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Ubiquitination and Abnormal Phosphorylation of Paired Helical Filaments in Alzheimer's Disease

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Contents

Abstract

The most characteristic cellular change in Alzheimer's disease is the accumulation of aberrant filaments, the paired helical filaments (PHF), in the affected neurons. There is growing evidence from a number of laboratories that dementia correlates better with the accumulation of PHF than of the extracellular amyloid, the second major lesion of Alzheimer's disease. PHF are both morphologically and biochemically unlike any of the normal neurofibrils. The major polypeptides in isolated PHF are microtubule-associated protein tau. Tau in PHF is phosphorylated differently from tau in microtubules. This abnormal phosphorylation of tau in PHF occurs at several sites. The accumulation of abnormally phosphorylated tau in the affected neurons in Alzheimer's disease brain precedes both the formation and the ubiquitination of the neurofibrillary tangles. In Alzheimer's disease brain, tubulin is assembly competent, but the in vitro assembly of microtubules is not observed. In vitro,

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the phosphate groups in PHF are less accessible than those of tau to alkaline phosphatase. The in vitro dephosphorylated PHF polypeptides stimulate microtubule assembly from bovine tubulin. It is hypothesized that a defect in the protein phosphorylation/dephosphorylation system is one of the earliest events in the cytoskeletal pathology in Alzheimer's disease. Production of nonfunctional tau by its phosphorylation and its polymerization into PHF most probably contributes to a microtubule assembly defect, and consequently, to a compromise in both axoplasmic flow and neuronal function.

Index Entries: Alzheimer's disease; mechanisms of neuronal degeneration; neurofibrillary changes; paired helical filaments: biochemistry; microtubule-associated protein tau; abnormal phosphorylation; ubiquitination; microtubule assembly; axoplasmic flow; protein phosphorylation/dephosphorylation.

Introduction

Alzheimer's disease is the single major cause of dementia in adults in industrialized societies. About 55% of cases of senile dementia are of the Alzheimer's type. Another approx 20% of senile dementia cases suffer from a combination of Alzheimer's disease and cerebrovascular dementias. Alzheimer's disease is the fourth leading cause of death in adults in the United States after heart disease, cancer, and stroke, claiming more than 100,000 lives a year. At present, neither the etiology nor the pathogenesis of this neurodegenerative disorder is understood. An understanding of the basic mechanism(s) of degeneration of neurons in Alzheimer's disease will help in development of rational therapeutic approaches to reverse or arrest the progression of the disease.

Neurofibrillary degeneration is one of the most characteristic brain lesions of Alzheimer's disease. Many of these affected neurons may be only partially functional, and in some areas of the brain, such as neocortex, many of them may eventually die, leaving behind tangled masses of abnormal fibrils, the "ghost tangles." Unlike the intraneuronal tangles, the ghost tangles in the extracellular space are made mostly of ~15 nm straight filaments and less-defined amorphous filmentous material admixed with paired helical filaments (PHF) and bundles of astroglial filaments (Okamoto et al., 1983; Yamaguchi et al., 1991; Tabaton et al., 1991). However, all neuronal degenerations that occur because of Alzheimer's disease might not necessarily involve neurofibrillary changes. Furthermore, in certain areas of the brain in Alzheimer's disease, such as some of the hypothalamic nuclei, neurons affected by cytoskeletal protein alterations may undergo minimal degeneration (Swaab et al., 1991). In this article (an update of Iqbal and Grundke-Iqbal, 1991), we attempt to identify a sequence of some key molecular changes associated with neurofibrillary degeneration that might constitute a major mechanism of neuronal degeneration in Alzheimer's disease.

Topography and Intracellular Location of Neurofibrillary Changes

Microtubules and neurofilaments are the two principal fibril components of the cytoskeleton of a normal mature neuron. In the brain of individuals with Alzheimer's disease, the cytoskeleton of many neurons is progressively disrupted and displaced by bundles of PHF (Kidd, 1964), which are morphologically unlike microtubules and neurofilaments (Wisniewski et al., 1984). These Alzheimer's neurofibrillary tangles (ANT) of PHF are found mostly in cerebral cortex, especially in the hippocampal pyramidal neurons of Sommers sector and in small pyramidal neurons in the outer laminae of fronto-temporal cortex. Subcortical nuclei, such as the nucleus basalis of Meynert, which provides a major cholinergic output to the cerebral cortex, are also affected by

these neurofibrillary changes. The tangles have not been observed in cerebellar cortex, spinal cord, peripheral nervous system, or extraneuronal tissues. Accumulations of PHF are most prominent in the neuronal perikarya, filling almost all the cytoplasm of the affected cells. Bundles of PHF are also seen in the dystrophic neurites surrounding a core or wisps of extracellular amyloid in the neuritic (senile) plaques. Thus, both the tangles and the plaques, which are the two histopathological hallmarks of Alzheimer's disease, contain PHF. PHF are also found as bundles in neurites in the neuropil of the telencephalic cortex. At this third location, PHF occur in small, inconspicuous profiles, the neuropil threads, scattered throughout both allo-cortical and isocortical areas (Braak et al., 1986).

Specificity of Tangles and Plaques to Alzheimer's Disease

Neither the ANT nor the plaques are unique to Alzheimer's disease. ANT are also found in great abundance in Guam-Parkinsonism dementia complex, dementia pugilistica, postencephalitic parkinsonism, adults with Down syndrome, and in small numbers in a few cases of subacute sclerosing panencephalitis, Hallervorden-Spatz disease, and neurovisceral lipid storage disease (for review, *see* Wisniewski et al., 1979; Iqbal and Wisniewski, 1983). The neuritic (senile) plaques are also seen in Down syndrome, aged humans, and in some species of animals. Unlike the tangles, which are present in only very small numbers in nondemented elderly and absent in animals, the plaques are seen frequently in both aged human and animal brains. The number of plaques in nondemented aged humans is sometimes similar to that seen in Alzheimer's disease cases (Katzman et al., 1988). Recent studies have shown that most of the plaques found in nondemented elderly, unlike in Alzheimer's disease, are free of PHF in the dystrophic neurites (Dickson et al., 1988; Barcikowska et al., 1989).

Relationship Between Tangles and Plaques

Both ANT and amyloid are congophilic, and because of this common staining property, a close relationship between the two lesions has been suggested. However, it has been shown that this property is most likely owing to β-pleated sheet structure (Glenner et al., 1974), which can be induced in many unrelated polypeptides. PHF and amyloid are different ultrastructurally and biochemically, and in several staining properties other than those observed with Congo red and thioflavin S (Table 1).

Heterogeneity of Alzheimer's Neurofibrillary Changes

ANT are heterogenous in both morphology and solubility. Most ANT are composed of PHF. In only a few Alzheimer's disease cases have tangles of 15-nm straight filaments or of these filaments admixed with PHF been observed (Shibayama and Kitoh, 1978; Yagishita et al., 1981). PHF are stable in both fresh and frozen autopsy tissue, and are resistant to solubilization in aqueous buffer in the absence of detergents or denaturants. Two general populations of ANT, ANT I and ANT II, have been identified on the basis of solubility and insolubility, respectively, in 2% SDS at room temperature for 3–5 min (Iqbal et al., 1984). However, ANT II are solubilized on repeated extractions in SDS and β-mercaptoethanol at 90–100°C, or more effectively, by ultrasonication followed by heating in 1% each of SDS and β-mercaptoethanol. Although native PHF are resistant to proteolysis, PHF isolated by SDS-treatment are digested by proteases (Iqbal et al., 1986a). In agreement with biochemical studies, ultrastructural studies of PHF have revealed that there are indeed two general populations of PHF, i.e., PHF with right-handed helices and PHF with left-handed helices (Wisniewski et al., 1986). The right-handed PHF are larger than the left-handed PHF, both in diameter and in periodicity of the helices.

Table 1
Relationship Between PHF and Plaque Amyloid

Characteristic	PHF/Tangles	β-Amyloid
Fibril diameter	10–22 nm	7nm
Congophilia	+	+
Argentophilia	+	– +
Periodic acid Schiff	–	+
Solubility in SDS	+	–
Mol wt (SDS-PAGE)	45–70 kDa	4 kDa
Antibodies to PHF	+	Rare
Antibodies to β-peptide	*	+

*Reacts with a few tangles, mostly ghost tangles.

Isolation and Polypeptide Composition of PHF

Different approaches to isolate PHF have been based on the sparing solubility of PHF in detergents, their resistance to proteolysis, or both (Iqbal et al., 1984, 1986a; Ihara et al., 1983; Masters et al., 1985a; Rubenstein et al., 1986; Wischik et al., 1988; Greenberg and Davies, 1990; Lee et al., 1991). Highly purified PHF are isolated from autopsied tissue by a combination of sucrose density gradient centrifugation and SDS treatment of neuronal cell body-enriched preparations at room temperature (Iqbal et al., 1984). Because native PHF are apparently resistant to proteolysis, PHF have also been isolated from crude tissue fractions without detergents by a combination of protease digestion and centrifugation on sucrose or $CsCl_2$ gradients, or both (Wischik et al., 1988; Grundke-Iqbal et al., 1988). Although PHF isolated by the protease treatment are highly purified, fragments of PHF polypeptides are lost as a result of proteolysis in the fibrils prepared by this technique (Wischik et al., 1988). PHF prepared by any of the above methods are contaminated to a certain degree with amyloid, lipofuscin, and some amorphous or granular tissue debris.

Unlike the brain amyloid in Alzheimer's disease, which has been shown to be composed of a 40–42 amino acid peptide, the β peptide (Glenner and Wong, 1984; Masters et al., 1985b), the biochemistry of the PHF has not been completely established. On SDS-polyacrylamide gels, the protein composition of the highly purified PHF bulk isolated from Alzheimer's disease brain is complex. The major polypeptides are in the 45–70 kDa region (Iqbal et al., 1984; 1986a; Grundke-Iqbal et al., 1984,1985a; Lee et al., 1991). In addition, a varying number of low-molecular-weight polypeptides are found in each PHF preparation. The third component of PHF is high-molecular-weight aggregates, which stay at the top of the gel during electrophoresis (Iqbal et al., 1984,1986a). Immunostaining of the 45–70 kDa polypeptides with antibodies to PHF and immunoabsorption of the tangle-staining antibodies with these polypeptides have made possible the determination of their PHF origin (Grundke-Iqbal et al., 1984,1985a,b). Immunochemical crossreactivity and coelectrophoresis on SDS-polyacrylamide gels of the 45–70 kDa PHF polypeptides with the family of microtubule-associated polypeptides known as tau and the labeling of isolated PHF and of PHF in tissue sections with antibodies to tau have suggested that tau is a major subunit protein of PHF J (Grundke-Iqbal et al., 1986a,b,1988; Iqbal et al., 1989, Lee et al., 1991). The immunocytochemical labeling of PHF in tissue sections with antibodies to tau has been confirmed by several other laboratories (Brion et al., 1985; Kosik et al., 1986; Ihara et al., 1986; Wood et al., 1986; Delacourte and Defossez, 1986; Yen et al., 1987). The presence of tau in PHF has also been confirmed by amino acid sequencing of tau fragments isolated from highly puri-fied PHF (Wischik et al., 1988; Kondo et al., 1988; Lee et al., 1991).

A group of investigators has also reported the presence of sequences of amyloid β-protein in preparations enriched in neurofibrillary tangles both from patients with Alzheimer's disease and with Guam-Parkinsonism dementia (Masters et al., 1985a; Guiroy et al., 1987). However, neither data on yields of the protein obtained from the PHF preparations nor data on the percentage of protein sequenced were reported. Furthermore, no definitive immunostaining of PHF with antibodies to amyloid protein or of amyloid with

antibodies to PHF has been shown to date. On the other hand, both the mRNA (Bahmanyar et al., 1987) for the amyloid β-peptide precursor and β-peptide immunoreactivity (Grundke-Iqbal et al., 1989) have been demonstrated to be present intraneuronally. Unlike the tangles, which are fibrillar, the amyloid reactivity in both Alzheimer's and normal cases is localized mainly to lipofuscin in different types of neurons, including the neurons with the neurofibrillary tangles (Grundke-Iqbal et al., 1989; Bancher et al., 1989b). A varying numer of ghost tangles ranging from a few (Hyman et al., 1989; Tabaton et al., 1991) to almost all (G. Perry, in preparation) are labeled with antibodies to β-amyloid, depending on the tissue fixation conditions. This immunostaining, however, is mostly restricted to β-amyloid and to the unidentified amorphous filamentous material adhering to tangle PHF in the extracellular space (Yamaguchi et al., 1991; Tabaton et al., 1991; Grundke-Iqbal and Iqbal, 1991). It thus remains to be determined whether the amyloid peptide is actually a component of the PHF or a contaminant of the PHF preparations employed for sequencing.

Abnormal Phosphorylation and Ubiquitination of PHF Polypeptides

In addition to tau, the presence of ubiquitin in isolated PHF and the immunostaining of PHF with antibodies to ubiquitin (Mori et al., 1987; Perry et al., 1987; Grundke-Iqbal et al., 1988) have been demonstrated. Ubiquitin, which might be a protease itself (Fried et al., 1987), is believed to be a part of the cellular defense system that tags abnormal proteins for the action of ATP-dependent nonlysosomal proteases (Hershko and Ciechanover, 1982). Monoclonal antibodies 3-39 and 5-25 raised against PHF (Wang et al., 1984) have been shown to recognize ubiquitin. On Western blots, these antibodies, the epitopes of which reside in the amino acid residues 50–65 and 64–76 of the ubiquitin sequence, respectively (Perry et al., 1989), label PHF polypeptides with the same molecular weights as tau. However, they do not react with tau from normal and Alzheimer's brain cytosol (Grundke-Iqbal et al., 1988; Koepke-Secundo et al., 1990). Furthermore, the monoclonal antibodies to PHF react much more strongly with PHF polypeptides than with free ubiquitin (Grundke-Iqbal et al., 1988). It thus appears that some of the tau in PHF might be ubiquitinated. Alternatively, ubiquitin-ubiquitin conjugates, comigrating with tau polypeptides on SDS-polyacrylamide gels, might be associated with PHF.

One of the modifications of tau in PHF is its abnormal phosphorylation (Grundke-Iqbal et al., 1986b). Unlike normal tau, in PHF, this protein is inaccessible to the monoclonal antibody Tau-1 (Grundke-Iqbal et al., 1986b), the epitope of which resides in amino acid residues 196–214 (Kosik et al., 1988) of the cDNA-derived sequence (Himmler et al., 1989) of bovine tau. Both on tissue sections and on immunoblots of PHF, the labeling of PHF polypeptides with this antibody is markedly increased when the sections or blots have been treated with alkaline phosphatase before immunolabeling. The exact nature of this abnormal phosphorylation of tau in Alzheimer's disease is not presently understood. The abnormal tau isolated from Alzheimer's disease brain contains up to 12 mol of phosphate/mol of protein, which is about four times the level in normal brain tau. Tau in PHF is abnormally phosphorylated at multiple sites. Two amino terminal sites (Iqbal et al., 1989; Brion et al., 1991) and a carboxy terminal site (Lee et al., 1991) of tau in PHF have been shown to be phosphorylated differently from tau in normal brain. Although in vitro several of these phosphorylation sites are accessible to alkaline phosphatase, the overall accessibility to the phosphatase in PHF is less than in normal microtubule tau (Iqbal and Grundke-Iqbal, 1990a).

Like PHF, in cytosol treated for in vitro assembly of microtubules from Alzheimer's disease brains, tau is abnormally phosphorylated. This abnormal phosphorylation is most prominent in the molecular species of tau with the slowest elec-

trophoretic mobility (Grundke-Iqbal et al., 1986b; Iqbal et al., 1986b). The presence of the abnormally phosphorylated tau in Alzheimer's disease brain has also been confirmed in several laboratories (Ihara et al., 1986; Nukina and Ihara, 1986; Wood et al., 1986; Flament et al., 1989; Flament and Delacourte, 1989; Zhang et al., 1989; Ksiezak-Reding et al., 1990).

The aberrant phosphorylation in Alzheimer's disease brains might be selective to a few neuronal proteins and not be a part of a generalized hyperphosphorylation. Levels of both total free phosphate and phosphoprotein phosphate are normal in Alzheimer's disease brain (Iqbal and Grundke-Iqbal, 1990a,b). At present, neither the nature of all the phosphorylation sites nor the protein phosphorylation/dephosphorylation system responsible for the abnormal phosphorylation of tau in Alzheimer's disease is known. Preliminary studies have revealed the presence of phosphoserine, phosphothreonine, and phosphotyrosine in isolated PHF, suggesting that more than one protein kinase might be involved in the phosphorylation of PHF (Murthy and Iqbal, 1990).

The abnormal phosphorylation of tau appears to represent one of the earliest changes leading to Alzheimer's neurofibrillary pathology. Tau-1 reactivity is seen after dephosphorylation in a number of apparently morphologically normal neurons of the neocortex in the nondemented aged and in cases with Alzheimer's disease, but not in normal young brains (Bancher et al., 1989a, 1991). At the electron microscopic level, the immunoreactivity is found in association with granular material, a few scattered PHF, and 15–20 nm straight filaments. This so-called embryonic stage of tangles is neither stained with silver impregnations nor labeled by antibodies to ubiquitin or to cytoskeletal proteins other than tau. Furthermore, abnormally phosphorylated tau isolated biochemically from cytosol of Alzheimer's disease brain does not show ubiquitin immunoreactivity, as tested by Western blots (Koepke-Secundo et al., 1990). Thus, both immunocytochemical and biochemical studies indicate that the accumulation of abnormally phosphorylated tau precedes the incorporation of ubiquitin into neurofibrillary tangles.

Effect of Cytoskeletal Changes on Biological Activity

One of the vital functions of the neuron is the transport of materials between the cell body and the nerve endings. Microtubule assembly, which is necessary for this intracellular transport, might be defective in Alzheimer's disease (Iqbal et al., 1986b,1987). Microtubules can be assembled in vitro from the cytosol of normal fresh autopsy brain obtained within 5 h postmortem. No in vitro assembly of microtubules is observed from cytosol of Alzheimer's disease brain (Iqbal et al., 1986b). Furthermore, heat-stable, microtubule-associated proteins, which include tau, prepared from Alzheimer's disease brain do not stimulate microtubule assembly from porcine tubulin in vitro (Nieto et al., 1990). The in vitro assembly of microtubules from the Alzheimer's disease tissue, however, is induced by the addition of DEAE dextran, a polycation that mimics the effect of tau for microtubule assembly. Tau stimulates microtubule assembly by polymerizing with tubulin (Weingarten et al., 1975) and maintains the microtubule structure (Drubin and Kirschner, 1986). Because tau in Alzheimer's brain cytosol is abnormally phosphorylated (Grundke-Iqbal et al., 1986b; Iqbal et al., 1986b) and phosphorylation of tau depresses tau's ability to promote microtubule assembly (Lindwall and Cole, 1984), it appears that this alteration of tau in the Alzheimer's brain might contribute to the microtubule assembly defect. PHF-tau dephosphorylated with alkaline phosphatase stimulates in vitro microtubule assembly. These findings confirm the role of the abnormal phosphorylation in microtubule assembly defect in Alzheimer's disease (Iqbal et al., 1991). Binding of guanosine triphosphate (GTP) to the β-subunit of tubulin, which initiates microtubule assembly, is stimulated by tau. Lack of functional tau in Alzheimer's disease brain might lead to decreased GTP bind-

ing and, consequently, decreased assembly of microtubules (Khatoon et al., 1990).

The concentrations of tubulin may decrease with age (Yan et al., 1985). As is the case in vitro, a critical concentration of brain tubulin is probably required for in vivo microtubule assembly. Any change in tubulin or in microtubule-associated proteins in the affected neurons that would decrease the efficiency of microtubule assembly would therefore be critical in the aged brain. The presence of abnormally phosphorylated tau might thus mean that this threshold is reached in the affected neurons in Alzheimer's disease, the result being reduced microtubule assembly and, consequently, impaired axoplasmic flow and the onset of neuronal degeneration. Because tau in PHF is abnormally phosphorylated, it seems that the altered tau might be catabolized inefficiently, thereby accumulating as PHF in the affected neurons. A disturbance in axoplasmic flow, both anterograde and retrograde, should lead to accumulations of components of the axoplasmic flow in both the perikaryon and the nerve terminals. PHF accumulate at both of these locations, i.e., ANT and plaque neurites. The amount of accumulation of the affected proteins depends on the rates of their transport, synthesis, and degradation by the cell. Thus, several neuronal components that are normally transported between the cell body and the terminals and are not rapidly degraded can be expected to accumulate in the affected neurons. However, only one or a few of these polypeptides might be capable of polymerizing into PHF. Immunocytochemical staining of ANT has been shown with antibodies to several proteins (Ishii et al., 1979; Anderton et al., 1982; Dahl et al., 1982; Gambetti et al., 1983; Yen et al., 1983; Grundke-Iqbal et al., 1985c; Kosik et al., 1984; Perry et al., 1985; Roberts et al., 1985; Sternberger et al., 1985; Nukina and Ihara, 1986). However, with the exception of tau and ubiquitin (*see above*), these proteins have not been observed in PHF treated with detergents/denaturants to remove nonspecific proteins trapped between the fibrils. Discoveries of the abnormal phosphorylation of tau in Alzheimer's disease brain, the presence of abnormal tau and of ubiquitin in PHF, and the failure to induce in vitro microtubule assembly in Alzheimer's disease brain cytosol lead us to hypothesize (1) that the protein phosphorylation–dephosphorylation system is defective in Alzheimer's disease brain, leading to abnormally phosphorylated tau and some other neuronal proteins, and (2) that the abnormal phosphorylation of tau contributes to a microtubule assembly defect, and consequent impairment of axoplasmic flow and neuronal degeneration (Fig. 1).

Protein phosphorylation is one of the major mechanisms for regulation of cellular function (for review, *see* Nairn et al., 1985). The state of phosphorylation of substrate proteins depends on the relative activities of proteinkinases and phosphoprotein phosphatases. Our studies (Grundke-Iqbal et al., 1986b; Iqbal et al., 1989; Iqbal and Grundke-Iqbal, 1990a) showing the dephosphorylation of the abnormally phosphorylated sites of tau after treatment with alkaline phosphatase in vitro suggest that the protein phosphorylation/dephosphorylation defect might be the result, in part, of a deficiency in a protein phosphatase system or systems in the affected neurons in Alzheimer's disease.

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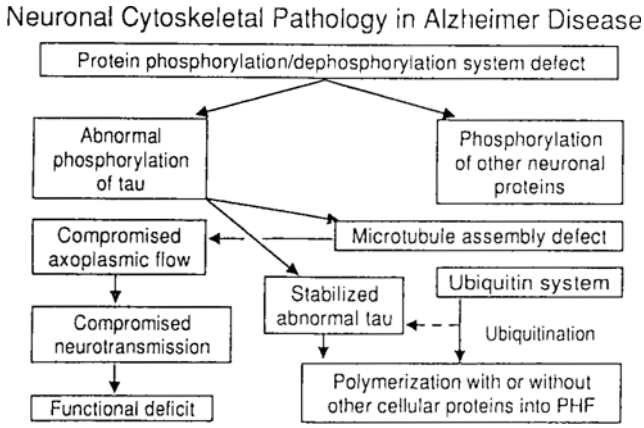

Fig. 1. A hypothetical scheme of sequence of major molecular events that might be involved in the pathogenesis of neurofibrillary degeneration in Alzheimer's disease.

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