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Hypothesis: Microtubules, a key to Alzheimer disease

(brain/tubulin/microtubule-associated protein)

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ABSTRACT **Alzheimer disease (AD) is a clinicopathologic syndrome of unknown etiology with numerous abnormalities in neuronal and nonneuronal cells. A review of the literature suggests that a common basic intracellular defect may underlie many of the reported abnormalities. We hypothesize impairment of the microtubule (MT) system as one explanation for the pathogenesis of AD. Evidence in support of the hypothesis includes the following: MTs are ubiquitous and vital cell components, unequally distributed, with the highest concentration in the brain; various abnormalities, including the key neuropathologic lesions, can be explained by impairments of the MT system; and experiments utilizing pharmacologic agents known to disrupt MTs have reproduced certain abnormalities observed in AD. The hypothesis provides a framework for systematic investigations of MTs at the cellular and molecular levels as well as the basis for *in vivo* diagnostic tests for AD.**

Alzheimer disease (AD) remains a disorder of unknown etiology without effective treatment. None of the hypotheses proposed (1–4) accounts for the gradually progressing deterioration and variable clinical presentations (5, 6), encompasses the diversity of neuronal and nonneuronal abnormalities (7–14), and elucidates how a disorder characterized by so much variability unequivocally has the brain as its most vulnerable target organ.

We postulate that impairment of the microtubule (MT) system provides a unifying hypothesis. MTs are ubiquitous cellular components with multiple intracellular roles, and the brain has a far higher tubulin content than any other tissue examined (15, 16). Our reasons for hypothesizing the involvement of MTs in AD may be understood following a brief overview of the MT system.

MTs are labile dynamic polymers that rapidly exchange subunits with the soluble tubulin pool (17, 18). Various tubulin isoforms have been reported and are equivalent in their ability to form MTs (19). The biosynthesis of tubulin follows the central dogma for the flow of genetic information, and impairment may occur at numerous levels. Beyond that, impairment may occur in posttranslational modification(s) of tubulin and the dynamics of polymerization and depolymerization of MTs.

Multigene families encode tubulins (20). Thus, lesions in any one of the structural genes for tubulin may result in new isoforms or the absence of specific isoforms. Further, new isoforms could be the result of lesions in normally quiescent genes [during cell differentiation, there is a selective transcriptional activation of tubulin genes (21, 22)] or in one or more of the tubulin pseudogenes. Lesions in the regulatory sequence for any one of the tubulin genes could increase, decrease, or eliminate a specific isoform. Or, changes at the DNA level could lead to differences in the level of translationally active mRNAs as a result of transcriptional control and/or mRNA instability. There may also be rapid and selective degradation of mRNA species in the nucleus, a defect in the processing of mRNA, or an impairment in its transport into the cytoplasm.

In the cytoplasm, mRNAs are translated into tubulin polypeptides, which are assembled into MT polymers. Numerous factors are known to affect MT assembly *in vitro* (23–25). The tubulin subunit pool autoregulates its synthesis (26) and each isoform may regulate its own intracellular level (27). Further, tubulin also undergoes posttranslational modifications, such as phosphorylation, which inhibits its ability to self-assemble into MTs (28, 29).

Adding to the heterogeneity of MTs are microtubule-associated proteins (MAPs), which copurify with MTs and are known to favor polymerization and to enhance the stability of MTs (30). One MAP of particular interest in AD is tau, which exhibits microheterogeneity due in part to different degrees of phosphorylation (31). Tau phosphorylation also modulates MT assembly and dephosphorylation increases the ability of tau to stimulate MT assembly (32).

The neuron is an extensively cross-linked compartment and the various forms and functions of each neuronal compartment may be related to the distinctive localization of its components, including different proportions of the isoforms of tubulin. MAPs, too, are compartmentalized within cells, tau being axon specific, and there is much less MAP-2 in axons than in dendrites and soma. In retinal ganglion cells only two of the many tau proteins seem to be transported with MTs in axons (33). It is unknown if these cells synthesize only the two tau proteins or synthesize all types but selectively transport only two. Similarly, it remains to be established if each type of cell synthesizes MTs with specific combinations of MAPs. Whether different combinations specify differences in MT stability or some as yet undefined functional aspect remains to be determined.

The proportion of tubulin isoforms varies in different brain regions (34), and this may be attributed to heterogeneity of the cell populations. Indeed, brain α- and β-tubulins appear more heterogeneous than tubulins in other cells/organs, and a single neuron may express multiple tubulin isoforms (35). The biological significance of these multiple isoforms has not as yet been established by either structural or functional differences at the MT polymer level, although it has been suggested that structurally different forms of MTs have functionally different roles (35).

In a quantitative and qualitative analysis of the composition of slow axonal transport compartments, striking differences emerged in the amounts of neurofilament, tubulin, and

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Abbreviations: AD, Alzheimer disease; MT, microtubule; MAP, microtubule-associated protein; PHF, paired helical filament; NGF, nerve growth factor; DS, Down syndrome; PMN, polymorphonuclear leukocyte; NFT, neurofibrillary tangle; EM, electron microscopic.

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MAPs transported between retinal ganglion cells, dorsal root ganglion cells, and ventral motor neurons (36). If different cellular systems in the brain have different MT polymers, this heterogeneity may provide a biological basis for the selective vulnerability of certain neuronal populations in AD.

Specifically, we propose the MT hypothesis as a unitary explanation for the pathogenesis of AD and for the brain's primary vulnerability to this disorder.

Hypothesis

Impairment of the MT system, regardless of cause, leads to impaired cellular functioning; the effects accumulate with time and eventually reach threshold levels for the symptomatic behavioral manifestations and neuropathologic lesions of AD. The underlying basis for MT impairment may be the result of genetic and/or nongenetic influences; in some individuals a genetic defect may predominate to the exclusion of nongenetic agents, whereas in others combinations of factors may interact.

Evidence Supporting the Hypothesis

In developing the MT hypothesis, we attempted to integrate the limited literature on impairment of the MT system. Clearly, systematic investigation of the MT system in AD is required to confirm or reject our hypothesis. Meanwhile, however, a number of abnormalities reported in AD can be explained by MT dysfunction, including the following:

Aneuploidy. Abnormality in spindle MTs can explain the chromosomal imbalance leading to the increased aneuploidy reported in lymphocytes of AD patients (37, 38). The increased intercellular DNA variability in peripheral blood mononuclear cells from AD patients compared to controls (11) is consistent with increased aneuploidy.

Association with Down syndrome (DS). (See refs. 39 and 40 for reviews.) DS is primarily the result of trisomy 21, and most individuals with DS develop AD neuropathologic changes by the age of 35 years (41, 42). An 80% reduction in a 55-kDa protein (immunologically identified as primarily tubulin) was reported in temporal cortex of older DS brains (43) as compared to only a 35% reduction in AD brains (44). The basis for the difference is unknown since histologically the temporal cortices appeared equivalent, and the tubulin decrease appeared to be associated with neurofibrillary tangles and neuronal loss. Spindle MT dysfunction has been suggested as a mechanism underlying DS (45).

Altered goal-directed cell migration. Reduced philothermal (46) and chemotactic responses of polymorphonuclear leukocytes (PMNs) (S.S.M., T.-K. Fu, and L.F.J., unpublished data) have been noted in AD patients. Since MTs play an important role in directed cell migration (47, 48), these responses are compatible with MT system impairment. A common underlying abnormality at the level of MTs is congruent also with the defective PMN chemotaxis described in DS (49, 50).

Neuropathologic lesions. The hallmark lesions of AD are neurofibrillary tangles, neuritic or senile plaques, and neuronal loss. Other neuropathologic lesions, such as granulovacuolar degeneration, occur as well. However, none of the lesions is unique to AD (51–53). Tangles and plaques, for example, are also found in the brains of normal older individuals (e.g., ref. 54) and appear morphologically identical to those observed in AD brains. It has not as yet been established whether they are also biochemically identical.

(*i*) Paired helical filaments (PHFs) are the primary components of neurofibrillary tangles (NFTs), and reports abound that the MAP tau is a major constituent of PHFs (55–63). Tau protein, localized to the axonal compartment of neurons, promotes MT assembly and stabilizes MTs (64–67). In AD, the distribution of tau immunoreactivity is dramatically altered; it is found in dendrites, neuronal cell bodies, and presynaptic regions (none of which shows detectable levels in normals) but appears to be absent from its usual axonal sites (68). This shift in tau immunoreactivity, presumably the result of the disruption of the MTs of cortical axons and tau accumulation, may lead to tau incorporation into PHFs. However, it is also possible that the shift may be the result of the unmasking of epitopes.

The PHF core (i.e., denuded of the fuzzy outer coat by Pronase treatment) contains a repeating three-domain structural subunit, tau protein fragments accounting for ≈10% of the subunit (69). The presence of tau in PHFs could result from sequestration of the tau pool within the cell, followed by failure of the cytoskeletal transport system as suggested in the original article, or from the reverse order of events—i.e., an abnormality in MT assembly preceding and initiating the failure of the transport system, which in turn leads to accumulation of excess tau protein in the neuronal perikaryon and its subsequent localization in PHFs. We suggest the latter sequence of events, the defective MT assembly being primary. One mechanism may be abnormal phosphorylation of tau (70), and the accumulation of phosphorylated tau may precede NFT formation (71). We await further data on the temporal sequence of tau phosphorylation and MT assembly in AD. Alternatively, an early immature form, with reduced efficiency for MT assembly (72), may be reexpressed.

(*ii*) Senile plaques consist of a plaque core (A4 amyloid peptide with a focal deposit of aluminosilicates) surrounded by dystrophic nerve processes and terminals that contain mitochondria and lysosomes (73). An impaired MT system can explain the accumulation of the organelles, since movement of lysosomes and mitochondria has been correlated with the integrity of MTs (74). The amyloid precursor protein in senile plaques (75) contains a sequence resembling a segment of a serine protease inhibitor (76–78) and may inhibit the proteolytic degradation of cellular debris, thereby promoting plaque formation. It may also inhibit dephosphorylation so that abnormally phosphorylated proteins result—e.g., the abnormally phosphorylated MAP tau found in AD.

(*iii*) Neuronal loss is also encompassed by the MT hypothesis since decreased MT polymerization or increased depolymerization results in disruption of axoplasmic and dendritic transport and neuronal death (79–82). The significant reduction in tubulin (44) mentioned earlier may be related to, and perhaps precede, neuronal loss. In a Northern analysis of levels of tubulin mRNA in AD cortex, the signal intensity for tubulin was 47% of control values (83). This decrease may be due to loss of neurons.

(*iv*) In an immunocytochemical investigation of granulovacuolar degeneration, the granules exhibited "tubulin-like immunoreactivity," but not actin, MAP-2, or neurofilament immunoreactivity (84). Use of a β-tubulin cDNA probe yielded no evidence of excess accumulation of the tubulin mRNA in brain tissue of AD patients (85), leading the authors to conclude that the sequestration of tubulin was not due to overproduction of tubulin but might be caused by its abnormal degradation. Disruption of the MT network and subsequent abnormal tubulin degradation could lead to sequestration of tubulin in granules.

(*v*) Neuritic masses in the olfactory epithelium noted in AD (86), which may be analogous to neurites surrounding plaques, stained positively with the monoclonal antibody to tau and the monoclonal antibody ALZ-50, which cross-reacts with tau. These abnormal masses of neurites together with the increase in tau immunoreactivity may be the result of disruption of MTs containing tau.

(*vi*) Electron microscopic (EM) studies of biopsy samples from patients with AD are also compatible with the MT hypothesis. Thus, it was noted that there was a precise

inverse relationship between the presence of PHFs and MTs so that when PHFs were present in the dendrites and perikarya, the array of MTs observed in dendrites of normal brain tissue, or of other neurons in AD brains, was absent (87). Although this finding does not necessarily imply that MTs are converted into PHFs, it raises the question of the fate of tubulin and MAPs. Further, the dendritic MTs in AD brain, when present, frequently exhibit an abnormal spatial distribution and a reduced number compared to dendrites from control brains. Dendritic MTs from biopsied frontal cortex (88) were significantly reduced in AD as well as abnormally distributed (i.e., packing in one sector with depletion in other areas vs. orderly distribution in controls).

Another EM study found hypertrophied neurofilamentous networks without MTs (89). Further, as the amount of the neurofilamentous networks decreased, PHFs and PHF-like strands increased in neuronal profiles. These investigators suggest phase transition, in which neuronal cytoskeletal proteins are posttranslationally modified (there is no evidence for a new gene product) into PHF-like strands.

Cholinergic system and nerve growth factor (NGF). The importance of the MT system for transport of neurotransmitters suggests that MT impairment would result in a wide range of neurotransmitter abnormalities. Indeed, cholinergic, noradrenergic, serotonergic, glutamatergic, GABAminergic (where GABA = γ-aminobutyric acid), and somatostatin systems have all been implicated in AD (90–92). The cholinergic system has been the most extensively investigated anatomically (93) as well as biochemically. The basis for the loss of this neuronal population is unknown. It has been postulated that lack of NGF is the reason since NGF is necessary for survival and maintenance of basal forebrain cholinergic neurons (94–97). The mechanism of action of NGF requires elucidation.

Neuronal terminals in the hippocampus and neocortex, areas with high concentrations of NGF and its precursor mRNA, take up and retrogradely transport NGF to the cell body. In the absence of a cytoskeletal substrate, no transport can occur, and neurons die. In experiments in which NGF was added to pheochromocytoma (PC12) cells, the most impressive effect was the induction of neurite outgrowth with promotion of MT assembly and the appearance of MT bundles extending from the cell body to the neurite tips (98–100). There was an associated parallel increase in total tubulin levels as well as tau and MAP-1. NGF was also necessary for maintenance of the differentiated state of PC12 cells. Consistent with the MT hypothesis, the action of NGF may be mediated through its promotion and maintenance of MT bundles.

Aluminum. Even though aluminum has been localized in senile plaques (101) and NFTs (102, 103), one argument against its having a key role in the pathogenesis of AD is that in dialysis dementia neither plaques nor tangles are seen despite the toxic concentrations of aluminum in the brain (104). However, aluminum may alter tubulin gene expression by increasing the binding of histones to DNA, resulting in increased chromatin compaction and reduced transcription (105). Aluminum also affects MT assembly; aluminum ions promote tubulin polymerization into MTs, effectively competing with magnesium, the physiologic mediator (association constant for Al^{3+} is 10^7 times Mg^{2+}). MTs formed with Al^{3+} were indistinguishable by EM study from those produced by Mg^{2+}. Nevertheless, sensitivity to calcium ion-induced depolymerization was markedly lower for the Al^{3+} MTs (106). Thus, Al^{3+} MTs may lack the regulatory controls that maintain the sensitive dynamics between polymerization and depolymerization.

Colchicine. Finally, results of experiments using colchicine, a well-known MT-specific disrupting agent, also support the hypothesized MT dysfunction.

(*i*) When infused into the rat hippocampus, colchicine destroyed hippocampal cells, impaired spatial memory (T-maze learning), and reduced activity in the cholinergic neurotransmitter system (specifically, the enzyme choline acetyltransferase, ChAT), mimicking the abnormalities seen in AD (107). Further, bilateral intracerebral injection of colchicine into the area of the nucleus basalis impaired learning and, concomitantly, decreased ChAT activity and levels of biogenic amines in the neocortex (108). These effects resemble the results produced by ibotenic and kainic acid except that, unlike these two agents, colchicine did not produce cell loss. Thus, neither the decreased ChAT activity nor the reduced levels of biogenic amines can be attributed to loss of neuronal populations, but they may represent the result of MT disruption with disturbance of axonal transport. Some colchicine-induced learning deficits were attenuated by some cholinergic agents (physostigmine, nicotine, and the arecoline analog RS-86). Clinically, cholinergic agents have been reported to improve the condition of some AD patients, at least temporarily.

(*ii*) Treatment of experimental animals and cultured neurons with MT inhibitors (colchicine, vinblastine, maytanprine) resulted in depolymerization of cytoplasmic MTs and subsequent accumulation of 10-nm filaments as well as formation of neurofilament tangles (109–112).

(*iii*) Colchicine treatment of PMNs obtained from cognitively intact humans suppressed the philothermal response in a dose-dependent fashion and resulted in a spatial distribution of responding cells that mimicked the pattern observed in cells from patients with AD (113).

(*iv*) Following colchicine treatment, skin fibroblasts from AD patients showed delayed reappearance of the cellular MT network compared to controls (12, 13). When examined under the light microscope, the cytoplasmic MT network of AD fibroblasts did not differ from that seen in control fibroblasts either prior to or following recovery from colchicine treatment. In view of the specificity of colchicine binding to tubulin and the slow and almost irreversible dissociation of this complex (114), an assembly-competent tubulin pool would not be available. Thus, the delayed reappearance suggests an impairment in tubulin gene expression.

(*v*) AD lymphoblasts also exhibit a slower rate of MT repolymerization following exposure to colcemid (M. S. Krawczun, personal communication).

Discussion

The data, though sparse, are compatible with the hypothesis that impairment of the MT system constitutes an important element in the pathophysiology of AD. The highest concentration of MTs is found in the brain, the organ most prominently affected in AD. Moreover, MTs are ubiquitous cellular components so that a basic defect in the MT system could also account for the numerous abnormalities reported in nonneuronal systems. Unequal distribution and great hetergeneity of MTs make them attractive for explaining the differential vulnerability of cellular systems.

Whether the MT impairment is a primary defect in AD or an epiphenomenon remains to be established. In either case, the hypothesis gives rise to a number of questions, including the following:

(*i*) Since MTs are ubiquitous throughout the body's cells, why is not every cellular system affected in AD? Several possible explanations emerge. It may be that only certain types of MTs are affected (e.g., MT specificity based on tubulin isotype and/or MAP composition). Outcome of MT impairment may depend on the role of MTs in particular cells. Thus, impaired ability to anterogradely transport nutrients can lead to accumulation of cellular metabolites eventually resulting in neuronal death, a readily detectable conse-

quence. By contrast, other roles may preclude detection of an impairment. For example, myocyte contraction was readily inhibited by dibutyryl cyclic AMP (115). This effect was reversed by removing the agent or adding colchicine or colcemid (but not lumicolchicine). EM and immunofluorescent studies revealed that dibutyryl cyclic AMP realigned MTs in a parallel array, suggesting that this arrangement conferred a physical constraint on cells and thus inhibited contraction. Yet, contrary to expectation, colchicine-treated cultures continued to contract and were morphologically similar to untreated cells. As expected, following colchicine treatment, intact MTs were not observed; hence, an organized MT network may not be needed for contraction but MT organization can influence contraction.

In an investigation of axonal transport of dopamine β-hydroxylase in rabbit and bullfrog axons, low temperatures were used to depolymerize MTs. Axonal transport was markedly impaired only after losses of >30% of MTs in rabbit axons and 65% in frog axons (116). These findings suggest that axons contain more MTs than needed for normal transport. In AD, there may be loss of neuronal MTs until the critical minimal level is exceeded (that level may differ intercellularly). The ensuing neuronal transformations (89) may underlie the threshold model proposed for dementia in AD (117).

(*ii*) Does variability in the MT–MAP complex lead to increased/decreased vulnerability to intra- or extraneuronal influences?

(*iii*) Why is tau expressed only in neurons and not fibroblasts or other peripheral tissues?

(*iv*) Do other disorders result from MT system impairment? The MT disarray in cortical dendrites of children with neurobehavioral retardation of unknown etiology (118) could point to one such MT disorder. Among non-brain diseases worth examining are disorders in which impaired goal-directed cell migration (chemotactic as well as philothermal) may reduce the efficacy of host defense, such as the primary ciliary dyskinesias, disorders in which depressed motility of PMNs has been reported (119). Although there is little information on brain function in such patients, one patient with Kartagener syndrome was described as suffering from "mental debility," and another, from "endogenous depression."

(*v*) Finally, questions arise as to the expected frequency of MT disorders. Impairment of a cellular system so vital and functioning so diversely might be expected to lead to a host of diseases and disorders. Its very importance to the organism, however, may preclude viable births—e.g., spontaneous abortions may include MT abnormalities. Further, the assumption that a large number of isoforms imply a large number of disorders is not necessarily warranted. Mutations in any one of the multiple tubulin genes may remain silent. Indeed, the availability of numerous isoforms may serve to "buffer" the system, especially if isoforms efficiently substitute for one another. In AD, the late onset and progressive deterioration may reflect a system that is no longer able to serve as a buffer, thus triggering a cascade of events resulting in AD. Systematic investigations of the MT system at basic biochemical, genetic, and molecular levels are needed.

The MT hypothesis opens a new approach to the study of AD and other disorders. It offers the possibility of developing *in vivo* diagnostic tests. It raises numerous researchable questions and provides a framework for inquiry into the pathogenesis and etiology(ies) of AD.

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