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Short Communication

Immunohistochemical Identification of Thrombospondin in Normal Human Brain and in Alzheimer's Disease

Luc Buée,*|| Patrick R. Hof,*†
David D. Roberts,‡ André Delacourte,||
John H. Morrison,*† and Howard M. Fillit*†

From the Department of Geriatrics and Adult Development, and the Fishberg Research Center for Neurobiology,† Mount Sinai School of Medicine, New York, New York; the Laboratory of Pathology,‡ National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and INSERM U156,|| Lille, France*

Thrombospondin is part of a family of adhesive glycoproteins and is involved in a number of physiologic processes such as angiogenesis and neurite outgrowth. Immunohistochemical localization of thrombospondin in normal human brains was investigated in the hippocampus and inferior temporal cortex. Two antibodies (one polyclonal and one monoclonal) against thrombospondin-labeled microvessels, glial cells, and a subpopulation of pyramidal neurons. The distribution of thrombospondin staining in patients with Alzheimer's disease was found to be comparable to control subjects. However, in patients with Alzheimer's disease a subset of pyramidal neurons that may be vulnerable in Alzheimer's disease exhibited decreased staining. This decrease in the intensity of labeling might constitute a marker for a neuronal population prone to early degeneration. In addition, thrombospondin staining was demonstrated in senile plaques in Alzheimer's disease. These results suggest that thrombospondin may be involved in the process of neuronal degeneration and senile plaque formation. (Am J Pathol 1992, 141:783–788)

Thrombospondin (TSP) is a large, multifunctional glycoprotein, and part of a family of adhesive proteins generated by alternative splicing and gene duplication.[1] TSP interacts with certain components of the cell surface and the extracellular matrix such as sulfated glycolipids, glycosaminoglycans (GAGs), heparan sulfate proteoglycan (HSPG), fibrinogen, fibronectin, collagen, histidine-rich glycoproteins, and plasminogen.[1,2] Thrombospondin promotes cell adhesion and migration and modulates responses of several cell types to growth factors.[1,2] Through its ability to modulate endothelial cell adhesion, motility, and growth, it is a potential angiogenesis regulatory factor.[3,4] One of the cell surface ligands for thrombospondin is HSPG, which may mediate some of the biological effects of thrombospondin on cells.[5–7] Heparan sulfate proteoglycan produced by tumor cells of neuroectodermal origin has also been shown to bind to TSP with high affinity.[8] Thrombospondin is found in cultured human brain glial cells[9] and is involved in the migration of cerebellar granule cells,[10] and neural crest cells.[11] Thrombospondin is also present in the embryonic retina, and promotes retinal neurite outgrowth,[12] making it a likely regulator of axonal growth in the embryonic nervous system.[13]

The biochemical and molecular analysis of the characteristic lesions of Alzheimer's disease (AD), neurofibrillary tangles (NFT), and senile plaques (SP), demonstrated that these pathologic changes contain a variety of abnormal elements. Neurofibrillary tangles have been shown to contain altered cytoskeletal proteins.[14,15] Within SP and congophilic angiopathy, the main component described is the β-protein or A4 peptide (βPA4).[16] Other components have also been detected such as α_1-antichymotrypsin,[17] laminin,[18] and GAG and/or proteoglycans (PG).[19,20]

To further investigate the possible events leading to the formation of the typical lesions of AD, we used antibodies against TSP, a heparin-binding protein, to exam-

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Address reprint requests to Dr. Howard Fillit, Department of Geriatrics, The Mount Sinai Medical Center, Box 1070, New York, NY 10029-6574.

ine its distribution in the cerebral cortex of demented and neurologically normal elderly patients. The antibodies used in this study were developed against TSP1, a product of the first described gene.[21] We do not have any evidence that they react with TSP2, a product of the second gene.[22] However, in the mouse, both TSP1 and TSP2 are expressed in fetal and adult brain.[23] It is not known if both genes are expressed in human brain. If they are expressed, both TSP proteins may be recognized with the polyclonal antibody because they have many consensus amino-acid sequences. Because monoclonal antibody A6.1 recognized the same elements as the polyclonal antibody in our materials, both TSP proteins may be detected with A6.1. We observed that TSP is found in the normal human brain. In AD, it is present within SP and in a particular neuronal subpopulation that is likely to be vulnerable in the degenerative process.

Materials and Methods

The brains of five patients with no history of neurologic or psychiatric disorders (72.8 ± 6.0 years, postmortem delay less than 6 hours), and of nine patients with senile dementia of the Alzheimer type (80.8 ± 2.1 years postmortem delay less than 6 hours) were obtained at autopsy. The diagnosis of possible AD was established clinically according to NINCDS-ADRDA criteria[24] and confirmed neuropathologically by the presence of high densities of SP and NFT in the hippocampus and the neocortex. The brains were processed as described by Hof et al.[25]

Forty micrometer thick sections were pretreated with 3% H_2O_2 in methanol (1:3, V:V) to eliminate the endogenous peroxidase activity. Then, they were incubated overnight at 4°C with either a polyclonal[26] (used at a working dilution of 1:1000) or the monoclonal A6.1[27] (used at a working dilution of 3.0 to 3.5 μg/ml) antibodies against a segment of TSP designated as type II, calcium dependent. After incubation, the sections were processed with an avidin-biotin kit (Vector Laboratories, Burlingame, CA), and diaminobenzidine, and then intensified by serial baths of 0.005% osmium tetroxide, 0.5% thiocarbohydrazine, and 0.005% osmium tetroxide.[25] Some TSP-labeled sections were counterstained with a 1% aqueous thioflavine S solution to simultaneously identify NFT and SP.

Rabbit polyclonal anti-TSP antiserum was prepared by immunizing rabbits with purified human platelet TSP. Its specificity has been demonstrated previously.[26] The mouse monoclonal antibody A6.1, which recognizes a calcium-dependent epitope in the 70 kda core of thrombospondin,[27] was provided by Dr. William Frazier, Washington University, St Louis. The antibody was purified by protein A affinity chromatography.

Results

In the cortex of control cases, the polyclonal antibody to TSP stained a subpopulation of pyramidal neurons (Figure 1A). Similar neurons were also stained with the monoclonal antibody A6.1 (Figures 1B, 2A–B). Thrombospondin-immunoreactive neurons were found in the pyramidal

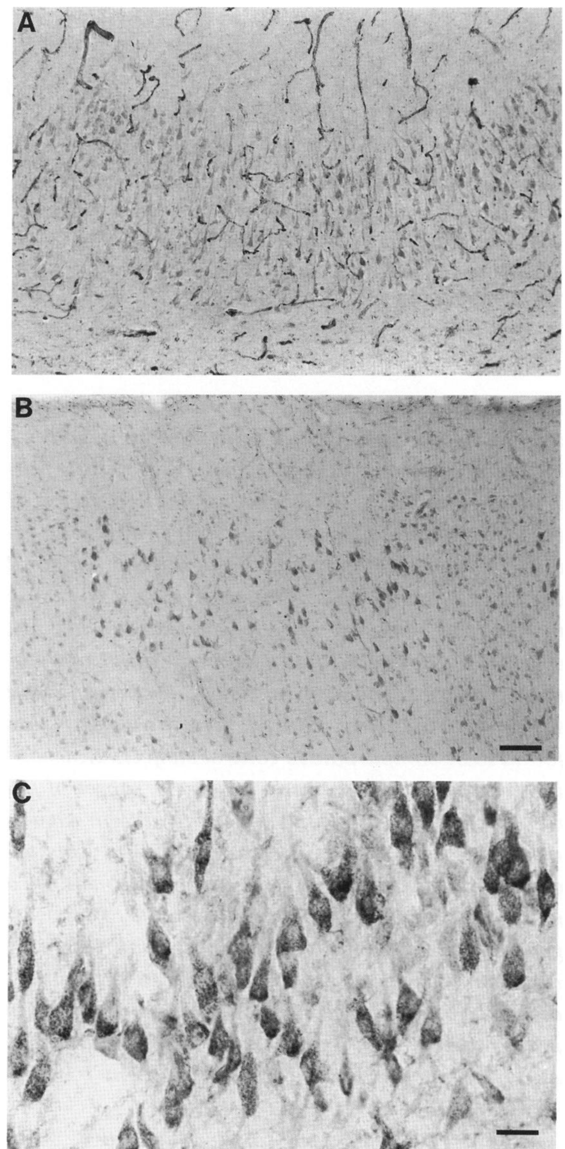

Figure 1. A: *Pyramidal neurons and microvessel staining using a polyclonal antibody against TSP in a control case.* **B:** *Layer II of the entorhinal cortex in a control case stained with the monoclonal antibody A6.1; note the neuronal labeling. Magnification bar = 100 μm.* **C:** *High magnification of CA3 pyramidal neurons stained with the monoclonal antibody A6.1. Note the dark granular staining. Magnification bar = 25 μm.*

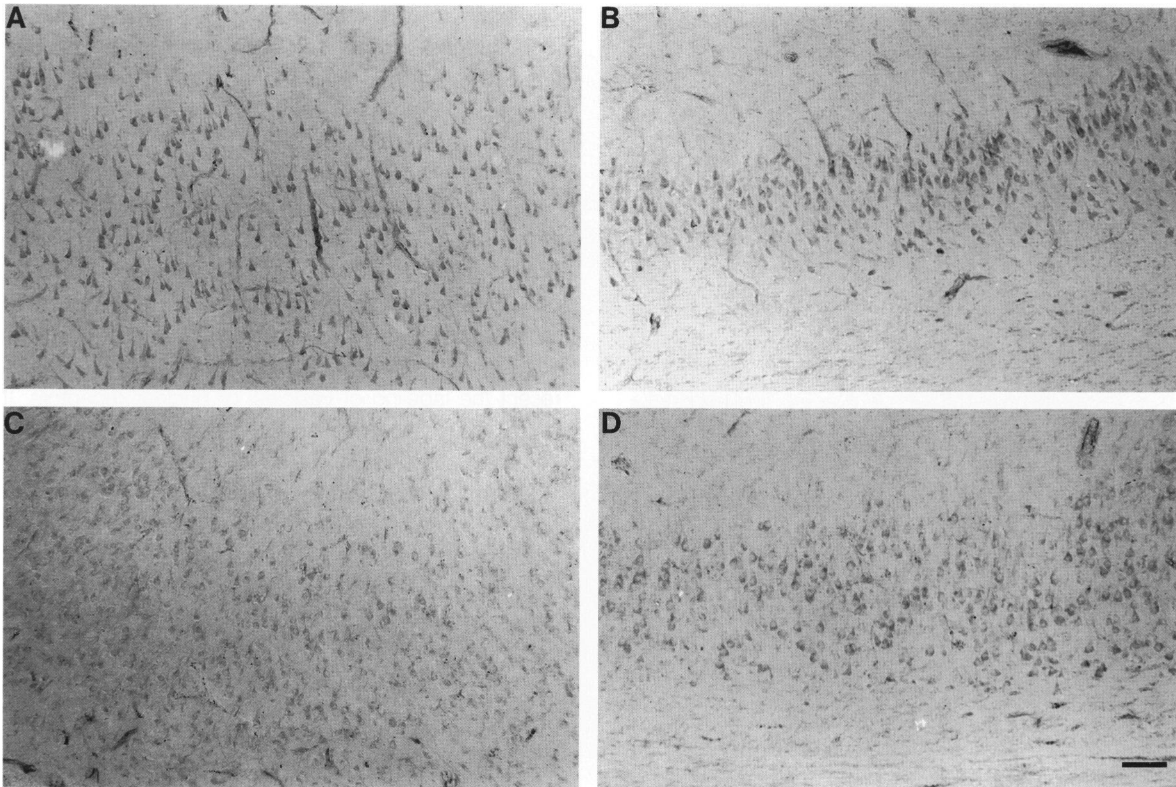

Figure 2. **A**: *CA1 field in a control case.* **B**: *CA3 field in a control case. Note the dark staining of pyramidal neurons in both areas. Some microvessels are also labeled.* **C**: *CA1 field in AD patient; the neuronal staining is decreased, whereas a few microvessels are labeled.* **D**: *CA3 field in AD patient; the neuronal staining is decreased. Materials were stained with the monoclonal antibody A6.1. Magnification bar = 100 μm.*

layer of the CA1-CA4 fields (Figure 2A–B) of the Ammon's horn and in the large polymorphic neurons of the hilus of the dentatus gyrus. In the entorhinal cortex, layer II demonstrated the darkest neuronal staining (Figure 1B), followed by layer III, and finally layers V and VI. In the inferior temporal cortex, pyramidal neurons of the layers III and V were also darkly labeled. At higher magnification, the labeling pattern of the pyramidal neurons appeared to be finely granular (Figure 1C). With the monoclonal antibody, the neuronal staining was much stronger than with the polyclonal antibody. Glial cells were also labeled, especially in the white matter surrounding blood vessels, as well as diffuse elements in the neuropil (not shown). Staining of glial cells was weaker than neuronal staining. A few microvessels were stained with both antibodies.

The neuronal staining was markedly decreased in all AD cases as compared with control cases. For instance, using antibody A6.1, pyramidal neurons were darkly stained in control cases in CA1 and CA3 fields (Figure 2A–B), whereas in AD patients, neuronal staining was almost absent in CA1 (Figure 2C) and severely decreased in CA3 (Figure 2D). Similar decreases in staining intensity in AD brains were observed in subiculum and in layers III and V of the inferior temporal cortex. In AD cases, no TSP staining of pyramidal neurons was observed in the entorhinal cortex. Staining of glial cells and fibrillar formations was comparable to that observed in control cases. Microvascular staining appeared to be weaker in AD as compared with control cases.

In AD, SP consistently stained for TSP and were observed in all cortical areas investigated. Both monoclonal and polyclonal antibodies against TSP-labeled SP (respectively Figure 3A–B). As demonstrated by double labeling, SP stained by thioflavine S was also labeled by anti-TSP antibodies (Figure 3B–C). The staining pattern of some SP also suggested that TSP may accumulate in dystrophic neurites surrounding the plaques.

Discussion

Our observations suggest that a subpopulation of TSP-immunoreactive neurons that appear to be vulnerable in AD is decreased or lost. This conclusion is based on the fact that weakly stained TSP-immunoreactive neurons or their absence in AD are found in the areas where neuron loss preferentially occurs in AD.[25,28,29] Furthermore, comparable glial and vascular staining was observed in control subjects as well as AD patients.

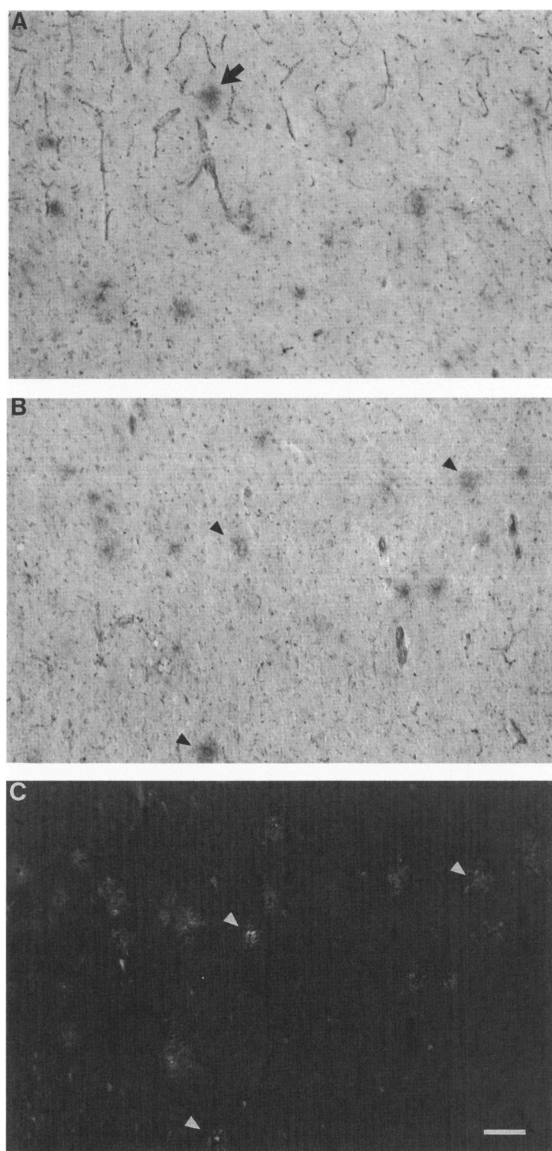

Figure 3. A: *Using the polyclonal antibody against TSP, SP (arrow) and microvessels are stained in AD cases.* **B, C:** *Double labeling of amyloid deposits using the monoclonal antibody A6.1 against TSP* **(B)** *and thioflavine S under fluorscence lightning conditions* **(C)**. *Note that all SP were stained by both thioflavine S and the antibody against TSP (arrowheads). Magnification bar = 100 μm.*

The presence of TSP in the central nervous system is compatible with immunochemical and biochemical analyses of human glial cells in culture that have demonstrated that these cells are able to synthetize TSP.[9] Thrombospondin staining was noted in glial cells in normal brain[9] but has not been previously reported in neuronal cells. We observed staining of glial cells in the brains of both control and AD cases. Furthermore, TSP has been shown to be involved in a number of physiologic processes in primary cell cultures of embryonic neurons,[13] or in the developing nervous system.[10–12] It may play a unique role in development by creating local foci of controlled balance between proteases and protease inhibitors necessary for migration and neurite outgrowth. TSP may have a similar function in the normal adult brain.

In AD cases, the granular TSP staining of pyramidal neurons appears decreased compared with control subjects. Since it is likely that the antibodies used in the present study crossreact with both TSP gene products, neuronal labeling may be related to the presence of neuron-specific TSP, which has not yet been characterized. This granular pattern could be intracytoplasmic and represent the labeling of vesicles, or nuclear receptors. It could also represent staining of surface receptors as observed in fibroblasts.[30] Thus, the loss of neuronal TSP staining in AD may have two explanations. Since neurons have at least two classes of TSP receptors,[12] it may simply reflect the loss of surface receptors, or it could result from the loss of these particular TSP-immunoreactive neurons that may contain a neuron-specific TSP. Pyramidal neurons are lost in AD.[25,28,29] Furthermore, in AD, this granular TSP staining was better preserved in CA3 than in CA1. These data are suggestive of a regional pattern of vulnerability. In this respect, CA1 contains more NFT than CA3 in AD. Layer II of the entorhinal cortex did not show any TSP staining in AD whereas in control subjects, staining of pyramidal cells was common. These neurons located within layer II of the entorhinal cortex form the perforant pathway that project to the gyrus dentatus. This circuit has been shown to be severely affected in AD.[31] Thus, the pattern of TSP-immunoreactive pyramidal cell loss parallels the neuronal degeneration and cell loss observed in AD.[25,28,29] Interestingly, the TSP neuronal subpopulation affected in AD appears to be comparable to a population of pyramidal neurons that contain high density of non-phosphorylated neurofilament protein.[25] However, double-labeling studies will be necessary to determine the degree to which TSP-positive pyramidal cells also contain high levels of non-phosphorylated neurofilament protein.

A number of extracellular components such as GAG/PG,[19] laminin,[18] or protease inhibitors[17] are found in SP. The presence of TSP, as well as these other proteins in SP may result from vascular and/or neuronal degeneration and participation in formation of SP. Biological effects of TSP are diverse and can stimulate or inhibit proliferation depending on the cell type. However, in the central nervous system, thrombospondin appears to be expressed at sites of proliferation or migration.[10–13] Since synaptic loss is one of the early events in AD pathology,[32] TSP may accumulate in these areas of synaptic loss to promote regeneration of injured cells. This may explain the fact that TSP staining of SP appears to be primarily in dystrophic neurites and also that TSP neuronal staining is

decreased. In addition, as previously demonstrated, microvasculature changes are found in AD.[20,33] TSP also plays a role in the regulation of the perivascular extracellular matrix and is an inhibitor of the angiogenesis.[3] There is evidence that components of the extracellular matrix are involved in SP.[18] Thus, the presence of TSP within SP may also result from vascular pathology. Biochemically, the presence of TSP within SP could reflect its high affinity for HSPG[8] that is always found to coexist with βPA4 in SP in AD.[18,20]

In conclusion, TSP is found in normal human brain, especially in a subpopulation of pyramidal neurons. Its decreased staining or its loss in AD might be a neuronal marker of early neuronal degeneration. These data suggest that pathologic changes linked to AD may involve a loss of specific chemically identifiable neuronal populations. Finally, TSP is found in SP and may be involved in a number of mechanisms during regeneration that may lead to formation of SP.

Acknowledgments

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