Selective Loss of Cerebral Keratan Sulfate in Alzheimer’s Disease*

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Birgitta Lindahl‡, Lars Eriksson§, Dorothe Spillmann¶, Bruce Caterson†, and Ulf Lindahl‡
From the ‡Department of Medical and Physiological Chemistry and §Department of Pathology, University of Uppsala, S-751 23 Uppsala, Sweden and the ¶School of Molecular and Medical Biosciences, University of Wales, UK-Cardiff CF1 3US, United Kingdom

Proteoglycans, especially heparan sulfate-substituted species, are known to be associated with the deposition of amyloid in Alzheimer’s disease. We previously found that heparan sulfate from afflicted brains, and from control subjects, differed minimally in quantity and structure (Lindahl, B., Eriksson, L., and Lindahl, U. (1995) Biochem. J. 306, 177-184). In the present study, a glycosaminoglycan fraction, shown to contain heparan sulfate and keratan sulfate, was radiolabeled by partial N-deacetylation (hydrazinolysis) followed by re-N-acetylation using [3H]acetic anhydride. Quantitation of the [3H]-labeled polysaccharides, based on digestion with heparitinase I from Flavobacterium heparinum and keratanase from Pseudomonas sp., revealed that the amounts of keratan sulfate in Alzheimer cerebral cortex are reduced to less than half of control values. Moreover, a monoclonal antibody against a highly sulfated keratan sulfate epitope bound to the majority of the neurons in normal cortex but not in the diseased tissue. The lack of highly sulfated keratan sulfate structures may reflect a specific functional defect of the cells.

Alzheimer’s disease (AD) is characterized by amyloid deposition in the cerebral parenchyma, the occurrence of neurofibrillary tangles in the perinuclear cytoplasm of neurons, and by neuronal degeneration. Proteoglycans, especially heparan sulfate (HS)-substituted species, are known to be associated with the amyloid deposits (1). In a previous study, we found that heparan sulfate isolated from afflicted brains, and from control subjects, differed minimally in quantity and structure (2). Keratan sulfate (KS), originally demonstrated in cornea and cartilage, was more recently identified in brain (3). KS is a linear polymer of β1,3-linked N-acetyl-lactosamine (Galβ1,4GlcNAc) disaccharide units that are sulfated at a variable degree on the C-6 positions of either the glucosamine or galactose residues (Fig. 1). While a limited number of KS-proteoglycans have since been isolated from brain (4), there are no reports to our knowledge concerning the distribution or characteristics of KS or KS-proteoglycans in AD.

Here we report that contrary to HS, KS, isolated together with the HS glycosaminoglycan fraction, is dramatically decreased in cerebral cortex of Alzheimer patients. This finding is paralleled by the loss of epitopes recognized by a monoclonal antibody with specificity for highly sulfated KS. The specific staining of normal neurons and the lack of these highly sulfated KS structures in afflicted neurons may reflect a specific functional defect of the neurons in AD.

MATERIALS AND METHODS

Isolation and Radiolabeling of Glycosaminoglycans—Sulfated glycosaminoglycans (GAGs) were isolated as described (2) from samples of cerebral cortex obtained at autopsy of control subjects and of individuals with the clinical diagnosis and typical neuropathological findings (2, 5) of AD. Briefly, the procedure involved lipid extraction, proteolytic digestion, and anion-exchange chromatography. Material more retarded on the ion-exchanger than a hyaluronan standard was recovered and digested with chondroitin ABC and nuclease (2). Residual polysaccharide (15-25 µg of hexuronic acid, as determined by the carbazole reaction (6)) was radiolabeled as described (2) by partial N-deacetylation (hydrazinolysis at 96 °C for 30 min) of N-acetylgalactosamine units, followed by re-N-acetylation using [3H]acetic anhydride. The products showed a specific activity of (1-2) × 106 dpm of [3H]µg of hexuronic acid (however, see heterogeneity of labeled material under “Results”).

Enzymatic Degradation of Glycosaminoglycans—Heparitinase I (EC 4.2.2.8) from Flavobacterium heparinum, and keratanase 1,4-ß-D-b-glactanohydrolase (keratanase) (EC 3.2.1.103) from Pseudomonas sp. were purchased from Sekagaku. Samples of N-(acetyl-3H) glycosaminoglycans (~100 × 106 dpm) were digested with 5 milliunits of heparitinase I in 150 µl of 50 mM Hepes, 1 mM CaCl2, pH 7.0, at 43 °C for 9 h, or with 50 milliunits of keratanase in 100 µl of 50 mM Tris-HCl, pH 7.4, at 37 °C for 15 h. The digestion products were separated by gel chromatography on a column (1 × 96 cm) of Sephadex G-100, equilibrated with 0.2 M NH4HCO3, and effluent fractions were analyzed for radioactivity by scintillation counting.

Antibodies and Immunohistochemistry—The mouse anti-KS monoclonal antibodies (mAbs), 2-D-3, 3-D-4, and 8-C-2, were as described (7). Two of these mAbs, 2-D-3 and 8-C-2, recognize low sulfated KS, whereas mAb 3-D-4 binds to highly sulfated KS sequences (8). Brain tissue was recovered at autopsy and fixed in 4% formaldehyde solution. Deparaffinized tissue sections were incubated overnight at 4 °C with the primary antibody, mAb 3-D-4, in dilution 1:50, and were then stained using a secondary, biotinylated, rabbit anti-mouse antibody (1:200; Dakopatts), followed by avidin-biotin-peroxidase complex and 3,3-diaminobenzidine (Sigma) as substrate/chromogen. The sections were then briefly counter stained with Mayer’s hematoxylin.

RESULTS

Samples of cerebral cortex were obtained at autopsy of individuals with AD and of control subjects. The pathological specimens were selected on the basis of the clinical diagnosis of AD, verified by the typical neuropathology at autopsy with neurofibrillary tangles, senile plaques with a central core of amyloid, and amyloid deposits in the vessel walls (5). Individuals without dementia and without these histopathological findings were used as controls. Sulfated GAGs were isolated from the tissues as described under “Materials and Methods.” Following

1 The abbreviations used are: AD, Alzheimer’s disease; GAG, glycosaminoglycan; HS, heparan sulfate; KS, keratan sulfate; mAb, monoclonal antibody.

2 B. Caterson, unpublished observation.

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removal of galactosaminoglycans by digestion with chondroitinase ABC, the polysaccharides were radiolabeled by partial N-deacetylation followed by re-N-[3H]acetylation. Fractionation of such N-acetyl-3H]polysaccharide preparations by anion-exchange chromatography yielded two major peaks, one of which emerged slightly after a hyaluronan standard, whereas the other one appeared immediately before chondroitin sulfate (Fig. 1). The more retarded of the separated 3H-labeled components was susceptible to deaminative cleavage with HNO2 (pH 1.5) and to digestion with bacterial heparitinase (type I; F. heparinum) (2). The less retarded component was resistant to these treatments, but was instead degraded upon incubation with Pseudomonas sp. keratan sulfate 1,4-β-D-galactanohydrolase (keratanase) and with Bacillus sp. keratan sulfate endo-β-N-acetylglucosaminidase (keratanase II) (data not shown). The two N-acetyl-3H]GAGs thus were identified as HS and KS.

Preliminary observations based on anion-exchange chromatography suggested that the amounts of KS, as related to those of HS, were decreased in cortex from AD brains as compared with control material (Fig. 1). To verify this finding, unfraccionated samples of chondroitinase-resistant 3H-labeled GAGs were digested with bacterial heparitinase (a–f) or with bacterial keratanase (g–l), and the products were analyzed by gel chromatography on Sephadex G-100, as described under "Materials and Methods." The chromatograms show control samples (a and g, 76-year-old individual, parietal cortex; b and h, 26 years, parietal cortex; c and i, 59 years, frontal cortex) and AD samples (d and j, 79 years, pooled from different locations; e and k, 80 years, parietal cortex; f and l, 75 years, parietal cortex). To ascertain complete digestion, all incubations with heparitinase contained a sample of N-acetyl-14C-labeled kidney HS (2) that was quantitatively converted into oligosaccharides (data not shown). Representative elution patterns of undigested 3H-labeled polysaccharides (●) are superimposed in panels a and c; the latter figure also shows the subdivision of peak areas used in assessing the proportion of material susceptible to degradation.

FIG. 1. Anion-exchange chromatography of polysaccharides derived from AD and control cerebral cortex. Sulfated GAGs were isolated from cerebral cortex obtained at autopsy of control subjects (●) and of individuals with the clinical diagnosis and typical neuropathological findings of AD (○). After fractionation by anion-exchange chromato-
most of the $^3$H label emerged as degradation products (g–i). When $[3H]$GAGs from AD brains were subjected to the same treatment the picture was reversed, with relatively large proportions of heparitinase-sensitive as compared with keratanase-sensitive material (compare d–f with j–l). Heparitinase-resistant components were completely degraded to labeled oligosaccharides upon subsequent incubation with keratanase (data not shown). Quantitative estimates of $[^3H]KS/[^3H]HS$ ratios, based on relative peak areas, indicated that KS accounted for $70 \pm 8\% (n = 3)$ of the $^3$H-labeled control polysaccharide, but only for $45 \pm 11\% (n = 6$; samples derived from five brains, one of which provided two samples from different locations) of the AD material. Thus, the ratio of labeled KS to HS would be $\sim 2.3:1$ in controls, whereas $\sim 0.8:1$ in AD samples. Since the concentration of HS in dry, defatted cortex was almost the same in AD ($\sim 110 \mu g$ of hexuronic acid/g) as in control material ($\sim 90 \mu g$ of hexuronic acid/g) (2), we conclude that the concentration of KS in AD cerebral cortex is reduced to less than half of that in control tissue. The decrease in KS contents of AD cortex would appear to be related to the pathological state of the tissue, and not to the age of the individuals, since all control subjects, regardless of age, yielded highly similar HS/KS profiles (Fig. 2).

Three mouse anti-KS mAbs with different saccharide epitopes (see “Materials and Methods”) were used in immunohistochemical characterization of AD and control cerebral cortex. Two of the mAbs, 2-D-3 and 8-C-2, which recognize low sulfated KS, stained neurons in control as well as in AD specimens (data not shown). mAb 5-D-4, on the other hand, which binds to highly sulfated KS sequences, generally differentiated normal (control) from diseased cortex. The extracellular matrix of control (Fig. 3, a and b), but not of AD (Fig. 3, c and d), tissue thus gave a faint but significant reaction with the antibody. More conspicuously, mAb 5-D-4 also gave a positive reaction for most neurons in control brains ($n = 6$) (Fig. 3, a and b) and in a case with dementia of the multiinfarction type (not shown). By contrast, the majority of neurons in 9 out of 11 AD brains failed to bind mAb 5-D-4 (examples shown in Fig. 3, c and d), and, in fact, could not be distinguished from those of control sections that had been stained in the absence of the primary antibody (mAb 5-D-4) (Fig. 3e). All specimens gave a strong positive reaction in the corpora amylacea (9) (not shown).

**DISCUSSION**

The radiochemical experiments demonstrate that the amounts of KS are decreased in cerebral cortex from AD as compared with control individuals, but do not directly indicate the absolute concentrations of KS in the tissue. However, this parameter may be indirectly assessed, using HS, previously quantified (see “Results” and Ref. 2), as an internal reference compound. Control experiments with purified preparations of $[^3H]$-labeled KS and HS (separated by anion-exchange chromatography) ascertained that the rate of N-deacetylation during hydrazinolysis was the same for both polysaccharides (data not shown). The extent of N-deacetylation achieved before re-N-[$^3H$]acetylation was $\sim 40\%$ (data not shown) for both HS and KS; hence, the N-acetyl groups of the resultant radiolabeled polysaccharides would exhibit similar specific radioactivity. Calculations based on the distribution of the $^3$H label between HS and KS, and the estimated abundance of N-acetyl groups in HS ($\sim 50\%$ of all disaccharide units) and KS ($100\%$ of all disaccharide units), thus indicated a KS content for normal cerebral cortex approximately equal to that of HS.

The cause of the selective loss of KS in AD is not known. The highly similar results seen for differently aged controls (Fig. 2) argue against a general effect of age on the chemical composition of KS in brain. A structural difference between AD and
control KS was suggested by immunohistochemical analysis using antibody 5-D-4, whereas antibodies against less sulfated structures did not reveal any alterations. However, a general impairment of sulfation reactions during GAG biosynthesis appears unlikely in view of previous findings regarding HS structure in AD (2). While we cannot exclude that defective KS sulfation is a secondary effect of neuronal degeneration, it is noted that neurons from a case of advanced vascular dementia stained strongly with mAb 5-D-4 (not shown). Moreover, and in accord with the previously recognized heterogeneity of the disease, so did 2 out of 11 cases that had been diagnosed as AD by clinical and histopathological criteria.

KS belongs to the poly-N-acetyl-lactosamine family, which shows remarkable changes during development and differentiation (10–12). Also KS-containing proteoglycans in the nervous system exhibit developmental changes (4). Interestingly, the distribution of mAb 5-D-4 in control samples (Fig. 3b) points to a largely intracellular location of the epitope. It is therefore of interest that a neuron-specific KS-substituted protein has been demonstrated in the Torpedo electric organ and in rat brain (13). This protein (“synaptoglycan” (4)) is located intracellularly in synaptic vesicles and has been implicated with the transport of acetylcholine (14). Conceivably, binding of the positively charged neurotransmitter to highly sulfated regions of KS may be critical to normal transport, which thus would be perturbed in AD.

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Note Added in Proof—After submission of this communication, we became aware of a recent report by Snow et al. (Snow, A. D., Nochlin, D., Sekiguchi, R., and Carlson, S. S. (1996) Exp. Neurol. 138, 305–317) that demonstrates the presence of the KS-containing SV2 proteoglycan in normal human brain and at sites of amyloid plaques in AD.

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3 It should be noted that the radiochemical analysis was restricted to the fraction of sulfated GAGs emerging after hyaluronan on anion-exchange chromatography and thus would not provide any information about less sulfated poly-N-acetyl-lactosamine species.