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Molecular pathology of Alzheimer neurofibrillary degeneration

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Abstract. The most characteristic brain lesion of Alzheimer disease is the accumulation of paired helical filaments (PHF) in the affected neurons. Based on solubility in detergents there are two general populations of PHF, the readily soluble (PHF I) and the sparingly soluble (PHF II) types. The major polypeptides of PHF are the microtubule associated protein tau. Tau in PHF is present in abnormally phosphorylated forms. In addition to the PHF, the abnormal tau is also present in unpolymerized form in the AD brain. Small amounts of ubiquitin (%) are associated with PHF II but neither with PHF I nor with the unpolymerized abnormally phosphorylated tau in AD brain. Furthermore, the pretangle neurons can readily be immunolabeled for abnormally phosphorylated tau but not for ubiquitin. The level of tau in neocortex is several-fold higher than in AD aged control cases, but this increase is in the form of the abnormally phosphorylated protein. The microtubule associated proteins from AD brain do not promote the assembly of microtubules in vitro, whereas the in vitro dephosphorylated PHF polypeptides stimulate the binding of GTP to the exchangeable site of tubulin and the assembly of microtubules. *In vitro* the phosphate groups in PHF are less accessible than those of tau to alkaline phosphatase. It is suggested that a defect in the protein phosphorylation/dephosphorylation system leads to hyperphosphory-lation of tau. The altered tau contributes to a microtubule assembly defect and consequently compromises the axoplasmic flow and leads to neuronal degeneration.

Key words: paired helical filaments: biochemistry, microtubule-associated protein tau, ubiquitination, microtubule assembly, protein phosphorylation/ deposphorylation

INTRODUCTION

Alzheimer disease is the single major cause of dementia in adults in industrialized societies. At present, neither the etiology nor the pathogenesis of this neurodegenerative disorder is understood. Alzheimer disease probably has polyetiology, which includes genetic, environmental, and metabolic factors. Major form of Alzheimer disease is sporadic, whereas a small percentage of cases are familial. To date, in only less than 3% of all the familial Alzheimer disease cases tested a mutation in the ß-amyloid precursor protein gene has been linked to the disease. Thus the etiology of most of the Alzheimer disease cases remains unknown.

Histopathologically, Alzheimer disease is characterized by the presence of two brain lesions, paired helical filaments (PHF) in the neurons and ß-amyloid in the extracellular space. The PHF accumulate in neuronal cell bodies as neurofibrillary tangles, in neurites as neuropil threads (Braak et al. 1986), and in the dystrophic neurites surrounding wisps or a core of ß-amyloid in neuritic (senile) plaques. In addition to the neuritic plaques, ß-amyloid also accumulates in the wall and the lumen of the brain vessels. Deposits of ß-peptide, polymers of which form amyloid are also seen as diffuse plaques throughout the affected areas of the brain. At present, the exact relationship between PHF and ß-amyloid in the pathogenesis of Alzheimer disease is not understood. However, there is growing evidence from a number of laboratories that dementia in Alzheimer disease patients is associated with neurofibrillary degeneration (Dickson et al. 1988, Katzman et al. 1988, Barcikowska et al. 1989). A detailed review on this subject was published recently (Iqbal and Grundke-Iqbal 1991). In this paper an update of studies on the pathogenesis of neurofibrillary degeneration is summarized.

METHODS

Methods have been described elsewhere and are only referred here. These references are: monoclonal antibodies 5-25 and 3-39 to PHF (Wang et al. 1984, Grundke-Iqbal et al. 1985b), epitope mapping of antibodies 5-25 and 3-39 (Perry et al. 1989), monoclonal antibody Tau-1 (Binder et al. 1985), polyclonal antibodies to tau (Grundke-Iqbal et al. 1986a, 1988) and amino terminal tau peptide, Peptide-1 (Iqbal et al. 1989), isolation of PHF (Iqbal et al. 1984), isolation of microtubule associated protein tau (Grundke-Iqbal et al. 1986a), dephosphorylation of abnormally phosphorylated tau/PHF-tau (Grundke-Iqbal et al. 1986b, Iqbal et al. 1986b, 1989), *in vitro* microtubule assembly from Alzheimer disease and control brain extracts (Iqbal et al. 1986b), GTP binding to tubulin (Khatoon et al. 1990) and radioimmunoassay for normal and abnormally phosphorylated tau in brain homogenates (Khatoon et al. 1992a).

RESULTS AND DISCUSSION

Identification of tau as a major protein subunit of PHF

We have observed that Alzheimer neurofibrillary tangles (ANT) are heterogenous in both morphology and solubility (Iqbal et al. 1984). Most ANT are composed of PHF. In some ANT PHF are admixed with a large number of 2.1 nm tau filaments (Ruben et al. 1991, 1992, 1993). PHF are stable in both fresh and frozen autopsy tissues and are resistant to solubilization in aqueous buffer in the absence of detergents or denaturants (Iqbal et al. 1984). 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). ANT I are insoluble in sarkosyl but are readily soluble in SDS (Rubenstein et al. 1986). 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.

Different approaches to isolate PHF have been based on the sparing solubility of PHF in detergents or their resistance to proteolysis, or both (Ihara et al. 1983, Iqbal 1984, 1986a, Masters et al. 1985, 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 (Grundke-Iqbal et al. 1988, Wischik 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.

On SDS-polyacrylamide gels, the protein composition of the highly purified PHF bulk isolated from Alzheimer disease brain is complex. The major polypeptides are in the 45 kDa-70 kDa region (Grundke-Iqbal et al. 1984, 1985a, Iqbal et al. 1984, 1986a). Immunostaining of the 45 kDa-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 kDA-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 (Grundke-Iqbal et al. 1986a,b, 1988, Iqbal et al. 1989). The immunocytochemical labeling of PHF in tissue sections with antibodies to tau has been confirmed by several other laboratories (Brion et al. 1985, Delacourte and Defossez 1986, Ihara et al. 1986, Kosik et al. 1986, Wood et al. 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 purified PHF (Kondo et al. 1988, Wischik 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 disease and with Guam-Parkinsonism dementia (Masters et al. 1985, 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 and normal cases is localized mainly to lipofuscin in different types of neurons, including the neurons with the neurofibrillary tangles (Bancher et al. 1989b, Grundke-Iqbal et al. 1989). A varying number of ghost tangles ranging from a few (Hyman et al. 1989, Tabaton et al. 1991) to almost all (Perry et al., in preparation) are labeled with antibodies to ß-amyloid, depending on the tissue fixation conditions. This immunostaining, however, is mostly restricted to ß-amyloid and to the unidentified amorphous material adhering to tangles in the extracellular space (Grundke-Iqbal and Iqbal 1991, Tabaton et al. 1991, Yamaguchi et al. 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.

Association of ubiquitin with PHF

In addition to tau, we have observed the presence of ubiquitin in isolated PHF and the immunostaining of PHF with antibodies to ubiquitin (Grundke-Iqbal et al. 1988, Wrzolek et al. 1992). These findings are in agreement with other laboratories (Mori et al. 1987, Perry et al. 1987). 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 non-lysosomal proteases (Hershko and Ciechanover 1982). Monoclonal antibodies 3-39 and 5-25 raised against PHF generated in our laboratory (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 brain cytosol (Grundke-Iqbal et al. 1988, Koepke-Secundo et al. 1990). Furthermore, the monoclonal antibodies to PHF react much stronger with PHF polypeptides than with free ubiquitin (Grundke-Iqbal et al. 1988). It thus appears that some of the tau in PHF, especially in the ghost tangles, might be ubiquitinated. Alternatively, ubiquitin-ubiquitin conjugates, comigrating with tau polypeptides on SDS-polyacrylamide gels, might be associated with PHF. Employing enzyme-linked immunoassays we have observed that the brain ubiquitin levels are markedly elevated in Alzheimer disease cases and this increase is also measured in both the lumbar as well as the ventricular cerebrospinal fluid of these patients (Wang et al. 1991a,b, Kudo et al. 1992). The amount of ubiquitin in isolated PHF is less than 5% (Grundke-Iqbal and Iqbal 1992). These findings suggest that although only minor amounts of ubiquitin are associated with the PHF, it might be involved in the pathobiology of Alzheimer disease, especially the neurofibrillary degeneration.

Abnormal phosphorylation of tau in Alzheimer disease brain

Tau in Alzheimer disease brain is phosphorylated differently from normal brain tau (Grundke-Iqbal et al. 1986b, Iqbal et al. 1986b). In PHF practically all tau isoforms are present in abnormally phosphorylated forms (Grundke-Iqbal et al. 1986b, Iqbal et al. 1989). Unlike normal tau, this protein is inaccessible in PHF 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 abnormal tau isolated from Alzheimer disease brain contains up to 12 moles of phosphate/mole of protein, which is about four-times the level in normal brain tau. Tau in PHF is phosphorylated at multiple sites. In addition to the Tau-1 site, we discovered an amino terminal site at which tau in PHF is phosphorylated differently from tau in normal brain (Iqbal et al. 1989). Subsequent to these studies several other sites at which tau in PHF is phosphorylated have been reported (Brion et al. 1991, Lee et al. 1991, Hasagawa et al. 1992). 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, the cytosolic tau is also abnormally phosphorylated in Alzheimer disease brain. This abnormal phosphorylation is most prominent in the molecular species of tau with the slowest electrophoretic mobility (Grundke-Iqbal et al. 1986b, Iqbal et al. 1986b). The presence of the abnormally phosphorylated tau in Alzheimer 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).

Employing a radioimmunoassay we have discovered that the levels of tau in brain homogenates from Alzheimer disease patients are approximately eight-fold higher than in age-matched controls, and that this increase is in the form of abnormally phosphorylated protein (Khatoon et al. 1992a). In brain cytosol the levels of normal tau in Alzheimer disease cases are approximately 50% of corresponding values in age-matched controls (Khatoon et al., in preparation).

The aberrant phosphorylation in Alzheimer disease brains might be selective to a few neuronal proteins and not be a part of a generalized hyperphosphorylation because the overall levels of phosphoprotein phosphate in Alzheimer disease brain homogenates are not altered significantly (Iqbal and Grundke-Iqbal 1990a,b). At present, neither the nature of all the phosphorylation site/s nor the protein phosphorylation/dephosphorylation system responsible for the abnormal phosphorylation of tau in Alzheimer disease is completely understood. Studies from our laboratory 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). We have also shown that in vitro bovine tau can be phosphorylated stoichiometrically up to six additional moles of phosphates per mole of the protein and an upwards electrophoretic mobility shift with casein kinase-1, calcium-calmodulin dependent protein kinase (CaM kinase-II), CaM kinase-Gr and protein kinase A; stoichiometric phosphorylation of bovine tau with protein kinase C did not induce a mobility shift (Singh et al. 1992). The phosphorylation of bovine tau by none of these kinases caused the blockage of the Tau-1 epitope. These studies have demonstrated that the mobility shift alone is not a valid criterion for the Alzheimer disease-like abnormal phosphorylation of tau. Other laboratories have shown the blockage of the Tau-1 epitope on phosphorylation with a tubulin-dependent protein kinase (Ishiguro et al. 1992), and with two mitogen-activated protein (MAP) kinases (Drewes et al. 1992, Roder and Ingram 1992). However, the time kinetics of the Tau-1 blockage reported by these investigators are very slow, suggesting that either the Tau-1 site is not a preferred substrate for the kinases employed or the reaction conditions are not optimal.

The abnormal phosphorylation of tau appears to represent one of the earliest changes leading to Alzheimer neurofibrillary pathology. Tau-1 reactivity is seen after dephosphorylation in a number of apparently morphologically normal neurons of the neocortex in the non-demented aged and in cases with Alzheimer 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 and 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 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.

Role of abnormally phosphorylated tau in the breakdown of the microtubule system in Alzheimer disease

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 disease (Iqbal et al. 1986b, 1987). We have found that microtubules can be assembled in vitro from the cytosol of normal fresh autopsy brain obtained within 5 h postmortem, but no assembly of microtubules is observed from identically treated brains of Alzheimer disease cases (Iqbal et al. 1986b). These findings have been confirmed by Nieto et al. (1990), who showed that

heat-stable microtubule-associated proteins, which include tau, prepared from Alzheimer disease brain do not stimulate microtubule assembly from porcine tubulin *in vitro*. We have found that the *in vitro* assembly of microtubules from the Alzheimer disease tissue, however, is induced by the addition of DEAE dextran, a polycation that mimics the effect of tau for microtubule assembly (Iqbal et al. 1986b). 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 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 brain might contribute to the microtubule assembly defect. We have shown that both PHF-tau (Iqbal et al. 1991) and the abnormal tau from Alzheimer disease brain cytosol (Alonso et al. 1992) when dephosphorylated with alkaline phosphatase stimulate in vitro microtubule assembly. These findings confirm the role of the abnormal phosphorylation in microtubule assembly defect in Alzheimer disease. 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 disease brain might lead to decreased GTP binding and, consequently, decreased assembly of microtubules (Khatoon et al. 1992b).

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 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, Kosik et al. 1984, 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 non-specific proteins trapped between the fibrils. Discoveries of the abnormal phosphorylation of tau in Alzheimer disease brain and of the presence of abnormal tau and of ubiquitin in PHF and the failure to induce in vitro microtubule assembly in Alzheimer disease brain cytosol lead us to hypothesize (1) that the protein phosphorylation-dephosphorylation system is defective in Alzheimer 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 protein kinases and phosphoprotein phosphatases. Our studies (Grundke-Iqbal

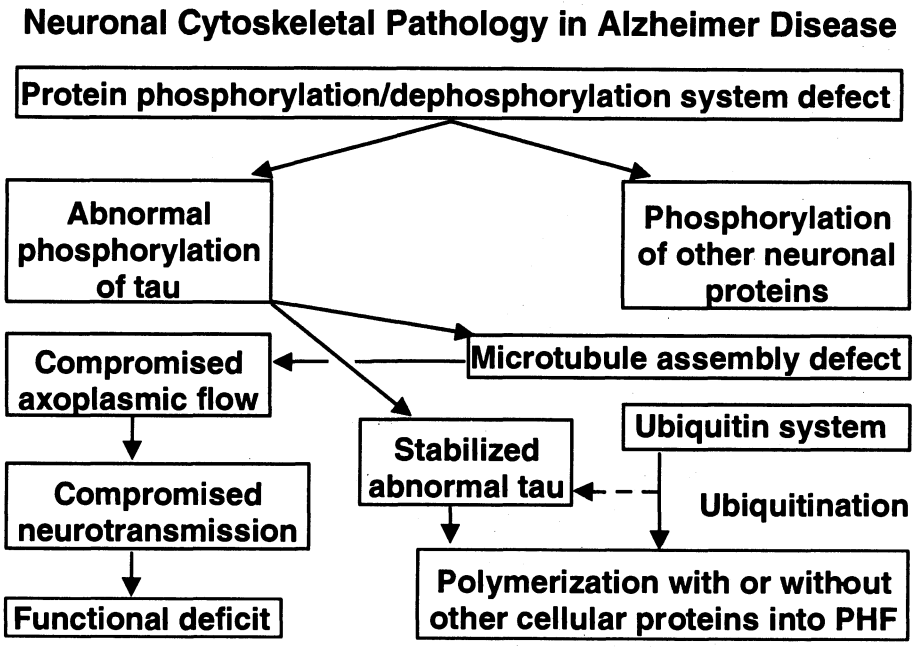

Fig. 1. A hypothetical scheme of sequence of major molecular events that might be involved in the pathogenesis of neurofibrillary degeneration in Alzheimer disease. (Figure reproduced with permission from Iqbal and Grundke-Iqbal, Mol. Neurobiol. 5: 399-410, 1991).

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 disease. We have found that both phosphoprotein phosphatase 1 and 2A activities towards phosphorylase kinase are decreased in brains of Alzheimer disease cases (Gong et al. 1992).

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