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David A. Dewitt
Liberty University, dadewitt@liberty.edu

Jerry Silver

David R. Canning

George Perry

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Chondroitin Sulfate Proteoglycans Are Associated with the Lesions of Alzheimer's Disease

DAVID A. DEWITT,* JERRY SILVER,* DAVID R. CANNING,* AND GEORGE PERRY*,†

*Department of Neurosciences and †Division of Neuropathology, Institute of Pathology
Case Western Reserve University, Cleveland, Ohio 44106-4901

Chondroitin sulfate proteoglycans (CSPG) are extracellular matrix proteins inhibitory to neurite outgrowth *in vitro* and correlated with decreased neurite outgrowth after CNS injury. Previously, heparan sulfate proteoglycan and dermatan sulfate proteoglycan have been shown to be associated with senile plaques (SPs) and neurofibrillary tangles (NFTs) but CSPG was not. In an immunocytochemical study, three monoclonal antibodies to different sulfation states of the chondroitin glycosaminoglycan were used to localize CSPG in cases of Alzheimer's disease. Chondroitin 4-sulfate was found in both SPs and NFTs. An antibody to unsulfated chondroitin strongly immunostained intracellular NFTs and the dystrophic neurites of SPs. Chondroitin 6-sulfate was found in NFTs and the area around SPs. These results suggest that CSPG, in addition or as an alternative to β-amyloid protein, could be responsible for the regression of neurites around senile plaques in Alzheimer's disease. © 1993 Academic Press, Inc.

INTRODUCTION

Alzheimer's disease is a severe mentally debilitating disease characterized by two principle lesions, the neurofibrillary tangle (NFT) and the senile plaque (SP). Senile plaques consist of a core of amyloid β-protein (Aβ), a 39- to 42-amino acid peptide (13) derived from a larger amyloid precursor protein (APP) (8). Dystrophic neurites containing APP (9) and processes of reactive astrocytes extend radially toward the Aβ core. Whether the neurites are growing into the SP or are dying and/or moving away from the plaque has not been determined. The status of neurites is compounded by *in vitro* studies in which Aβ has been shown to have both trophic and toxic effects on neurons (29).

Proteoglycans are highly anionic proteins with one or more glycosaminoglycans attached to the protein core. They are one of the principle components of the extracellular matrix. Enzymatic digestion of glycosaminoglycans leaves a distinct antigenic sugar stub specific for each type of glycan.

APP has been shown to be involved in the growth regulation of different cell types (12, 17). In cultured neurons, APP has been shown to be associated with the extracellular matrix (11) and to mediate cell–cell and cell–surface adhesion (4). Recently, APP was shown to be a chondroitin sulfate proteoglycan core protein (19). While the presence of chondroitin 4-sulfate (C-4) was determined associated with APP, neither chondroitin 6-sulfate (C-6) nor unsulfated chondroitin (C-0) was found attached to APP.

Chondroitin sulfate proteoglycans (CSPGs), C-6 in particular, can inhibit neurite outgrowth *in vitro* (23, 24). CSPG may serve as an inhibitory barrier during development providing for axon guidance (5). In adult animals, CSPGs and reactive astrocytes have been correlated with decreased neurite outgrowth in response to injury to the central nervous system (14). Glial cells make and secrete CSPGs (15, 19). Additionally, a subpopulation of neurons have CSPG lining their perimeter (3). The exact function of this neuronal proteoglycan is unknown.

Previously, proteoglycans have been found in SPs and NFTs. Heparan sulfate proteoglycan has been found in both SPs (21, 22, 26) and NFTs (16, 18, 26). Decorin, a dermatan sulfate proteoglycan, was reported to line the periphery of SPs and was localized to paired helical filaments within NFTs (20). However, CSPGs have not previously been found in either lesion.

In this immunohistochemistry study, we demonstrate the presence of three differentially sulfated CSPGs in the lesions of Alzheimer's disease.

MATERIALS AND METHODS

Sections from frontal cortex or hippocampus from six cases of Alzheimer's disease confirmed by pathological examination (ages 69–85; postmortem interval 2–7 h) and two controls (ages 57 and 78; postmortem interval 15–18 h) were studied. Tissue was fixed in methacarn (methanol:chloroform:acetic acid, 6:3:1) and embedded in parafin. Immunostaining was done by the peroxidase anti-peroxidase method with 3′,3′-diaminobenzidine as

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the cosubstrate (25). To detect C-6 CSPG core protein, the monoclonal antibody 3B3 was used (7). Methacarn-fixed sections were incubated with chondroitinase ABC (ICN) or with chondroitinase AC (Sigma) in Tris–acetate buffer (pH 8 and pH 7.6, respectively) for 2 h at 37°C or overnight at 20°C followed by overnight incubation at 4°C with antibody. Heparinase and heparitinase treatments were also used as controls. SPs were verified by Congo red or staining adjacent sections with 4G8, (10) a monoclonal antibody to Aβ. Antibodies to C-0 and C-4 (1B5 and 2B6, respectively) were used to determine whether these forms of CSPG were also present. NFTs were identified by double immunostaining with an antisera to τ (27). Anti-τ utilized biotin–avidin-conjugated alkaline phosphatase with Fast Blue as the substrate.

RESULTS

The antibody to unsulfated chondroitin strongly stained dystrophic neurites around SPs (Fig. 1D) and intracellular NFTs (Figs. 1A and 1D). The staining of NFTs was distinct from other neurons in the same section and from controls. Consistent with a previous report (3) a subset of neurons stained for unsulfated chondroitin as a wide band along the neural perimeter (data not shown).

Chondroitin 4-sulfate was localized to the perimeter of Congo red-positive SPs (Fig. 1H), NFTs (Fig. 1B), and dystrophic neurites (Figs. 1E and 1F). In order to verify that the 2B6 antibody was recognizing C-4 instead of dermatan sulfate, the sections were treated with chondroitinase AC, which does not digest dermatan sulfate (28). There was no apparent difference in staining between either chondroitinase enzyme treatment (Figs. 1E and 1F) which suggests that chondroitin 4-sulfate is in fact associated with both SPs and NFTs.

C-6 was localized with the 3B3 antibody. Intense staining was found at the immediate periphery of Aβ deposits in senile plaques (Fig. 1G). In addition, more diffuse staining occurred further out around the SPs. Most of the C6 immunoreactivity around SPs appeared as an extracellular deposit around the SP. SPs were identified by Congo red (Fig. 1I) and adjacent sections stained with 4G8, an antibody which recognizes Aβ. C-6 CSPG was also localized to τ-positive NFTs (Fig. 1C).

C-4 and C-6 were predominately confined to the area around the SP core—the same area as dystrophic neurites and astrocytic processes although diffuse, light staining occurred throughout the neuropil. None of the chondroitin antibodies stained the core. Essentially every NFT immunostained for τ was also stained for all three CSPGs with the CSPG staining much stronger, making identification of τ sometimes difficult. Almost without exception these NFTs were intraneuronal since the cytoplasm surrounding nuclei was apparent, indicating that CSPG is associated primarily with intraneuronal tangles. None of the antibodies to CSPG immunostained any structure without previously treating the sections with chondroitinase to digest the sugars. Treatment with heparinase or heparitinase did not result in staining by the antibodies either. Immunoblots of A68, τ, ubiquitin, P-component, and neurofilaments did not show binding of chondroitin antibodies with or without chondroitinase digestion.

DISCUSSION

In this study, we have demonstrated the presence of three differentially sulfated CSPGs in the lesions of Alzheimer's disease. Unsulfated chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate were all found associated with SPs, NFTs, and dystrophic neurites.

Prior reports indicated that dermatan sulfate was present in senile plaques and NFTs (20) while chondroitin sulfate was not (21). The antibodies used in these previous studies to identify chondroitin sulfate were raised to the glycosaminoglycan chains of CSPG. We also saw no immunoreactivity with antibodies raised to the CSPG sugar residues (data not shown). Here, antibodies which recognize the chondroitinase-digested stub have been shown to recognize both SPs and NFTs. Perhaps the sugar residues are masked by another protein or proteoglycan which prevents antibody binding but not enzyme activity, thus allowing for exposure of the digested epitope.

Enzymatic digestion with chondroitinase AC specifically demonstrates the presence of C-4 as opposed to dermatan sulfate. Chondroitinase ABC digests both chondroitin and dermatan sulfate sugars while chondroitinase AC only digests chondroitin. Since both enzymes yielded a similar staining pattern, the antibody is recognizing chondroitin. Dermatan sulfate would not be stained after chondroitinase AC treatment. These results suggest that C-4 is present in SPs and NFTs. The 3B3 antibody to C-6 can also bind to C-0 albeit with a much lower affinity (1). 1B5, which specifically binds to C-0 sites, did not stain SPs as strongly as 3B3 or with the same pattern, suggesting that the immunolocalization of 3B3 is specific for C-6, at least around SPs.

The presence of C-6 around SPs is significant because this glycosaminoglycan is particularly associated with decreased neurite outgrowth *in vitro* and in response to injury within the central nervous system. Large amounts of C-6 around SPs could deter neurites from growing in toward the SPs and may actually be causing or mediating neuritic dystrophy. Interestingly, the area which contains C6 immunoreactivity around the SP overlaps with the area known to show a decrease in neuritic density (2). Abnormal CSPG accumulation could disrupt cell adhesion or growth factor utilization.

It is unclear whether each antibody is recognizing a different CSPG core protein or the same core protein

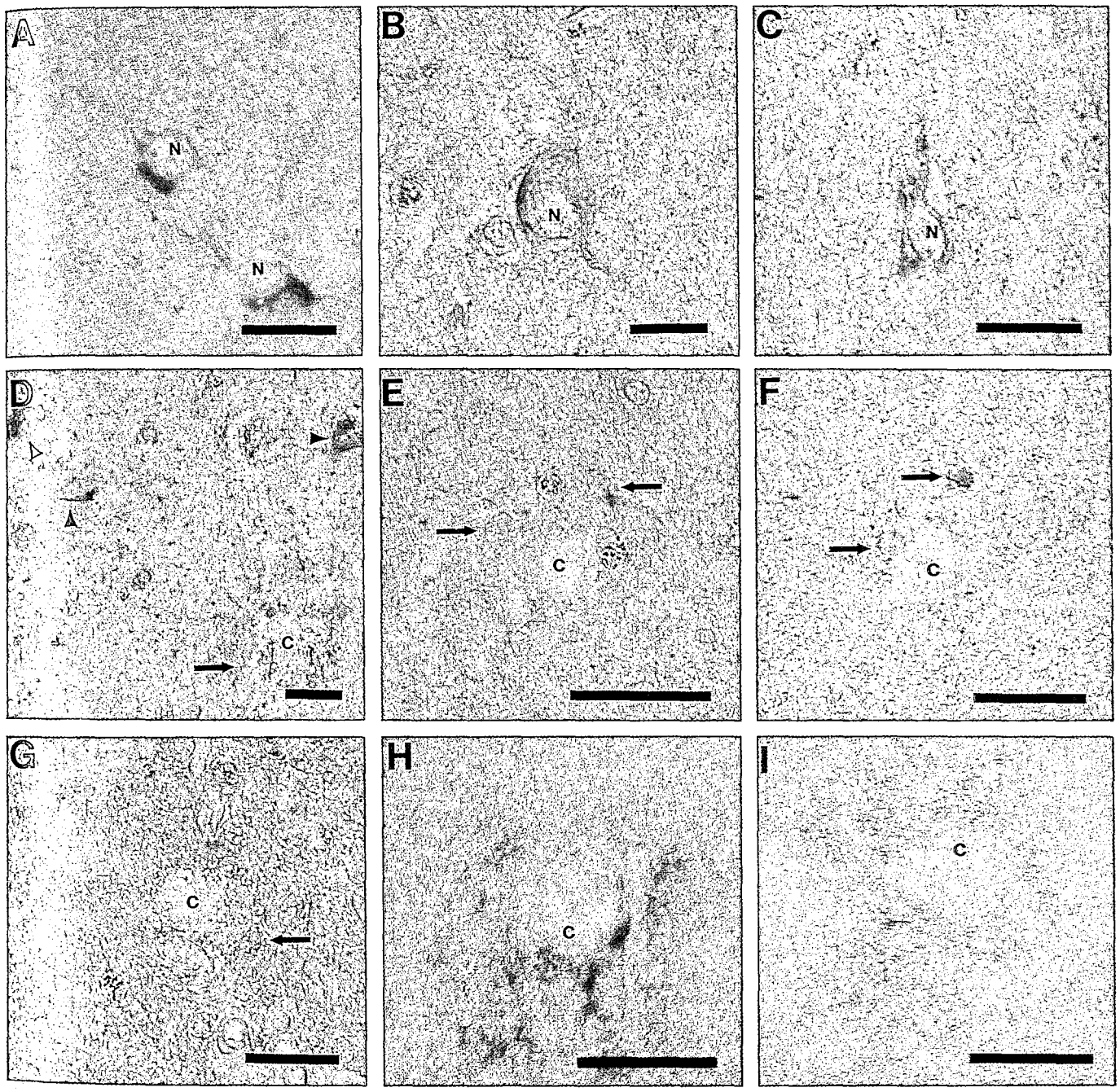

FIG. 1. Neurofibrillary tangles were identified with antisera to τ (purple). Sections were treated with chondroitinase ABC prior to addition of antibody. Monoclonal antibodies indicate different sulfation states of chondroitin sulfate proteoglycan (brown). Unsulfated chondroitin (A), chondroitin 4-sulfate (B), and chondroitin 6-sulfate (C) are present in intracellular neurofibrillary tangles (N, nucleus). CSPG can be found in the periphery of senile plaques. Note the absence of CSPG immunoreactivity in Aβ core of the senile plaque (c, core in D through I). Dystrophic neurites are immunostained for τ (D through G, arrows). Unsulfated chondroitin primarily stained dystrophic neurites (D) and neurofibrillary tangles (arrowheads). Chondroitin 4-sulfate CSPG occurred with both chondroitinase ABC (E) and chondroitinase AC (F). Chondroitin 6-sulfate surrounds the Aβ core (G). Senile plaques immunostained for CSPG were verified by Congo red (H and I). Congo red birefringence of the Aβ core is seen as a green and yellow cross under polarized light. Scale bars = 20 μm.

with differentially sulfated chondroitin. Additionally, the same proteins could have other glycosaminoglycans not digested by chondroitinase. These questions are significant because there could be multiple sources in brain for the different CSPGs or different forms may occur at various stages of neuronal degeneration.

We have found in additional studies that astrocytes accumulate CSPGs in response to Aβ (6), therefore, glia

may be the source for some of the CSPGs. However, since primarily intraneuronal NFTs were also stained, a potential source of CSPG could be neuronal. In the case of NFTs, CSPG accumulation may occur concurrently with NFT formation. In the vicinity of SPs, both neurons and glia could interact synergisticly and accumulate various proteoglycans to produce a terrain refractory to neural regeneration.

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