Association of Heparan Sulfate Proteoglycan with the Neurofibrillary Tangles of Alzheimer's Disease

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The major intracytoplasmic lesion of Alzheimer's disease is the neurofibrillary tangle (NFT), which is primarily composed of paired helical filaments (PHFs). The mechanism responsible for the formation of PHFs, as well as their insolubility and apparent heterogeneity, is unknown. We found that basic fibroblast growth factor (bFGF) binds to heparinase-sensitive sites in NFTs. bFGF binding is due to a heparan sulfate proteoglycan (HSPG) immunocytochemically identified in NFTs. In the presence of polycations (e.g., Ca^{2+}), HSPG will bind to free carboxyl groups in NFT proteins. HSPG binding may play a role in transforming normal soluble proteins into insoluble PHFs.

The composition and mode of formation of the neurofibrillary tangle (NFT), a distinctive neuronal lesion, composed of paired helical filaments (PHFs) in Alzheimer's disease (AD), is controversial (Perry, 1987). Two features that make the understanding of the NFT difficult are the complexity of their composition and their insolubility. Although it has recently been demonstrated that a soluble form of PHF is solely composed of the microtubule-associated protein tau (Greenberg and Davies, 1990; Lee et al., 1991), NFTs are largely insoluble and contain other antigens distinct from tau, including neurofilaments (Gambetti et al., 1987; Mulvihill and Perry, 1989) and tropomyosin (Galloway et al., 1990). The mechanism responsible for the incorporation of these presumably associated proteins into NFTs is unknown.

In a previous study of the role of growth factors in the formation of senile plaques, we found that basic fibroblast growth factor (bFGF) binds to NFTs (Perry et al., 1990a,b; Siedlak et al., 1991). We now show that bFGF is bound to a heparan sulfate proteoglycan (HSPG) that is also present in NFTs. We found that HSPG binds to the free carboxyl groups of the PHFs and suggest that similar interactions may mediate the binding of other proteins to NFTs and render them insoluble.

Materials and Methods

Tissue. The hippocampal gyrus was taken from nine pathologically confirmed (Khachaturian, 1985) cases of AD (mean age, 82 yr; range, 73–88 yr) and two controls (60 and 64 yr) and placed in methacarn (methanol 6:chloroform 3:acetic acid 1), a fixative that makes no covalent modification, for 24–48 hr before dehydration and embedding in paraffin. Tissue from two AD cases (69 and 73 yr) was frozen in liquid nitrogen-chilled isopentane. We also used a Bouin's-Hollande fixed, paraffin-embedded sample taken from the frontal cortex of an AD case (74 yr) taken at biopsy (gift of Dr. S. Chou, Cleveland Clinic Foundation). Paraffin-embedded sections were cut at 6 μm , and frozen sections, at 10 μm .

Antibodies. The following antibodies were used: (1) monoclonal antibody (Ig fraction) to an epitope located in the carboxyl terminal of bFGF, 48.1 (J. M. Scardina, unpublished observations), and (2) rabbit antiserum to an HSPG core protein (Ledbetter et al., 1987; Noonan et al., 1988).

Protein preparations. The following proteins were used: (1) human bFGF (154 amino acid form) recombinantly produced in *Escherichia coli* and purified to homogeneity by using anion exchange chromatography, (2) heparan sulfate proteoglycan core protein isolated from Engelbreth-Holm-Swarm tumor (Ledbetter et al., 1985, 1987; Noonan et al., 1988), and (3) ovalbumin obtained from Sigma.

Preparation of peroxidase-protein conjugates. Peroxidase-conjugated proteins were prepared by coupling horseradish peroxidase (HRP) to ovalbumin (as a control), bFGF, or HSPG core protein. Briefly, proteins were dialyzed into 0.9% NaCl in 1 M NaHCO_3 , pH 9.5, and incubated for 16 hr at room temperature (RT) with activated peroxidase Pierce at a ratio adjusted for the molecular weight of the protein to be coupled. Excess reactive groups were blocked with 0.2 M Tris, and 1% bovine serum albumin, followed by dialysis in 150 mM NaCl and 50 mM Tris, pH 6.8.

Staining. Sections were immunostained with the antibodies by employing the peroxidase-antiperoxidase procedure (Sternberger, 1986) with 3,3'-diaminobenzidine as cosubstrate. Prior to immunostaining, sections were treated with 3% H_2O_2 in methanol for 30 min to block endogenous peroxidase. HRP-protein conjugates were visualized by development for peroxidase activity as above. In some experiments, tissue sections were treated with 0.2 M NaOH at RT for 1 hr prior to staining. In control experiments, primary antibodies were omitted and the results compared with our experimentals. The extent of ligand binding was evaluated by comparing staining intensities.

Competition, adsorption, and binding experiments. In order to ascertain the specificity of bFGF binding to NFT in sections, we incubated bFGF at 4°C for 22 hr with either 100 $\mu\text{g}/\text{ml}$ heparin, 5 mg/ml polylysine, or 30 $\mu\text{g}/\text{ml}$ protamine and then applied the solutions to the sections followed by immunostaining with monoclonal antibody to bFGF 48.1. In other experiments, we applied 0.5 $\mu\text{g}/\text{ml}$ bFGF-HRP together with various concentrations of bFGF (0.15 ng to 160 $\mu\text{g}/\text{ml}$ serially diluted 1:4) to the section and incubated for 16 hr at 4°C. In adsorption experiments, the antibody was incubated with antigen for 16 hr at 4°C before application to tissue sections. Binding of bFGF or HSPG to NFT without HRP labeling was assessed by incubating the tissue section with ligand, rinsing with the same buffer used for incubation, fixing with 3.7% formaldehyde for 5 min, and then immunostaining with an antibody to the ligand, but at a greater dilution that does not identify endogenous

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Figure 1. bFGF binds to neurofibrillary tangles (arrows), senile plaque neurites (large arrowheads), and neuropil threads (small arrowheads) as well as to amyloid deposits (*). In control sections (not shown), bFGF binding was limited to blood vessels and the few age-related NFTs. Scale bar, 25 μ m.

Figure 2. NFTs recognized by an antibody to HSPG; note that the NFTs recognized are intraneuronal. Nucleus is indicated by arrowhead. Additionally, intraneuronal granules are stained. No staining of NFTs or granules was seen in the absence of primary antibody. Scale bar, 25 μ m.

HSPG. In control experiments, the sections were stained with the same antibody dilution minus the ligand preincubation.

pH and Ca^{2+} dependence of bFGF and HSPG binding. The influence of pH on bFGF or HSPG binding to tissue sections was evaluated by incubating ligand or ligand-HRP at pH 3.1, 4.1, 5.0, 6.0, 7.0, 7.6, 8.0, 9.0, 9.9, and 10.8 by using 0.1 M Bis-Tris-HCl from pH 3.1 to 6.0, 0.1 M Tris-HCl for pH 7.0–9.0, and 0.05 M cyclohexylaminopropane sulfonic acid-HCl for pH 9.9 and 10.8 as buffers. The dependence on Ca^{2+} was determined by using Ca^{2+} /EGTA buffers and ratios of 0.3, 0.5, 0.75, 0.9, 1.0, 1.05, 1.25, 1.5, and 3.0 with 2 mM EGTA (Potter and Gergely, 1975) in 150 mM NaCl and 50 mM Tris-HCl, pH 7.85. Greater than 1 mM free Ca^{2+} solutions omitted EGTA.

Protein and sugar modification treatments. Paraffin-embedded tissue sections were incubated with one of the following: (1) 0.05 U/ml of either heparinase (0.05 U/ml, ICN, or 1 U/ml, Sigma), heparinase 2 (1 U/ml, Sigma), heparitinase (0.05 U/ml, ICN or Seikagaku), or a combination of heparinase and heparitinase at RT for 22 hr in 20 mM $CaCl_2$ and 10 mM Tris, pH 7.0 (Schubert et al., 1988). The specificity of heparinase or heparitinase treatment for heparan removal from sections was shown by comparison with the effect of 0.01 μ g/ml chondroitinase ABC (ICN) in 20 mM $CaCl_2$ and 10 mM Tris, pH 8.0, incubated at RT for 22 hr or 400 μ g/ml trypsin (Worthington) at 37°C for 10 min in 300 mM NaCl, 20 mM $CaCl_2$, and 50 mM Tris, pH 7.6. As controls for any residual protease activity in the heparinase preparation, the inhibitor 0.5 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, or exogenous protein (1 mg actin/ml) was used. (2) Two percent periodic acid for 16 hr at RT to modify sugar residues (Behrouz et al., 1989); (3) nitrous acid consisting of 0.24 M sodium nitrite in 1.8 M acetic acid for 90 min at RT to modify N-linked sulfates (Hirabayashi et al., 1989); (4) 4 U/ml neuraminidase (Sigma, type VI) in 0.2 M acetate, pH 5.4, for 2 hr at 37°C to remove sialic acid residues (Szumanska et al., 1987); (5) 0.1 M ethanolamine with the addition of 20 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (carbodiimide) after 10 min at 37°C to block and convert carboxyl groups to secondary amines (Taniuchi et al., 1986); (6) 10 mM paraformaldehyde with 60 mM sodium cyanoborohydride, 10 mM EDTA, and 10 mM phosphate, pH 7.0, for 2 hr at 37°C to block amino groups (Peterson et al., 1979); (7) 400 μ g/ml alkaline phosphatase (Sigma, type VII-S, bovine intestinal) in 0.1 M Tris, pH 8.0, with 0.01 M phenylmethylsulfonyl fluoride for 18 hr followed by 2

U/ml acid phosphatase (Sigma; prostatic, 3200 U/mg) in 0.1 M acetate, pH 5.0, for 3 hr at 37°C (Ueda et al., 1990); or (8) an unembedded methacarn-fixed tissue block of 3 mm thickness treated with 50% hydrofluoric acid for 48 hr at 4°C (Mayor et al., 1990) to remove phosphate groups followed by paraffin embedding.

Results

bFGF bound to NFTs (Fig. 1) as well as to amyloid deposits of senile plaques, an extracellular filamentous lesion, and vessel walls in both control and AD cases. However, the binding was more prominent in the AD cases because of the larger number of NFTs and senile plaques. bFGF binding, regardless as to whether it was with NFTs, amyloid deposits, or vessels, had the characteristics shown in Table 1. bFGF showed saturable binding that appeared to be charge dependent since Ca^{2+} or other polycations as well as anions inhibited the binding. Since bFGF is known to bind to its receptor as well as to HSPG (Rifkin and Moscatelli, 1989; Yayon et al., 1991), bFGF binding to NFTs suggested that HSPGs might be present. The blocking of bFGF binding by treatment of tissue with heparinases or heparitinase

Table 1. Characteristics of bFGF and HSPG binding to NFTs

	bFGF binding	HSPG binding
pH	7.0–10.0	7.0–9.0
Ca^{2+}	$<9.0 \times 10^{-6}$ M	1–25 mM
Saturation	0.5 μ g/ml	—
Inhibitors	Heparin, polylysine, protamine	—

Figure 3. Intraneuronal NFTs, as well as extracellular NFTs (not shown), bind HSPG (HSPG–HRP). NFTs and intraneuronal granules recognized in neuronal cell bodies containing a nucleus are indicated by arrowheads. Differential interface contrast microscopy. Scale bar, 25 μ m.

and the finding that an antiserum to an HSPG immunoreacted with NFTs (Fig. 2) supported bFGF interaction with HSPG in NFTs.

In order to understand how HSPGs are incorporated into NFTs, we characterized the HSPG-binding sites in NFTs by studying the properties of HSPG binding to NFTs (Fig. 3). The binding characteristics (Tables 1 and 2) suggest that Ca^{2+} acts as a bridge between anionic groups on HSPG, for example, removal of N-linked sulfate groups by nitrous acid or sugar residue oxidation with periodate blocked HSPG binding to NFTs, and anions in NFTs. Consistent with the nonspecific nature of the polycation requirement, protamine (30–100 μ g/ml) can sub-

stitute for Ca^{2+} . The Ca^{2+} inhibition of bFGF binding to HSPG (anion) in NFTs, is probably mediated by the cationic region of bFGF (Seno et al., 1990). HSPG binding to NFTs is probably to cationic groups in NFT; candidates include sugar residues of P-component, a serum glycoprotein (Kalaria and Grahovac, 1990; Kalaria et al., 1991), HSPG, phosphate, or carboxyl residues in PHF proteins. To distinguish among these possibilities, we treated sections with a variety of reagents that alter either sugar residues or amino acid side chains (Table 2). The same treatments were performed to characterize bFGF-binding sites. Alteration of sugar residues with reagents such as periodic acid or nitrous acid, neuraminidase, or heparinases generally increased HSPG binding to NFT. Thus, not only did HSPG not seem to be bound to sugar residues in NFTs, but the removal of sugar residues seemed to unmask new binding sites. In contrast, sugar-modifying treatments generally reduced bFGF binding. Moreover, treatment with formaldehyde–cyanoborohydride, to block amino groups, or with phosphatases or hydrofluoric acid (Mayor et al., 1990), to remove phosphate groups, did not decrease HSPG binding, indicating that free amino and phosphate groups in NFTs did not play a role in HSPG binding. Treatment with carbodiimide, which had no effect on bFGF binding, blocked HSPG binding, indicating that free carboxyl groups are required for HSPG binding to NFT.

A perplexing aspect of HSPG binding was that although HSPG is a protein of the extracellular matrix, HSPG was found in intraneuronal NFTs. Two alternative possibilities are that the association of HSPG with NFTs has occurred during postmortem autolysis or after extracellularization of NFTs. Both possibilities are unlikely because identical findings were obtained with the tissue obtained at biopsy (not shown), and we found that neurons with abundant cytoplasm and the nucleus had HSPG immunoreactivity (Fig. 2) and HSPG-binding sites (Fig. 3). Therefore, HSPG must be associated with intracellular NFTs *in vivo*.

Discussion

It has been well established that extracellular amyloid deposits of AD associate with molecules present in the extracellular matrix (Snow et al., 1988; Snow and Wight, 1989). However, there

Table 2. Effect of modifying treatments on bFGF and HSPG binding to NFTs

Treatment	Modification	bFGF	HSPG
Heparinases	Heparan removal	↓	—
Heparinase 2	Heparan removal	↓	—
Heparitinase	Heparan removal	↓	—
Heparitinase + heparinase	Heparan removal	↓	—
Heparitinase + heparitinase with protease inhibitor	Heparan removal	↓	—
Chondroitinase ABC	Chondroitin removal	—	—
Neuraminidase	Sialic acid removal	↓	—
Trypsin	Proteolysis	—	—
Periodic acid	Sugar oxidation	↓	↑
Nitrous acid	N-linked sulfate removal	↓	↑
Carbodiimide	Carboxyl blockage	—	↓
Formaldehyde	Amino blockage	—	—
Phosphatases	Phosphate removal	—	↑
Hydrofluoric acid	Phosphate removal	—	—

Effect on binding: ↑, increased; —, no effect; ↓, decreased.

is considerable evidence that a glycoprotein, possibly a proteoglycan, is also associated with NFTs (Mann et al., 1988; Rosenblatt et al., 1989; Snow et al., 1989, 1990). The present study confirms and extends these findings by characterizing the biochemical basis for HSPG interaction with NFT. We have found that HSPG- and bFGF-binding sites are present in NFTs. Both these components are likely to be bound to an anion in NFTs provided by HSPG for bFGF and by carboxyl residues for HSPG. HSPG incorporation into NFTs occurs intraneuronally and is therefore not an artifact resulting from contamination of extracellular NFTs.

The presence of HSPG in NFTs may provide an explanation for two puzzling features of these inclusions: number of the components and the insolubility. HSPG is present in all known amyloid filaments in systemic and cerebral amyloidoses, and HSPG deposition is concomitant with amyloid filament formation (Snow et al., 1987a). Although amyloid filaments form *in vitro* by self-assembly, *in vivo* HSPG is likely to be required as an initiator of amyloid formation because of the low concentration of free amyloidogenic subunits (Kisilevsky and Snow, 1988; Snow and Wight, 1989). HSPG presumably plays this role because of its high negative charge, which allows it to concentrate and protect ligands from proteolysis while making insoluble complexes, as it apparently does for bFGF (Schreiber et al., 1985; Gospodarowicz and Cheng, 1986; Saksela et al., 1988).

HSPG may play a similar role in the formation of the abnormal filaments of NFTs. It is known that HSPG can bind proteins other than bFGF; some of these proteins such as amyloid precursor protein (Schubert et al., 1988, 1989; Klier et al., 1990), the asymmetric form of AChE (Brandon et al., 1985; Mesulam and Moran, 1987), and P-component (Hamazaki, 1987; Duong et al., 1989; Kalaria and Grahovac, 1990; Kalaria et al., 1991) have been found in NFTs. Therefore, the compositional complexity of NFTs may be due to the binding of proteins with multiple binding sites triggered by the presence of HSPG, a phenomenon similar to that occurring in the extracellular matrix in which various proteins self-assemble through multiple hetero- and homologous bonds (Yurchenco and Johannes, 1990). Moreover, the binding of HSPG to carboxyl groups may explain why the identified PHF constituents, tau, neurofilaments, and tropomyosin, are abundant in glutamate and aspartate residues (Cleveland et al., 1977; Geisler et al., 1983; Matsumura et al., 1985). Consistent with this interpretation, antibodies to tau block HSPG binding (S.L. Siedlak and G. Perry, unpublished observations). While HSPG has not been detected in enriched PHF fractions (Sparkman et al., 1990; Lee et al., 1991; P. Mulvihill and G. Perry, unpublished observations), its association with PHF may depend on polycationic bridges not maintained in the isolation procedure. Although PHF may be primarily a polymer of tau, PHF formation may require incorporation of multiple components that are rendered insoluble as they are incorporated. HSPG's interaction with PHF proteins may provide the key to the understanding of these mechanisms.

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