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RAPID COMMUNICATION

The Presence of Heparan Sulfate Proteoglycans in the Neuritic Plaques and Congophilic Angiopathy in Alzheimer's Disease

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Two immunocytochemical probes were used to specifically identify and localize heparan sulphate proteoglycans (HSPGs) in 17 cases of Alzheimer's disease (AD). A monoclonal (HK-102) and an affinity-purified polyclonal antibody, each recognizing specific domains on the protein core of a basement membrane-derived HSPG, localized HSPGs to the amyloid fibrils present in neuritic plaques (NPs) and congophilic angiopathy (CA) in the brains of Alzheimer's patients, with weak to no immunostaining in neurofibrillary tangles from the same tissues. HSPGs were also demonstrated in "primitive plaques," suggesting that their accumulation takes place during early stages of plaque development. Immunolocalization of HSPGs to subsets of astrocytes and neuronal cells, particularly those in close proximity to NPs and CA, suggested possible involvement of these two cell types in deposition of HSPGs into the amyloidotic lesions. The current study not only identifies a new component (HSPGs) present in the amyloid deposits of NPs and CA but also suggests that astrocytes, neurons, or both may be involved in its deposition at these sites. (Am J Pathol 1988, 133:456–463)

BY FAR THE MAJOR emphasis in Alzheimer's disease (AD) research has been characterizing and identifying the various proteins and other constituents present within the Alzheimer's lesions. Although a number of components have been found within the substructure of the neurofibrillary tangles (NFTs), including neurofilaments,[1–3] the family of microtubule-associated phosphoproteins known as "tau,"[4–6] and ubiquitin,[7,8] only a few protein components have been identified in the Alzheimer amyloid deposits in neuritic plaques (NPs) and congophilic angiopathy (CA). These include the A4 or beta-protein,[9–11] and most recently alpha 1-antichymotrypsin, a serine protease inhibitor.[12]

Previous histochemical studies[13,14] have suggested that highly sulfated glycosaminoglycans (GAGs) are found specifically co-localized to all types of amyloid deposits irrespective of the identity of the amyloid protein present. In addition, highly sulfated GAGs were also demonstrated to be localized to the NPs, CA, and NFTs in the brains of Alzheimer's patients.[15,16] Although these investigations suggest that

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highly sulfated proteoglycans (PGs) are present in the AD lesions, the specific nature of the PGs and GAGs involved has not been demonstrated. In the present study we have used two well-characterized antibodies directed against the protein core of basement membrane-derived heparan sulfate proteoglycans (HSPGs) to identify and localize this macromolecule to NPs and CA in Alzheimer's disease.

Materials and Methods

Autopsy Material

Brain tissue (hippocampus, amygdala, and frontal cortex), obtained within 3–4 hours after death, from 17 cases of AD (confirmed at autopsy) were used in the present study.

Fixation and Tissue Processing

For light microscopy, tissues were fixed in 10% formalin or Bouin's fixative[17] for 24 hours followed by routine processing and embedding in paraffin. Additionally, tissues were fixed in Carnoy's solution[18] or in a solution of 3% paraformaldehyde and 0.25% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4), for 4 hours before routine paraffin embedding. From each block, 6-μ serial sections were cut and placed on gelatin-coated slides. For electron microscopy, small pieces of hippocampus and amygdala were fixed in a solution of 3% paraformaldehyde and 0.25% glutaraldehyde in 0.05 M phosphate buffer and processed as described previously.[19]

Heparan Sulfate Proteoglycan Antibodies

Two different antibodies recognizing the protein core of a basement membrane HSPG produced by the Englebreth-Holm-Swarm tumor were used for immunohistochemical studies: 1) an affinity purified polyclonal antibody (used at a dilution of 1:10 and 1:50) that recognizes the core protein of either the low or high density HSPG,[20,21] and 2) a monoclonal antibody (HK-102) (used undiluted or at a dilution of 1:10) that recognizes the core protein of the low density HSPG.[22,23] Both these antibodies have been well-characterized and found to immunostain basement membranes in a variety of normal tissues.[22,24]

Histochemical and Immunocytochemical Detection of Amyloid and HSPGs

Congo red staining[25] was used on paraffin sections to detect and localize the NPs, NFTs, and CA in each of the cases. Additionally, antibodies raised to the major low molecular weight amyloid protein (gift of Dr. Dennis Selkoe) also were used (1:200 dilution) to identify amyloid deposits in NPs and CA.[26] Adjacent serial sections were used for immunocytochemical staining with the anti-HSPG antibodies, allowing us to determine whether the same sites positive for amyloid were also positive for HSPGs.

Immunostaining of tissue sections was accomplished using the peroxidase-antiperoxidase method.[27] For immunocytochemical staining the primary antibody was used initially through a series of dilutions to obtain the best specificity with the least background staining. Only the optimum dilutions of primary antibody are reported. Best results were found with both HSPG antibodies on tissues fixed with either Carnoy's solution or 3% paraformaldehyde and 0.25% glutaraldehyde in 0.05 M phosphate buffer. Additionally, we found that we can greatly enhance the immunodetection of HSPGs localized to amyloidotic sites by pretreating formalin-fixed tissue sections for 5 minutes with 80% formic acid before immunostaining.[28]

Controls for Immunocytochemistry

To rule out nonspecific binding and to ensure specificity of the polyclonal and monoclonal antibodies, sections were treated with anti-HSPG antibody (either polyclonal or monoclonal) after pre-incubation (overnight at 4 C) in the presence of HSPG antigen at an antigen:antibody protein concentration of 7:1. Isolation of the HSPG antigens used for the immunoabsorbtion experiments was described previously.[21,22] In addition, irrelevant antibodies of the same IgG class were also used as controls including: 1) rat anti-macrophage 1 (anti-MAC 1, dilution of 1:100) (Hybritech, San Diego, CA), 2) rat anti-laminin (1:1000, ICN Biochemicals, Costa Mesa, CA), as well as 3) Tris-buffered saline (TBS). Controls for the polyclonal anti-HSPG antibody included anti-Factor VIII (1:200, Hybritech) and TBS.

Immunogold Labeling at the Ultrastructural Level

For electron microscopic immunogold labeling,[19] nickel grids containing sections were floated on drops of 3% hydrogen peroxide for 5 minutes followed by rinsing on four separate drops of distilled water. Grids were then incubated on drops of 10% normal goat serum (20 minutes), and floated on 50 μl drops containing either 1) the rat anti-HSPG monoclonal antibody (undiluted or 1:10 dilution), 2) the rabbit anti-HSPG polyclonal antibody (1:5, 1:10, or 1:50 dilution), 3) either the monoclonal or polyclonal antibody preabsorbed with excess HSPG antigen,[21,22] or 4) any of the

other negative controls described above. Sections were incubated overnight at 4 C. Sections were then rinsed on drops of filtered TBS, followed by incubation for 1 hour in goat-anti-rat IgG (1:100) for the monoclonal antibodies, or goat-anti-rabbit IgG (1:100) for the polyclonal antibodies, both conjugated to 10 nm gold in TBS. Grids were then rinsed 10 times with filtered TBS, followed by rinsing under distilled water. Samples were dried, counterstained with uranyl acetate and lead citrate, and viewed using a JOEL 100B electron microscope at 60 kv.

Results

Light Microscopic Immunolocalization of Amyloid and HSPGs in Alzheimer's Brain

Amyloid containing NPs, identified by either Congo red staining under polarized light or by positive immunostaining with the anti-beta-amyloid protein antibody (Figure 1A), on adjacent serial sections were positively immunostained by either the monoclonal (Figure 1B) or polyclonal (not shown) anti-HSPG antibodies. In NPs, anti-HSPG immunostaining was most prevalent in the region of the amyloid core (Figure 1B). No staining of NPs was observed with any of the control antisera. Although rat-anti laminin showed positive immunostaining of normal blood vessels (not shown), no positive immunostaining of NPs was observed (Figure 1C). Virtually every plaque positive with Congo red or anti-beta-amyloid protein antibody was found on adjacent sections to be positive with either of the HSPG antibodies in all 17 cases of AD examined. In many cases, primitive plaques (those having little to no amyloid cores) also were found to show positive immunostaining with either of the HSPG antibodies (Figure 1D). Because the primitive plaques are believed to represent an early stage of plaque maturation,[29] these observations suggest that HSPGs are present in plaques even at early stages of development.

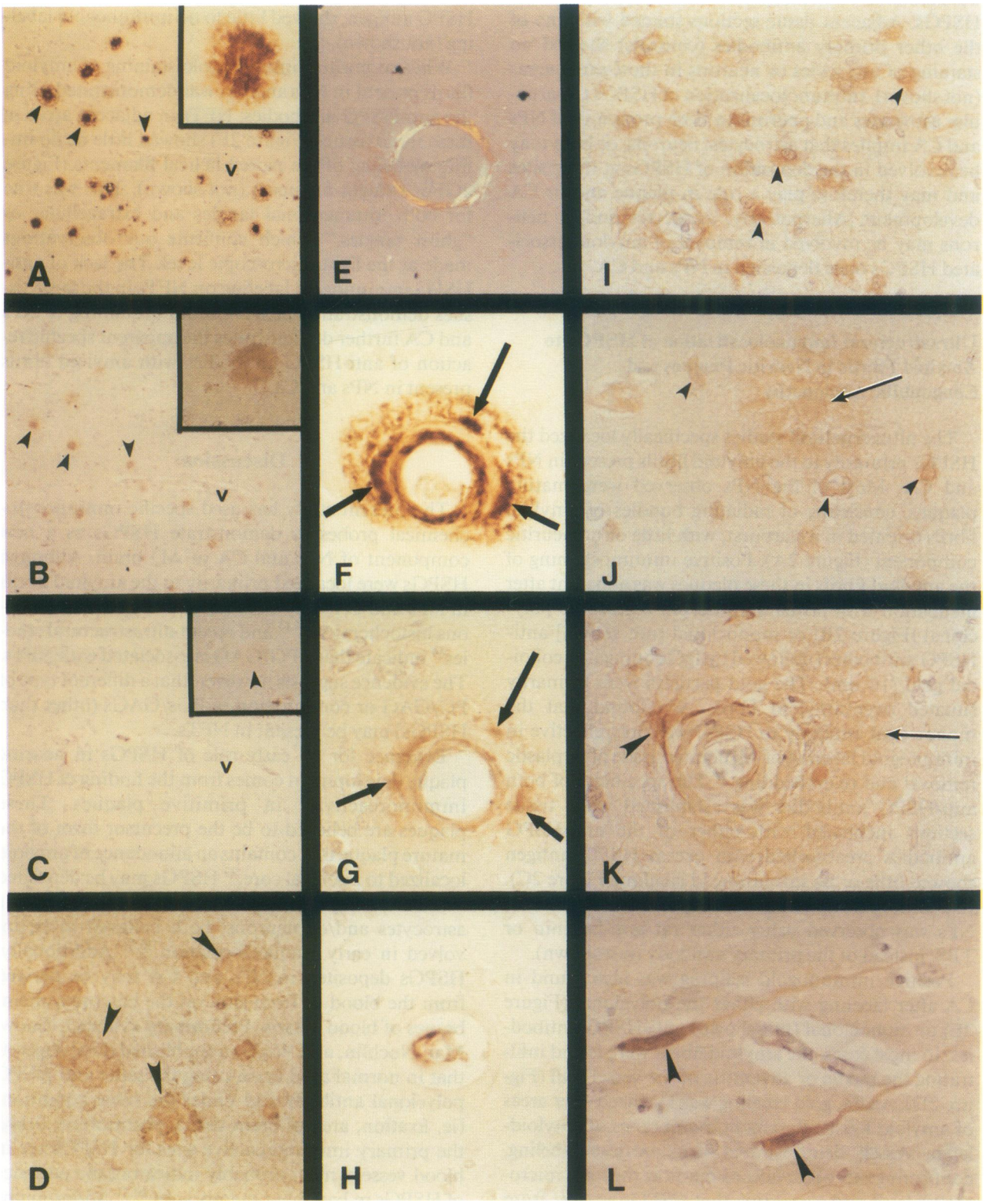
Practically every vessel found to contain amyloid deposits by either positive Congo red staining (Figure 1E) or anti-beta-amyloid protein immunostaining (Figure 1F), also was strongly positive for HSPGs with either the monoclonal (Figure 1G) or polyclonal (not shown) anti-HSPG antibody. This was true for all cases of AD examined. Control antisera or TBS instead of the primary antibody (Figure 1H) exhibited no staining.

In most of the AD cases Congo red staining also revealed an abundance of NFTs. Adjacent sections immunostained with either of the HSPG antibodies revealed very weak or no immunostaining of tangles (not shown).

The polyclonal anti-HSPG antibody immunostained blood vessels (including capillaries, arterioles, and small arteries) within the brain parenchyma, primarily in areas of AD brain that did not contain NPs, NFTs or CA (not shown). In areas of AD brain tissue containing abundant NPs, CA, and/or NFTs much of the HSPG antigenicity was lost from the nonamyloid containing blood vessels. This is believed to either represent a loss of HSPGs from blood vessel basement membranes or a change in the HSPG conformation at these sites. The monoclonal anti-HSPG was found not to react with any vascular constituents found in the normal human brain.

The anti-HSPG antibodies also showed strong cytoplasmic immunostaining of fibrous astrocytes in AD brain (Figure 1I). This observation is significant because fibrous astrocytes have been shown previously to increase in brains of patients with AD in comparison to age-matched controls.[30] In addition, astrocytes positive for HSPG were observed in close proximity to either amyloid-containing vessels (Figure 1K) or areas containing NPs (Figure 1J). In many instances, certain neurons, which did not contain NFTs, also were found to show strong immunostain-

Figure 1—Immunolocalization of heparan sulfate proteoglycans in Alzheimer's brain. Hippocampus obtained within 3 hours after death from a male patient (aged 72) with AD (Panels A–C, I, L). Calcarine cortex obtained within 4 hours after death from a female patient with AD (aged 92, Panels D–H, J, K). All sections stained with the antibodies were pretreated for 5 mins. with formic acid prior to immunostaining. **A**—Immunostaining of plaque amyloid (arrowheads) with anti-amyloid protein antibody (1:200). Unstained vessel (v) serves as landmark for serial sections. ×220 Inset shows higher power of plaque amyloid. ×800 **B**—Adjacent serial section of Panel A demonstrating positive immunostaining of plaque amyloid (arrowheads) with anti-HSPG (monoclonal, undiluted culture medium of hybridoma). ×220 Inset shows higher power of plaque amyloid stained with monoclonal anti-HSPG antibody. ×800 **C**—Adjacent serial section (of Panel B) stained with TBS shows lack of immunostaining of NP amyloid. ×220 Inset shows lack of immunostaining of plaque core amyloid (arrowhead) using rat anti-laminin (1:1000). ×800 **D**—Positive immunostaining of "primitive plaques" (arrowheads) with anti-HSPG (polyclonal, 1:5) antibody. ×960 **E**—Congophilic angiopathy (CA) in an arteriole demonstrated by positive Congo red staining under polarized light. ×800 **F**—Adjacent serial section (of Panel E) stained with anti-amyloid protein (1:200) detects amyloid deposits in wall of vessel and in periphery surrounding vessel (arrows). ×800 **G**—Adjacent serial section stained (of Panel F) with anti-HSPG (undiluted monoclonal) showed positive staining in exact locales as amyloid deposits (arrows). ×800 **H**—Adjacent serial section (of Panel G) stained with TBS instead of the primary antibody shows no staining of the same vessel. ×800 **I**—Anti-HSPG (monoclonal) immunostaining of fibrous astrocytes (arrowheads) in AD brain. Positive staining is localized to the cytoplasm of astrocytes. ×920 **J**—Positive immunostaining of neuritic plaque containing amyloid core (arrow) and adjacent astrocytes (arrowheads) with anti-HSPG (monoclonal) antibody. ×880 **K**—Positive immunostaining of both perivascular plaque (arrow) and adjacent astrocyte (arrowhead) with anti-HSPG antibody (monoclonal). Astrocytic processes are seen attached to the vessel wall. ×980 **L**—Anti-HSPG (monoclonal) immunostaining of pyramidal neurons (arrowheads). Strong staining of extending processes is also seen. These neurons were found not to contain tangles (Congo red negative on adjacent section). ×945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ing for HSPGs (Figure 1L). Most of these neurons staining positive for HSPG were found primarily in regions adjacent to NPs, whereas some areas, such as in the pyramidal layer of the hippocampus (which did not contain NFTs), also were occasionally positive for HSPGs. Adjacent tissue sections stained with any of the other primary antibodies (controls) showed no staining of astrocytes or neurons in these same areas (not shown). Immunolocalization of HSPGs to particular astrocytes and neurons in close proximity of NPs and CA implies that astrocytes, neurons, or both may be involved in the deposition of HSPGs at these sites and may therefore play a role in plaque and/or CA development. Alternatively, astrocytes and/or neurons may be involved in removal of amyloid-associated HSPGs once deposited in NPs and CA.

Ultrastructural Immunolocalization of HSPGs to Amyloid Fibrils in Neuritic Plaques and Congophilic Angiopathy

The ultrastructural studies specifically localized the HSPGs primarily to the amyloid fibrils present in NPs and CA. Most of the NPs observed were mature plaques, consisting of radiating bundles of amyloid fibrils oriented in a starburst, with little or no neuritic component (Figure 2A). Positive immunostaining of the amyloid fibrils in these plaques was apparent after incubation with primary antiserum (either the polyclonal [Figure 2B] or monoclonal [not shown] anti-HSPG antibodies), followed by IgG conjugated colloidal gold (10 nm). The gold particles were primarily situated over amyloid fibrils. We found that the monoclonal antibody to HSPG was only effective in immunogold labeling of Alzheimer tissue after plastic removal and re-embedding.[19] The specificity of both anti-HSPG antibodies was established when tissue sections incubated with either of the anti-HSPG antibodies preabsorbed with excess HSPG antigen showed little or no immunogold labeling (Figure 2C). In addition, no apparent labeling of amyloid fibrils in NPs was observed using either rat anti-laminin or TBS, instead of the primary antibody (not shown).

Positive immunogold staining was also found in CA after labeling with either the polyclonal (Figure 2E) or monoclonal (not shown) anti-HSPG antibodies. In most cases, the amyloid fibrils were found infiltrating the media or adventitia of the vessel wall (Figure 2D). Again, gold labeling was localized over areas of amyloid fibrils. Basement membranes of amyloid-laden vessels demonstrated weak immunolabeling, confirming our observations made at the light microscopic level and suggesting that HSPGs are lost from blood vessel basement membranes in amyloid-laden vessels or that a change in the HSPG conformation occurs at these sites. Tissue sections incubated with either the anti-HSPG polyclonal antibody or the anti-HSPG monoclonal antibody, preabsorbed with excess HSPG antigen, showed little to no immunogold labeling (not shown).

Whereas positive immunogold staining of amyloid fibrils present in NPs and CA was demonstrated using the anti-HSPG antibodies, NFTs in adjacent areas in these same tissues (Figure 2F) showed little or no immunolabeling of the paired helical filaments (Figure 2G) or straight filaments (not shown). This was true for both intraneuronal tangles and extracellular or "ghost tangles," which confirms our observations made at the light microscopic level. The lack of anti-HSPG immunogold labeling on NFTs in the same tissues demonstrating positive immunostaining of NPs and CA further demonstrates the apparent specific reaction of anti-HSPG antibodies with amyloid fibrils present in NPs and Ca.

Discussion

The present study has used specific immunocytochemical probes to demonstrate HSPGs as a new component of NPs and CA in AD brain. Although HSPGs were localized primarily to the amyloid fibrils in NPs and CA, they were not found in NFTs. Previous histochemical[14,15] and recent ultrastructural studies[16] indicate that a PG/GAG is associated with NFTs. The evidence suggests however, that a different type of PG/GAG or combination of PGs/GAGs (other than HSPGs) may be present in NFTs.

Evidence for an early role of HSPGs in neuritic plaque development comes from the finding of HSPG immunoreactivity in primitive plaques. These plaques are believed to be the precursor form of the mature plaque that contains an abundance of amyloid localized to a central core.[29] HSPGs may be deposited into the developing plaque by adjacent and infiltrating astrocytes and/or neurons, both believed to be involved in early plaque formation.[30,31] Additionally, HSPGs deposited in NPs and CA may be derived from the blood or from sites on the basement membranes of blood vessels. Preliminary evidence (Snow, Mar, Nochlin, and Wight, unpublished data) suggests that in normal aged brain using the same anti-HSPG polyclonal antibody and under the same conditions (ie, fixation, area of brain tissue, postmortem delay) the primary immunoreactive sites for HSPGs are in blood vessels, most probably indicating the presence of HSPGs in basement membranes.[20,21] Additionally,

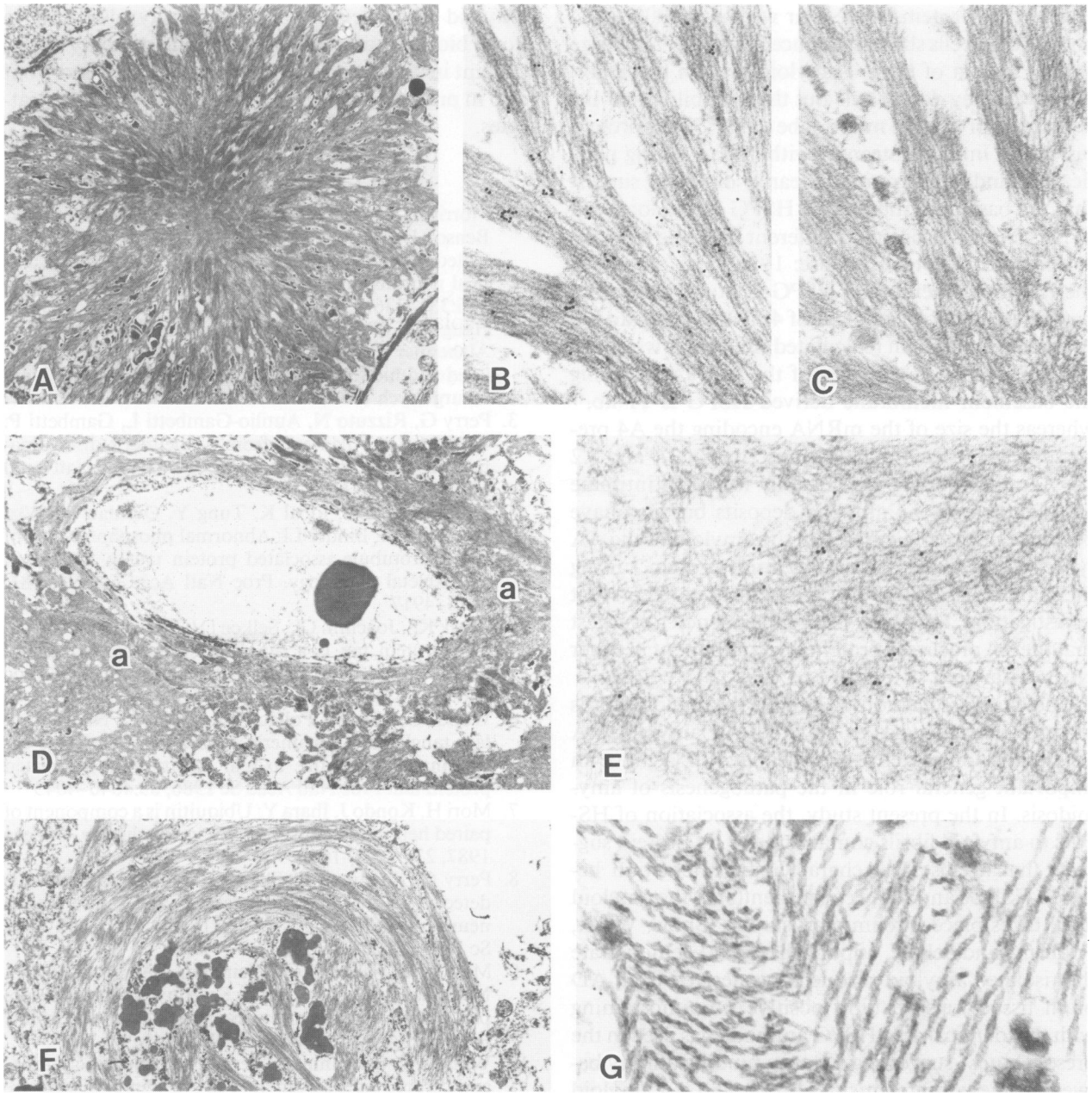

Figure 2—Immunogold localization of heparan sulfate proteoglycans to amyloid fibrils in neuritic plaques and congophilic angiopathy in Alzheimer's disease. All electron micrographs shown are from the hippocampus of a 72 year old male patient with AD. Tissue was obtained within 3 hours after death. **A**—Star-shaped "mature" plaque with radiating bundles of amyloid fibrils. ×6000 **B**—Anti-HSPG (polyclonal, 1:5) immunogold labeling of star-shaped plaque shows 10 nm gold particles are specifically associated with amyloid fibrils with less gold labeling apparent in areas between amyloid fibrils. ×40,000 **C**—Only a few gold particles remain in amyloid plaque after serial section (of Panel B) was stained with anti-HSPG antibody (polyclonal, 1:5) pre-adsorbed with excess HSPG antigen. ×35,000 **D**—Low-power electron micrograph showing cerebrovascular amyloid (a) present in the adventitia of an arteriole. ×7000 **E**—Immunogold labeling of cerebrovascular amyloid in the adventitial portion of the arteriole shown in Panel D with the polyclonal (1:5) anti-HSPG antibody. Immunogold labeling is localized over areas of amyloid fibrils. ×40,000 **F**—Low-power electron micrograph of intraneuronal neurofibrillary tangle. ×6000. **G**—Anti-HSPG (polyclonal) immunostaining of intraneuronal NFT in same tissue section as Panels A and D. Only a few gold particles are associated with the paired helical filaments. ×50,000

astrocytes in normal aged brain show weaker immunostaining than observed in AD brain, whereas neurons show very weak immunoreactivity.

Once deposited in the plaque, HSPGs may serve as a trophic factor in attracting neurites to the area of the plaque, thereby serving as a nidus for further plaque development to occur. Previous *in vitro* studies have demonstrated that HSPGs can activate and promote neuritic outgrowth.[32–35]

A recent article by Schubert et al[36] suggests that the

HSPG core protein (molecular weight, 80,000) present in PC 12 cells shows sequence homology and a size similar to that of the A4 amyloid protein precursor. However, they do not rule out the possibility that the A4 precursor protein may not be a HSPG core protein but that it interacts strongly with HSPG during purification and gel filtration. Clearly, our data suggest that the basement membrane HSPG found localized to NPs and CA is probably different from the reported A4 precursor protein because: 1) the core protein of the basement membrane HSPG is much larger (approximate molecular weight of 400,000)[21,22] than the A4 precursor protein (calculated molecular weight of about 79,000),[37] 2) the size of the mRNA encoding the basement membrane derived HSPG is 11 kb,[39] whereas the size of the mRNA encoding the A4 precursor was reported to have transcripts of 3.4 and 3.2 kb,[37] and 3) HSPGs are not only found in intimate association with A4 amyloid deposits but also have been recently demonstrated in the amyloid of the AA type[38,40] and those containing prion protein[41] using the same HSPG antibodies as described in the present investigation. There has been no evidence to suggest that the A4 precursor protein is a component in other types of amyloid.

The close association of HSPGs to amyloid fibrils in three differnt types of amyloid (A4 amyloid, AA amyloid, prion protein) suggests that HSPGs have an important general role in the pathogenesis of amyloidosis. In the present study, the association of HSPGs to amyloid fibrils containing the A4 protein suggests that a close and characteristic interaction between HSPGs and some constituent(s) of the amyloid fibril takes place. Preliminary studies (Snow, Wight, Podlisny, and Selkoe, unpublished data) demonstrate that isolated amyloid plaque cores derived from AD brain tissue[26] also exhibit positive immunostaining with the identical anti-HSPG antibodies as used in the present study, suggesting a strong binding affinity between some components of HSPGs and A4 amyloid fibrils. It is even possible that part of the HSPG molecule may become incorporated into or attached to a portion of the amyloid fibril. The intimate association of HSPGs to the amyloid fibrils in these lesions may also stabilize amyloid once formed and may protect it from subsequent degradation.

Although the current study identifies and localizes one type of PG to the amyloid fibrils in NPs and CA in AD, it is possible that others are also present. Combinations of PGs present in these lesions may be instrumental in determining the ultimate morphologic appearance of the plaque in AD and in the prion diseases. These studies may not only be important in identifying possible cell types involved in plaque or amyloid fibril development, but may also lead to novel biochemical markers that could not only be important in diagnosis and monitoring of the disease but also in providing clues as to possible therapeutic strategies.

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