement membrane permeability and integrity (Groffen et al., 1999), lipoprotein metabolism (Libeu et al., 2001), cancer and angiogenesis (Kleeff et al., 1998; Iozzo and San Antonio, 2001; Sasisekharan et al., 2002), wound healing (Bernfield et al., 1999) and Alzheimer’s disease (van Horssen et al., 2003; Snow and Wight, 1989). In the near future, further insights into the exact pathophysiological role of specific HSPGs will be provided by transgenic and knockout animal models.

**Distribution of heparan sulfate proteoglycans in Alzheimer’s disease brains**

The first reports on accumulation of GAGs in AD lesions were published in 1987. By using the (sodium sulfate) Alcian blue method, Snow and colleagues demonstrated the presence of sulfated GAGs in neuritic plaques, NFTs and CAA-affected vessels (Snow et al., 1987a). Later, cationic dyes like Cuprolinice blue and Ruthenium red were used to study the ultrastructural localization of sulfated GAGs in AD lesions (Young et al., 1989; Watson et al., 1997). Together, these histochemical and ultrastructural data suggest that sulfated GAGs may play a role in the pathogenesis of AD.

Snow and coworkers were the first to demonstrate the presence of HSPGs in both diffuse and neuritic plaques and cerebrovascular amyloid by using immunohistochemistry (Snow et al., 1988; Snow et al., 1990a). Additionally, colocalization of HS and HSPGs with neurofibrillary tangles, even during the earliest stages of neurofibrillary pathology was demonstrated (Perry et al., 1991). These studies were performed with antibodies recognizing either GAGs or core proteins of basement membrane HSPGs (Snow et al., 1988; Snow et al., 1994b; Young et al., 1989; Snow et al., 1990a; Perlmutter et al., 1990) (Su and Cummings, 1992). The exact specificity of these antibodies (often raised against HSPGs isolated from Engelbreth-Holm-Swarm (ESH) tumor (Snow et al., 1988; Snow et al., 1990a; Buee et al., 1993) or glomerular tissue (Perlmutter et al., 1990; Buee et al., 1993) was often not well documented (Snow et al., 1990a; Spillantini et al., 1999). Nowadays, a variety of well-characterized mono- and polyclonal antibodies is available. Herewith, it is possible to identify which specific HSPG subtypes are associated with various AD lesions.

**The role of heparan sulfate proteoglycans in Alzheimer’s disease pathology**

**Heparan sulfate proteoglycans and interaction with APP and Aβ**

It has been suggested that both intact HSPG and HSPG core protein bind to APP-695 with similar affinities (Narindrasorasak et al., 1991). In this respect, it is intriguing that this specific APP isoform is brain specific. It is conceivable that the interaction between APP and HSPGs might influence the generation of Aβ from APP. However,
the functional role of this interaction, with regard to the formation of Aβ in vivo, is largely unknown and remains to be elucidated yet. In fact, APP itself can occur in a CSPG form (Shioi et al., 1992; Shioi et al., 1993) and there are indications that APP might occur as an HSPG as well (Schubert et al., 1988). Until now, agrin and perlecan are the only HSPGs that have been shown to interact directly with the amyloid β protein (Castillo et al., 1997; Cotman et al., 2000) but a similar role has been suggested for glypican (Schulz et al., 1998). It was also found that glypican and a yet unidentified HSPG species bind with high affinity to APP (Williamson et al., 1995; Williamson et al., 1996; Buee et al., 1993; Lindahl et al., 1999).

The question remains whether deposition of HSPGs in AD lesions is preceded by accumulation of Aβ cq. hyperphosphorylated tau or vice versa. The finding that in adolescent brains of patients with Down syndrome antibodies directed against HSPGs clearly stained diffuse primitive senile plaques strongly indicates that HSPG accumulation is an early event in the formation of senile plaques (Snow et al., 1990a). In addition, accumulation of GAGs occurred virtually at the same time and at the same location as amyloid deposition in experimental amyloidosis (Snow and Kisilevsky, 1985). One explanation for these findings could be that the presence of amyloid interferes with HSPG biosynthesis.

Diffuse SPs, which are primarily composed of non-fibrillar Aβ, are thought to gradually transform into classic SPs with a central core of fibrillar amyloid, surrounded by a halo of reactive astrocytes and glial cells (Selkoe, 1991; Dickson, 1997). It has been demonstrated that both perlecan and agrin promote the conversion of non-fibrillar Aβ into fibrillar Aβ and may thus contribute to the enhanced neurotoxic properties of Aβ (Castillo et al., 1997; Snow et al., 1994a; Cotman et al., 2000). It is yet unknown if other HSPG subtypes have similar catalytic properties.

**Heparan sulfate proteoglycans and resistance to Aβ degradation**

When HSPGs and GAGs are bound to basic fibroblast growth factor (bFGF) the latter is protected against proteolytic degradation by extracellular proteases (Saksela et al., 1988). Likewise, HSPGs might protect Aβ against proteolytic degradation. In 1995, Gupta-Bansal and colleagues demonstrated that both ESH tumor-associated HSPGs and CSPGs isolated from bovine nasal septum cartilage inhibit proteolytic breakdown of fibrillar Aβ. These PGs may thus contribute to the accumulation and persistence of SPs (Gupta et al., 1995). In particular agrin may act this way, because this HSPG contains nine follistatin-like protease inhibitor domains (Groffen et al., 1998; Biroc et al., 1993). HSPGs and CSPGs could function as chaperones forming a protective shield against enzymatic attacks by proteases. Besides degradation by naturally occurring proteases, microglial uptake and subsequent degradation may mediate Aβ clearance. It has been suggested that microglial cells in the vicinity of SPs are capable of phagocytic uptake of fibrillar Aβ. In this respect, CSPGs were shown to inhibit the removal of Aβ by microglia (Shaffer et al., 1995). Whether HSPGs have a similar effect is not yet clear. Conversely, it was shown that Aβ1-40 inhibited the
activity of hepanasases and thereby prevented proteolytic breakdown of HSPGs (Bame et al., 1997). This may indirectly contribute to the stability and persistence of SPs.

In conclusion, HSPGs may a) bind to Aβ b) catalyze the polymerization of Aβ and thereby contribute to formation of neurotoxic fibrillar Aβ deposits and c) serve as chaperone proteins and thereby protect Aβ accumulations against proteolytic cleavage (Figure 3).

**Figure 3. The putative role of heparan sulfate proteoglycans in amyloid deposition**

Schematic overview of the putative role of heparan sulfate proteoglycans in Aβ fibril formation and stabilization. Aβ naturally forms large Aβ fibrils. This fibrillation process is accelerated by heparan sulfate proteoglycans, which function as catalyst. On the other hand, heparan sulfate proteoglycans protect Aβ against proteolytic degradation by forming a protective shield around the fibrils. Since, CSPGs have been demonstrated to inhibit the removal of Aβ by microglia, such a role can probably also be attributed to HSPGs. Additionally, Aβ 1-40 inhibits the degradation of HS by hepanasases and thus indirectly leads to the persistence and stability of SPs. Finally, Aβ deposits in the brain parenchyma and vessel wall may lead to neurotoxicity and vascular degeneration, respectively.

**Interaction of heparan sulfate proteoglycans with tau protein**

The close association between sulfated GAGs and neurofibrillary tangles was already described in 1987 (Snow et al., 1987b), but it was not until 1996 when Goedert et al unraveled the possible role of these complex structures in the formation of tangles (Goedert et al., 1996). They showed that sulfated GAGs had the ability to induce the formation of paired helical filaments. Moreover, the presence of sulfated GAGs increased the ability of several kinases to phosphorylate tau protein, causing tau to dissociate from microtubules. Additionally, when highly sulfated GAGs bind to the microtubule-binding repeat region of the tau protein, binding of tau to microtubules is prevented. Thus GAGs might cause a conformational change of the tau protein, promoting microtubule disassembly and polymerization of tau molecules, ultimately leading to the formation of insoluble paired helical filaments (Hasegawa et al., 1997; Hernandez et al., 2002; Perry et al., 1991). Finally, HSPGs may play an important
role in the stabilization of tau deposits by providing protection against proteolytic degradation and removal (Snow and Wight, 1989).

**Other proteoglycans associated with Alzheimer’s disease lesions**

Besides HSPGs, several other proteoglycans accumulate in SPs, NFTs and vascular amyloid. Decorin, a small dermatan sulfate proteoglycan, weakly binds to Ab and clearly localizes to SPs and tangles (Snow et al., 1995; Snow et al., 1992). In contrast to HSPGs, which are evenly distributed over the neuritic plaque, decorin was primarily localized in the periphery of senile plaques. This staining pattern may indicate that the deposition of decorin is associated with later stages of senile plaque development, or is part of a secondary reaction to the plaques.

Chondroitin sulfate (CS) core proteins have not been identified in AD lesions yet. However, immunohistochemical analysis demonstrated the presence and specific distribution of three different chondroitin sulfates (CS) side-chains in SPs and NFTs. Chondroitin-4-sulfate was localized in both SPs and NFTs, whereas chondroitin-6-sulfate was predominantly observed in NFTs and in the area around neuritic plaques. In addition, unsulfated chondroitin was present in tangles and dystrophic neurites. It has been demonstrated that CS is able to mediate neuritic dystrophy and thereby contributes to the pathogenesis of senile plaques (DeWitt et al., 1993).

Keratan sulfate proteoglycans (KSPGs) are the last group of proteoglycans associated with SPs. Antibodies directed against synaptic vesicle KSPG/KS clearly stained synapses and dystrophic neurites in the circumference of neuritic plaques. Conversely, KSPG/KS were not found in diffuse plaques, cerebrovascular amyloid and tangles in AD brains (Snow et al., 1996). Staining for synaptic vesicle-associated KSPGs showed a reduced immunoreactivity in AD neocortex compared to age-matched controls, probably as a result of decreased synaptic density in AD brains. Additionally, radiochemical experiments demonstrated that the amount of highly sulfated KS in AD cerebral cortex was reduced to even less than half of the control values. On the other hand, monoclonal antibodies directed against less sulfated structures did not show any alterations (Lindahl et al., 1996).

Taken together, the above-mentioned studies have demonstrated variable expression of four distinct classes of PGs/GAGs in association with NFTs, SPs and CAA. KSPG and DSPG as well as CS decorated the periphery of senile plaques, whereas HSPGs are so far the only group of PGs associated with all three AD lesions. These data suggest that this particular subset of PGs/GAGs plays a pivotal role in the formation and persistence of these lesions. The exact function of KSPGs, DSPGs and CSPGs in the pathogenesis of tangles and CAA remains to be elucidated, preferably in cultured cell systems or experimental animal models.
Heparan sulfate proteoglycan involvement in other amyloidoses and in prion diseases

Heparan sulfate proteoglycans in amyloidoses
The first reports on the association of sulfated GAGs with amyloidoses date back to the 1960s and early 70s (Snow et al., 1989; Bitter and Muir, 1966; Bitter and Muir, 1965; Dalferes, Jr. et al., 1967; Pennock, 1968; Pennock et al., 1968; Pras et al., 1971; Sasai, 1970; Berenson et al., 1969; Pomerance et al., 1976). GAGs have consistently been found in all kinds of amyloid (e.g. AA or AL amyloidosis) regardless the type of amyloidogenic protein deposited (Hasegawa et al., 1997; Snow and Wight, 1989; Snow and Kisilevsky, 1985; Snow et al., 1987a; Snow et al., 1987b). Young and colleagues showed a distribution pattern of highly sulfated PG in fibrillar Aβ deposits that was identical to amyloid fibrils of four unrelated amyloidoses (Young et al., 1989). HSPG accumulation in amyloidotic lesions seemed to occur after initial deposition of amyloid, but in an early stage already. This close spatiotemporal relationship between PGs and diverse amyloid proteins suggests that PGs play a general role in amyloidogenesis. The availability of well-defined antibodies directed against specific proteoglycan subtypes might shed light onto the distribution pattern of these molecules in several types of amyloidoses.

Heparan sulfate proteoglycans in prion diseases
The amyloid depositions found in prion diseases are composed of protease-resistant prion protein (PrP). Already in 1989, Snow and his colleagues demonstrated the presence of sulfated GAGs in amyloid plaques in prion diseases like Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome, kuru and scrapie (Snow et al., 1990b) Additionally, immunohistochemical surveys, using antibodies directed against HS and HSPG, revealed that PrP plaques contain HSPGs as a common constituent (Snow et al., 1990b; McBride et al., 1998). Interestingly, polysulfated compounds were able to inhibit deposition of PrP in scrapie-infected neuroblastoma cells, suggesting that these inhibitors were capable of blocking the interaction between PrP and endogenous HS/HSPG (Caughey and Raymond, 1993). For a further understanding of the role of HSPGs in the process of prion disease, elucidation of the interactions between PrP and HSPGs are important. Recently, Warner et al. identified several regions of the cellular prion protein with HS binding sites (Warner et al., 2002). More research is needed not only to identify which HSPG subtypes accumulate in PrP plaques, but also to gain insight into the functional role of HS/HSPGs in the pathogenesis of prion diseases.

Heparan sulfate proteoglycans in α-synucleinopathies and tauopathies
Parkinson’s disease and dementia with Lewy bodies are characterized by the presence of Lewy bodies and Lewy neurites, which are composed of α-synuclein aggregates (Baba et al., 1998). It has been indirectly shown that, based on the binding of basic fibroblast growth factor to heparinase-sensitive sites, Parkinson’s disease-associated Lewy bodies contain HSPGs (Perry et al., 1992). Moreover, several chondroitin sulfate proteoglycans colocalize with both Lewy bodies and Lewy neurites (DeWitt et al., 1994). GAGs, in particular heparan sulfate, have the ability to induce the formation of α-synuclein fibrils in vitro (Cohlberg et al., 2002). HSPGs and
HS were present in a variable fraction of tangles associated with progressive supranuclear palsy, Pick’s disease and other tauopathies (Spillantini et al., 1999; Perry et al., 1992; Odawara et al., 1998). Moreover, antibodies directed against chondroitin sulfate proteoglycans decorated reactive astrocytes in the caudate nucleus of Huntington’s disease brains (DeWitt et al., 1994). Together, these findings demonstrate HSPGs/HS are differentially expressed in a variety of neurodegenerative disorders with intracellular amyloid protein deposits.
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Chapter 2


Chapter 2


Chapter 2


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Het bekijken van histologische coupes is in essentie kijken naar een patroon van genexpressie, op een topografische controleerbare wijze
Chapter 3

Heparan sulfate proteoglycan expression in cerebrovascular amyloid β deposits in Alzheimer's disease and hereditary cerebral hemorrhage with amyloidosis (Dutch) brains

Jack van Horssen¹, Irene Otte-Höller¹, Guido David², Marion L.C. Maat-Schieman³, Lambert P.W.J. van den Heuvel⁴, Pieter Wesseling¹,⁵, Robert M.W. de Waal¹, Marcel M. Verbeek¹,⁵

From the Departments of Pathology¹, Pediatrics⁴, Neurology⁵, University Medical Center, Nijmegen, The Netherlands; The Center for Human Genetics², University of Leuven and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium; Department of Neurology³, Leiden University Medical Center, The Netherlands.

Abstract

Cerebrovascular deposition of amyloid β protein (Aβ) is a characteristic lesion of Alzheimer’s disease (AD) and hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D). Besides Aβ, several other proteins and proteoglycans accumulate in CAA. We have now analyzed the expression of the heparan sulfate proteoglycan subtypes agrin, perlecan, glypican-1, syndecans 1-3 and HS GAG side-chains in CAA in brains of patients with AD and HCHWA-D. Here, specific well-characterized antibodies directed against the core protein of these HSPGs and against the glycosaminoglycan (GAG) side-chains were used for immunostaining. Glypican-1 was abundantly expressed in CAA both in AD and HCHWA-D brains, whereas perlecan and syndecans-1 and -3 were absent in both. Colocalization of agrin with vascular Aβ was clearly observed in CAA in HCHWA-D brains but only in a minority of the AD cases. Conversely, syndecan-2 was frequently associated with vascular Aβ in AD, but did not colocalize with vascular Aβ deposits in HCHWA-D. The three different syndecans, agrin, glypican-1 and HS GAG, but not perlecan, were associated with the majority of senile plaques in all brains. Our results suggest a role for agrin in the formation of SPs and of CAA in HCHWA-D, but not in the pathogenesis of CAA in AD. Both syndecan-2 and glypican, but not perlecan, may be involved in the formation of CAA. We conclude that specific HSPG species may be involved in the pathogenesis of CAA in both AD and HCHWA-D, and that the pathogenesis of CAA and senile plaques may differ with regard to the involvement of HSPG species.


Introduction

The most frequent cause of dementia in the elderly is Alzheimer’s disease (AD), a progressive neurodegenerative disorder characterized by the presence of neurofibrillary tangles (NFTs), senile plaques (SPs) and cerebral amyloid angiopathy (CAA) [42]. NFTs are intraneuronal accumulations composed of hyperphosphorylated tau protein [28]. The major component of both senile plaques and CAA is the amyloid β protein (Aβ), a 39- to 42-amino-acid protein formed by proteolytic processing of the amyloid β precursor protein [17].

Deposition of Aβ can be observed in the media and adventitia of cortical and medium-sized and small leptomeningeal vessels. Vascular amyloid is also found in the brains of individuals with Down’s syndrome and with hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D) [32]. HCHWA-D is a rare autosomal dominant amyloid β disease with extensive deposition of amyloid β protein in the brain vasculature and of diffuse SPs in the brain parenchyma. Immunohistochemical analysis has shown that CAA in AD and HCHWA-D consists of both Aβ1-40 and Aβ1-42/43 [18,23,32]. In advanced stages of CAA, Aβ1-40 may become the predominant species, but Aβ1-42 is the isoform that is initially deposited [2,43].

Besides Aβ itself, several Aβ-associated proteins such as inflammatory proteins [57], apolipoprotein E (ApoE) [35,41,48] and heparan sulfate proteoglycans (HSPGs) [37,45,46,56,58] have been identified in senile plaques. HSPGs are a heterogeneous family of biological macromolecules consisting of linear sulfated polysaccharides, covalently linked to a protein core. Apart from their chemistry they can be divided into two classes depending on their localization in tissues. The first type of HSPG species resides in the cell membrane and includes the syndecans and glypicans, which differ in core protein structure and type of attachment to the cell membrane [8]. Syndecans have a transmembrane and cytoplasmatic domain [4], whereas the glypicans are anchored to membrane lipids via glycosylphosphatidylinositol [27]. The second class of HSPGs contains molecules associated with the extracellular matrix, such as agrin and perlecan. Perlecan was first isolated from the Engelbreth-Holm-Swarm tumor in 1980 [21] and consists of a large core protein, carrying three heparan sulfate side-chains [6]. Agrin, one of the major basement membrane-derived HSPGs of the brain, plays an important role in the synaptogenesis at the neuromuscular junction [36,60].

Previously, we investigated the expression of several HSPGs in cerebral senile plaques and NFTs [58]. We observed that agrin is the major HSPG present in SPs and NFTs, whereas glypicans and syndecan are less frequently associated with these lesions, and perlecan is entirely absent. Furthermore, our data suggested that glycosaminoglycan side-chains of the HSPGs might play an important role in the formation and persistence of both senile plaques and neurofibrillary tangles.
Although both SPs and CAA primarily consist of Aβ, the pathogenesis of senile plaques differs from that of CAA in many aspects, e.g. Aβ isoform composition, degree of Aβ fibrillization and the toxic effects of Aβ towards vascular cells and neurons [57]. Furthermore, it has been demonstrated in previous studies that HSPGs are present in CAA, but the specific nature of these HSPGs has not been well documented [45,46]. Therefore, it was the aim of the present study to examine the association of different HSPGs with CAA. We used a panel of well-characterized antibodies directed against the core proteins of syndecans 1-3, glypican-1, perlecain and agrin, as well as antibodies directed against heparitinase-digested HSPGs and the glycosaminoglycan side-chains of HSPGs. In this way, the role of these biological macromolecules in CAA of AD and HCHWA-D brains was investigated and a comparison with the expression of these HSPG species in senile plaques could be made.

Materials and Methods

Autopsy Material
Tissue samples from frontal, temporal, parietal and occipital neocortex were obtained after a rapid autopsy procedure and immediately frozen in liquid nitrogen. Seven AD patients (4 male, 3 female) were selected for the presence of CAA (age 81.6 ± 8 years; post mortem delay 3.4 ± 0.9 hours). Three cases without neurological disease (age 83 ± 1.5 years; post mortem delay 3 ± 1 hours) were selected as controls. Additionally, serial sections from frontal neocortex of four HCHWA-D patients (1 male, 3 female) (age 51.2 ± 2.4 years; post mortem delay 10.3 ± 6.2 hours) were available.

Antibodies
Primary antibodies and their sources are listed in Table 1. We used biotin-labeled horse anti-mouse as secondary antibody with the exception of primary incubations with BL31 (biotin-labeled donkey anti-goat), 1948 (biotin-labeled rabbit anti-rat) and JM13 and JM403 (biotin-labeled goat anti-IgM). All secondary antibodies and the avidin-biotin-peroxidase complex (ABC) were obtained from Vector (Burlingame, CA).

Immunohistochemistry
To examine the expression patterns of the various HSPGs in CAA in AD and HCHWA-D brains, large numbers of serial sections (4 μm) were used. At regular intervals sections were stained with anti-Aβ, to allow for a comparison with the HSPG stainings. Serial cryosections of the four cortical regions were air-dried, fixed in acetone for 5 minutes and subsequently for 5 minutes in acetone containing 0.15% H2O2 to block endogenous peroxidase activity. In case of staining with 3G10, a monoclonal antibody directed against heparitinase-digested HSPGs, sections were pretreated with 50 mU heparitinase (heparinase III, EC 4.2.2.8; Sigma Chemical Co., St Louis, MO) diluted in 10 mM HEPES and 2 mM CaCl2 (pH 7.0) at 37 °C for 1 hour. Then, sections were preincubated for 30 minutes with 20% animal serum, the type of which was determined by the specific secondary antibody used. Afterwards, sections were incubated overnight at 4 °C with the appropriate primary antibody (Table 1). Subsequently, the sections were incubated with the secondary biotin-
labeled antibody for one hour at room temperature and with ABC according to the manufacturer's description. Diaminobenzidine (DAB) was used as chromogen. Between the incubation steps, sections were thoroughly washed with PBS. Copper sulfate (0.5%) in saline solution was used to enhance DAB-staining. After a short rinse in tap water the preparations were incubated with hematoxylin for 3 minutes and extensively washed with tap water for 10 minutes. Finally, sections were dehydrated with ethanol followed by xylol. All antibodies were diluted in PBS containing 0.1% bovine serum albumin, which also served as a negative control. In each series one 10 µm section was used for Congo red staining. For this staining, acetone-fixed sections were dehydrated with increasing concentrations of ethanol (0-80%) and subsequently preincubated in a solution of 3% NaCl in 80% ethanol. Finally, sections were incubated in the same solution containing 0.5% Congo red and counterstained with hematoxylin.

<table>
<thead>
<tr>
<th></th>
<th>Specificity</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>6C6</td>
<td>Aβ</td>
<td>1:400</td>
<td>Elan pharmaceuticals</td>
</tr>
<tr>
<td>AT8</td>
<td>Tau</td>
<td>1:200</td>
<td>Innogenetics</td>
</tr>
<tr>
<td>JM 72</td>
<td>Agrin (core protein)</td>
<td>1:500</td>
<td>[53]</td>
</tr>
<tr>
<td>BI 31</td>
<td>Agrin (core protein)</td>
<td>1:750</td>
<td>[54]</td>
</tr>
<tr>
<td>95J10</td>
<td>Perlecan (core protein)</td>
<td>1:500</td>
<td>[19]</td>
</tr>
<tr>
<td>1948</td>
<td>Perlecan (core protein)</td>
<td>1:500</td>
<td>Chemicon</td>
</tr>
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<td>3G10</td>
<td>Pan-HSPG</td>
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<td>[9]</td>
</tr>
<tr>
<td>JM13</td>
<td>Heparan sulfate side-chains</td>
<td>1:100</td>
<td>[52]</td>
</tr>
<tr>
<td>JM403</td>
<td>Heparan sulfate side-chains</td>
<td>1:300</td>
<td>[51]</td>
</tr>
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<td>10H4</td>
<td>Syndecan-2 (ectodomain)</td>
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<td>[10,30]</td>
</tr>
<tr>
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<td>Syndecan-1,-3 (cytoplasmatic domain)</td>
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</tr>
<tr>
<td>1C7</td>
<td>Syndecan-3 (ectodomain)</td>
<td>1:100</td>
<td>[30,31]</td>
</tr>
<tr>
<td>S1</td>
<td>Glypican-1 (core protein)</td>
<td>1:50</td>
<td>[11,12]</td>
</tr>
</tbody>
</table>

**Table 1.**

Primary antibodies used in this study

Fractions positive in immunostaining (percentage of amyloid laden vessels stained by a specific antibody compared with the anti-Aβ stainings) were independently scored in any of the following categories 0, 0-50, 50-100, or 100%, by two of the authors (JvH and MMV). When the scores differed more than one scale unit consensus was obtained after re-evaluation.
Results

CAA in AD

6C6, a monoclonal antibody directed against Aβ was used to study Aβ expression in HCHWA-D brains and in four different regions of the AD brain. Both cortical and leptomeningeal vessels were affected by deposition of Aβ in the vascular wall and were strongly stained by mAb 6C6. In large leptomeningeal vessels, compact Aβ deposits were localized in the outer media at the junction with the adventitia. Cortical and smaller leptomeningeal vessels showed Aβ accumulation throughout the blood vessel wall. Besides the amyloidogenic vessels, also senile plaques were strongly stained by the anti-Aβ mAb 6C6. Additionally, Congo red staining revealed that mAb 6C6-positive vessels contained fibrillar Aβ (not shown). Although CAA was slightly more abundant in the occipital part of AD brains, only minor quantitative differences between the four examined regions were observed.

Agrin

JM-72 and BL-31, two antibodies directed against the core protein of agrin, showed similar staining patterns. When compared with Aβ-staining, nearly all senile plaques were intensely stained by both JM-72 and BL-31. Only in a minority of the sections (15%) colocalization of agrin with Aβ deposits in some leptomeningeal and cortical vessels was observed (Figure 1b), whereas in a majority of the patients no expression (Figure 1d) or only a very weak association of agrin with Aβ in the vascular wall was observed. However, the basement membranes of Aβ-negative cerebral blood vessels were prominently stained. No significant differences in intensity and frequency of agrin expression in CAA were found within one patient among the four examined regions. The results of the immunohistochemical staining for agrin and other HSPGs in AD brains are summarized in Table 2.

<table>
<thead>
<tr>
<th>HSPG species</th>
<th>normal vessels</th>
<th>CAA in AD</th>
<th>CAA in HCHWA-D</th>
<th>SPs in AD</th>
<th>SPs in HCHWA-D</th>
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<tbody>
<tr>
<td>Agrin</td>
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<td>-/+/-/+3</td>
<td>++/-3</td>
<td>++/++++</td>
<td>++/+++</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
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<td>+</td>
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<td>-</td>
<td>+/++++</td>
<td>+</td>
</tr>
<tr>
<td>Syndecan-3</td>
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<td>-/+</td>
<td>-/+</td>
<td>+/++++</td>
<td>+</td>
</tr>
<tr>
<td>Glypican-1</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>++/+++</td>
<td>++</td>
</tr>
<tr>
<td>HS GAG</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>HS Stub</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Table 2. Expression of HSPGs in normal vessels, CAA and SPs

Positive fractions (percentage of amyloid laden vessels stained by a specific antibody compared with the anti-Aβ stainings) were scored in the following categories: 0 (-), 0-50% (+), 50-100% (++) or 100% (+++), by two of the authors (JvH and MMV). 1 Restricted to staining of the basement membrane. 2 Restricted to staining of vascular Aβ. 3 Expression varied between the different patients.
Perlecan
Similar to the results with the anti-agrin antibodies, the two antibodies directed against different epitopes of perlecan also strongly stained the basement membranes of the cerebral vessels (Figure 1f). However, neither 95J10 nor 1948 stained either amyloidogenic vessels (Figure 1f) or senile plaques.

![Image](image_url)

**Figure 1.** Immunohistochemical staining of cerebral amyloid angiopathy in serial sections from AD brain for A\(\beta\) (mAb 6C6) (a,c,e), agrin (mAb JM72) (b,d) and perlecan (mAb 95J10) (f). Frequently, agrin was not expressed in CAA (d). Sporadically agrin (mAb JM72) colocalized with A\(\beta\) deposits in the vessels (b). Cerebral senile plaques were also stained by mAb JM72 (b and d). In all cases perlecan (mAb 95J10) was not associated with vascular A\(\beta\) (f), whereas mAb 95J10 did stain the basement membrane in cerebral (micro)vasculature (f). Original magnifications: a-f x250
**Syndecans**

Three antibodies directed against different members of the syndecan family, 2E9 (anti-syndecan-1,-3), 10H4 (anti-syndecan-2) and 1C7 (anti-syndecan-3), were used to investigate expression of these cell-membrane-associated HSPGs in CAA. Staining by 10H4 demonstrated expression of syndecan-2 in CAA in a majority of the cases (Figure 2b). In two patients only a weak association of syndecan-2 with vascular Aβ was observed (not shown). Furthermore, colocalization of syndecan-2 with senile plaques, especially the amyloid core, was found (not shown). Syndecan-2 expression was absent in unaffected cerebral vessels. Syndecan-3 rarely colocalized with deposits in the vascular wall, as demonstrated by 1C7 staining. However, 1C7 stained cerebral senile plaques and endothelial cells (Figure 2d). Reactive astrocytes, especially those surrounding senile plaques and amyloidotic vessels, were intensely stained by 1C7 (Figure 2d). In all cases 2E9 neither stained CAA nor Aβ-negative vessels (Figure 2f), whereas a variable fraction of cerebral senile plaques was stained.

![Syndecan Images](image-url)
Figure 2.  
Immunohistochemical staining of cerebral amyloid angiopathy in serial sections from AD brain for Aβ (mAb 6C6) (a,c,e,g), syndecan-2 (mAb10H4) (b), syndecan-3 (mAb 1C7) (d), syndecan-1,3 (mAb 2E9) (f) and glypican-1 (mAb S1) (h). Both syndecan-2 (b) and glypican-1 (h) accumulate in the amyloidotic vessels, whereas syndecan-1,3 does not colocalize with vascular Aβ (f). Syndecan-3 is not expressed in amyloidotic vessels. Syndecan-3 is strongly expressed by reactive astrocytes associated with CAA and by endothelial cells (d). Original magnifications: a-h x250

Glypican-1
In the present study we used one anti-glypican antibody, mAb S1. This antibody, directed against the glypican core protein, stained a majority of the senile plaques. In particular, the amyloid cores of the senile plaques were strongly stained. In all cases glypican-1 expression was observed in CAA (Figure 2h), although not always with the same intensity. Aβ-negative cerebral blood vessels remained unstained.
HS GAGs
Three different antibodies were used to investigate the expression of HS GAG side chains in AD brains: JM403 and JM13, both recognizing different epitopes in the HS polysaccharide side chain and 3G10, an antibody directed against heparitinase-digested HSPGs. All three antibodies stained cerebral senile plaques and normal blood vessels. Additionally, 3G10 generally demonstrated intense staining of CAA in AD brains (Figure 3b). JM13 and JM403 also frequently stained the vascular Aβ deposits (Figure 3d) (approximately 75% of the vessels stained by 6C6 were JM403/13 positive), although not with the same intensity as observed with 3G10.

Figure 3.
Immunohistochemical staining of cerebral amyloid angiopathy in serial sections from AD brain for Aβ (mAb 6C6) (a,c), heparitinase-digested HSPG (mAb 3G10) (b) and HS GAG side-chains (JM403) (d). Original magnifications: a-d x250
Expression of HSPGs in HCHWA-D brain

Expression in senile plaques
Senile plaques in HCHWA-D brain are predominantly of the diffuse type. Only sporadically classic senile plaques were observed by using 6C6 and Congo red staining. We observed many similarities in the expression of HSPGs in HCHWA-D and in AD senile plaques. Virtually all senile plaques, as compared to 6C6 staining (Figure 4a), were immunopositive for agrin (Figure 4b) and HS GAG (not shown). Furthermore, all syndecan and glypican-1 antibodies stained senile plaques albeit less intense than the senile plaques in AD brains. Like in AD brains, perlecan expression in senile plaques in HCHWA-D brains was not observed.

Expression in CAA
In general, the expression of HSPGs in CAA of HCHWA-D brains was similar to that in CAA of AD brains. For example, like in AD brains, both perlecan and syndecan-1,-3 did not colocalize with vascular Aβ in this specific type of CAA, whereas HS GAGs and glypican-1 did. Occasionally, colocalization of syndecan-3 with vascular Aβ deposits was observed (data not shown). In contrast to the absence of agrin in most cases of CAA in AD, we found that agrin clearly colocalized with vascular Aβ in three HCHWA-D brains (Figure 4b). In two of these patients agrin was expressed in approximately 70 percent of the amyloid laden vessels, whereas in one patient all amyloid laden vessels were stained by mAb JM72. Furthermore, no expression of agrin was observed in amyloidogenic vessels of the 4th patient. Finally, syndecan-2 expression in CAA of HCHWA-D brains was absent (Figure 4c), whereas in AD brains syndecan-2 frequently colocalized with vascular Aβ deposits. A summary of immunohistochemical stainings for HSPGs on HCHWA-D brain tissue sections is listed in Table 2.
Discussion

Relatively little attention in AD research has been given to the pathogenesis of CAA, probably because this lesion was considered to be of minor importance. Nevertheless, the accumulation of Aβ in the vascular wall leads to degeneration of endothelial cells, vascular smooth muscle cells and pericytes, ultimately resulting in impaired functioning of the blood-brain barrier [24], capillary obliteration and a compromised cerebral blood flow [38,61]. It has been demonstrated that HSPGs are able to form complex structures with Aβ and therefore inhibit the proteolytic breakdown [7,20]. These findings strongly indicate that the interaction between Aβ and HSPGs may be important for the progression of Aβ depositions in patients with AD. Furthermore, immunohistochemical studies have demonstrated an association of HSPGs with senile plaques, neurofibrillary tangles and CAA [37,45,46,56,58,]. In these studies, antibodies directed against the GAG side-chains of HSPGs were used. In addition, antibodies against HSPGs purified from different sources have been employed. However, in most of these studies the exact nature of the HSPG species to which the antibody was raised, was unknown. We used a panel of well-characterized antibodies, directed against different HSPGs, to investigate the expression of various HSPG species in the characteristic AD lesions, with a focus on CAA.

We demonstrated that agrin is variably expressed in CAA, which is unexpected in view of recent findings from our laboratory and from others [13] that agrin was the major HSPG deposited in SPs and NFTs in AD brains [58]. It was reported that agrin is able to accelerate Aβ fibril formation, to bind fibrillar Aβ via a mechanism that involves the HS GAG side-chains of agrin, and protect Aβ (1-40) from proteolysis [7]. All these studies implicated an important role for agrin in the pathogenesis of SPs.
and NFTs. However, a similar role for agrin in the pathogenesis of CAA seems to be unlikely, given the frequent absence of agrin expression in this lesion.

Several previous studies reported that perlecan is expressed in SPs, NFTs and CAA [45]. Furthermore, based on animal studies, it has been suggested that perlecan may affect Aβ fibrillization [47]. However, in contrast to these data, we recently demonstrated, and confirmed, that perlecan is not present in SPs and NFTs [58]. In the current study we describe absence of perlecan expression in CAA. The monoclonal anti-perlecan antibodies 95J10 and 1948 strongly stained normal cerebral blood vessels, indicating that the absence of staining of CAA is not due to a lack of sensitivity. Our data are in conflict with those of Snow et al. who reported immunostaining of CAA with an antibody, HK102, raised against mouse perlecan [45]. However, we previously demonstrated that this antibody only reacts with mouse perlecan and does not cross-react with human perlecan [58]. In conclusion, we did not find any evidence that perlecan is involved in any of the characteristic lesions of the AD brain, i.e. CAA, SPs or NFTs.

In our previous study we demonstrated that the syndecans 1-3 and glypican-1 are all, to a variable degree, expressed in SPs. Here we describe that only syndecan-2 and glypican-1 colocalize with vascular Aβ, indicating that they may be involved in the pathogenesis of CAA. These data are in disagreement with those of Van Gool et al. [56], who observed absence of syndecan-2 and glypican-1 expression in CAA. This discrepancy might be explained by the fact that these authors used tissue that was formalin-fixed and paraffin- embedded, which may have resulted in the destruction of antigenic epitopes. The absence of the syndecans-1 and -3 in CAA indicates that these HSPGs do not afflict the pathogenesis of CAA. The similarity between the expression profiles of glypican-1 and syndecan-2 in CAA and SPs is remarkable, since both HSPGs were predominantly observed in the amyloid cores of the cerebral classic SPs. Both the Aβ deposits in the core of the classic SPs and those in the vascular wall primarily consist of fibrillar Aβ, suggesting that syndecan-2 and glypican-1 are particularly associated with fibrillar Aβ. However, both HSPGs are also expressed in diffuse SPs, which predominantly consist of non-fibrillar Aβ. Future in vitro studies may elucidate the association of syndecan-2 and glypican-1 with different Aβ-isoforms.

Castillo et al. demonstrated that sulfated GAGs not only have the capacity of binding to Aβ, but more essentially have the ability to enhance amyloid fibril formation, an important key event in amyloidogenesis [5]. Furthermore, Kisilevsky et al. observed that some low-molecular-weight anionic sulfate compounds that interfere with the interaction of HSPG with Aβ are able to inhibit Aβ fibril aggregation [26]. Snow et al., demonstrated immunoreactivity in SPs and CAA using an antibody (HK-249) directed against an epitope in the HS side-chain of the HSPG [46]. These observations are in line with our results, since JM13 and JM403, both antibodies directed against the HS GAG side-chain, also stained SPs and CAA. From these data, we conclude that GAG side-chains are involved in the pathogenesis of CAA, similar to their role in the pathogenesis of SPs [46]. Furthermore, it is likely that the GAG side-chains, accumulating in vascular amyloid, are predominantly associated with glypican-1 and syndecan-2, or other as yet unidentified HSPG species.
Van Duinen et al. [55] described the expression of various extracellular matrix deposits in HCHWA-D brains. More specifically, they found that a monoclonal antibody directed against HSPG was unreactive or only weakly reactive with vascular Aβ. Furthermore, they demonstrated colocalization of HSPG with only a minority of the senile plaques, suggesting that HSPGs are present in small quantities only, if at all, in both vascular amyloid and Aβ-positive diffuse plaques. In contrast to their observations, we observed expression of agrin, glypican-1 and HS GAG side-chains in amyloid-laden vessels, suggesting a possible role of these HSPGs in the formation of CAA in HCHWA-D. Similarly, we demonstrated the presence and, therefore, a possible involvement of several HSPGs in diffuse senile plaques in HCHWA-D brains.

Aβ accumulating in HCHWA-D brain differs from Aβ in AD brain, because of a point mutation in the gene encoding for the amyloid β precursor protein [29,50]. Theoretically, this may lead to a change in the specific interaction between Aβ and HSPG molecules, and explain the observed differences in HSPG deposition in CAA between HCHWA-D and AD patients. Changes in Aβ-HSPG interactions due to structural alterations in the Aβ peptide have been described [34], but the interaction of Aβ, carrying the HCHWA-D mutation, with HSPG remains unstudied so far.

The cellular source of Aβ in CAA is not yet known. Aβ may be produced locally in the vascular wall by endothelial cells, pericytes or smooth muscle cells, but may be derived from the circulation as well. Alternatively, as suggested by Weller et al. [62], Aβ, produced by neurons, may be transported by interstitial fluid drainage towards the vascular wall. This hypothesis is supported by Calhoun et al., who have shown in APP transgenic mice that a neuronal source of APP is sufficient to induce CAA, demonstrating that vascular APP production is not required for vascular Aβ formation [3]. Similarly, HSPGs may be produced either locally or by neurons and astrocytes and subsequently transported to the vasculature by this mechanism. Additional research is needed to elucidate the cellular source of HSPGs.

In this regard it is remarkable that HSPGs, normally associated with the vascular basement membrane i.e. agrin and perlecan, are not found in CAA, whereas other HSPGs, in particular glypican-1 and syndecan-2, that are not expressed by vascular cells, are observed in association with CAA. These observations indeed suggest that the HSPGs in CAA are not synthesized locally, but are transported from a distant cellular source in the brain parenchyma towards the vascular wall. Although agrin is also produced by neurons, it is possible that, due to its very high molecular weight (>212 kDa) relative to glypican-1 and the syndecans (<100 kDa), it cannot be transported over such a relatively long distance.

In conclusion, our results suggest involvement of glypican-1 in the pathogenesis of CAA in AD and HCHWA-D brains. A remarkable difference was noted for syndecan-2, which may play a distinct role in CAA associated with AD, whereas absence of syndecan-2 in amyloid-laden vessels in HCHWA-D brains suggests no significant effects for this HSPG species in this disease. In contrast to syndecan-2, agrin frequently colocalized with vascular Aβ in HCHWA-D brains, but is not or only sporadically expressed in CAA in AD. Both perlecan and the syndecans-1 and -3
probably do not contribute to the pathogenesis of vascular Aβ deposits. Finally, we observed differences between the expression of several HSPGs in CAA and senile plaques. Therefore we speculate that, although Aβ is the major component of both senile plaques and CAA, the pathogenesis of these lesions with regard to the role of HSPGs is different.

**Acknowledgments**

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References


Chapter 4

Accumulation of heparan sulfate proteoglycans in cerebellar senile plaques

Jack van Horssen¹, Johanneke Kleinnijenhuis¹, Cathy N. Maass¹, Annemieke A.M. Rensink¹, Irene Otte-Höller¹, Guido David², Lambert P.W.J. van den Heuvel², Pieter Wesseling¹, Robert M.W. de Waal¹, Marcel M. Verbeek¹,4

From the Departments of Pathology¹, Pediatrics³, Neurology⁴, University Medical Center, Nijmegen, The Netherlands; The Center for Human Genetics², University of Leuven and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium.

Abstract

Alzheimer’s disease (AD) brains are characterized by the presence of senile plaques (SPs), which primarily consist of amyloid β protein (Aβ). Besides Aβ, several other proteins with the ability to modulate amyloid fibril formation accumulate in SPs, e.g. heparan sulfate proteoglycans (HSPGs). Cerebellar SPs are predominantly of the diffuse type, whereas fibrillar SPs are rarely observed. Furthermore, because of the spatial separation of non-fibrillar and fibrillar SPs in the cerebellum, this brain region provides a model for the study of the association of Aβ-associated factors with various stages of SP formation. In the present study, we performed an immunohistochemical analysis to investigate the expression of the HSPG species agrin, perlecan, glypican-1 and the syndecans 1-3 as well as glycosaminoglycan side-chains in cerebellar SPs. We demonstrated that agrin and glypican-1 were expressed in both non-fibrillar and fibrillar cerebellar SPs, whereas the syndecans were only associated with fibrillar cerebellar SPs. Perlecan expression was absent in all cerebellar SPs. Since fibrillar and non-fibrillar SPs may develop independently in the cerebellum, it is likely that agrin, glypican-1 as well as heparan sulfate glycosaminoglycans may contribute to the formation of both cerebellar plaque types, whereas syndecan only seems to play a role in the generation of cerebellar fibrillar plaques.
Introduction

Brains of individuals with Alzheimer's disease (AD) contain a number of pathological lesions such as neurofibrillary tangles (NFTs) [22], senile plaques (SPs) and cerebral amyloid angiopathy (CAA) [30]. The major component of SPs and CAA is amyloid-β protein (Aβ), a 39- to 43-amino-acid peptide, that is derived from the amyloid precursor protein (APP) [15]. SPs can be categorized as either classic SPs or diffuse SPs. The first type is characterized by the presence of an amyloid core consisting of fibrillar Aβ and surrounded by a wreath of dystrophic neurites and reactive microglial cells. In contrast, diffuse SPs are primarily composed of non-fibrillar Aβ. However, fine bundles of amyloid fibrils may be observed in these plaques by using electron microscopy [8]. In AD cortex both types of SPs are frequently associated with neuritic and glial changes.

Selkoe hypothesized that non-fibrillar Aβ deposits gradually transform into fibrillar SPs in AD brains [30]. This theory is supported by observations in Down Syndrome (DS) brains, where non-fibrillar SPs can already be found at the age of 20 years, clearly preceding other types of SPs. Therefore, it was suggested that this specific type of plaque represents the earliest stage of SP formation in AD and that during a protracted process fibrillar SPs can be formed. On the other hand, in brain tissue of transgenic mice, both cored SPs and diffuse SPs appear simultaneously at 11-13 months of age, suggesting that in mouse brains, either plaque type may develop independently, and that diffuse plaques do not necessarily transform into cored plaques [19].

Although both types of SPs are abundantly present throughout the cerebral cortex in AD brains, SPs in the cerebellum are predominantly of the non-fibrillar type and the number of cored plaques is limited [20,46,47]. Dickson divided SPs in a number of subtypes with regard to their amyloid deposition morphology and presence and type of neuritic degeneration [11]. Cerebellar diffuse SPs consisting of non-fibrillar Aβ are, in contrast to cerebral diffuse SPs, not associated with paired helical filament (PHF)-type neurites. However, ubiquitin-immunoreactive dystrophic neurites can be found. Cerebellar classic plaques are described as "burnt-out" or "cored" plaques and are characterized by central deposits of compact fibrillar amyloid and the absence of dystrophic neurites [11]. The various plaque types in the cerebellum are found at different locations. Non-fibrillar cerebellar plaques are primarily located in the molecular layer of the cerebellar cortex, whereas fibrillar cerebellar SPs are mostly observed in the granular and Purkinje cell layers.

It remains unclear why SPs in the cerebellar molecular layer rarely contain an amyloid core. It is possible that factors present in the cerebrum, but lacking in the cerebellum, are necessary to trigger Aβ fibrillation. A number of Aβ-associated proteins are known to enhance fibril formation, for instance heparan sulfate proteoglycans (HSPGs). Therefore, the cerebellum with its spatial separation of non-fibrillar and fibrillar SPs provides a unique in vivo model to investigate the association and role of HSPGs in the various types of plaques.
Heparan sulfate proteoglycans are biological macromolecules characterized by a core protein to which glycosaminoglycan side-chain (GAG side-chains) are covalently attached. These GAG side-chains are composed of repeating disaccharide units of 1→4 linked iduronic acid/glucuronic acid and N-acetyl glucosamine. Two subfamilies of HSPGs can be distinguished; they are either associated with the cell membrane or with the extracellular matrix. Syndecans and glypicans belong to the first group and differ from each other in core protein structure and type of attachment to the cell membrane. Syndecans have a transmembrane domain and a cytoplasmic domain, which contains 3-5 heparan sulfate side-chains. However, hybrid forms containing both heparan sulfate chains and chondroitin sulfate chains also exist. Glypicans are linked to the cell membrane via a glycosylphosphatidylinositol-anchor and are characterized by cystein-rich globular ectodomains and the presence of 2-3 heparan sulfate chains. Agrin and perlecans belong to the basement membrane-associated HSPGs. Agrin is highly expressed in the brain and plays an important role in the synaptogenesis at the neuromuscular junction [2].

The initial reports on the description of HSPGs in cerebellar SPs did not specify the subtype of HSPG expressed in these plaques [34]. In previous studies we demonstrated by immunohistochemical analysis expression of syndecan 1-3, glypican-1, agrin and GAG side-chains in cerebral SPs, NFTs and CAA, suggesting that these HSPG species are involved in AD pathology [42,43]. By using these antibodies we studied the distribution of these HSPGs in the various types of SPs in the cerebellum. These expression patterns were compared to those found in the cerebrum.

**Materials and Methods**

**Autopsy Material**
Tissue samples from cerebellum and cerebrum were obtained during rapid autopsy procedure and snap-frozen in liquid nitrogen. Eight AD patients (4 male, 4 female) were selected for the presence of SPs in the cerebellum by immunostaining for Aβ (age 75.8 ± 11.2 years; post mortem delay 3.6 ± 1.1 hours, (mean ± SD)). Frontal cortex of nine AD patients (3 male, 6 female; age 82.2 ± 9.0 years; post mortem delay 3.7 ± 0.9 hours) were used and three cases without neurological disease (age 83 ± 1.5 years; post mortem delay 3 ± 1 hours) were used as controls.

**Antibodies**
Primary antibodies and their sources are listed in Table 1 (for more details about their reactivity, see also Verbeek et al. [43]). We used biotin-labeled horse anti-mouse as secondary antibody with the exception of primary incubations with BL31 (biotin-labeled donkey anti-goat), 1948 (biotin-labeled rabbit anti-rat) and JM13 and JM403 (biotin-labeled goat anti-mouse IgM). All secondary antibodies and the avidin-biotin-peroxidase complex (ABC) were obtained from Vector (Burlingame, CA).
**Immunohistochemistry**

To examine the expression of the various HSPGs in cerebellar and cerebral plaques, serial cryosections (4 μm) were used. At regular intervals, sections were stained with anti-АВ, to allow for topographical alignment with the HSPG stainings. Furthermore, in each series three 10 μm sections were used for Congo red staining to detect fibrillar АВ. For this staining, acetone-fixed sections were dehydrated with increasing concentrations of ethanol (0-80%) and subsequently preincubated in a solution of 3% NaCl in 80% ethanol. Finally, sections were incubated in the same solution containing 0.5% Congo red and counterstained with hematoxylin.

For immunohistochemical staining of HSPGs, sections were air-dried, fixed in acetone for 5 minutes and subsequently for 5 minutes in acetone containing 0.15% H₂O₂ to block endogenous peroxidase activity. In case of staining with 3G10, a monoclonal antibody directed against heparitinase-digested HSPGs, sections were pre-treated with 50 mU heparitinase (heparinase III, EC 4.2.2.8; Sigma Chemical Co., St Louis, MO) diluted in 10 mM HEPES and 2 mM CaCl₂ (pH 7.0) at 37°C for 1 hour. Then, sections were preincubated for 30 minutes with 20% animal serum, the source of which was determined by the specific secondary antibody used. Afterwards, sections were incubated overnight at 4°C with the appropriate primary antibody (Table 1). Subsequently, sections were incubated with the secondary biotin-labeled antibody for one hour at room temperature and with ABC according to the manufacturer's description. Diaminobenzidine (DAB) was used as chromogen. Between incubation steps, sections were thoroughly washed with PBS. Copper sulfate (0.5%) in saline solution was used to enhance DAB-staining. After a short rinse in tap water the preparations were incubated with hematoxylin for 3 minutes and extensively washed with tap water for 10 minutes. Finally, sections were dehydrated with ethanol followed by xylol. All antibodies were diluted in PBS containing 0.1% bovine serum albumin, which also served as a negative control.

The percentage of SPs stained by a specific antibody compared with the anti-АВ stainings was independently scored in any of the following categories 0%, 0-25%, 25-50%, 50-75%, 75-100% by two of the authors (JvH and MMV). When scores differed more than one scale unit consensus was reached after re-evaluation.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>6C6</td>
<td>АВ</td>
<td>1:4000</td>
<td>Elan pharmaceuticals</td>
</tr>
<tr>
<td>AT8</td>
<td>Tau</td>
<td>1:200</td>
<td>Innogenetics</td>
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<tr>
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<td>Agrin (core protein)</td>
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<td>[39]</td>
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<td>Agrin (core protein)</td>
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<td>[40]</td>
</tr>
<tr>
<td>9G110</td>
<td>Perlecan (core protein)</td>
<td>1:500</td>
<td>[16]</td>
</tr>
<tr>
<td>1948</td>
<td>Perlecan (core protein)</td>
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<td>Chemicon</td>
</tr>
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<td>3G10</td>
<td>Pan-HSPG</td>
<td>1:300</td>
<td>[5]</td>
</tr>
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<td>JM13</td>
<td>Heparan sulfate side-chains</td>
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<td>[38]</td>
</tr>
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<td>Heparan sulfate side-chains</td>
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</tr>
<tr>
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</tr>
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<td>Syndecan-3 (ectodomain)</td>
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<td>[23,24]</td>
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<tr>
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<td>Glypican-1 (core protein)</td>
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<td>[7,9]</td>
</tr>
<tr>
<td>2E9</td>
<td>Syndecan-1,-3 (cytoplasmatic domain)</td>
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<td>[2,23]</td>
</tr>
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**Table 1.** Primary antibodies used in this study.
Results

Cerebellar and cerebral SPs
In the cerebellum of six patients a variable number of both non-fibrillar and fibrillar cerebellar plaques were observed by staining with the anti-Aβ mAb 6C6. One cerebellum contained a remarkably large number of fibrillar plaques, whereas in another cerebellum only non-fibrillar SPs were detected. Non-fibrillar SPs were observed throughout the molecular layer and showed a tendency for orientation perpendicular to the surface of the folia (Fig 4A). On the other hand, fibrillar SPs were predominantly located in the granular and Purkinje cell layer (Fig 1C). Sporadically, fibrillar SPs were found in the molecular layer. All cerebral sections revealed numerous non-fibrillar (Fig 1E) and fibrillar SPs (not shown) throughout the cortical grey matter.

Agrin and perlecan
JM-72 and Bl-31, two anti-agrin core protein antibodies were used to investigate the expression of agrin in SPs. Furthermore, 95J10 and 1948, two antibodies directed against the core protein of perlecan were used to study perlecan expression in AD cerebellum. In approximately 70% of the non-fibrillar cerebellar SPs agrin expression was found (Figure 1B), whereas this molecule was expressed in virtually all non-fibrillar cerebral SPs (Fig 1F). Furthermore, agrin expression in non-fibrillar SPs was much less intense in the cerebellum than in the frontal cortex (Figure 1B, 1F). In a majority of the sections agrin was only expressed in a part of the cerebellar senile plaque (Figure 1B). In all cases fibrillar SPs (Congo red-positive, not shown) in the cerebellum were strongly stained by the two anti-agrin antibodies (Figure 1D). Furthermore, both JM-72 and BL-31 stained the cerebellar vasculature (Figure 1B, 1D). The anti-perlecan mAbs 95J10 and 1948 neither stained non-fibrillar nor fibrillar SPs in the cerebellum (not shown), whereas cerebellar blood vessels were strongly stained indicating that the antibody was reactive. The results of the immunohistochemical staining for agrin, perlecan and other HSPGs in AD brains are summarized in Table 2.
<table>
<thead>
<tr>
<th>HSPG species</th>
<th>cerebral non-fibrillar SPs</th>
<th>cerebral fibrillar SPs</th>
<th>cerebellar non-fibrillar SPs$^1$</th>
<th>cerebellar fibrillar SPs$^2$</th>
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</thead>
<tbody>
<tr>
<td>Agrin</td>
<td>++++</td>
<td>++++</td>
<td>+++$^3$</td>
<td>++++</td>
</tr>
<tr>
<td>Perlecan</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Syndecan-1,-3</td>
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<td>++/+++$^4$</td>
<td>-</td>
<td>++++</td>
</tr>
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<td>-</td>
<td>++</td>
</tr>
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<td>++</td>
<td>+++/++$^4$</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>Glypican-1</td>
<td>++++</td>
<td>+++</td>
<td>+++$^3$</td>
<td>++++</td>
</tr>
<tr>
<td>HS GAG</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
</tr>
</tbody>
</table>

Table 2. Expression of HSPGs in cerebral and cerebellar SPs
Positive fractions (percentage of senile plaques stained by a specific antibody compared with the anti-Aβ stainings) were scored in the following categories: 0% (-), 0-25% (+), 25-50% (++), 50-75% (+++), or 75-100% (+++). $^1$ in molecular layer, $^2$ in granular and Purkinje cell layer, $^3$ only a part of the plaque was stained, $^4$ Expression varied between the different patients.
Syndecans and glypican-1

Three different anti-syndecan mAbs were used in the present study: 2E9 (anti-syndecan-1,-3), 10H4 (anti-syndecan-2) and 1C7 (anti-syndecan-3). None of the syndecans were expressed in cerebellar non-fibrillar SPs, whereas syndecan-1 and -3 were expressed in all fibrillar SPs (Figure 2B,C), and syndecan-2 was observed in approximately 40%-50% of these SPs (Figure 2A). All syndecans accumulated in both types of SPs in the cerebral cortex, however less frequent and intense than agrin and glypican-1 (Table 2). The distribution of glypican-1 was studied by using S1, a mAb directed against the core protein of glypican-1. Glypican-1 was abundantly expressed in both non-fibrillar and fibrillar SPs in the cerebrum. However, glypican-1 was only weakly expressed in non-fibrillar cerebellar SPs and often only a part of the plaque area was stained, similar as observed with the anti-agrin mAbs (Figure 3B). A more pronounced expression was observed in fibrillar cerebellar SPs (Figure 3D).
Figure 3.
Immunohistochemical staining of senile plaques in serial sections from AD cerebellum for Aβ (mAb 6C6) (a,c), glypican-1 (mAb S1) (b,d). Glypican-1 was expressed in both non-fibrillar (b) and fibrillar cerebellar senile plaques (d), however only a part of the non-fibrillar plaque was weakly stained, similar as observed with the anti-agrin mAbs (see also figure 1). Original magnifications: a,b x300; c,d x1200. bar = 25 μm

HS GAGs
Three different antibodies were used to investigate the expression of HS GAG side-chains in AD cerebellum: JM403 and JM13, both recognizing different epitopes in the HS polysaccharide side-chain, and 3G10, an antibody directed against heparitinase-digested HSPGs. In all cases all non-fibrillar and fibrillar SPs in the frontal cortex were stained with the above-mentioned mAbs (not shown). Also, in cerebellum all SPs were immunopositive for HS GAG side-chains (Figure 4B,C,E,F). Remarkably, in the cerebellum all anti-GAG mAbs stained the non-fibrillar SPs more intensely and frequently than the other HSPG-mAbs (compare Figure 4B,C to Figure 1B and Figure 3B).
Discussion

Evidence for an important role of HSPGs in the pathogenesis of AD is accumulating. HSPGs are able to enhance Aβ fibrillation \textit{in vitro} \cite{4} and in rat brains \textit{in vivo} \cite{34}. Furthermore, HSPGs protect Aβ fibrils from proteolysis \textit{in vitro} \cite{4} by forming complex structures that are relatively resistant to proteolytic attacks by Aβ-degrading enzymes. In addition, it has been demonstrated that HSPGs colocalized with Aβ-containing deposits both in diffuse and classic cerebral SPs and in amyloid-laden vessels \cite{26,32,33,41,42,48}.

Recently, we demonstrated agrin expression in virtually all cerebral SPs and NFTs, suggesting an important role for this molecule in the formation and persistence of SPs and NFTs in AD neocortex \cite{43}. These previous observations are supported by Cotman et al. \cite{4} and Donahue et al \cite{12}, who also observed agrin expression in cerebral SPs. In the present study we found that, in the cerebellum, agrin accumulates in general in both non-fibrillar and fibrillar SPs. However, we observed that non-fibrillar cerebellar SPs are less frequently and less intensely stained than non-fibrillar cerebral SPs. Zhan et al. only described agrin expression in fibrillar cerebellar SPs \cite{48}. It is possible that this discrepancy with our results is caused by a lower sensitivity of the staining techniques used by Zhan et al., which did not allow the detection of low levels of agrin. We conclude that agrin is expressed in NFTs and
in non-fibrillar and fibrillar SPs in both cerebrum and cerebellum, albeit at a lower level in cerebellar non-fibrillar SPs.

Glypican-1 was identified and cloned as one of the cell surface HSPGs of human lung fibroblasts. Schulz et al. demonstrated a decrease in Aβ toxicity in pheochromocytoma PC12 cells pre-treated with phosphatidylinositol-specific phospholipase C, an enzyme that specifically cleaves the GPI-anchors, suggesting that a member of the glypican family is involved in mediating Aβ toxicity [29]. In previous studies we demonstrated expression of glypican-1 in NFTs and cerebral SPs [43] and in vascular amyloid deposits in AD brains [42]. Here, we demonstrate that glypican-1 is expressed in both non-fibrillar and fibrillar cerebellar SP.

We speculate that agrin and glypican-1 are involved in the formation of both fibrillar and non-fibrillar cerebellar SPs and possibly in the transformation of diffuse to classic SPs. Furthermore, agrin may protect Aβ fibrils against proteolytic degradation, because it contains nine protease-inhibiting domains [43], and may thereby contribute to the persistence of Aβ deposits in AD brains.

The present study demonstrates that the expression of syndecans in cerebellum is limited to fibrillar SPs. The absence of syndecan expression in non-fibrillar cerebellar SPs is remarkable in view of our previous finding that syndecan-1, -2 and -3 are expressed to some extent in non-fibrillar neuritic cerebral SPs [43]. Therefore, the syndecans, in contrast to agrin and glypican-1, do not seem to be involved in the deposition of non-fibrillar Aβ in the cerebellum. Non-fibrillar SPs in the cerebral cortex are often associated with glial and neuritic changes. It is possible that this is related to increased syndecan production. This may explain why syndecan is not expressed in cerebellar non-fibrillar SPs, since such cellular activation is not observed there.

The absence of perlecan expression in both non-fibrillar and fibrillar cerebellar SPs was in line with our previous observations that perlecan did not accumulate in any of the cerebral AD lesions [42,43]. Antibodies directed against perlecan (1948 and 95J10) stained the cerebellar vasculature, showing their reactivity. Although previous studies reported perlecan involvement in the pathogenesis of AD [32,33,34] and in particular in cerebellar SPs [35], we were not able to confirm these data.

The potency of HSPGs to accelerate Aβ fibril formation is mainly attributed to the GAG side-chains of the proteoglycans [3,4]. In the present study antibodies directed against the GAG side-chains and to heparitinase-digested HSPGs intensely stained both non-fibrillar and fibrillar SPs in the cerebellar cortex. Interestingly, staining for GAGs was much more intense and widespread than the HSPG core protein stains. Therefore, we speculate that either free GAG side-chains or other subtypes of HSPGs, e.g. collagen XVIII [17] and/or other members of the glypican family [18], might accumulate in non-fibrillar cerebellar Aβ deposits as well.

Besides HSPGs, several other Aβ-associated proteins have been identified in cerebral SPs and CAA [10]. For example, complement factors [14], apolipoprotein E [25], α1-
antichymotrypsin [1,27], and amyloid P component [13,22,48] are expressed in cerebral SPs. Reports on expression of these Aβ-associated components in cerebellar non-fibrillar SPs are conflicting [21,28,31,36], suggesting that their expression level is low or even zero. The cytokine-inducible adhesion molecule ICAM-1, which is expressed in all types of cerebral plaques and in fibrillar cerebellar SPs, was consistently not detected in cerebellar non-fibrillar SPs [44,45].

In conclusion, HSPGs are a group of molecules that have the ability to a) bind to Aβ and thereby immobilize these deposits and b) induce the conversion of non-fibrillar into fibrillar SPs in AD brain. The results of our study confirm the association of HSPGs with various types of plaques and suggest that in particular, agrin, glypican-1 and GAG side-chains are involved in the formation of both fibrillar and non-fibrillar cerebellar SPs, whereas the syndecans are only associated with fibrillar cerebellar SPs.

Acknowledgement

We thank Dr. Schenk for the 6C6 antibody. Prof. Dr. J.H. Berden (Department of Nephrology, U.M.C., Nijmegen) for the antibodies JM403 and JM13 and Dr. R. Koopmans, Dr. J. H. M. Cox-Claessens, and Dr. G. Woestenburg (Psychogeriatric Centers "Joachim en Anna" and "Margriet," Nijmegen, The Netherlands) for their cooperation in the rapid autopsy protocol. Financial support was obtained from the "Internationale Stichting Alzheimer Onderzoek".
References


Chapter 5

Collagen XVIII: a novel heparan sulfate proteoglycan associated with vascular amyloid depositions and senile plaques in Alzheimer’s disease brains

Jack van Horssen¹, Micha M.M. Wilhelmus¹, Ritva Heljasvaara², Taina Pihlajaniemi², Pieter Wesseling¹, Robert M.W. de Waal¹, Marcel M. Verbeek³.

Departments of Pathology¹ and Neurology², University Medical Center, Nijmegen, The Netherlands and Collagen Research Unit, Biocenter and Department of Medical Biochemistry², University of Oulu, Finland.

Abstract

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.
Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder and the most prevalent form of late-life mental failure in humans. Histopathologically, three 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 Verbeek et al. (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 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 two 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 two 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. Here, 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 eleven 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 five 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, CA) except the FITC-labeled sheep anti-mouse antibody, which was obtained from ICN Pharmaceuticals (Costa Mesa, CA).

**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% H₂O₂ 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, CA). 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).

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 and G). Severe CAA was found in 7 patients, moderate 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 and C) nor in NFTs (Figure 2B and 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 and F) and classic SPs (Figure 1H and 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).
Figure 1. Immunofluorescence staining of senile plaques in sections from AD brains for Aβ (mAb 6C5) (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). Original magnifications: a-i x630. For color figure see appendix.

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 (c). Original magnifications: a-c x630. For color figure see appendix.
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 cannot 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, e.g. 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 has 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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References


Chapter 6

Absence of heparan sulfate proteoglycans in Lewy bodies and Lewy neurites in Parkinson’s disease brains

Jack van Horssen¹, Rob A.I. de Vos², Ernst N.H. Jansen Steur³, Guido David⁴, Pieter Wesseling¹, Robert M.W. de Waal¹, Marcel M. Verbeek⁵

From the Departments of Pathology¹ and Neurology⁵, University Medical Center, Nijmegen, Laboratorium Pathologie Oost Nederland², Enschede, The Netherlands; Department of Neurology³, Medisch Spectrum Twente, Enschede, The Netherlands; The Center for Human Genetics⁴, University of Leuven and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium

Abstract

α-Synuclein is the major constituent of Lewy bodies and Lewy neurites in Parkinson’s disease (PD) and dementia with Lewy bodies (DLB). Relatively little is known about the exact mechanism of α-synuclein deposition and fibrillation in these α-synucleinopathies. In order to better understand the pathogenesis of α-synucleinopathies it is important to identify molecules that regulate the fibrillation of α-synuclein. Since it has been demonstrated that heparan sulfate proteoglycans (HSPGs) and glycosaminoglycans (GAGs) promote the conversion of non-fibrillar amyloid β-protein (Aβ) into neurotoxic fibrillar Aβ in Alzheimer’s disease, they might also be involved in α-synuclein aggregation. It was the aim of our study to examine the distribution pattern of these macromolecules in PD brains and the possible association with Lewy bodies and Lewy neurites. Although HSPGs clearly colocalized with senile plaques, we were unable to identify HSPGs or GAGs in Lewy bodies and Lewy neurites and therefore concluded that it is likely that α-synuclein fibrillation and stabilization occurs independently of the presence of HSPGs or GAGs.
Introduction

The neuropathological diagnosis of both Parkinson’s disease (PD) and dementia with Lewy bodies (DLB) relies on identification of intraneuronal Lewy bodies and Lewy neurites [8]. These inclusions occur exclusively in specific types of neurons of select regions of the human nervous system [2, 31]. It was demonstrated that α-synuclein is the major constituent of Lewy bodies and Lewy neurites, and that anti-α-synuclein antibodies immunostained Lewy bodies and Lewy neurites in PD and DLB brains [1]. α-Synuclein is also the main component of the glial and neuronal inclusions of multiple system atrophy [13].

α-Synuclein was first isolated and purified from the electric organ of Torpedo californica [17]. It was suggested that α-synuclein plays a role in neuronal plasticity because its expression in songbirds increased during learning [10]. In normal functioning neurons, α-synuclein is synthesized in the perikaryon and transported to presynaptic terminals. α-Synuclein is usually localized in synaptic boutons, typically in close proximity of, or bound to synaptic vesicles [17]. The native protein likely plays a role in maintenance and stabilization of fully matured synapses [18]. However, its normal localization seems to be disturbed in PD/DLB, where reallocation occurs resulting in accumulation of α-synuclein in the cell body and in more proximal parts of the neurites. Monomeric α-synuclein can form fibrils in solution [10, 12]. During this fibrillization process, native unfolded α-synuclein undergoes conformational changes resulting in partially folded intermediates and toxic proteofibrils. It has been reported that heparan sulfate proteoglycans (HSPGs) and glycosaminoglycans (GAGs) promote the conversion of non-fibrillar Aβ into neurotoxic fibrillar Aβ [3, 5, 23] and may protect Aβ against proteolytic degradation [5, 22]. Furthermore, HSPGs/GAGs have consistently been found in all kinds of amyloid, regardless of the specific type of amyloidogenic protein deposited [23, 26, 27, 28, 29, 30]. Therefore, it might be expected that these complex macromolecules play a similar role in α-synuclein fibrillogenesis and stabilization. It was the aim of our study to examine the distribution pattern of HSPGs and GAGs in PD brains in order to collect evidence for a specific in vivo association of HSPGs/GAGs with α-synuclein.

Materials and Methods

Autopsy Material
Brain tissue from patients with clinically diagnosed and neuropathologically confirmed PD (with or without dementia) was obtained at autopsy. All brains displayed the presence of α-synuclein-immunoreactive Lewy bodies and Lewy neurites, while at the same time being free of intracytoplasmic inclusions related to non-PD α-synucleinopathies. After the routine autopsy procedure, tissue samples from the anterior cingulate cortex were obtained and snap frozen in liquid nitrogen. Cingulate cortex sections of 10 PD patients (7 male, 3 female) were investigated for the presence of HSPGs and GAGs (age 74.8 ± 7.9 years; post mortem delay 20.1 ± 4.2 hours). This study was conducted with due regard for the restrictions of the Dutch legislation on the use of human tissue material for research purposes (informed
consent was obtain of a legal representative of the patient). Samples were anonymized leaving only clinical data accessible.

Antibodies

Mouse monoclonal antibody synuclein Ab-2 (syn211, NeoMarkers, Fremont, CA), a highly specific mAb for α-synuclein that does not cross-react with β- and γ-synuclein, was used as primary antibody. Polyclonal antibody 40-4, directed against Aβ (generous gift of Dr. W.E. van Nostrand, Stony Brook, NY [7]) was used to demonstrate the presence of senile plaques. Additionally, specific antibodies directed against heparinase-digested HSPGs (3G10), heparan sulfate side-chains (JM13, JM403), agrin core protein (JM72) and glypican-1 core protein (S1) were used to study the expression of these molecules in PD brains. The characterization of these antibodies has been described in previous reports [6, 24]. Anti-glypican-1 (S1) and anti-agrin (JM72) antibodies were selected because both mAbs strongly stain a variety of amyloidogenic lesions such as neurofibrillary tangles, senile plaques and amyloid-laden vessels. We used biotin-labeled horse anti-mouse as secondary antibody with the exception of primary incubations with JM13 and JM403, in which case biotin-labeled goat anti-mouse IgM was used. All secondary antibodies and the avidin-biotin-peroxidase complex (ABC) were obtained from Vector (Burlingame, CA).

Immunohistochemistry

For immunohistochemical double-staining, 4 μm cryosections were air-dried, fixed in acetone for 5 minutes, subsequently for 5 minutes in acetone containing 0.15% H₂O₂ to block endogenous peroxidase activity, and preincubated for 30 minutes with 20% normal horse serum with the exception of staining with JM13 and JM403 in which case we used 20% normal goat serum. Sections were incubated overnight at 4 °C with either 3G10, JM13, JM403, S1 or JM72 at concentrations of 10 μg/ml. In case of staining with 3G10, sections were pretreated with 50 mU heparitinase III, EC 4.2.2.8; Sigma Chemical Co., St Louis, MO) diluted in 10 mM HEPES and 2 mM CaCl₂ (pH 7.0) at 37 °C for 1 hour. Subsequently, sections were incubated with either biotin-labeled horse anti-mouse antibody in case of primary staining with 3G10, JM72 and S1 or biotin-labeled goat anti-mouse IgM in case of primary staining with JM13 and JM403, for 30 minutes at room temperature and with avidin-labeled Texas Red for 45 minutes. Between incubation steps, sections were thoroughly washed with phosphate buffered saline (PBS). After the last rinse in PBS, sections were first incubated with a mixture of rabbit anti-mouse alkaline phosphatase and rabbit anti-mouse horse radish peroxidase for 30 minutes (dilution 1:20), to block residual free binding sites on the mouse antibodies from the first cycle. The success of this blocking procedure was verified by omitting secondary antibodies from the first cycle. Then, sections were washed with PBS and incubated with either anti-α-synuclein Ab-2 or anti-Aβ 40-4 at a concentration of 10 μg/ml. Finally, sections were incubated with FITC-labeled sheep anti-mouse or swine anti-rabbit antibodies, respectively, for one hour at room temperature. Selected sections were also incubated with ToPro-3 for nuclear staining (dilution 1:1000, Molecular probes). Between incubation steps, sections were thoroughly washed with PBS and finally mounted in Vectashield (Vector, Burlingame, CA). All antibodies were diluted in PBS.
containing 0.1% bovine serum albumin, which also served as a negative control. Sections were examined using a confocal laser-scanning microscope (Leica, Wetzlar, Germany).

**Results**

Ab-2, a monoclonal antibody directed against α-synuclein, clearly stained Lewy bodies and Lewy neurites throughout the cingulate cortex (Figure 1 and 2). Antibodies directed against HS side-chains (JM13, JM403) and heparinase-digested HSPGs (3G10) intensely stained both parenchymal and leptomeningeal vessels. However, no colocalization with α-synuclein in Lewy bodies and Lewy neurites was observed (Figure 1A and B).

![Figure 1.](image)

**Figure 1.**
Immunofluorescence staining of Lewy bodies and Lewy neurites in sections from PD brains for α-synuclein (mAb Ab-2, green) (a,b), HSPG core protein (3G10, red) (a) and HSGAG (JM403, red) (b). In a nuclei were stained blue. Cortical LBs and LNs are stained by the anti-α-synuclein antibody. Both 3G10 and JM403 strongly stained the cerebral (micro)vasculature. Neither HSPG core-protein nor HSGAGs colocalize with α-synuclein (a,b). Autofluorescent lipofuscin deposits are clearly visible. Original magnifications: a,b x630. For color figure see appendix.

Senile plaques were observed in only one PD patient using anti-Aβ antibody 40-4 (Figure 2A). In contrast to the absence of HSPGs and GAGs in Lewy bodies and Lewy neurites, senile plaques were clearly decorated with both JM 403 (data not shown) and 3G10 (Figure 2B and C). Anti-glypican-1 (S1) and anti-agrin (JM72) antibodies did not stain Lewy bodies or Lewy neurites (data not shown). However, JM 72 clearly stained the basement membranes of cerebral vessels, indicating that the absence of Lewy bodies and Lewy neurites staining was not due to technical problems.
Discussion

Both Lewy bodies and neurofibrillary tangles are associated with cytoskeletal changes leading to accumulation of altered or misfolded proteins. Lewy bodies and Lewy neurites are characterized by inclusions of aggregated α-synuclein, whereas neurofibrillary tangles and dystrophic neurites are composed of microtubule-associated tau protein. Several studies demonstrated that heparan sulfate proteoglycans and heparan sulfate are present in both senile plaques and tangles, promote Aβ aggregation in vitro, and are involved in the stabilization of these amyloid deposits by providing protection against proteolytic degradation and subsequent removal [14, 15, 19, 23, 30]. A large number of components, also observed in association with senile plaques, including ubiquitin, neurofilaments and αB-crystallin have been identified in Lewy bodies. It is unclear, however, if this is a passive association or if these proteins affect α-synuclein fibrillogenesis. It has been described that some pesticides and metals may accelerate the rate of formation of α-synuclein fibrils in vitro [24]. Recently, it was found that tissue transglutaminase, by cross-linking α-synuclein, contributed to the generation of α-synuclein aggregates [16].

Relatively little is known about the role of heparan sulfate proteoglycans in α-synuclein fibrillogenesis [21]. Recently, Cohberg and colleagues demonstrated that glycosaminoglycans, in particular heparan sulfate, heparin and other highly sulfated components, stimulated the formation of α-synuclein fibrils in vitro [4]. Apart from its catalytic function, heparin was also incorporated into the fibrils. Based on the binding
of basic fibroblast growth factor to heparinase sensitive sites in Lewy bodies, it was suggested that Parkinson disease-associated Lewy bodies contain heparan sulfate proteoglycans [19]. Additionally, several chondroitin sulfate proteoglycan subtypes accumulated in Lewy bodies [9]. We were unable to confirm the accumulation of heparan sulfate proteoglycans/glycosaminoglycans in Lewy bodies and Lewy neurites, suggesting that these sulfated molecules, in contrast to their role in AD pathology, are not involved in α-synuclein fibrillization and stabilization and suggesting that the binding of basic fibroblast growth factor to Lewy bodies may be based on a different type of interaction than the binding to heparan sulfate proteoglycans. In line with our previous observations that heparan sulfate proteoglycans are observed in senile plaques in AD brains (reviewed in [29]), we demonstrated that heparan sulfate proteoglycans and heparan sulfate colocalized with extracellular Aβ in senile plaques in PD brains. Furthermore, we previously have demonstrated that heparan sulfate proteoglycans accumulate in intracellular tangles [30]. Given the absence of heparan sulfate proteoglycans in Lewy bodies, the present study suggests that heparan sulfate proteoglycans are not always associated with any type of intracellular protein aggregation. In this respect, the pathogenesis of α-synuclein aggregation seems to differ from that of tau aggregation.

Although we cannot exclude that heparan sulfate proteoglycans and heparan sulfate are involved in early stages of Lewy bodies formation, such an involvement was not observed in post-mortem material as a clear colocalization, indicating that either Lewy body formation is independent of the presence of heparan sulfate proteoglycans and heparan sulfate, or that the early stages of Lewy body formation are not represented in our material. Therefore, future research, e.g. using animal models, is warranted to provide more insight into the exact mechanism of α-synuclein fibrillization and a possible role of heparan sulfate proteoglycans and glycosaminoglycans.

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References


Chapter 7

Induction of heparan sulfate proteoglycan production by Aβ

Jack van Horssen¹, Ivon J.M. Van der Linden¹, Irene Otte-Höller¹, Micha M.M Wilhelms¹,², Guido David³, Leo A. B. Joosten⁴, Robert M.W. de Waal¹, Marcel M. Verbeek²

From the Departments of Pathology¹ and Neurology², University Medical Center, Nijmegen; The Center for Human Genetics³, University of Leuven and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium and Rheumatology Research Laboratory and Advanced Therapeutics⁴, University Medical Center Nijmegen, Nijmegen.
Abstract

Senile plaques (SPs), neurofibrillary tangles (NFTs) and cerebral amyloid angiopathy (CAA) are three major histopathological hallmarks of Alzheimer’s disease brains. Next to the presence of abnormal Aβ protein aggregates, several other proteins accumulate in these lesions, including heparan sulfate proteoglycans. Here, we studied the cellular source of amyloid-associated heparan sulfate proteoglycans and demonstrated that several cerebral cell types produce a variety of heparan sulfate proteoglycans in vitro. Since the molecular pathways leading to the excessive production and deposition in Alzheimer’s disease brains are largely unknown we studied the influence of Aβ1-40 carrying the Dutch mutation (DAβ1-40) on heparan sulfate proteoglycan metabolism. We showed by 35S-sulfate labeling, western blot analysis and (electron) microscopy that treatment of human brain pericytes with DAβ1-40 resulted in an increase of both highly glycosylated glypican-1 and agrin. Future studies at the mRNA level will provide information whether DAβ1-40 acts as an inducer of heparan sulfate proteoglycan production, by enhancing HSPG gene expression.
Introduction

Neuropathological examination of Alzheimer’s disease (AD) brains demonstrates three distinctive lesions: senile plaques (SPs), neurofibrillary tangles (NFTs) and cerebral amyloid angiopathy (CAA). The major component of both SPs and CAA is amyloid β protein (Aβ), a proteolytic cleavage product of amyloid precursor protein (APP). NFTs are mainly composed of fibrillar intraneuronal hyperphosphorylated tau protein (1-4). Next to the presence of these abnormal protein aggregates, several other proteins accumulate in AD lesions, including inflammatory proteins (5-8), amyloid-P component (9), apolipoprotein E (10-12), α₁-antichymotrypsin (13-16) and heparan sulfate proteoglycans (HSPGs) (17-21).

HSPGs are complex macromolecules involved in a variety of biological processes such as cellular proliferation, migration and differentiation (22;23). They are composed of a long sulfated polysaccharide chains covalently attached to a specific serine residue of the core protein. These heparan sulfate glycosaminoglycan (HSGAG) side-chains may undergo a series of enzymatic modifications leading to a unique variable sequence with highly sulfated regions alternated with relatively largely unmodified regions (22;24;25). HSPGs can be divided into two major groups: the extracellular matrix-associated proteoglycans including perlecan, agrin and collagen XVIII (26;27), and the cell surface proteoglycans of the syndecan and glypican families (22).

Recent immunohistochemical studies have demonstrated differential expression patterns of several HSPG subtypes in AD lesions. Agrin and glypican-1 immunopositivity has been observed in all types of AD lesions (19;20;28;29), whereas collagen XVIII was only found in classic SPs and amyloid-laden vessels in the cerebral cortex (21). A variable number of SPs and tangles were immunostained by syndecan 1-3 antibodies, while the expression of syndecans in the cerebellum was limited to fibrillar SPs (19;20). Syndecan-2, collagen XVIII and glypican-1 were the predominant HSPG species accumulating in CAA-affected vessels (18). Previous studies reported the involvement of perlecan in the pathogenesis of AD but we were not able to confirm these data (19;20).

Although the distribution of HSPGs in AD lesions has been a subject of interest, both the cellular source of amyloid-associated proteoglycans and their molecular pathways leading to the excessive production in AD brains remain largely elusive. Therefore, the aim of our study was to investigate the cellular origin of these Aβ-associated HSPGs and the possibility that HSPG production is regulated by Aβ. By using a panel of well-defined antibodies for western blot analysis and immunocytochemistry we studied the production and expression of several HSPG subtypes by human brain pericytes (HBP), glioma cells, astrocytoma cells and neuroblastoma cells. Additionally, we examined the possible influence of Aβ on HSPG production by metabolic labeling and immuno-electron microscopy.
Materials and Methods

Antibodies
Primary antibodies and their sources are listed in Table 1. For immunohistochemistry we used biotin-labeled horse anti-mouse as secondary antibody with the exception of primary incubations with QH 48.18 (biotin-labeled goat anti-rabbit). For immunoblot analysis we used peroxidase-labeled rabbit anti-mouse antibody as secondary conjugate, again with the exception of primary incubations with QH 48.18 (peroxidase-labeled swine anti-rabbit). All secondary antibodies and the avidin-biotin-peroxidase complex (ABC) were obtained from Vector (Burlingame, CA).

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Specificity</th>
<th>Dilution IHC/WB</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM 72</td>
<td>Agrin</td>
<td>1:2000/1:500</td>
<td>(37)</td>
</tr>
<tr>
<td>DL-101</td>
<td>Syndecan-1 ectodomain</td>
<td>1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>1C7</td>
<td>Syndecan-3 ectodomain</td>
<td>1:500</td>
<td>(38;39)</td>
</tr>
<tr>
<td>S1</td>
<td>Glypican-1</td>
<td>1:500/1:50</td>
<td>(40)</td>
</tr>
<tr>
<td>QH48.18</td>
<td>Collagen XVIII</td>
<td>1:2000</td>
<td>(41)</td>
</tr>
</tbody>
</table>

Table 1. Primary antibodies used in this study. IHC: Immunohistochemistry, WB: western blot analysis

HCHWA-D Aβ1-40-protein
HCHWA-D Aβ1-40 (DAβ1-40), 89 % pure, was obtained from Biosource (Etten-Leur, The Netherlands). This Aβ isoform contains the Dutch mutation (Glu22→Gln22). Lyophilized DAβ1-40 was dissolved in sterile water (pH 7) at 250 μM and stored at −80 °C prior to use. HBP were treated with 25 μM DAβ1-40 during 3 to 6 days to examine the effects on expression and production of HSPGs by immunohistochemistry and western blot analysis, respectively.

Cell cultures
HBP were isolated and characterized as described previously {32}. In brief, neocortical brain tissue was obtained after rapid autopsy and collected in ice-cold phosphate-buffered saline (PBS) supplemented with several anti-microbial compounds. For HBP, brain tissue was homogenized in Earl’s modified essential medium (EMEM, Bio-Whittaker, Verviers, Belgium), centrifuged, and resuspended in EMEM containing 15 % dextran (T70). After centrifugation the sedimiented capillaries were treated with 1% collagenase. After collagenase digestion capillary clusters were seeded on fibronectin-coated culture dishes and after 5-7 days HBP migrated from the capillary clumps and firmly attached to the coated dish. For further experiments HBP were maintained in EMEM with L-glutamine containing 10% human serum, 20 % newborn calf serum, 0.1 % basic fibroblast growth factor and 2 % penicillin/streptomycin. Prior to experiments, HBP were incubated in EMEM containing 0.1% bovine serum albumin (BSA) and antibiotics for at least 4 hours. Next, cells were incubated in fresh EMEM/0.1% BSA, either supplemented or not with 25 μM synthetic DAβ1-40 peptide for the indicated number of days. Glioma cells were maintained in Dulbecco’s modified essential medium (DMEM) with 10 % fetal calf serum (FCS) and 2 % penicillin/streptomycin. U373 astrocytoma cells were
maintained in 45 % HAM’s F10 medium with L-glutamine, 45 % DMEM with 4.5 g/l glucose and L-glutamine, 10 % FCS and 2 % penicillin/streptomycin. IMR32 neuroblastoma cells were maintained in RPMI 1640 medium supplemented with 25 mM L-2-hydroxyethylpiperazine–L-2-ethanesulfonic acid (HEPES), L-glutamine, 10 % FCS, 1% sodiumpyruvate and 2 % penicillin/streptomycin. All media and sera were obtained from Bio-Whittaker Europe. Astrocytoma cells (U373) were a kind gift of Dr. R. Veerhuis (Dept. of Pathology, Free University, Amsterdam, The Netherlands), neuroblastoma cells (IMR-32) were a kind gift of Dr. G. Bosman (Dept. of Biochemistry, UMC Nijmegen, The Netherlands) and glioma cells (E2) were a kind gift of Dr. Bernsen and Prof. Van der Kogel (Dept. of Radiotherapy, UMC Nijmegen, The Netherlands).

**Western blot analysis**

After removal of the media, cells were thoroughly rinsed three times with physiological salt solution. Subsequently, cells were treated with 8 M urea (Sigma), and aliquots of cell lysates (similar protein concentrations) were mixed with equal amounts of dithiothreitol (DTT)-containing reducing sample buffer (2.3 % sodiumdodecyl sulfate (SDS), 12.5 % Tris-HCl (0.5 M, pH 6.8), 77 % glycerol, 3% DTT and bromophenol blue indicator in milliQ). This mixture was heated for 5 minutes at 100 °C and proteins were subsequently separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred onto nitrocellulose membranes (Hybond, Amersham Corp.) in blotting buffer, containing 25 mM Tris (pH 8.6), 192 mM glycine and 20% methanol. Blots were incubated for 15 minutes in blocking solution (PBS/0.05% Tween-20 (PBS-T) containing 5% low fat milk powder, rinsed three times in PBS-T and subsequently incubated overnight at 4°C with specific primary antibodies in PBS-T with 0.5% low fat milk powder (Table 1). The blots were then rinsed three times in PBS-T and incubated with the appropriate secondary antibody in PBS-T with 0.5% low fat milk powder for 1 hour at room temperature. Blots were washed three times in PBS-T and finally rinsed in PBS. Detection was performed by chemiluminescence according to the manufacturer’s protocol (Boehringer, Mannheim, Germany) and exposed to Kodak X-OMAT-R films. Each experiment was repeated three times.

**Immunofluorescence staining of DAβ1-40-treated HBP**

To study the effects of DAβ1-40 on the expression of agrin and glypican-1 at the immunocytochemical level, HBP were cultured in sterile gelatin-coated 8-well chamber slides, and incubated either with or without DAβ1-40 for 3 days. After fixation with periodate-lysine-paraformaldehyde (PLP) for 10 minutes, cells were incubated overnight at 4°C with either anti-agrin (JM72) or anti-glypican-1 (S1). Finally, cells were incubated with FITC-labeled sheep anti-mouse for 1 hour and mounted in Vectashield (Vector, Burlingame, CA). Between incubation steps, cells were thoroughly washed with PBS. All antibodies were diluted in PBS containing 0.1% bovine serum albumin, which also served as a negative control. Nuclei were stained with To-Pro-3 (Molecular probes, Eugene, Oregon, dilution 1:1000). Immunofluorescence stainings were examined using a confocal laser-scanning microscope (Leica, Wetzlar, Germany).
**Immuno-electron microscopical analysis**

HBP were cultured on fibronectin-coated foil bottom dishes (WillCo Wells BV, Amsterdam, The Netherlands) in Eagle’s minimal essential medium (EMEM, Bio-Whittaker, Verviers, Belgium) supplemented with 10% human serum, 20% FCS and 0.1% basic fibroblast growth factor to confluency. Cells were incubated overnight with EMEM containing 0.1% BSA. Subsequently, cells were incubated for three days with fresh EMEM-BSA with or without 12.5 µM DAB$_{1-40}$ peptide. After washing twice with PBS, pericytes were fixed in PLP for 2 hours, followed by washing with PBS and blocking with 20% goat serum in PBS with 0.1% BSA for 1 hour. Cell preparations were pre-treated with 0.05% Triton-X in PBS for 30 minutes to increase permeability of the cells. Incubation with antibody JM72 was performed overnight at 4°C. After washing four times with PBS, samples were incubated with ultra-small gold conjugated goat anti-mouse antibodies (Aurion, Wageningen, The Netherlands) overnight at 4°C. Again, samples were washed with PBS and fixed for 10 minutes in 2% glutaraldehyde, washed with PBS, followed by a 10 minutes post-fixation with 2% osmium tetroxide. After intense washing with PBS and ultrapure water the preparations were silver-enhanced with the Aurion R-Gent SE-EM kit and the reaction was stopped after 20 minutes in ultra pure water. Then, cells were washed in 30% ethanol, en-bloc stained with 2% uranyl acetate in 30% ethanol for 30 minutes at 4°C and washed again in 30% ethanol. Subsequently, samples were dehydrated with increasing concentrations of ethanol followed by mixtures of epon with 100% ethanol and finally pure epon resin. The foil was cut with a razor blade and embedded in pure epon resin. Ultrathin sections were cut on a Leica Ultracut and stained with uranyl acetate and lead citrate. Photographs were taken on a Jeol 1200 EX/II electron microscope at 60 kV.

**Assessment of $^{35}$S-sulfate incorporation**

To study effects of DAB$_{1-40}$ on proteoglycan synthesis, HBP were cultured for 24 h in EMEM/0.1% BSA, in the presence or absence of 12.5 µM synthetic DAB$_{1-40}$ peptide. Transforming growth factor-β1 (TGF-β1) increased the production of various extracellular matrix proteins, including HSPGs (30) and therefore was used as positive control (2 ng/ml). Subsequently, $^{35}$S-labeled sulfate (25 µCi) (PerkinElmer, Boston, MA, USA) was added to the medium for 24 h. Then, cells were washed in EMEM three times and digested for 2 h at 65°C in a papain solution containing chondroitin sulfate as carrier. Cells were centrifuged for 10 minutes at 1000 g to remove cell debris and 0.2% cetylpyridinium chloride (CPC) solution was added to the supernatant in equal amounts and incubated for 2 h at 37°C to precipitate the GAGs. After incubation the mixture was centrifuged for 10 minutes at 10,000 g to collect the precipitate. Finally, the precipitated residue was rinsed twice and dissolved in Lumasolve (Omnilabo, Breda, The Netherlands). Subsequently, the dissolved precipitated residue was diluted in Optifluor (Packard BioScience, Groningen, The Netherlands) and $^{35}$S-sulfate incorporation was measured by liquid scintillation counting (PerkinElmer 1450 Microbeta, Boston, MA, USA) and expressed as counts per minute (cpm). One-sided unpaired t-tests were performed to compare the effects of TGF-β1 or DAB$_{1-40}$ on $^{35}$S-GAG incorporation. All values are expressed as means ± SD. Each experiment was repeated three times.
Results

Western blot analysis
The expected 64 kDa band for glypican-1 core protein was present in the urea extracts of HBP, U373 astrocytoma cells and IMR32 neuroblastoma cells, but undetectable in glioma extracts (Figure 1A). Agrin was detected with monoclonal antibody JM72 as a band with an approximate molecular weight of 212 kDa particularly in cell lysates of HBP. Additionally, glioma cells, astrocytoma cells and neuroblastoma cells produced considerable amounts of agrin. A second immunoreactive band just above 212 kDa was observed in astrocytoma cell lysates (Figure 1B). Both anti-glypican-1 and anti-agrin antibodies also recognized high molecular weight smears, that probably represented glycosylated variants of glypican-1 and agrin (Figure 2A and B). Detection of syndecan-1 with the DL-101 antibody resulted in a band with approximate weight of 100 kDa in extracts of all cell types (Figure 1C). 1C7, a monoclonal antibody directed against syndecan-3 detected characteristic bands at 50 kDa in extracts of HBP, glioma cells and astrocytoma cells, whereas no syndecan-3 band was detected in lysates of neuroblastoma cells (Figure 1D). Collagen XVIII core protein (180 kDa) was observed in cell lysates of HBP and astrocytoma cells, whereas neither glioma cells nor neuroblastoma cells produced detectable amounts of collagen XVIII (Figure 1E).

To examine possible effects of DAβ1-40 on HSPG metabolism, HBP were treated with 12.5 μM DAβ1-40 for 3 to 6 days. Treatment of cultured HBP with DAβ1-40 induced signs of cellular degeneration, like disruption of the cell membrane. Interestingly, both glypican-1 and agrin expression levels were significantly increased upon DAβ1-40 treatment (Figure 2A and B). Moreover, DAβ1-40 treatment led to an increase in the expression of high molecular weight smears (> 200 kDa), which could be detected with both anti-agrin and anti-glypican-1 antibodies. Such high molecular smears are characteristic for heavily glycosylated proteoglycans. Addition of DAβ1-40 to HBP did not result in differential expression of collagen XVIII, syndecan-1 or syndecan-3 (data not shown).
Figure 1. Western blot analysis of heparan sulfate proteoglycans from human brain pericytes (HBP), glioma cells (E2), astrocytoma cells (U373) and neuroblastoma cells (IMR32).

Figure 2. Western blot analysis of glypican-1 and agrin extracted from control (lane 1) and DAβ1,4G-treated cultured human brain pericytes (lane 2).
**Immunocytochemical and immuno-electron microscopical analysis**

The anti-agrin antibody demonstrated a perinuclear distribution pattern (Figure 3A), while glycan-1 antibody stained the membrane of control HBP (Figure 3C). In line with the western blot results we observed an increased agrin (Figure 3B) and glycan-1 (Figure 3D) expression. In addition, DAβ1-40 treatment resulted in an intense cell membrane associated staining pattern (Figure 3B). Immuno-electron microscopy (IEM) analysis of untreated cells showed agrin immunopositivity within lysosomal structures (Figure 4A), whereas upon DAβ1-40 treatment, agrin was predominantly found in close association with DAβ1-40 fibrils on the cell surface and in DAβ1-40-containing phagosomes (Figure 4B). Glycan-1 immunoreactivity in control cells was mainly observed at the cell surface and sporadically in the cytoplasm (Figure 4C). After DAβ1-40 treatment glycan-1, like agrin, colocalized with amyloid fibrils on the cell surface. Additionally, glycan-1 immunoreactivity was also detected throughout the cytoplasm (Figure 4D).

![Figure 3.](image)

Immunofluorescence staining of control (a, c) and DAβ1-40 treated (b, d) cultured human brain pericytes (HBP) for agrin (a,b) and glycan-1 (c,d). Protein expression of agrin and glycan is clearly increased upon incubation with DAβ1-40. Original magnification 630x. For color figure see appendix.
Figure 4.
Immuno-electron microscopic analysis of control (a,c) and DA\(\beta\)-treated (b,d) human brain pericytes (HBPs) for localization of agrin (a,b) and glypican-1 (c,d). In untreated pericytes agrin is mainly found within lysosomes (a, arrow), and glypican in close association with the cell surface (c). After DA\(\beta\)-treatment agrin colocalized with DA\(\beta\) fibrils located on the cell surface (b, arrow) (e) and in phagosomal structures (b, arrowhead) (f). Glypican-1 immunoreactivity was associated with DA\(\beta\) fibrils on the cell surface (d,d, arrow) and distributed throughout the cytoplasm. Original magnification a,b 12,000x, c-f 25,000x
The effect of DAβ1-40 on $^{35}$S-incorporation
To study the influence of DAβ1-40 on $^{35}$S-sulfate incorporation, HBP were cultured for 24 h either supplemented or not with 12.5 μM synthetic DAβ1-40. Incorporation of radioactive sulfate serves as an indication of glycosaminoglycan (GAG) synthesis. Incorporation of $^{35}$S-sulfate was significantly enhanced in TGF-β1-treated HBP, compared to control HBP. Addition of DAβ1-40 even resulted in a 3-fold increase in $^{35}$S-sulfate incorporation (Figure 5).

![Figure 5.](image)

**Effect of DAβ1-40 on $^{35}$S-glycosaminoglycan incorporation in human brain pericytes.** TGF-β1 treatment markedly enhanced $^{35}$S-sulfate incorporation (****: p < 0.01), compared to control HBP. Incubation with DAβ1-40 resulted in a 3-fold significant increase in $^{35}$S-sulfate incorporation (****: p < 0.01).

Discussion

Several immunohistochemical studies have demonstrated that specific heparan sulfate proteoglycan (HSPG) subtypes are involved in the formation of cerebral and cerebellar senile plaques, tangles and cerebrovascular amyloid depositions in brains of individuals with Alzheimer’s disease (AD). In AD brain, neuritic plaques are surrounded by reactive astrocytes and activated microglial cells. Astrocytes are known to synthesize and release a range of molecules among which are growth factors, neurotransmitters and extracellular matrix components like PGs (31), making this specific cell type a possible source of neuritic SP-associated PGs. Alternatively, neurons and microglial cells, particularly those in close vicinity of neuritic plaques, may also serve as a source of HSPGs. Previous reports have shown that both neurons, glial cells and reactive astrocytes produce agrin, glypican-1 and syndecan -3 (18;32-34). In line with these studies we demonstrated that neuroblastoma, glioma and astrocytoma cells express various HSPG subtypes *in vitro*. Although syndecan-3 is ubiquitously present in CNS tissue and produced by neurons we were unable to detect syndecan-3 expression by IMR32 neuroblastoma cells. This discrepancy might be explained by the use of a neuroblastoma cell line instead of cultured primary neurons. Specific HSPG subtypes that accumulate in CAA-affected vessels e.g. collagen XVIII and glypican-1 may be locally produced by vascular cell types, like HBP. It has been reported that both endothelial cells and pericytes produce collagen
XVIII (35). Here we also show that human brain-derived pericytes express other HSPG species such as agrin, syndecan-1 and syndecan-3, in vitro.

Western blot analysis showed the expected bands for glypican-1 (64 kDa), syndecan-1 (80 kDa), agrin (212 kDa) and collagen XVIII (180 kDa). Although syndecan-3 has a core protein of 80 kDa we observed a band at about 50 kDa in cell lysates. This band may represent a common breakdown product of syndecan-3 containing the antibody epitope. Surprisingly, we predominantly detected HSPG core proteins and not high molecular smears, which are characteristic for proteoglycans carrying GAG side-chains. It is likely that HSPGs produced by above-mentioned cell types under non-stimulated conditions contain relatively few GAG side-chains.

Accumulation of GAGs occurred virtually at the same time and at the same location as amyloid deposition in experimental amyloidosis (36). One explanation for the close spatiotemporal relation between GAGs and amyloid may be that amyloid interferes with the rate of biosynthesis of HSPGs. Here we studied the effect of DAB1_{1-40} on the production of several HSPGs and demonstrated that addition of DAB1_{1-40} to HBP may result in an increase of both highly glycosylated glypican-1 and agrin. However, to confirm this finding, future research will be needed to study the regulation of agrin and glypican-1 expression at the mRNA level. These experiments will provide quantitative PCR data, which may prove our hypothesis that DAB1_{1-40} influences the production of agrin and glypican-1. Interestingly, DAB1_{1-40} treatment did not affect the synthesis of syndecan-1, syndecan-3 and collagen XVIII, suggesting that agrin and glypican-1 play a specific role in AD pathogenesis. DAB1_{1-40} treatment caused an increase in agrin and glypican-1 production, which may contribute to the deposition of these specific HSPG subtypes in AD lesions. Immuno-electron microscopy revealed that both agrin and glypican-1 were found in close proximity of DAB1_{1-40} fibrils on the cell. These cell surface-associated amyloid fibrils might function as a HSPG reservoir thereby explaining the excessive accumulation of HSPGs in AD lesions. In this respect, it is interesting that both agrin and glypican-1 are the most predominant HSPG species accumulating in senile plaques, vascular amyloid and neurofibrillary tangles (18-20).

Today, evidence is accumulating that prefibrillar oligomeric structures, or protofibrils, may be the key toxic species in AD and responsible for cell death, whereas mature fibrils may essentially be neuroprotective (30). It has been shown that HSPGs enhance the conversion of prefibrillar Aβ protofibrils into mature Aβ fibrils. If Aβ treatment indeed leads to an increase in HSPG production, it may actually be a neuroprotective response to the exposure of Aβ.

In summary, we demonstrated that several brain specific cell types produce a variety of HSPGs in vitro. Both [35S]-sulfate labeling, western blot analysis and (electron) microscopy revealed that treatment of HBP with DAB1_{1-40} resulted in a marked increase of both highly glycosylated glypican-1 and agrin. We therefore hypothesize that regulation of HSPG expression may be closely linked to Aβ formation and that this mechanism may modulate AD pathogenesis. Future studies at the mRNA level will provide information whether indeed acts as an inducer of HSPG production.
Additional research is also needed to study the effects of wild type $\beta_{1-40}$ and $\beta_{1-42}$ on HSPG metabolism.

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References


Een blot in de morgen geeft soms een week vol zorgen
Chapter 8

Cerebral amyloid angiopathy with severe secondary vascular pathology: a histopathological study

Jack van Horssen\textsuperscript{1*}, Danielle de Jong\textsuperscript{2}, Robert M.W. de Waal\textsuperscript{1}, Cathy Maass\textsuperscript{1}, Irene Otte-Höller\textsuperscript{1}, Berry Kremer\textsuperscript{2}, Marcel M. Verbeek\textsuperscript{2}, Pieter Wesseling\textsuperscript{1}

From the Departments of Pathology\textsuperscript{1} and Neurology\textsuperscript{2}, University Medical Center, Nijmegen, The Netherlands.

Submitted
Abstract

Cerebral amyloid angiopathy (CAA) is a common neuropathological finding and is characterized by deposition of fibrillar amyloid in cortical and leptomeningeal vessels. In this study we describe the macroscopic and microscopic neuropathological findings of five patients with severe CAA-associated secondary vascular changes, including smooth muscle cell degeneration, hyalinization, double-barreling phenomenon, macrophage infiltration, and aneurysmal dilatation of the vessel wall. In three of the five patients these vascular changes were associated with multiple small hemorrhages, whereas in two patients areas of ischaemic necrosis were observed. However, none of these patients suffered from large (lobar) hemorrhagic accidents. Nevertheless, severe cerebral amyloid angiopathy, particularly when associated with secondary vascular pathology, may lead to vascular dementia-like ischaemic changes. Hence, the distinction between cerebral amyloid angiopathy patients from those suffering from vascular dementia (VaD) can be difficult. We speculate that CAA, particularly when associated with secondary vascular pathology, although not resulting in large hemorrhages, may contribute to cognitive decline. The functional impact of CAA and CAA-related secondary vascular changes on cognitive performance warrants further exploration.
Introduction

Cerebral amyloid angiopathy (CAA) is a common finding in the aging brain. It is observed in 80-90% of patients with neuropathologically confirmed Alzheimer’s disease (AD), in 15-20% of patients who present with lobar hemorrhages, as well as in elderly patients with small cortical infarcts or punctate cortical hemorrhages [3,15,16,17,39]. CAA is characterized by deposition of amyloid in the media and adventitia of medium-sized and small cortical and leptomeningeal vessels [41]. Except for rare cases of cerebral amyloidosis, caused by depositions of cystatin C [27], transthyretin [14], gelsolin [19], amyloid-Bri [13] or prion protein [4], in the vast majority of cases the main constituent of these amyloid deposits is the amyloid \( \beta \)-protein (A\( \beta \)) [6,30].

Although CAA is a well-defined neuropathological finding, the clinical impact of this phenomenon in the context of AD is not yet clear. Its best-known clinical presentation is lobar cerebral hemorrhage [5,16,18,43]. In fact, the term CAA is often equated with a distinct disease entity that predisposes to lobar hemorrhages, rather than with a strictly defined neuropathological finding that may be encountered in various diseases [39]. However, severe CAA may be present without the occurrence of lobar hemorrhages [11,42].

Depending on the degree of damage of the vessel wall, CAA can be histologically classified as mild, moderate or severe [42]. In most AD cases, the damage is microscopically mild and limited to deposition of amyloid with replacement of vascular smooth muscle cells (SMCs) or pericytes and thickening of the vessel wall. In some cases, however, secondary changes may occur such as (several types of) inflammation, aneurysmal dilatation, and bleeding. Although the histopathology of CAA is relatively well-documented, little attention has been given to secondary vascular changes. Mandubur described for the first time the occurrence of several secondary microvascular changes in cases of CAA [23]. Other reports on the association of AD- or age-related CAA and secondary vascular changes are rare [39,42]. In this paper, we present the neuropathological findings of five patients with severe CAA and extensive secondary vascular changes, but without lobar cerebral hemorrhage.

Materials and methods

Autopsy material
On the basis of a previous immunohistochemical study on the distribution of heparan sulfate proteoglycans in CAA-affected vessels [35], five patients with extremely extensive CAA and severe secondary vascular changes were identified (3 men, 2 women: age 77.2 ± 7.2 years; post mortem delay 4.4 ± 0.9 hours). Formalin-fixed, paraffin-embedded tissue samples from cerebellum, hippocampus, frontal and occipital neocortex were stained and vascular changes systematically and semiquantitatively scored (see below). Similar material of 5 age-matched, pathologically confirmed AD patients (age 78.4 ± 6.8 years; post mortem delay 4.7 ±
0.8 hours) was used as control tissue. Relevant clinical information was retrieved from patient files.

**Histology and immunohistochemistry**

Macroscopic changes in the central nervous system were studied by both external examination and at cut surface after coronal slicing of the brain. Microscopic cerebrovascular pathology was studied in detail by staining of serial sections with hematoxylin-eosin (H&E) and a combined H&E and Luxol Fast Blue (LFB) staining for myelin. Calcification of the vessel wall was analyzed using the Von Kossa staining. Additionally, immunohistochemical stainings were performed for the detection of amyloid β-protein (anti-Aβ antibody A4; DAKO, Glostrup, Denmark), hyperphosphorylated tau protein (AT8; Innogenetics, Ghent, Belgium), α-smooth muscle actin (αSMA; DAKO) and the macrophage marker CD68 (DAKO).

**Quantification of histological abnormalities**

Frontal and occipital blood vessels were particularly laden with amyloid and hippocampal and cerebellar vessels were relatively spared of CAA. Therefore, we focused for this study on the frontal and occipital regions with regard to the secondary vascular changes in these vessels. In analogy to Vinters et al. [40] the following secondary changes in CAA-affected vessels were scored in a semiquantitative way: 1) hyalinization/fibrosis; 2) smooth muscle cell degeneration; 3) double barreling phenomenon; 4) macrophage infiltration within the vessel wall; 5) multinucleated giant cells in or around the vessel wall; 6) perivascular chronic inflammation; 7) calcification; 8) necrosis of the vessel wall; 9) microaneurysms; 10) recent micro-hemorrhages; and 11) iron and hemosiderin deposition.

Histopathological slides of frontal and occipital neocortex were scored as follows: absence (-) or sporadic (+), moderate (++), frequent (+++) occurrence of CAA-associated lesions. Additionally, we analyzed the distribution and frequency of NFTs according to the Braak and Braak method (1), and the presence of SPs was scored as absent (-), sporadic (+), moderate (++), or frequent (+++). Above-mentioned histopathological features were evaluated and scored independently by two investigators (JvH, PW). Discrepancies were evaluated and consensus was obtained after re-evaluation.

**ApoE genotyping and APP mutation screening**

DNA was extracted from small pieces of cerebral cortex using a DNA isolation kit (Biozym, Landgraaf, the Netherlands). Apolipoprotein E (APOE) genotypes were determined by polymerase chain reaction (PCR) and HhaI restriction analysis as described by Hixson and Vernier [12]. Screening for APP mutations was performed by PCR and sequencing of exon 16 and 17 of chromosome 21 according to previously described methods [7,25].
Results

Clinical features
All five patients had suffered from a progressive dementing illness, clinically diagnosed in four of the five cases as AD or vascular dementia. However, retrospective application of the NINCDS-ADRDA criteria would lead us to redefine the clinical diagnosis of probable AD for patients 1-3 and 5, whereas none of the patients fulfilled established criteria for vascular dementia or CAA. In four patients the disease duration was five to ten years, but in patient 1, with a relatively early disease onset, the disease duration was 19 years. Neuroimaging (T2 gradient echo) revealed small cerebral hemorrhages only in patient 3, whereas none of the patients suffered from large cerebrovascular accidents. Only one patient, case 4, had a history of an atypical, rapidly progressive dementia with myoclonus and extensive leukoencephalopathy on MRI (Table 1).

<table>
<thead>
<tr>
<th>Patient</th>
<th>No./ Sex/ Age (yr)</th>
<th>Family History of Dementia</th>
<th>Vascular Factors</th>
<th>Clinical Symptoms</th>
<th>Age at Onset (yr)</th>
<th>Disease Duration (yr)</th>
<th>Progression of Decline</th>
<th>Compatible with clinical diagnostic criteria</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Risk</td>
<td>Stroke</td>
<td>Gradual</td>
<td></td>
<td></td>
<td>AD¹ VaD² CAA¹</td>
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<tr>
<td>1/M/75</td>
<td></td>
<td>0</td>
<td>HChol, D, BP, E*</td>
<td>-</td>
<td>56</td>
<td>19</td>
<td>Gradual A, LE</td>
<td>yes no no</td>
</tr>
<tr>
<td>2/N/78</td>
<td></td>
<td>0</td>
<td>HT, D, BP</td>
<td>-</td>
<td>68</td>
<td>10</td>
<td>Gradual A</td>
<td>yes no no</td>
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<tr>
<td>3/V/89</td>
<td>3 S and P</td>
<td>0</td>
<td>Pacemaker, D, BP, E*, D, BP, E*</td>
<td>-</td>
<td>80</td>
<td>9</td>
<td>Gradual cortical</td>
<td>yes no no</td>
</tr>
<tr>
<td>4/M/74</td>
<td></td>
<td>0</td>
<td>MI, HT, M*</td>
<td>-</td>
<td>66</td>
<td>7</td>
<td>Stable, last year fast</td>
<td>A, LE, I (incl. Lentiformis) LE</td>
</tr>
<tr>
<td>5/M/70</td>
<td></td>
<td>0</td>
<td>D, BP, E*</td>
<td>-</td>
<td>61</td>
<td>9</td>
<td>Gradual A</td>
<td>yes no no</td>
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</table>

Table 1. Clinical features of CAA patients
S = sister, P = father, HChol = hypercholesterolaemia; HT = hypertension; MI = myocardial infarction; D = dementia; BP = behavioral problems, E = epilepsy, M = myoclonia, A = atrophy, LE = leukoencephalopathy, I = infarction, AD = Alzheimer’s disease, VaD = vascular dementia, CAA = cerebral amyloid angiopathy *during last stage of disease; ¹NINCDS-ADRDA criteria for probable AD; ²NINDS-AIREN criteria for probable vascular dementia; ³Boston criteria for probable CAA.

Macroscopy
Macroscopic examination revealed multiple small petechial hemorrhages in the cerebral neocortex of three patients (Figure 1). Atherosclerosis of basal arteries and cortical atrophy varied from absent to severe. Except for one patient, ventricles showed moderate to marked dilatation. Ischaemic necrotic lesions, sometimes accompanied by old hemorrhages were observed in four patients. Macroscopic observations are listed in Table 2.
Macroscopic examination revealed multiple small old and recent hemorrhages (arrow) in two patients.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Weight (g)</th>
<th>Atherosclerosis basal arteries</th>
<th>Cortical atrophy</th>
<th>Ventricles</th>
<th>Focal lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>250</td>
<td>severe</td>
<td>moderate to severe (especially in fronto-temporal region)</td>
<td>moderate dilatation</td>
<td>multiple small old ischaemic-necrotic lesions, occasional larger old ischaemic-necrotic lesions with dispersed old hemorrhages</td>
</tr>
<tr>
<td>2</td>
<td>1030</td>
<td>unknown</td>
<td>moderate</td>
<td>unknown</td>
<td>absent</td>
</tr>
<tr>
<td>3</td>
<td>890</td>
<td>moderate to severe</td>
<td>severe (especially in fronto-temporo-occipital region)</td>
<td>marked dilatation</td>
<td>large old ischaemic-necrotic lesions (occipital pole, 3 cm)</td>
</tr>
<tr>
<td>4</td>
<td>1310</td>
<td>moderate</td>
<td>absent</td>
<td>normal</td>
<td>multiple small, old and more recent, hemorrhages</td>
</tr>
<tr>
<td>5</td>
<td>880</td>
<td>absent</td>
<td>severe</td>
<td>marked dilatation</td>
<td>dispersed old ischaemic-necrotic lesions</td>
</tr>
</tbody>
</table>

Table 2. Macroscopic information of CAA patients.

Microscopy

Aβ and tau-immunoreactivity
Extensive Aβ deposits were observed in middle-sized and small cortical and leptomeningeal blood vessels with a predilection for the occipital and frontal cortex (Figure 2A-C). In the cerebellum, small and sporadically larger, leptomeningeal vessels were affected. Occasionally, large cortical and leptomeningeal vessels in the parahippocampal region were highlighted by the anti-Aβ antibody, but most of the hippocampal vessels were devoid of Aβ. Aβ-staining revealed abundant presence of both diffuse and classic senile plaques (SPs) throughout the frontal and occipital cortex of two patients (Figure 2B), whereas in the other three brains only a small number of SPs were found in these areas (Figure 2C). Small numbers of diffuse SPs
were observed in the hippocampus and cerebellum of all patients. AT8, an antibody directed against hyperphosphorylated tau protein, strongly stained tangles and dystrophic neurites in frontal and occipital neocortex as well as in hippocampal sections. According to the Braak and Braak method, four patients were classified as stage VI with NFTs located throughout the neocortex. Patient 4 was graded as stage III with only AT8 positive tangles in the hippocampus and not in the cerebral neocortex.

![Figure 2](image1.png)

**Figure 2.** Overview of immunohistochemical staining of cerebral amyloid angiopathy and senile plaques from frontal (a,b,c) neocortex for Aβ (mAb 6C6). A majority of the "double-barrel" leptomeningeal vessels only show infiltration of Aβ in the outer media/most abluminal part of the vessel (a; arrows), whereas sporadically the inner ring contains Aβ (a; small arrow). Both cortical and leptomeningeal vessels are laden with Aβ, either with (b) or without (c) the presence of multiple senile plaques. Original magnifications: a,b,c x50.

**Secondary degenerative changes**

Whereas α-SMA is normally extensively present in the tunica media of all types of cerebral vessels except capillaries, severe reduction of α-SMA expression was observed in heavily amyloid-laden parenchymal and leptomeningeal vessels in all patients (Figure 3A). Other prominent findings in the examined brain sections were the presence of vascular hyalinization (Figure 3B) and the double-barreling phenomenon (Figure 3C). In three patients this phenomenon was observed in both cortical and leptomeningeal vessels, whereas in the other two patients it was restricted to the leptomeningeal blood vessels. Neither calcified deposits nor fibrinoid necrosis were observed in CAA-affected vessels in any of the five patients. Large areas of ischaemic necrosis were only present in two patients in the vicinity of CAA-affected vessels with major secondary changes (Figure 3D). LFB staining demonstrated white matter lesions (myelin pallor), often accompanied by perivascular atrophy, in a majority of the sections (Figure 3E).
**Secondary inflammatory changes**

We observed a number of abnormalities indicative of an inflammatory reaction in and around amyloid-laden vessels. Macrophage infiltration within and around the walls of CAA-affected vessels was found in all patients (Figure 4A). Reactive astrocytes and gliosis were frequently seen around both amyloid-laden and hyalinized vessels. However, multinucleated giant cells were not observed and only sporadically perivascular lymphocytes were detected around affected vessels (Figure 4B).
Aneurysms, micro-hemorrhages and iron/hemosiderin accumulation
Microaneurysms were sporadically found in three brains (Figures 5A and B) and were often accompanied by the loss of both α-SMA and Aβ staining in the affected part of the vessel wall. Remnants of former hemorrhages in the form of intraparenchymal iron depositions were found in all patients (Figures 5C and D), whereas recent focal intraparenchymal extravasation of erythrocytes was only observed in two patients (Figure 5E). Overall results of the immunohistochemical stainings are summarized in Table 3.
APOE genotypes and APP mutations

Genetic analysis revealed no mutations in the Aβ region of APP. Moreover, we did not find an overrepresentation of a specific ApoE genotype associated with this small group of patients compared to the genotype distribution in the general population (Table 3).

AD patients

In the control group of AD patients, multiple characteristic AD changes were observed in all patients e.g. SPs, NFTs and mild to moderate CAA. In contrast to the patients with severe CAA, no secondary vascular changes were observed. Only sporadically macrophages were found in the vicinity of amyloid-laden vessels. Occasionally, reduced α-SMA expression was observed in CAA-affected intracortical and leptomeningeal vessels. None of the other secondary degenerative and inflammatory changes were present in these age-matched patients.
Discussion

In this study we describe the macroscopic and microscopic neuropathological findings in five patients with severe CAA-associated secondary vascular changes. All patients showed extensive amyloid angiopathy of both leptomeningeal and parenchymal vessels. Next to CAA, which is a common neuropathological finding in AD brains, secondary vascular pathology was the most striking observation. Although it has been demonstrated that severe CAA is an important factor in the genesis of lobar cerebral hemorrhages [39,40], none of the patients in our study suffered from such large hemorrhages. Today, diagnostic criteria as proposed by the Boston CAA group are only reserved for cases with CAA-related hemorrhages and full post mortem examination [20]. The fact that none of the patients in our study suffered from such large hemorrhages emphasizes the need for new more sensitive and specific diagnostic tests to diagnose these patients during lifetime.

CAA is also found in the brains of individuals with Down's syndrome [24] and carriers of the Flemish [32], London [34] and Dutch APP mutation [21,22]. Recently, Grabowski and colleagues identified a novel APP mutation associated with severe CAA [8]. Both SPs and CAA can be observed in brains of individuals with Down's syndrome and the Flemish mutation, whereas brains of patients with the Dutch mutation lack typical AD features such as NFTs and cored plaques. Patients with HCHWA-D suffer from recurrent hemorrhagic strokes with mid-life onset. These strokes result from severe CAA with secondary microvascular degeneration [40]. In addition, since it has been described that these patients may develop cognitive alterations before the onset of their first hemorrhage, CAA may also contribute to cognitive decline [26]. Patients in our study showed some similarity with HCHWA-D patients, since both groups of patients suffered from severe CAA accompanied by profound secondary vascular changes. In contrast, in our patients large cerebral bleedings were not encountered and APP mutations were absent.

Vascular dementia is defined as the loss of cognitive function resulting from ischaemic, ischaemic-hypoxic, or hemorrhagic brain lesions as a result of cerebrovascular disease and cardiovascular pathologic changes. Severe CAA with associated secondary vascular pathology might lead to similar ischaemic-like changes, making the distinction between patients with widespread CAA and those suffering from VaD difficult.

Today, the number of AD investigators that consider CAA as a potentially important contributor to the cognitive decline in AD is increasing. Evidence for a role of CAA in AD pathogenesis and dementia has come from large clinico-pathological studies, which describe that AD patients with concomitant CAA were more severely cognitively impaired than those without CAA [17,29]. Still the functional impact of vascular pathology in AD pathology in general and CAA-related secondary vascular changes in particular on cognitive performance is largely unclear and needs further elucidation.
Accumulation of Aβ in the vascular media and/or adventitia is associated with degeneration of endothelial cells [33], vascular smooth muscle cells [2] and pericytes [37], and ultimately results in loss of integrity of the blood-brain barrier, or even defects in the vessel wall [31]. We observed severe loss or even complete absence of α-SMA in most of the CAA-affected vessels, illustrating the ongoing degeneration of smooth muscle cells. Hyalinization of vessels was one of the most frequently observed secondary vascular abnormalities. Hyalinized vessels often showed a weak Aβ-immunoreactivity, suggesting that in course of time, vascular Aβ leads to degeneration of vascular cells, followed by a gradual replacement by hyalinized fibrous tissue in the vessel wall. Vessels with massive Aβ deposition in the tunica media and adventitia show the ‘double barreling’ phenomenon [5,39], in which Aβ-positivity is often absent from the inner vessel wall remnant. This appearance might be the consequence of the loss of SMC resulting in either splitting of the tunica media or separation of the tunica media from the tunica adventitia. Additionally, CAA is also associated with fibrinoid necrosis and microaneurysm formation. Ultimately, these degenerative changes may lead to impaired vessel wall integrity cumulating into rupture and cerebral hemorrhages.

Inflammatory reactions are thought to play a minor role in the pathogenesis of CAA [38]. Nevertheless, macrophages infiltrating the vessel wall were noted in all patients. It is tempting to speculate that these cells attempt to clear Aβ by phagocytosis. It has been suggested that vascular Aβ might be a trigger for a giant cell reaction [9], however, such multinuclear cells were absent in our group of patients.

Several reports described a strong association between the severity of CAA and APOE ε4, the major risk factor for sporadic and late-onset Alzheimer’s disease [10,28,44]. Other studies observed that the APOE ε2 allele is over-represented in patients with CAA-related hemorrhages. Although we investigated a relatively small group of patients we did not observe any association of a certain APOE genotype with the presence of secondary vascular lesions.

In summary, we describe the macroscopic and microscopic neuropathological findings of five CAA patients with severe secondary vascular changes in detail. Although the impact of CAA-related secondary vascular changes on cognitive performance warrants further clarification, it is tempting to hypothesize that these vascular changes might contribute to the cognitive decline. This would imply that the classic view of differentiating AD, VaD and CAA might need revision, since the borders between these “entities” are blurred.

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References


Chapter 9

Summarizing discussion and future perspectives
The role of heparan sulfate proteoglycans in Alzheimer’s disease and Parkinson’s disease pathogenesis

Proteoglycans are invariably associated with all kinds of amyloid deposits in the human body. These complex macromolecules, in particular heparan sulfate proteoglycans, have also been implicated in several aspects of the pathogenesis of Alzheimer’s disease, including the genesis of senile plaques, cerebrovascular amyloid and neurofibrillary tangles. Today, evidence is accumulating for the involvement of proteoglycans and glycosaminoglycans in the pathogenesis of Alzheimer’s disease. Several immunohistochemical surveys performed in the 80’s and 90’s demonstrated that specific heparan sulfate proteoglycan subtypes may be involved in the formation of cerebral and cerebellar senile plaques, tangles and cerebrovascular amyloid deposits in brains of individuals with Alzheimer’s disease. However, due to the lack of specific antibodies, the exact nature of these HSPGs remained largely elusive. Nowadays, we have the disposal of well-defined antibodies directed against specific heparan sulfate proteoglycans. Herewith it is possible to examine the distribution of these complex macromolecules in Alzheimer’s disease brains and other amyloid-related disorders. In the first part of this thesis we describe the expression profile of several HSPGs in AD lesions.

Extracellular matrix-associated heparan sulfate proteoglycans
In previous studies, perlecan has been identified as the major heparan sulfate proteoglycan involved in the pathogenesis of Alzheimer’s disease (1). Additionally, several reports showed perlecan expression in senile plaques, tangles and amyloid-laden vessels (2;3). However, we were unable to confirm these observations using well-defined monoclonal antibodies directed against the core protein of perlecan (4;5). Therefore, we suggest that it is unlikely that perlecan plays a major part in the pathogenesis of Alzheimer’s disease. In contrast to perlecan, agrin was found to be the predominant heparan sulfate proteoglycan subtype present in all types of cerebral and cerebellar senile plaques (5;6) (chapter 3 and 4). These observations are in line with other reports that described agrin expression in both diffuse and classic cerebral senile plaques (7;8). Anti-agrin antibodies also immunostained a variable number of tangles and amyloid-laden vessels in Alzheimer’s disease brains. Collagen XVIII was found to be a novel heparan sulfate proteoglycan associated with both classic senile plaques and vascular amyloid, whereas neither tangles nor diffuse plaques were stained by anti-collagen XVIII antibodies (9) (chapter 5).

Cell membrane-associated heparan sulfate proteoglycans
Glypican-1 and syndecans 1-3 are expressed in both diffuse and classic cerebral senile plaques and tangles (5). In the cerebellum, glypican-1 is present in both diffuse and classic senile plaques, whereas syndecan 1-3 are only associated with classic plaques (6). Remarkably, the most intense glypican-1 and syndecan-2 immunopositivity is observed in amyloid cores of classic plaques and in amyloid-laden vessels, suggesting that these heparan sulfate proteoglycan subtypes, like collagen XVIII, have a particular affinity for fibrillar Aβ (4).
Heparan sulfate glycosaminoglycan side-chains
Antibodies directed against the glycosaminoglycan-side chains and to heparitinase-digested heparan sulfate proteoglycans stained all types of senile plaques in cerebrum and cerebellum. Interestingly, diffuse cerebellar senile plaques are more intensely and extensively stained for glycosaminoglycans than for core proteins, suggesting that free glycosaminoglycan chains may accumulate in these amorphous lesions. Moreover, heparan sulfate glycosaminoglycans are abundantly present in amyloid-laden vessels (4-6;10;11). Table 1 provides a summary of the distribution of heparan sulfate proteoglycans in normal vessels, cerebral and cerebellar senile plaques, vascular amyloid and neurofibrillary tangles.

Expression of heparan sulfate proteoglycans in Parkinson’s disease brains
Parkinson’s disease brains are neuropathologically characterized by the presence of Lewy bodies and Lewy neurites. These intraneuronal structures are mainly composed of α-synuclein aggregates. Although relatively little is known about the exact mechanism of α-synuclein fibrillization it has been demonstrated that heparan and heparan sulfate glycosaminoglycans promote α-synuclein aggregation in vitro (12).

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<th>HSPG species</th>
<th>normal vessels</th>
<th>CAA in AD</th>
<th>Cerebellar diffuse SPs</th>
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Table 1: Summary of HSPGs accumulation in normal vessels, cerebral and cerebellar SPs, vascular amyloid and neurofibrillary tangles. Positive fractions (percentage of amyloid laden vessels, senile plaques or tangles stained by a specific antibody compared with the anti-Aβ or anti-tau stainings) were scored in the following categories: 0 (+), 0-50% (+), 50-100% (++), or 100% (+++). 3 Restricted to staining of the basement membrane. 4 Restricted to staining of vascular Aβ. 5 Expression varied between the different patients. HSPG: heparan sulfate proteoglycan, CAA: cerebral amyloid angiopathy, AD: Alzheimer’s disease, SP: senile plaque.

However, we were unable to identify these molecules in Lewy bodies and Lewy neurites and therefore concluded that it is unlikely that these sulfated molecules are involved in α-synuclein fibrillization and stabilization (chapter 6). Animal models for Parkinson’s disease may be useful to examine whether heparan sulfate proteoglycans
and heparan sulfate glycosaminoglycans are actively involved in the early stages of Lewy body and Lewy neurite formation.

The results of our immunohistochemical studies indicate that:

1. Several types of heparan sulfate proteoglycans, including agrin, glypican-1, collagen XVIII and several syndecans are differentially expressed in various Alzheimer’s disease lesions (Table 1).
2. In contrast to what was concluded in previous reports, perlecan does not play a role in the pathogenesis of cerebral amyloid angiopathy, neurofibrillary tangles or senile plaques.
3. Agrin and glypican-1 are the major heparan sulfate proteoglycan subtypes present in senile plaques and amyloid laden vessels in Alzheimer’s disease brains.
4. Collagen XVIII is a novel heparan sulfate proteoglycan and is only associated with fibrillar Aβ-containing classic senile plaques and vascular amyloid.
5. The pathogenesis of cerebral amyloid angiopathy and senile plaques differs with regard to the involvement of specific heparan sulfate proteoglycan species.
6. Heparan sulfate proteoglycans and heparan sulfate glycosaminoglycans are likely not involved in α-synuclein aggregation.

**Cellular origin of amyloid-associated heparan sulfate proteoglycans**

Although our immunohistochemical data clearly demonstrated that heparan sulfate proteoglycans are associated with the characteristic Alzheimer’s disease lesions it is yet unknown which specific cell types are responsible for the excessive production of these sulfated molecules in Alzheimer’s disease brains. We demonstrate that several brain specific cell types, like human brain pericytes, neuroblastoma cells, glioma cells and astrocytoma cells produce a variety of HSPGs *in vitro* (chapter 7). In the future, cell biological research using primary cultures of various neuronal and neural cells may provide additional information about the cellular source of amyloid-associated proteoglycans.

**Induction of heparan sulfate proteoglycan expression by DAβ₁₋₄₀**

To investigate the possible influence of Aβ on the production of heparan sulfate proteoglycans we used a model system of cultured human brain pericytes. Using these cells we were able to study several aspects of Aβ-mediated toxicity. For these studies we used cytotoxic Aβ₁₋₄₀ carrying the Glu22Gln mutation as in hereditary cerebral hemorrhage with amyloidosis of the Dutch type (HCHWA-D). We found that treatment of human brain pericytes with DAβ₁₋₄₀ may result in an increase of glypican-1 and agrin and, more specifically, the levels of glycosylated glypican-1 and agrin. Both agrin and glypican-1 colocalized with DAβ₁₋₄₀ fibrils on the cell surface of human brain pericytes (chapter 7). These cell surface-associated amyloid fibrils might function as a heparan sulfate proteoglycan reservoir and may thereby contribute to the excessive accumulation of HSPGs in AD lesions. In this respect, it is remarkable that agrin and glypican-1 are the most predominant HSPG species accumulating in senile plaques, vascular amyloid and neurofibrillary tangles. Future studies at the mRNA level are needed to confirm whether DAβ₁₋₄₀ indeed acts as an inducer of heparan sulfate proteoglycan production. These studies will focus on the
regulation of agrin and glypican-1 at the mRNA level and will provide quantitative PCR data, which may prove our hypothesis that DAβ1-40 influences the production of agrin and glypican-1. Additional research is also needed to study the effects of wild type Aβ1-40 and Aβ1-42 on HSPG metabolism. If wild type Aβ1-40 and Aβ1-42 are linked to the regulation of heparan sulfate proteoglycans as well, it would be a challenge to unravel the underlying molecular pathways responsible for the overproduction of HSPGs in Alzheimer’s disease brains.

**Heparan sulfate proteoglycans: Friend or foe?**

It has been generally accepted that the conversion of monomeric Aβ into mature amyloid fibrils is necessary for the neurotoxic properties of Aβ. HSPGs catalyze this polymerization process, thereby contributing to the formation and deposition of neurotoxic fibrillar Aβ aggregates. Additionally, these sulfated biological macromolecules may also serve as chaperone proteins and thereby protect Aβ accumulations against proteolytic attack and degradation. It has been suggested that proteoglycans predominantly bind to Aβ via GAG side-chains, but an additional role for the protein backbone in this binding process cannot be excluded (13;14). The binding site has been identified as the 13-16 region (HHQK) of the Aβ peptide and represents a unique target site for the development of inhibitors of Aβ fibril formation (15). Since proteoglycans may alter the proteolytic processing of fibrillar Aβ, drugs that inhibit the binding of these glycoconjugates to Aβ might increase the turnover of Aβ by increased exposure to naturally occurring proteases. Both *in vitro* and *in vivo* studies demonstrated a protective activity of small polysulfated compounds against Aβ-induced effects (16). Several underlying mechanisms can be responsible for the protective actions of these sulfated GAG mimetics. First, they may inhibit the formation of β-pleated sheets and thereby the binding of heparan sulfate to Aβ (17-19). Finally, these highly sulfated compounds may block the adherence of Aβ to the cell surface. Either effect could result in protection of neurons and vascular cells against Aβ-mediated cellular toxicity (17). For application in a therapeutic setting it is important that such compounds meet strict criteria like a good bioavailability, including the ability to cross the blood-brain barrier efficiently, and a strong safety profile. In this respect, it is promising that even after chronic exposure to GAG mimetics no intrinsic cellular toxicity was observed (17-20). Furthermore, such a therapeutic strategy has already been shown to be effective in a mouse model, where these molecules clearly reduced the progression of inflammation-associated amyloid (21). Phase II clinical trials with therapeutic inhibitors of amyloid formation are underway and may become potential anti-amyloid therapeutic agents in the future.

Previous reports mostly focused on fibrillar Aβ as the major culprit in the pathogenesis. However, the identity of the neurotoxic aggregate and the mechanism by which it initiates its destructive effects on neurons are unknown. Recent biophysical studies aimed at elucidating the mechanism of in vitro fibrilization together with animal model studies suggest that prefibrillar oligomeric structures, or protofibrils, may be the key toxic species in Alzheimer’s disease, whereas mature fibrils may essentially be inactive or even neuroprotective (22). It has been shown
that heparan sulfate proteoglycans enhance the conversion of prefibrillar Aβ into mature Aβ fibrils. If Aβ treatment indeed leads to an increase in heparan sulfate proteoglycan production, it may actually be a neuroprotective response to the exposure of Aβ. In addition, we recently demonstrated that co-incubation of heparin or heparan sulfate with DAβ1-40 significantly inhibits DAβ1-40-mediated cell death in a dose dependent manner. This preliminary finding and the possibility that DAβ1-40 treatment induces heparan sulfate proteoglycan production may change our current way of thinking about the role of heparan sulfate proteoglycans in Alzheimer’s disease pathology.

During the last decade much progress has been made in unraveling the exact role of proteoglycans in the pathogenesis of Alzheimer’s disease, and evidence for an important function in Alzheimer’s disease pathology is accumulating. Our immunohistochemical and cell biological data convincingly demonstrated that heparan sulfate proteoglycans are tightly involved in Alzheimer’s disease pathology. However, further research is warranted to reveal whether heparan sulfate proteoglycans may act as friend or foe in the pathogenesis of Alzheimer’s disease.

The role of vascular pathology in Alzheimer’s disease pathology
The second part of this thesis describes the neuropathological findings of a small group of patients with severe cerebral amyloid angiopathy accompanied by major secondary vascular changes. Cerebral amyloid angiopathy is characterized by the deposition of amyloid β-protein in the vessel wall and may lead to severe vascular damage. Still, the functional impact of cerebral amyloid angiopathy on the pathogenesis of Alzheimer’s disease is relatively unclear and often considered as a coincidental finding. We showed that severe cerebral amyloid angiopathy, particularly when aggravated by secondary vascular changes, leads to impaired vessel wall integrity and microhemorrhages. However, none of the patients suffered from large (lobar) hemorrhagic accidents. Since diagnostic criteria as proposed by the Boston cerebral amyloid angiopathy group are only reserved for cases with cerebral amyloid angiopathy-related hemorrhages, none of our patients met clinical criteria for cerebral amyloid angiopathy. The established clinical criteria for cerebral amyloid angiopathy therefore fail to recognize the patients in our study. This finding emphasizes the need for new, more sensitive and specific diagnostic tests to diagnose these patients during lifetime.

Although in some patients a clinical diagnosis of vascular dementia was suggested during the course of the disease, none of the patients met the established clinical criteria for vascular dementia as well. Nevertheless, neuropathological examination revealed ischaemic lesions, characteristic for vascular dementia, in the brains of three patients. Vascular dementia is defined as the loss of cognitive function resulting from ischaemic, ischaemic-hypoxic, or hemorrhagic brain lesions as a result of cerebrovascular disease and cardiovascular pathologic changes. We found that severe cerebral amyloid angiopathy, particularly when associated with secondary vascular pathology, might lead to similar ischaemic changes. Hence, the distinction
between cerebral amyloid angiopathy patients from those suffering from vascular dementia can be difficult.

We conclude that although neuropathological analysis demonstrated vascular dementia-like ischaemic lesions, cerebral amyloid angiopathy was the most predominant neuropathological finding in our group of patients. However, retrospective application of the NINCDS-ADRDA criteria lead to a clinical diagnosis of probable Alzheimer’s disease for four patients, whereas none of the patients fulfilled established criteria for vascular dementia or cerebral amyloid angiopathy. Our findings underscore the neuropathological heterogeneity of dementia and we propose that the classic view of differentiating Alzheimer’s disease, vascular dementia and cerebral amyloid angiopathy might need modification, since the borders between these “entities” seem to be blurred. Although the functional impact of vascular pathology and CAA-related secondary vascular changes in particular on cognitive performance warrants further clarification, it is tempting to speculate that cerebral amyloid angiopathy may be an important contributor to the cognitive decline (chapter 8).
References


Chapter 10

Nederlandse samenvatting
Mede door de ontwikkeling van antibiotica, gebalanceerde voeding en aandacht voor hygiène is de gemiddelde levensverwachting in de 20ste eeuw drastisch gestegen. Als gevolg hiervan is ook de incidentie van leeftijdsgere lateerde aandoeningen, zoals de ziekte van Alzheimer, toegenomen. Verwacht wordt dat er in Nederland tegen het midden van deze eeuw meer dan 300.000 patiënten aan de ziekte van Alzheimer zullen lijden. In 1906 was het de Duitse neuropsychiatre Alois Alzheimer die kenmerkende veranderingen ontdekte in het hersenweefsel van mensen die aan ernstige dementie waren overleden. Hij sprak over een vrouw die was overleden na jaren problemen te hebben gehad met haar geheugen, verward overkwam en moeite had met het begrijpen van vragen. Na haar dood verrichte Alzheimer onderzoek op haar hersenen en beschreef de kenmerkende Alzheimer lesies. Microscopische veranderingen werden zichtbaar gemaakt door een zilverkleuring. Hij vond kluwens van draadachtige structuren in de zenuwcellen en eiwitafzettingen buiten de zenuwcellen. Tevens vond hij ook eiwitphoping in de wand van hersenbloedvaten. Deze microscopische pathologische afwijkingen in de hersenen zijn tot op de dag van vandaag nog altijd de karakteristieke kenmerken van de ziekte van Alzheimer en de aanwezigheid van deze lesies is nog steeds nodig om de ziekte van Alzheimer definitief vast te stellen.


In de eerste hoofdstukken (3, 4 en 5) wordt de distributie van diverse heparansulfaat proteoglycanen in Alzheimer hersenen beschreven. Verschillende studies hebben laten zien dat perlecan in alle Alzheimer lesies voorkomt en blijkbaar dus een
belangrijke rol speelt in de ontwikkeling van amyloïde plaques, tangles en vasculair amyloid. Wij hebben deze resultaten niet kunnen bevestigen en laten daarentegen zien dat andere heparansulfat proteoglycanen, zoals agrine en glypican-1, een veel belangrijkere rol spelen. Verder vinden we dat sommige heparansulfat proteoglycanen, zoals syndecan-2 en glypican-1, met name geassocieerd zijn met vasculair amyloid, terwijl agrine voornamelijk voorkomt in allerlei soorten amyloïde plaques. Het feit dat heparan sulfat proteoglycanen differentieel tot expressie komen in Alzheimer lesies bevestigt eerdere bevindingen dat de pathogenese van vascular amyloid en seniele plaques verschilt, ondanks het feit dat beide lesies grotendeels zijn opgebouwd uit dezelfde bouwsteen, namelijk het amyloid β-eiwit. In hoofdstuk 6 beschrijven we de expressie van heparansulfat proteoglycanen in hersenen van Parkinson patiënten. De afwijkingen in Parkinson hersenen worden gekekenmerkt door de aanwezigheid van α-synucleine bevattende Lewy lichaampjes. α-Synucleine heeft, net als het amyloid β-eiwit, de eigenschap fibrillen te kunnen vormen. Ondanks het feit dat andere onderzoekers hebben gevonden dat heparansulfat de fibrillisatie van dit eiwit bevordert, hebben we geen heparansulfat (proteoglycanen) gevonden in Lewy lichaampjes.

Samengevat laten de resultaten van onze immuunhistochemische studies het volgende zien:
1. Verschillende soorten heparansulfat proteoglycanen o.a. agrine, glypican-1, collageen XVIII en verschillende syndecans komen differentieel tot expressie in Alzheimer lesies.
2. In tegenstelling tot eerdere studies veronderstellen we dat perlecan een verwaarloosbare rol speelt in de pathogenese van de ziekte van Alzheimer.
3. Agrine en glypican-1 zijn de meest voorkomende typen heparansulfat proteoglycanen in seniele plaques en vascular amyloid.
4. Collageen XVIII is een nieuw amyloid-geassocieerd heparansulfat proteoglycaan en komt alleen voor in klassieke plaques en cerebrale amyloid angiopathie.
5. De pathogenese van cerebrale amyloid angiopathie en amyloïde plaques verschilt met betrekking tot de rol van specifieke heparansulfat proteoglycanen.
6. Heparansulfat proteoglycanen komen niet voor in α-synucleine positieve Lewy lichaampjes en spelen waarschijnlijk geen belangrijke rol bij de fibrillisatie van α-synucleine.

Toen we wisten welke heparansulfat proteoglycanen voorkomen in Alzheimer lesies zijn we op zoek gegaan naar de cellen in de hersenen die deze eiwitten produceren. In hoofdstuk 7 staat beschreven welke celtypen de amyloid-geassocieerde heparansulfat proteoglycanen bevatten. We hebben gevonden dat zowel neurionale als vasculaire celtypen een scala aan heparansulfat proteoglycanen aanmaken. Vervolgens hebben we gekeken naar de invloed van amyloid β-eiwit op de productie van heparansulfat proteoglycanen. Daarvoor hebben we gebruik gemaakt van een vasculair celtype, de pericyt genaamd. Deze cellen liggen rondom kleine hersenvaatjes en produceren tal van heparansulfat proteoglycanen. Met behulp van diverse technieken hebben we gevonden dat als we deze cellen behandelen met amyloid β-eiwit dit lijkt te leiden tot een opregulatie van twee specifieke heparansulfat proteoglycanen, namelijk glypican-1 en agrine. Opvallend is dan ook dat met name deze twee heparansulfat proteoglycanen voorkomen in Alzheimer
lesies. Om er zeker van te zijn dat amyloid β-eiwit inderdaad de productie van heparansulfaat proteoglycanen induceert moet er verder onderzoek uitgevoerd worden.

**Heparan sulfaat proteoglycanen: vriend of vijand?**

Over het algemeen wordt aangenomen dat de fibrillarisatie van amyloid β-eiwit essentieel is voor de schadelijke eigenschappen van dit eiwit. Uit eerder onderzoek is gebleken dat heparansulfaat proteoglycanen in staat zijn om dit proces te versnellen. Verder binden heparansulfaat proteoglycanen aan amyloid β-eiwit waardoor het amyloid β-eiwit minder snel wordt afgebroken. Heparansulfaat proteoglycanen bevorderen dus zowel de vorming als de stabilisatie van toxisch fibrillair amyloid β-eiwit bevattende plaques en cerebrale amyloid angiopathie. Hersenonderzoekers bestuderen met name de schadelijke effecten van fibrillair amyloid β-eiwit. Men weet echter nog steeds niet welke vorm van amyloid β-eiwit schadelijk is voor zenuwcellen. Er zijn tegenwoordig steeds meer aanwijzingen dat protofibrillen, een soort voorloper van de volwassen Aβ fibrillen, de meeste schade kunnen aanrichten. Het feit dat heparan sulfaat proteoglycanen de omzetting van protofibrillen in volwassen fibrillen kunnen bevorderen zou dus betekenen dat heparansulfaat proteoglycanen juist een beschermende functie hebben. Dit zou kunnen verklaren waarom de productie van heparansulfaat proteoglycanen wordt bevorderd door blootstelling aan amyloid β-eiwit. Toekomstig onderzoek is nodig om uit te wijzen of heparansulfaat proteoglycanen als vriend of vijand beschouwd moeten worden.

**Vasculaire pathologie**

In hoofdstuk 8 is beschreven hoe we hebben getracht meer inzicht te krijgen in de vasculaire component van de ziekte van Alzheimer. Tijdens het bestuderen van de distributie van heparan sulfaat proteoglycanen in vasculair amyloid, ontdekten we een kleine groep patiënten met ernstige cerebrale amyloid angiopathie en pathologische vaatveranderingen. Deze groep patiënten kenmerkte zich door uitgebreide amyloidophopingen in de wanden van zowel grote als kleine vaten. Naast deze amyloid angiopathie vonden we ook een aantal secundaire degeneratieve en inflammatoire vaatveranderingen zoals gladde spiercel degeneratie en infiltratie door macrofagen. Uiteindelijk kunnen deze vaatveranderingen leiden tot verzwakking en verwijding van de vaatwand en uiteindelijk resulteren in (kleine) bloedingen. Inderdaad vonden we bij drie patiënten meerdere kleine bloedingen, echter bij geen van de patiënten waren grote hersenbloedingen aantoonbaar. Aangezien het optreden van grote bloedingen een diagnostisch criterium is voor cerebrale amyloid angiopathie werd geen van onze patiënten gediagnosticeerd als lijdend aan cerebrale amyloid angiopathie. We concluderen dan ook dat de huidige klinische criteria niet adequaat zijn om deze groep van patiënten te herkennen en dat er dus nieuwe criteria geformuleerd moeten worden om deze patiënten in de toekomst beter te diagnosticeren en uiteindelijk te behandelen. In drie gevallen suggereerde het klinisch beeld en het neuropathologisch verslag een vasculaire dementie. Bij toepassing van strikte klinische criteria bleek echter dat geen van de patiënten volledig aan de klinische criteria voor vasculaire dementie. Deze vorm van dementie wordt veroorzaakt door een slechte doorbloeding van het hersenweefsel wat resultert in zuurstoftekort (hypoxie) in de hersenen. Uitgebreide cerebrale amyloid
angiopathie, gecombineerd met ernstige secundaire vaatveranderingen kan leiden tot soortgelijke hypoxische veranderingen, waardoor het moeilijk is onderscheid te maken tussen deze patiënten en mensen die leiden aan vasculaire dementie. Verder suggereren we dat ernstige cerebrale amyloïd angiopathie met secundaire vaatveranderingen, waarbij neuritische plaques vrijwel ontbreken, toch een belangrijke bijdrage kan leveren aan cognitieve stoornissen.
List of publications


Dankwoord

Het studentenleven net ontgroeid begon ik 1 oktober 1999 met het onderzoek beschreven in dit proefschrift. Ik heb in deze periode veel geleerd en nu is het dan zo ver, mijn boekje is af en ik ben er ontzettend trots op. Het moge echter duidelijk zijn dat ik dit onderzoek niet alleen heb uitgevoerd en ik wil hierbij dan ook iedereen bedanken voor zijn of haar inzet en betrokkenheid op wat voor manier dan ook. Een paar mensen wil ik in het bijzonder bedanken.

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Jack
Curriculum Vitae


Van 1 oktober 1999 tot eind oktober 2003 werd op de afdeling Pathologie van het Universitair Medisch Centrum Nijmegen onder begeleiding van Dr. Ir. M.M. Verbeek en Dr. R.M.W. de Waal het in dit proefschrift beschreven onderzoek verricht. Tijdens zijn promotieonderzoek was Jack betrokken bij de begeleiding van diverse studenten en stagiaires. Tevens presenteerde hij zijn eigen onderzoeksresultaten op diverse bijeenkomsten in binnen- en buitenland. Per 1 november 2004 is hij werkzaam als postdoc op de afdeling Moleculaire celbiologie en immunologie (Prof. Dr. C.D. Dijkstra en Dr. H.E. de Vries). Hij doet onderzoek naar cerebrovasculaire veranderingen in multiple sclerosis breinen.
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Chapter 5, Figure 1.
Immunofluorescence staining of senile plaques in sections from AD brains for Aβ (mAb 6C5) (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). Original magnifications: a-i x630.

Chapter 5, 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 x630.
Chapter 5, 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

Chapter 6, Figure 1.
Immunofluorescence staining of Lewy bodies and Lewy neurites in sections from PD brains for α-synuclein (mAb Ab-2, green) (a,b), HSPG core protein (3G10, red) (a) and HSGAG (JM403, red) (b). In a nuclei were stained blue. Cortical LBs and LNs are stained by the anti-α-synuclein antibody. Both 3G10 and JM403 strongly stained the cerebral (micro)vasculature. Neither HSPG core-protein nor HSGAGs colocalize with α-synuclein (a,b). Autofluoresent lipofuscin deposits are clearly visible. Original magnifications: a,b ×630.
Chapter 6, Figure 2.
Immunofluorescence staining of a senile plaque with polyclonal antibody 40-4 (anti-Aβ, green) (a) and monoclonal antibody 3G10 (anti-HSPG core-protein, red) (b). HSPG core-protein colocalized with the Aβ-positive senile plaque (c). Original magnifications: a-c x630.

Chapter 7, Figure 3.
Immunofluorescence staining of control (a,c) and DAβ1-40-treated (b,d) cultured human brain pericytes (HBP) for agrin (a,b) and glypican-1 (c,d). Protein expression of agrin and glypican is clearly increased upon incubation with DAβ1-40. Original magnification 630x.
Chapter 8, Figure 3.
H&E staining of paraffin sections demonstrated several secondary vascular changes. Frequently, amyloid-laden vessels are characterized by a decrease, or even absence of, α-SMA expression (a). Hyalinization of severely CAA-affected vessels is frequently observed (b). Both cortical and leptomeningeal vessels show the “double-barreling” phenomenon (c). Large necrotic area with amyloid-laden vessels, “double-barrel” vessels and hyalinized vessels (d). LFB&HE staining demonstrated loss of myelin, often referred to as myelin pallor, and major perivascular atrophy (e).
Original magnifications: a,c x50; b x200; d,e x12,5.
Chapter 8, Figure 4. Immunohistochemical staining of macrophages infiltrating the vessel wall (a). Note the narrow lumen caused by intimal proliferation. Perivascular lymphocytic infiltration around an amyloid-laden vessel (H&E) (b). Original magnifications: a x400; b x200.

Chapter 8, Figure 5. Aneurysms, in the deeper (a), but also in the superficial part of the cortex (b). Remnants of a ruptured vessel (c). Severely affected vessel characterized by perivascular depositions of iron and hemosiderin. Note the presence of foam cells within the vessel wall (d). Recent cerebral (micro)hemorrhage (H&E) (e). Original magnifications: a,b,d x100; c x 200; e x12.5.
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