American Journal of Pathology, Vol. 139, No. 3, September 1991

Sequestration of Tau by Granulovacuolar Degeneration in Alzheimer's Disease

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Antibodies directed against three regions of tau have been used in a histologic study of granulovacuolar degeneration (GVD) in Alzheimer's disease (AD). Granulovascular degeneration complexes, consisting of a dense granule in a less-dense vacuole, were found in hippocampal pyramidal neurons in all patients studied. Anti-tau antibodies directed against the N-and C-termini, and the repeat region of tau, were found to immunolabel the granule of the GVD complex. Intracellular neurofibrillary tangles also were labeled by these antibodies. In particular, MAb6.423, which recognizes tau protein sequestered in paired helical filaments (PHF) in AD, but not the normal tau proteins so far described in human brain, labeled GVD granules. Contrarily, a generic tau marker (MAb7.51), which immunolabels all known isoforms of isolated and expressed tau protein, including PHF-tau, did not label the GVD granule. These findings demonstrate that the entire tau molecule is sequestered within the GVD granule, and that the tau protein found in GVD complexes is antigenically related to that found in PHFs. There is, however, a difference in the way in which the repeat region of tau is incorporated into the two structures, making the MAb7.51 epitope unavailable in the GVD complex. These findings suggest that the formation of GVD complexes in hippocampal pyramidal neurons vulnerable to neurofibrillary degeneration may represent an alternative pathway for dealing with an aberrant molecular complex, which contributes to the formation of GVD granules and neurofibrillary tangles in AD. (Am J Pathol 1991, 139:641–647)

Granulovacuolar degeneration (GVD) refers to the presence of conspicuous structures—a dense granule (0.5 to 1.5 μ in diameter) in a vacuole-like, less dense area about twice the diameter found typically in the cytoplasm of hippocampal pyramidal cells.[1] The vacuoles, some 3 to 5 μ in diameter, appear to be empty in electron micrographs; the granules (0.5 to 1.5 μ in diameter) seem to be amorphous. This formation was first described in Alzheimer's disease (AD) by Simchowicz.[2]

The GVD complex is not specific for AD or for the hippocampus, although its association with both is characteristic and well established.[3,4] In nondemented elderly patients it was found in 0% to 7% of nerve cells in involved regions of the hippocampus.[3] Ball and Lo[4] showed that the number of hippocampal pyramidal cells involved, which was never more than 1%, increased gradually with age in the nondemented.

In AD, Tomlinson and Kitchener[3] found at least 9% of pyramidal cells involved in 22 of 25 (88%) demented patients with histologically confirmed AD. Granulovacuolar degeneration also has been described in Pick's disease,[4] multi-infarct dementia,[3] Guamanian amyotrophic lateral sclerosis and parkinsonism-dementia complex,[5] tuberous sclerosis,[6] progressive supranuclear palsy,[7] and Down's syndrome.[8,9] The presence of GVD in hippocampal pyramidal cells, like that of neurofibrillary tangles (NFTs), is typical of AD.

The relationship between GVD and NFTs in hippocampal cells in AD is unknown. Attempts to define the molecular composition of NFTs by immunohistochemical methods have lead to the identification of a large number of possible protein constituents, including the microtubule-associated protein, tau,[10–16] neurofilament proteins,[17–21] microtubule-associated protein,[22–23] vimentin,[24] tropomyosin,[25] and ubiquitin.[26–32] The combined presence of such proteins, although confusing, may signify a complex disturbance in neuronal cytoskeletal functioning, before or after paired helical filaments (PHF) formation. Likewise, attempts to define the molecular composition of the GVD complex immunohistochemically have produced equally confusing results, with claims made for the identification of tropomyosin,[25] neurofilament protein,[33,34] tubulin,[35] ubiquitin,[36] and tau protein.[11,34,37] Of these various proteins, only tau protein has

Supported by the Della Martin Foundation (Los Angeles) and the Charles Wolfson Charitable Trust (London).

Accepted for publication May 15, 1991.

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been established definitively on biochemical, ultrastructural, and immunobiochemical grounds as an integral component of the PHF.[38–40] Indeed the repeat region of tau, which is embedded within the pronase-resistant core of the PHF,[16] is immunochemically distinct from the corresponding segment of normal soluble forms of tau proteins so far described in the adult human brain.[41,42] N-and C-terminal portions of the tau molecule, located in the protease-sensitive outer coat of the PHF, are lost in the course of tangle degradation in the extracellular space, although the repeat region survives extensive proteolytic degradation of PHFs *in vitro*[16] and in the extracellular compartment *in vivo*.[43]

Because tau has been implicated also as a component of the GVD complex, we have undertaken a detailed immunohistochemical analysis of the involved parts of the tau molecule. We establish here that the whole of the tau molecule is incorporated into the GVD granule and that it is antigenically related to a distinctive form of tau protein found in PHFs in AD.

Methods and Materials

Brain tissues were obtained after death from eight patients who had died in hospital and satisfied DSM-III criteria for dementia[44] at some time before death. These same tissues have been used in other studies reported previously.[43] Abundant NFTs and senile plaques were found in the hippocampus and cerebral cortex in each patient, satisfying criteria established by an NINCDS-ADRDA work group[45] for definite AD. The mean age at death of the AD patients was 76.4 years (standard deviation [SD] = 6.5); the estimated duration of dementia varied from 1 to 11 years. Brains from five nondemented patients (aged 23, 35, 40, 51, and 86 years), showing no significant pathology on routine neuropathologic examination, served as controls.

Brains were removed 6 to 12 hours after death and divided in the midsagittal plane. From one half, specimens were taken from several areas including the posterior hippocampus and fixed overnight in 5% acetic acid in methanol or 10% neutral formalin in phosphate-buffered saline (PBS). Tissues were embedded in paraffin and sectioned serially at 10 μ.

Immunocytochemical Methods

Serial sections (10 μ) were deparaffinized in xylene, hydrated in the conventional manner, and treated with 1% aqueous hydrogen peroxide. They were treated with a solution of fat-free skimmed milk (2% in PBS) to block nonspecific labeling before being treated (1 hour) with a monoclonal or polyclonal (serum) antibody. Sections were treated with 1% horseradish peroxidase-conjugated rabbit immunoglobulin to mouse immunoglobulin (for monoclonal antibodies) or 1% peroxidase-conjugated swine immunoglobulin to rabbit immunoglobin (for BR133/134) in 10% human serum in PBS. Peroxidase was visualized with diaminobenzidine (DAB), activated with hydrogen peroxide. Sections were counterstained with Ehrlich's hematoxylin. Alternate sections, stained only with hematoxylin and eosin (H & E), assured the identification of the GVD complex. Details of these methods have been described.[43]

Monoclonal antibody (MAb) 6.423 was described previously.[46,47] This antibody was raised against a PHF-core preparation described by Wischik et al.[16] Monoclonal antibodies 11.57 (unpublished data) and 7.51[47] also were raised against PHF-core subfractions. Monoclonal antibody 6.423 has been shown to recognize the repeat region of tau released from the pronase-resistant core of the PHF,[16] but no other form of isolated or expressed tau.[46–48] Monoclonal antibody 11.57 likewise fails to recognize any of the known tau isoforms that have been expressed, or normal tau isolated from adult human brain. Like MAb6.423, MAb11.57 recognizes a distinct form of tau that is associated with PHFs (unpublished data). Monoclonal antibody 7.51 recognizes PHF-core–derived tau and native soluble tau.[47,48] BR133 and BR134 are antisera raised in rabbits immunized with synthetic peptides corresponding to residues 1–16 and 339–352, respectively, of human type I tau (unpublished data).

Three commercially available antitubulin antibodies were used. Monoclonal antitubulin antibodies against α-tubulin (N.356) and β-tubulin (N.357) were obtained from Amersham Arlington Heights, Illinois. A polyclonal anti-tubulin antibody (65-095) was obtained from ICN Irvine, California.

Two sections from each specimen served as controls. One was not treated with the primary antibody (Figure 1D); the other (in the case of the two polyclonals) was treated with antiserum plus the antigen used to generate it, before being treated with hydrogen peroxide and DAB. Specificities of the antisera were established by demonstrating the loss of immunoreactivity after preadsorption with the native antigen.

Results

The GVD complex was found in hippocampal pyramidal cells in all eight cases of AD studied. As previously noted, it was most commonly found in the cornu ammonis, in fields H1 and H2 of Rose, which include fields CA1 and CA2 of Lorente de No and which are sites of major NFT accumulation in AD. Pyramidal cells with GVD seemed

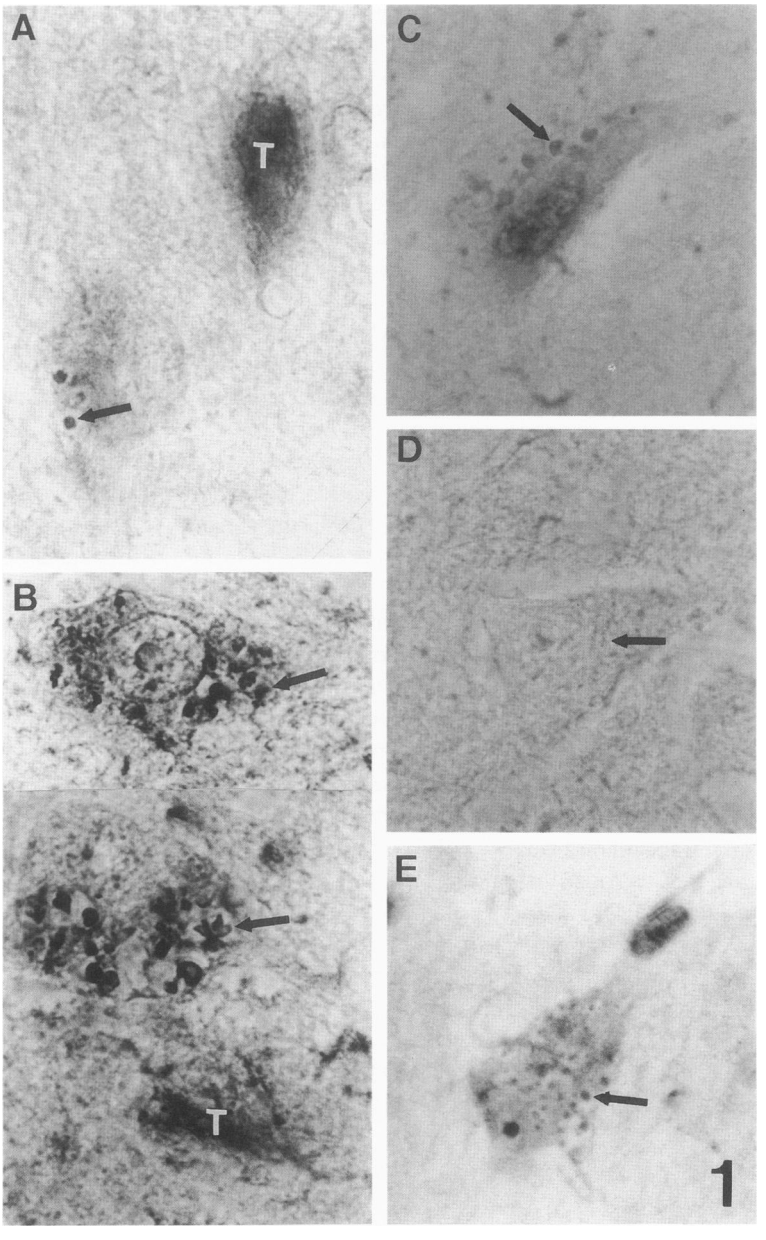

Figure 1. *GVD complex in pyramidal neurons in hippocampus in AD.* **A**: *GVD granules in pyramidal neuron in region CA1 immunolabeled by MAb6.423, visualized as dense DAB reaction product (arrow). MAb6.423, labeling of extracellular tangle* (T) *is of similar density. No counterstain;* ×*400.* **B**: *GVD granules (arrow) and neurofibrillary tangle* (T) *in region CA1, immunolabeled by MAb11.57, visualized as dense DAB reaction product. No counterstain;* ×*400.* **C**: *Unlabeled GVD granules (arrow) are distinctly stained in pyramidal neuron in region CA1, stained with hematoxylin (Ehrlich) and eosin, indicated by arrows.* ×*400.* **D**: *GVD granules (arrow) are barely visible in pyramidal neuron in region CA1 after immunolabeling procedure with omission of primary antibody. No counterstain;* ×*400.* **E**: *GVD granules (arrow) in pyramidal neuron in region CA2 immunolabeled by BR133, visualized as dense DAB reaction product. Counterstained with Ehrlich's hematoxylin,* ×*400.*

particularly prevalent in CA2, where we have previously described a selective abundance of intracellular NFTs.[43]

In unlabeled sections stained with H & E, normal-appearing pyramidal cells were found in fields H1 and H2, in which there was no indication of NFTs or the GVD complex. In other pyramidal cells, the GVD complex, with an intensity of staining similar to that characteristic of nuclei, was readily identified (Figure 1C). After immunostaining, selective labeling (of similar color and density) of NFTs and GVD granules become apparent in some pyramidal neurons. All antibodies that labeled GVD granules also labeled NFTs, and with the conditions used it appeared that such antibodies labeled all GVDs present in these cells, although it was not possible to establish, with certainty, the existence of unlabeled GVD granules. Most commonly, neurons contained either labeled NFTs or numerous, clearly labeled GVD complexes, although occasionally labeled GVD complexes were observed in neurons containing labeled NFTs, especially in area CA1. In such neurons, described by Tomlinson and Kitchener,[3] both the GVD complex and the NFTs were typically immunolabeled.

The fixation sensitivity of anti-tau antibodies that label NFTs has been discussed elsewhere.[43] BR133 and BR134 labeled both NFTs and GVD granules (Fig 1E) in sections fixed either with acetic acid-methanol or with formaldehyde. Contrarily, MAb6.423 optimally labeled GVD granules and intracellular NFTs (Figure 1A) only after formaldehyde fixation, and, after acetic acid-methanol fixation, only extracellular tangles. As discussed previ-

ously, this appears to reflect the 'deeper' location of the MAb6.423 epitope within the PHF core.[43,49] Labeling of GVD granules and NFTs by MAb11.57 (Figure 1B) and MAb5.25E was seen only after acetic acid-methanol fixation. Thus, in all cases, the conditions optimal for staining intracellular NFTs and GVD granules were identical.

Whereas immunolabeling of the GVD complex predicts the labeling of intracellular NFTs, the converse is not true. Monoclonal antibody 7.51, a universal tau marker directed against an epitope located in the last two repeats of all nine tau isoforms,[48] labeled both intracellular and extracellular NFTs, but not GVD granules. This unanticipated failure to label GVD granules was independent of the fixation conditions used.

We also have tested other antibodies implicated in the immunolabeling of GVDs. The anti-ubiquitin antibody, MAb5.25E, shown in a former study to label intracellular and extracellular tangles after acetic acid-methanol fixation,[43] gave ambiguous labeling of GVD granules with the same conditions. Thus we are unable to confirm the report of Love et al,[36] because in conditions in which tangles were strongly labeled, GVD granules were not.

We also have attempted to reproduce the findings of Price et al,[35] who reported tubulin immunoreactivity of tangles and GVD granules. The anti–α-tubulin and anti–β-tubulin MAbs (respectively, Amersham N.356 and N.357) labeled neither NFTs nor GVD granules. By contrast, the 'antitubulin' serum (ICN 6.095) labeled both NFTs and GVD granules. This antiserum, however, has equal potency against isolated tau and tubulin proteins in enzyme-linked immunosorbent assay.[50] We are therefore unable to confirm the presence of tubulin reactivity in the GVD complex with the antibodies used.

No pyramidal cells were found with the GVD complex in any of the nondemented subjects.

Discussion

It has been shown that the number of hippocampal neurons containing the GVD complex is correlated directly with those containing NFTs,[5,51] and inversely correlated with the number of pyramidal cells.[51] It has been convincingly demonstrated, therefore, that GVD is associated with the degeneration of neurons in AD, presumably by some process related to neurofibrillary degeneration.

The GVD complex and NFTs are clearly dissimilar ultrastructurally. Whereas the PHF has a highly regular macromolecular geometry,[52,53] the granule of the GVD complex appears 'amorphous' in electron micrographs.[54] Yet immunohistochemical studies demonstrate similarities between NFTs and the GVD complex. Although both structures are reported to be immunolabeled by antibodies directed against tau, however,[34,55] ubiquitin,[36] high-molecular-weight neurofilament protein,[33,34] and tropomyosin,[25] only tau has been shown unequivocally to be part of the core structure of the PHF.[16] In addition, immunohistochemical studies suggesting a relationship between PHFs and the GVD complex have been difficult to interpret. Firstly there have been problems of morphologic specificity. Grundke-Iqbal et al[11] showed tau-1 immunoreactivity of the GVD complex in some hippocampal pyramidal cells confined to 'darkly stained outlines of cytoplasmic vacuoles,' not granules; and Joachim et al[37] reported GVD granules 'variably immunostained' by an anti-tau MAb. Conversely, Love et al[36] have reported failure to find immunolabeling of GVD granules with Alz-50 and tau-1. Secondly there have been uncertainties regarding specificity of the antibodies used. For example, Dickson et al,[34] using RT97 and NP14, MAbs thought to be directed against high-molecular-weight neurofilament protein and tau, respectively, found labeling of GVD granules with both, but considered it unlikely that tau is a constituent of the GVD complex. In this study, we have focused our attention on structures that could be identified unambiguously as GVD complexes and have sought to define clearly the contribution of tau protein to their composition.

Tau has been shown to be incorporated into PHFs in such a way that its repeat region is embedded within the protease-resistant core of the PHF,[16] whereas its N-terminal 200 amino acids contribute mainly to the protease-sensitive, fuzzy outer coat.[38] The protease-resistant core, which accounts for the bulk of the molecular mass of the PHF (100 kd/subunit), contains no epitope associated with the N-terminal half of the tau molecule.[50] Likewise tangles that have undergone partial proteolytic degradation in the extracellular space lack N- or C-terminal tau

Table 1. *Epitopes and GVD-immunolabeling Properties of Antibodies*

Antibody	Epitope	Immunolabeling	Source (reference)
6.423	tau, 3-repeat region	+ + +	(40, 48)
11.57	tau, 3-repeat region	+ + +	Novak, et al
7.51	tau, 3-repeat region	–	(48)
BR133	tau, amino acids 1–16	+	(41)
BR134	tau, amino acids 339–352	+	(41)
N.356	α-tubulin	–	Amersham, Arlington Heights, IL
N.357	β-tubulin	–	Amersham, Arlington Heights, IL
65–095	tau? tublin?	+	ICN, Costa Mesa, CA

immunoreactivity, but retain epitopes associated with the tandem repeat region.[43]

Antibodies directed against these three portions of the tau molecule have been used in the present study. Antibodies directed against the amino terminus (BR133), the carboxy terminus (BR134), the repeat region (mAb6.423), and a segment adjacent to the repeat region (MAb11.57), all recognize the granule of the GVD complex. This shows unambiguously that the complete tau molecule is contained in the GVD granule as well as in PHFs.[16,40,56]

Not all tau-directed antibodies that immunolabel NFTs also label the GVD granule. In particular, MAb7.51, which is directed against an epitope contained in the last two repeats of all known tau isoforms,[46] labels NFTs[43] but does not label the GVD granule. In preparations of isolated PHFs, the MAb7.51 epitope appears to be hidden, whereas tau remains bound to the PHF. It is exposed when tau is released from the PHF core by acid treatment,[48,57] and by certain fixation conditions employed in immunohistochemical procedures.[43] The MAb7.51 epitope is contained within the minimum recognition unit required for MAb6.423 reactivity,[48] and no tau species have been released from the PHF core that are recognized by MAb6.423, but not by MAb7.51.[48] The unavailability of the MAb7.51 epitope in the GVD complex implies that the repeat region of tau is incorporated differently in the GVD granule and the PHF core.

The presence of the complete tau molecule in the GVD complex and NFTs suggests that the GVD granule may represent a site of tau sequestration, perhaps related to PHF formation in hippocampus. Although no temporal relationship between granulovacuolar degeneration and neurofibrillary degeneration has been demonstrated, the GVD complex might relate directly either to PHF formation or to degradation. The latter, suggested by Dickson et al,[34] is not supported by our findings.

We consider it unlikely that GVD formation follows the partial proteolytic degradation of PHFs, because this has been shown to be associated with the loss of BR133 and BR134 epitopes,[43] and these epitopes are present in the GVD granule. No data convincingly support a role for the GVD complex in PHF formation. The predominant segregation of NFT-containing neurons and neurons containing the GVD complex within regions vulnerable to neurofibrillary degeneration may signify that formation of the GVD complex is part of an alternative pathway for the metabolism of an aberrant molecular complex containing MAb6.423-reactive tau in AD.

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