Chitin-like Polysaccharides in Alzheimer's Disease Brains

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Abstract: The role of polysaccharides in the pathogenesis of Alzheimer disease (AD) is unclear. However, in light of studies indicating impaired glucose utilization in AD and increased activation of the hexosamine pathway that is seen with hyperglycemia, in the brains of patients with AD, aberrantly high levels of glucosamine may result in synthesis of glucosamine polymers such as chitin, a highly insoluble polymer of N-acetyl glucosamine, linearized by β1-4 linkages. To examine this further, we studied brain tissue at autopsy from subjects with sporadic and familial AD using calcofluor histochemistry. Calcofluor excites on exposure to ultraviolet light and exhibits a high affinity for chitin *in vivo* by interacting with β1-4 linkages. Amyloid plaques and blood vessels affected by amyloid angiopathy showed bright fluorescence. Moreover, treatment with chitinase, followed by β-N-acetyl glucosaminidase showed a decrease in calcofluor fluorescence. Since chitin is a highly insoluble molecule and a substrate for glycan-protein interactions, chitin-like polysaccharides within the brain could facilitate nucleation of amyloid proteins in various amyloidoses including AD.

Keywords: Alzheimer disease, amyloid, chitin, N-acetyl glucosamine.

INTRODUCTION

Controversy over the role of carbohydrates in the pathophysiology of amyloidosis dates back to Virchow [1] and the introduction of the term "corpora amylacea" (amyloid bodies) for material within the brain and spinal cord with an affinity for iodine. A few years later Friedreich and Kekule [2] used a similar term "amyloid" to describe extra-cellular accumulations in peripheral organs, and debated whether they were describing the same material that Virchow identified. It is now apparent that Virchow was describing CNS corpora amylacea, being intra-astrocytic accumulations of carbohydrate material, whereas the "amyloid" assessed by Friedreich and Kekule is a collection of extra-cellular, insoluble fibrils that are largely proteinaceous in composition, with positivity for thioflavin, and "apple green" birefringence on plane polarized light following Congo red staining [3], i.e. amyloid as it is presently understood.

The list of specific proteins capable of forming amyloid fibrils continues to expand [4]; noteworthy examples associated with specific disease states include amyloid-β, prion protein, transthyretin, serum amyloid A, immunoglobulin light chain, and gelsolin. While the role of glycans in amyloidoses such as Alzheimer disease (AD) has been studied using lectin histochemistry [5,6], and immunochemical and radiochemical detection of O-GlcNAc [7], the role of polysaccharides per se apart from glycoconjugates in the amyloidosis of AD has received little attention, with one recent exception of a study demonstrating the novel identification of amylose in the AD brain [8].

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Because of impaired glucose utilization in the AD brain [9], high levels of N-acetyl glucosamine and consequent production of glucosamine polymers in the AD brain are a potential result. Metabolic changes seen in the setting of diabetes mellitus are consistent with this concept [10]. Particularly noteworthy is chitin, a highly insoluble polymer of β(1-4) 2 acetamido-2-deoxy-D-gluco-pyranose (N-acetyl glucosamine) containing β1-4 linkages of N-acetyl glucosamine.

Calcofluor, a fluorochrome that excites on exposure to ultraviolet light, exhibits a high affinity for chitin *in vivo* by interacting with β1-4 linkages [11] and, as such, exhibits antifungal activity by disrupting chitin synthesis [12]. Therefore, to characterize the relationship between chitin and AD, in this study we used calcofluor to examine brain tissue at autopsy from subjects with late-onset sporadic and early onset familial AD (A431E).

MATERIALS AND METHODS

Tissue Preparation

Hippocampus and cerebral cortex from seven subjects with confirmed sporadic AD, Braak stage V-VI [13] [ages 73 to 90; post-mortem intervals (PMI) 6 to 15 hours; cerebral cortex from one subject carrying A431E presenilin 1 mutation and hippocampus and cerebral cortex from five controls without clinical neurodegenerative disease (ages 24 and 59; PMI 7 to 24 hours) were obtained at autopsy. Paraffin-embedded sections and 10 micron thick cryostat sections were prepared. Both paraffin-embedded sections and cryostat sections were used along with unstained controls and examined with the same fluorescent optics.

Control tissues were obtained from the University of Maryland Brain and Tissue Bank.

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Calcofluor Fluorescence Histochemistry

Sections are treated for 1 hr with 0.1% Calcofluor and then washed with water using several rinses. To distinguish specific calcofluor staining from tissue autofluorescence, untreated sections were analyzed in parallel. Sections were imaged on an Olympus BX-41 light microscope with fluorescence optics [100 watt mercury illuminator, UV (Ex 340-380 nm, Em 435-485 nm)].

Immunocytochemistry

Immunocytochemistry using antibodies against amyloid-β (Dako) and phosphorylated tau (AT8, Endogen) were performed on the same sections used for calcofluor histochemistry and also on serial sections to confirm the identity and location of senile plaques and neurofibrillary tangles, respectively.

AT8: following pretreatment with 0.03% H_2O_2 and acetone, slides were incubated with AT8 antibody diluted 1:1,000 for 1 hour at 37°C, followed by biotinylated secondary antibody, addition of avidin-biotin peroxidase complex. The reaction product was developed with Nova Red chromogen.

Amyloid-β: following pretreatment with formic acid for 3 minutes, sections were incubated with primary antibody (mouse monoclonal) for 1 hour at room temperature, followed by biotinylated secondary antibody, addition of avidin-biotin peroxidase complex, and developed with Nova Red chromogen.

Specificity Studies

In order to confirm specificity of calcofluor histochemistry for chitin and glucosamine accumulations, both frozen and fixed serial sections of AD brain were treated with chitinase (Sigma, C-7809) and /or β-N-Acetyl-glucosaminidase (Sigma) followed by calcofluor. Chitinase (synonyms: Poly(1,4-b-[2-acetamido-2-deoxy-D-glucoside]) glycanohydrolase; chitodextrinase) pretreatment consisted of 1 mg/ml in citrate buffer, pH = 6.0. β-N-Acetylglucosaminidase pretreatment consisted of 0.2 units/ml, pH 5.0. Sections were viewed using fluorescence optics and photomicrographed as above.

Light Microscopic Imaging

Immunocytochemical stains were imaged on a Nikon DXM1200 digital camera and ACT-1 software.

RESULTS

Calcofluor Histochemistry, Amyloid-β Immunohistochemistry, and Tau Immunocytochemistry in Sporadic AD

Calcofluor fluorescence was intense with predominantly a plaque pattern, as well as labeling neurofibrillary tangles, in all sporadic AD subjects (Fig. **1**). Blood vessel walls also showed calcofluor staining in a pattern identical to cerebral amyloid angiopathy (Fig. **1**). Double staining with amyloid-β immunocytochemistry showed striking colocalization to diffuse, neuritic, and cored plaques. The dystrophic neuritic

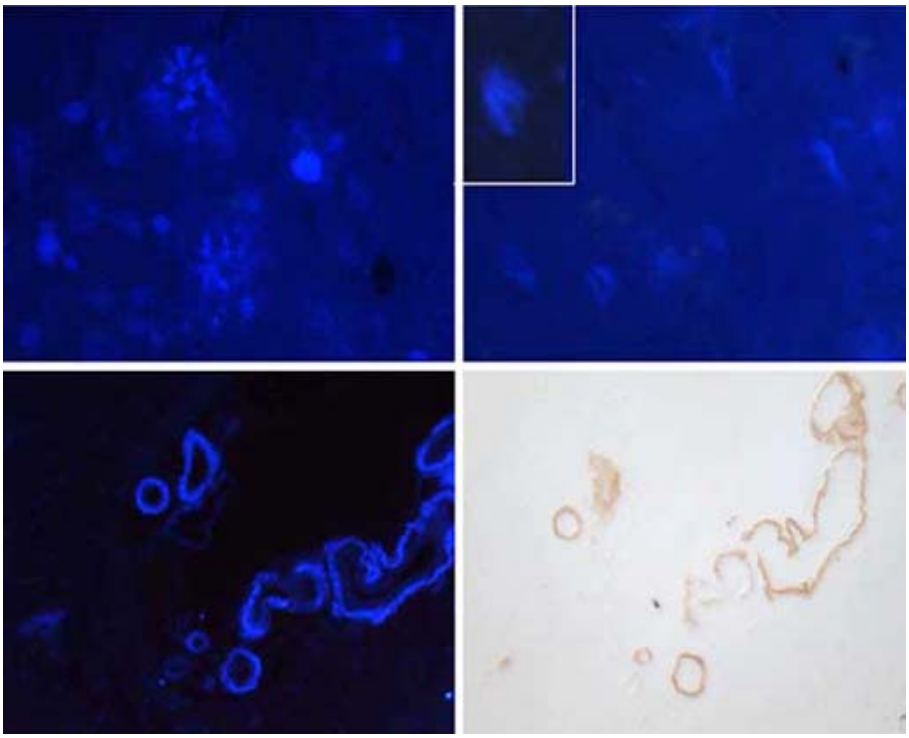

Fig. (1). Calcofluor fluorescence histochemistry. The upper left panel demonstrates calcofluor labeling of senile plaques in AD. Neurofibrillary tangles (upper right panel and inset) are also labeled. Calcofluor fluorescence histochemistry followed by immunostaining of the same sections with antibodies to amyloid-β, demonstrates complete overlap in the pattern of amyloid deposition in amyloid angiopathy using these two techniques (lower left - calcofluor stain, lower right – amyloid-β immunohistochemistry).

component of neuritic plaques remained unstained with calcofluor. Neurofibrillary tangles, while numerous in all AD subjects, were sometimes labeled with calcofluor, as confirmed with AT8 immunocytochemistry. No obvious staining of glial cells or corpora amylacea was detected in AD or control subjects. The control subjects contained no amyloid deposits or neurofibrillary pathology by amyloid-β and AT8 immunocytochemistry respectively, and no significant labeling with calcofluor.

Calcofluor Histochemistry, Amyloid-β Immunocytochemistry, and Tau Immunocytochemistry in AD Subject with Presenilin Mutation

Calcofluor staining again showed striking fluorescence that colocalized with amyloid plaques, including so-called cotton wool plaques that are particularly numerous in subjects with presenilin-1 mutations (Fig. **2**), and blood vessels affected by amyloid angiopathy in both familial and sporadic AD, while the staining patterns for amyloid-β and calcofluor were identical (Fig. **3**). No staining of neurofibrillary pathology (neurofibrillary tangles, neuropil threads, dystrophic neurites) or granulovacuolar degeneration was observed with double staining for phospho-tau (AT8). No glial cell or corpora amylacea staining was detected.

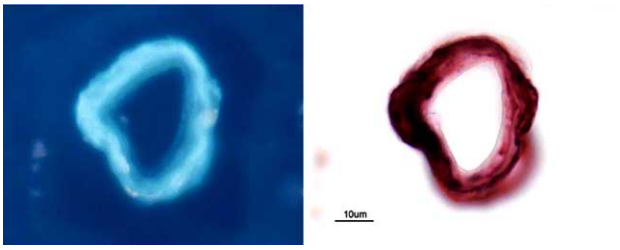

Fig. (2). High magnification photomicrograph of blood vessel in subject with A431E mutation, affected by amyloid angiopathy. Strong calcofluor staining (left) is accompanied by an identical reaction using amyloid-β immunocytochemistry (same section).

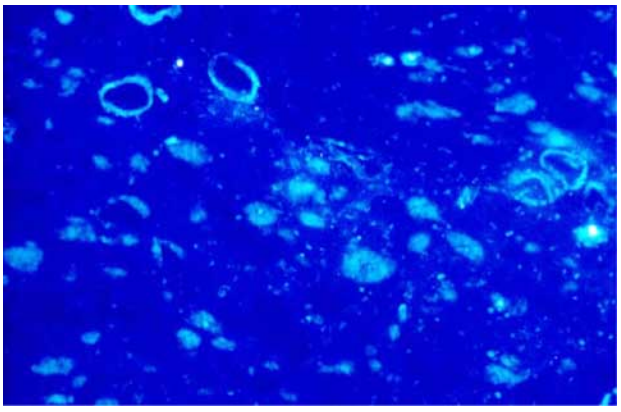

Fig. (3). Low magnification fluorescent micrograph of cerebral cortex from a subject with A431E presenilin mutation. Cotton-wool plaques and blood vessels affected by amyloid angiopathy are strongly fluorescent with calcofluor staining.

Specificity Studies using Chitinase and β-N-Acetylglucosaminidase

Treatment with either chitinase (which degrades chitin to chitobiose) or β-N-Acetylglucosaminidase alone demonstrated no differences with subsequent calcofluor histochemistry. Amyloid plaques and amyloid angiopathy observed on calcofluor histochemistry were equally intense with or without pretreatment with either enzyme. On the other hand, treatment with β-N-Acetylglucosaminidase following treatment with chitinase showed an overall decrease in subsequent calcofluor fluorescence as compared to untreated and chitinase pretreated slides (Fig. **4**).

DISCUSSION

Calcofluor staining strikingly colocalized with amyloid plaques and amyloid angiopathy of AD, in both sporadic and familial early onset cases. This finding, in addition to the finding that chitinase followed by β-N-acetylglucosaminidase diminishes calcofluor fluorescence, suggests that chitin-like polysaccharides, including chitobiose, comprise an integral component of pathological lesions (senile plaques, amyloid angiopathy) of AD.

Since chitin is highly insoluble, this study raises the question of whether protein is purely responsible for the chemical properties of amyloid, or whether chitin-like polysaccharides influence these features as well. It is noteworthy that commercial chitin stains red with Congo Red and also shows green birefringence with plane polarized light (data not shown). This, in addition to calcoflour staining of amyloid plaques and amyloid angiopathy, is consistent with the concept that chitin influences the histochemical properties of amyloid previously ascribed to protein secondary structure. It should also be noted that diffuse plaques, and cotton wool plaques of familial early onset AD, lack the characteristic filamentous amyloid by electron microscopy [14]. Likewise, calcofluor staining is more widespread than Congo red staining, in brain affected by AD. Thus, chitin-like polysaccharides overall are more widespread than protein fibrils of classically defined amyloid, suggesting that accumulation of N-acetyl glucosamine precedes formation of typical amyloid fibrils.

Whether brain chitin accumulation might provide a protective or a deleterious function remains an important unanswered question. Commercial chitin and one of its derivatives, chitosan, have applications in wound healing and enhancement of function of inflammatory cells and fibroblasts [15]; thus, chitin within the brain may stimulate inflammatory processes such as microglial cell activation and elaboration of soluble mediators of immunity, processes which are becoming increasingly apparent in AD pathogenesis [16]. On the other hand, the concept that amyloid plaques present an attempt at neuroprotection [17], and therefore that the chitin-amyloid-β interaction might be neuroprotective, should be considered in future studies.

Glucosamine, the basic unit of chitin and chitosaccharides, is formed from glucose via fructose and fructose-6-phosphate. High levels of glucose in the presence of the normal complement of cellular enzymes in turn lead to the production of glucose polymers (starch) as well as glu-

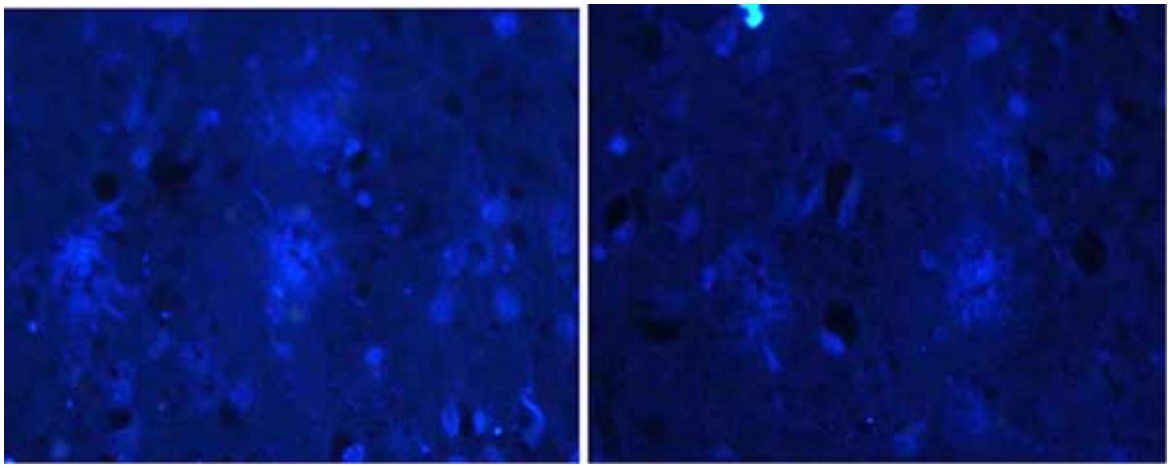

Fig. (4). The left photomicrograph demonstrates an amyloid plaque with calcofluor histochemistry. No differences were detected using pre-treatment with chitinase. However, upon *additional* treatment with N-Acetylglucosaminidase (right) a marked decrease in plaque fluorescence was observed.

cosamine polymers (chitin). The markedly impaired cerebral glucose metabolism in AD, as evidenced by, for example, reduced [18F] deoxyglucose utilization in position emission tomography [18], reduced densities of cortical glucose transporters [9], and altered cortical glucose metabolism in aged transgenic Tg2576 mice that demonstrate AD pathology [19], may contribute significantly to synthesis of chitin-like material within the brain by increasing the available pool of glucose and shifting cellular metabolism in favor of glucosamine synthesis.

It is important to recognize that chitin synthesis is an energy-dependent process, requiring chitin synthases. While no definitive mammalian chitin synthase has been identified, a potential developmental role of chito-oligosaccharides has been suggested in several studies [20-22]. Hyaluronan synthase-1 (HAS1) has been shown to convert activated glucosamine to chito-oligosaccharides *in vitro* using murine HAS1 gene product [23]. In addition, human synovial fluid of patients with rheumatoid or osteoarthritis contains high levels of a chitinase 3-like glycoprotein [24]. Thus, under pathological conditions, HAS1 or a related molecule may serve as a chitin synthase, converting activated glucosamine to chitin-like polysaccharides and facilitating the process of amyloidosis.

It is also noteworthy that chitin synthesis is initiated intracellularly via activated N-acetyl glucosamine and chitin synthase, and possibly also including a "priming" polypeptide [25]. The additional finding in this study of calcofluor labeling of neurofibrillary tangles raises interesting possibilities. Whether tau, amyloid-β, or some other molecule may serve as a priming polypeptide warrants further study.

In summary, we have demonstrated that calcofluor fluorescence colocalizes with amyloid-β in familial and sporadic AD, suggesting that chitin is an important component of the highly insoluble amyloid fibrils that characterize AD. Based on these data and the properties of chitin in general, we suggest that chitin or chitin-like polysaccharides provide scaffolding for protein accumulation and fibril formation, and may provide a neuroprotective function. While further studies are necessary to determine whether chitin is indeed protective, the data presented here suggest that chitin may be associated with AD and possibly other amyloidoses. The implications of our data in terms of treatment advances and understanding of disease pathogenesis of these progressive and fatal conditions are considerable.

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