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Activation of p38 Kinase Links Tau Phosphorylation, Oxidative Stress, and Cell Cycle-Related Events in Alzheimer Disease

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Abstract. The temporal association between oxidative stress and the hallmark pathologies of Alzheimer disease (AD) demonstrates that oxidative stress is among the earliest events in the disease. Nonetheless, neither the consequences of oxidative stress nor how oxidative stress relates to other pathological features of the disease are clear at this point. To begin to address these issues, we investigated p38 kinase, which is induced by oxidative stress, in the pathogenesis of AD. In hippocampal and cortical brain regions of individuals with AD, p38 is exclusively localized in association with neurofibrillar pathology. By marked contrast, these brain regions exhibit a low level of diffuse p38 staining in the neuronal cytoplasm in controls. We found a complete overlap of the immunostaining profiles of p38 and tau-positive neurofibrillary pathology and that the majority of p38 was activated in AD neurons, both of which support an association of p38 with the disease process. Moreover, the finding that PHF-tau co-immunoprecipitates with p38, and that p38 co-purifies with PHF-tau, strongly suggests that they are physically associated in vivo. Since p38 is also implicated in cell cycle regulation, our findings provide a link between the cell cycle re-entrant phenotype, cytoskeletal phosphorylation and oxidative stress in AD.

Key Words: Alzheimer disease; Cell cycle; Cytoskeleton phosphorylation; Oxidative stress; p38 kinase; Signal transduction.

INTRODUCTION

There is increasing evidence for the pivotal role played by oxidative stress in the pathophysiology of Alzheimer disease (AD) (1, 2), the leading cause of senile dementia. In fact, as one of the earliest events in AD, oxidative stress may be an important mediator in the onset, progression, and pathogenesis of the disease. Nonetheless, the mechanistic consequences of oxidative stress in the pathogenesis of AD are, as yet, unclear. One obvious and important question is the role of oxidative stress in relation to the 2 pathological hallmarks of the disease; namely, the neurofibrillary tangles (NFT), composed of highly phosphorylated tau (3), and amyloid-β containing senile plaques. Indeed, while oxidative stress is known to influence the phosphorylation state of tau protein (4) as well as regulate amyloid-β production (5), the precise underlying mechanisms are not completely understood. To further delineate this relationship, we investigated the role of p38, a stress activated protein kinase which phosphorylates tau protein at sites found in the NFT of AD (6, 7).

Alterations in gene expression and enzyme activity induced by cellular stress are mediated through the interplay of multiple signaling pathways. Among these are the mitogen activated protein kinase (MAPK) pathways, which are the central mediators that propagate signals from the membrane to the nucleus. The p38 kinase pathway, 1 of the 3 best described MAPK pathways, is primarily activated by cellular stresses, including oxidative stress, UV irradiation, chemotherapeutic drugs, pro-inflammatory cytokines, and heat shock (8, 9). In addition to being able to phosphorylate tau, p38 is the first kinase outside of the Cdk family shown to regulate the function of retinoblastoma protein in vivo and therefore provides a vital link between cellular stress and cell cycle regulation (10). In this regard, it is notable that a number of components of the cell cycle are upregulated in AD (11), which is suggestive of a re-entrant phenotype. Given that p38 has also been implicated in apoptosis (12, 13) it may play a crucial role in the decision between apoptosis and proliferation (i.e. re-entry into the cell cycle) in cells challenged by oxidative stress.

MATERIALS AND METHODS

Brain Tissue

Hippocampal, frontal cortical, and cerebellar brain tissue obtained postmortem was fixed in methacarn (methanol:chloroform:acetic acid; 6:3:1), embedded in paraffin and 6-μm-thick consecutive sections were prepared on silane-coated slides (Sigma, St. Louis, MO) for immunocytochemistry. Cases used in this study were chosen randomly and include AD (n = 18; ages = 69–91 yr; postmortem interval = 3–23 h) and control (n = 18; ages = 53–91 yr; postmortem interval = 3–48 h) cases, as based on clinical and pathological criteria established by CERAD and an NIA consensus panel (14, 15). There was no clinical history of dementia for all the controls used.

Immunocytochemical Procedures

Immunocytochemistry was performed by the peroxidase antiperoxidase protocol essentially as described previously (16, 17).

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Briefly, following immersion in xylene, hydration through graded ethanol solutions and elimination of endogenous peroxidase activity by incubation in 3% hydrogen peroxide for 30 min, sections were incubated for 30 min at room temperature in 10% normal goat serum (NGS) in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.6) to reduce non-specific binding. After rinsing briefly with 1% NGS/TBS, the sections were sequentially incubated overnight at 4°C with either (i) immunoaffinity purified rabbit polyclonal antibody to p38 (StressGen Biotechnologies Corporation, Inc., Victoria, BC), which recognizes an epitope corresponding to amino acids residues 341–360 of human p38 or (ii) immunoaffinity purified rabbit polyclonal antibody to phospho-p38 (New England Biolabs, Inc., Beverly, MA), which only recognizes p38 activated by dual phosphorylation at Thr180 and Tyr182, or (iii) mouse monoclonal AT8 antibody to phosphorylated cytoskeletal tau protein. The sections were then incubated in either goat anti-rabbit (ICN, Costa Mesa, CA) or goat anti-mouse (ICN, Costa Mesa, CA) antisera, followed by species-specific peroxidase anti-peroxidase complex (Sternberger Monoclonals Inc. and ICN, Cappel). 3-3′-Diaminobenzidine (DAB) was used as a chromagen. For some experiments, sections were double-labeled with 2 different antibodies. Rabbit antisera were localized using the PAP method with DAB as the chromogen. Monoclonal antibodies were localized using the alkaline phosphatase anti-alkaline phosphatase method using Fast Blue as the chromogen.

Absorption experiments were performed to verify the specificity of antibody binding. Briefly, the immunostaining protocol was repeated using absorbed antibody produced by an overnight incubation of primary antibody with purified p38 peptide (50 μg/ml) at 4°C (StressGen Biotechnologies Corporation, Inc). In parallel, absorption of anti-p38 with irrelevant peptide [TGF-β activating kinase 1 (TAK1) peptide (10 μg/ml) (StressGen Biotechnologies Corporation, Inc.)] or β protein precursor (0.1 mg/ml) (gift from Dr. Barry Greenberg) or irrelevant antibody (i.e. TAK1 antibody) (StressGen Biotechnologies Corporation, Inc.) with p38 peptide (StressGen Biotechnologies Corporation, Inc.) was performed as a control against artifactual absorption.

To determine the specificity of the phosphorylation-dependent antisera, some sections were treated with 2 U alkaline phosphatase (Type III; Sigma) in 100 μl Tris pH = 8.0 and 0.01 M PMSF at room temperature for variable times from 1–72 h prior to incubation in primary antibody.

Immunoblotting and Immunodotting

Tissue from the gray matter of temporal cortex of AD (n = 13) and control cases (n = 16) were homogenized in 10 vol. of TBS containing 0.02% Sodium azide, 0.5% Sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml antipain (lysis buffer). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10 μg/lane) and electroblotted onto Immobilon-P (Millipore, Bedford, MA) by standard procedures as previously described (17). Transferred blots were incubated sequentially with blocking agent (10% nonfat milk in TBS-Tween), rabbit anti-p38 antibody (StressGen Biotechnologies Corporation, Inc.) and affinity-purified goat anti-rabbit immunoglobulin peroxidase conjugate preabsorbed to eliminate human cross-reactivity. Blots were developed by the ECL technique (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's instruction. Blots were striped in stripping buffer (2% SDS, 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol, pH 6.8) for 30 min at 60°C and then probed with antibody against HO-2 (StressGen Biotechnologies Corporation, Inc.), which is constitutively expressed in neuronal cells. Quantification of the results was performed using a computer-assisted scanning system (PDI, Huntington Station, NY). The data obtained were expressed as optical densities and analyzed statistically using one-way analysis of variance.

Dot blots were prepared by applying 4 μg of tau protein (0.5–1.0 mg/ml), immunizing peptide of p38 (1 mg/ml), 4 μg of insoluble PHF (18), or a control fraction (19) directly onto Immobilon (Millipore) membrane and then air-dried. Human tau was prepared from a normal human brain by a modification of the procedure previously described by Lindwall and Cole (19). PHF-enriched fractions were prepared from 4 AD cases and 2 age-matched control cases according to previously described methods (18). The membrane was incubated sequentially with blocking agent (10% nonfat milk in TBS-Tween), rabbit anti-p38 and goat anti-rabbit peroxidase conjugate. Dot blots were developed using DAB.

Immunoprecipitation

Grey matter, which was visually dissected from white matter in frontal cortex in AD cases (n = 4) and control cases (n = 4) stored at −80°C, was homogenized in 10 vol. of TBS containing 0.02% Sodium azide, 0.5% Sodium deoxycholate, 0.1% SDS, 1% NP-40, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml antipain (lysis buffer). The protein concentration was determined by a Lowry assay (BCA Kit; Pierce, Rockford, IL). The homogenate was precleared by incubating with Protein A-agarose (Boehringer Mannheim Corp., Indianapolis, IN) at 4°C for 1 h, followed by centrifugation at 5,000 rpm for 10 min at 4°C. p38 antibody (StressGen Biotechnologies Corporation, Inc.) or, as a control, irrelevant Ras antibody (StressGen Biotechnologies Corporation, Inc.) was added to the supernatant and incubated at 4°C for 4 h with end-over-end rotation, followed by the addition of Protein A-agarose and incubation overnight. Following centrifugation at 4,000 rpm for 10 min at 4°C, the supernatant was carefully aspirated and discarded. The pellet was washed 4 times with lysis buffer, and the sample was boiled for 5–10 min prior to SDS-PAGE. For immunoblot analysis of the precipitate, 2 μl of bead slurry was used for precipitation of 100 μg of grey matter of protein homogenate. The entire precipitate was loaded in 1 lane, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transfer onto Immobilon-P (Millipore) by standard procedures as previously described (17). p38, PHF-1 (A gift from Dr. Barry Greenberg) and TG3 (which recognize phosphorylated epitopes in PHF and conserved phospho-epitopes in dividing cells) (20) antibodies were used to blot the precipitates.

RESULTS

Immunolabeling with anti-p38 antibody demonstrated intense staining of neurofibrillary tangles, neuritic senile plaques, granulovacuolar degeneration, and neuropil threads, the classic features of the neuropathology of AD

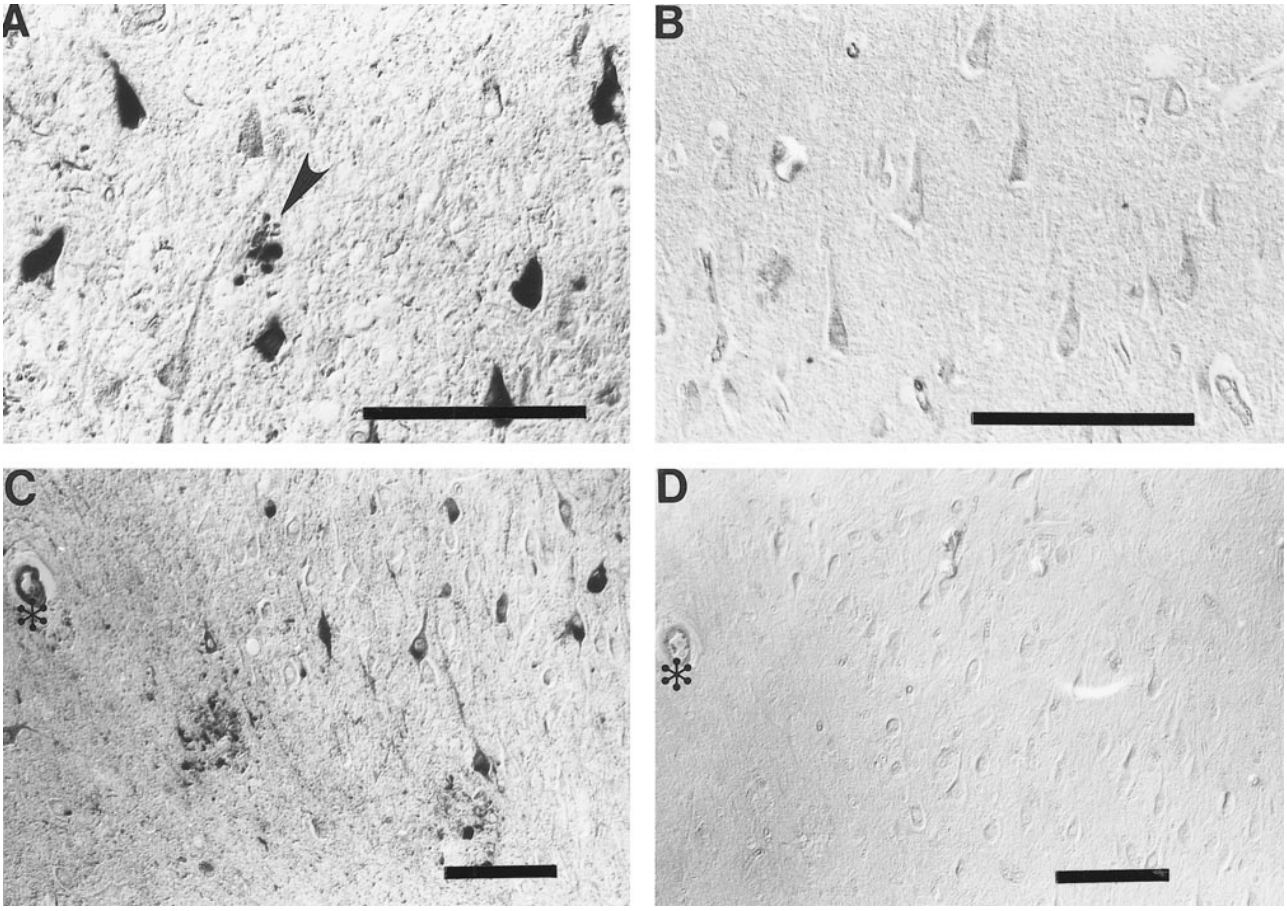

Fig. 1. Immunocytochemical localization of p38 in NFT-containing neurons of a hippocampal section (A) from an AD patient. (Arrowhead indicates granulovacuolar degeneration.) p38 staining is diffuse and much weaker in hippocampus (B) in most control cases. Neuronal immunostaining in the hippocampus with p38 antibody in AD (C) is abolished completely by adsorption with immunizing peptide (D). Asterisk indicates landmark blood vessel in adjacent sections. Scale bars: 100 μm.

brain, in both the hippocampus (Fig. 1A, C) and cortex (not shown). As assessed by double staining with Congo red, p38 was primarily localized to intracellular NFT and only a few extracellular NFTs (<20%). There was almost complete overlap of the immunostaining profiles of p38 and tau-positive neurofibrillary pathology as assessed by staining of adjacent sections (Fig. 2A–D) and double staining (Fig. 2E, F) using AT8, an antibody that only recognizes tau protein when serine 202 and threonine 205 are phosphorylated. Less than 10% AT8-positive NFTs did not label with p38. In marked contrast, p38 immunoreactivity in age-matched controls was localized in the cytosol, weaker, and more diffuse (Fig. 1B). As often found in normal aging, a few pyramidal neurons contained neurofibrillary pathology and, in these cells, p38 was present in the AD-like pattern (result not shown). Importantly, in the cerebellum, an area that is unaffected by AD, there was no difference in the staining pattern between AD and normal cases, with Purkinje cells being diffusely stained in all cases (result not shown). In the hippocampus of some AD and control cases and the cortex of all AD and control cases, non-neuronal cells, including astrocytes and microglia, exhibited diffuse p38 cytoplasmic labeling. In all cases, and for all 3 brain areas examined, there was no relationship between agonal status, cause of death or postmortem interval and p38 immunoreactivity.

To confirm the specificity of p38 immunocytochemistry, several control experiments were performed in parallel. Absorption of the p38 antibody with the immunizing peptide of p38 almost completely abolished immunostaining (Fig. 1D), whereas no effect was observed by the absorption of the (i) antibody to TAK1 with p38 peptide; (ii) antibody to p38 with TAK1 peptide, or (iii) antibody to p38 with β protein precursor (results not shown). To exclude the possibility that the p38 antibody was cross-reacting with tau protein, we used enriched human tau protein on dot blots and found no immunoreactivity (result not shown). Furthermore, there is no significant homology between p38 and tau protein.

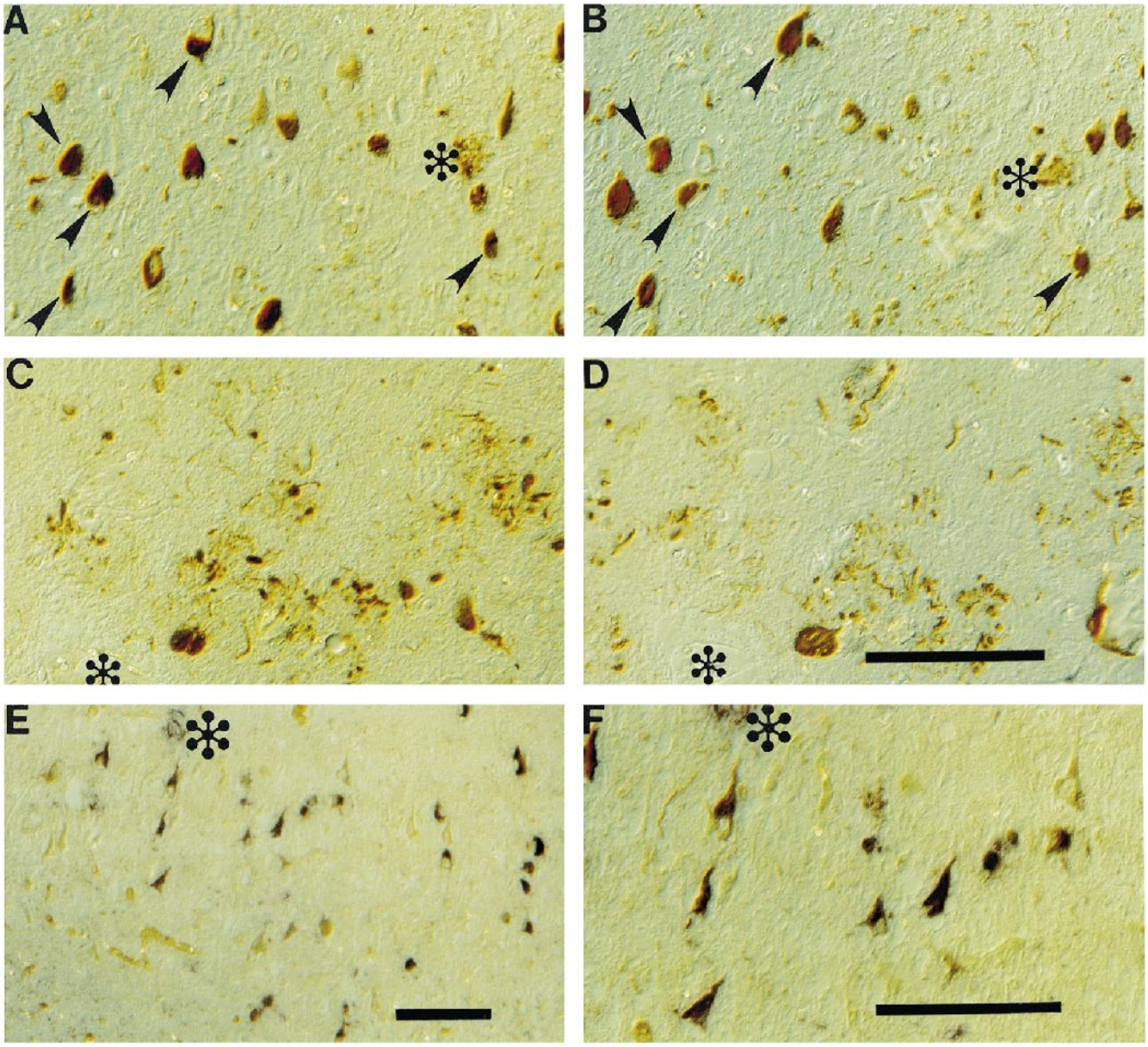

Fig. 2. Adjacent serial sections of the hippocampus of a case of AD immunostained with anti-p38 (A, C) and AT8 (B, D) with landmark vessel (*). A, B show an area of neurofibrillary tangles and C, D show an area of senile plaques in the same case. Most of the same neurofibrillary tangles (arrowheads) and senile plaques are labeled by both p38 and AT8. Double-immunostaining of pyramidal neurons in the hippocampus of an AD patient with AT8 (blue) and p38 (brown) (E, F). F is a higher magnification picture of the area marked with asterisk in (E). Most neurofibrillary tangles in AD contain both AT8 and p38. Scale bars: A–D = 100 μm; E, F = 100 μm.

To demonstrate activation of p38 in AD, antibody against phospho-p38 was used and, as with the nondiscriminatory p38 antibody, an intense immunoreactivity was associated with the neurofibrillary pathology in AD (Fig. 3B). By marked contrast, the diffuse staining displayed by the nondiscriminating antibody in controls was barely detectable with the phospho-p38 antibody (Fig. 3A). In fact, the pattern of phospho-p38 was essentially identical to that of p38 (Fig. 3B, C) indicating that the majority of p38 was activated in AD. To verify this finding, we pretreated the sections with alkaline phosphatase and while phospho-p38 staining was abolished (Fig. 3D–F), there was no change in p38 staining. Whereas NFT and SP staining was easily lost after 1 hour of treatment with alkaline phosphatase (Fig. 3E), staining of granulovacuolar degeneration was more stable and lost only

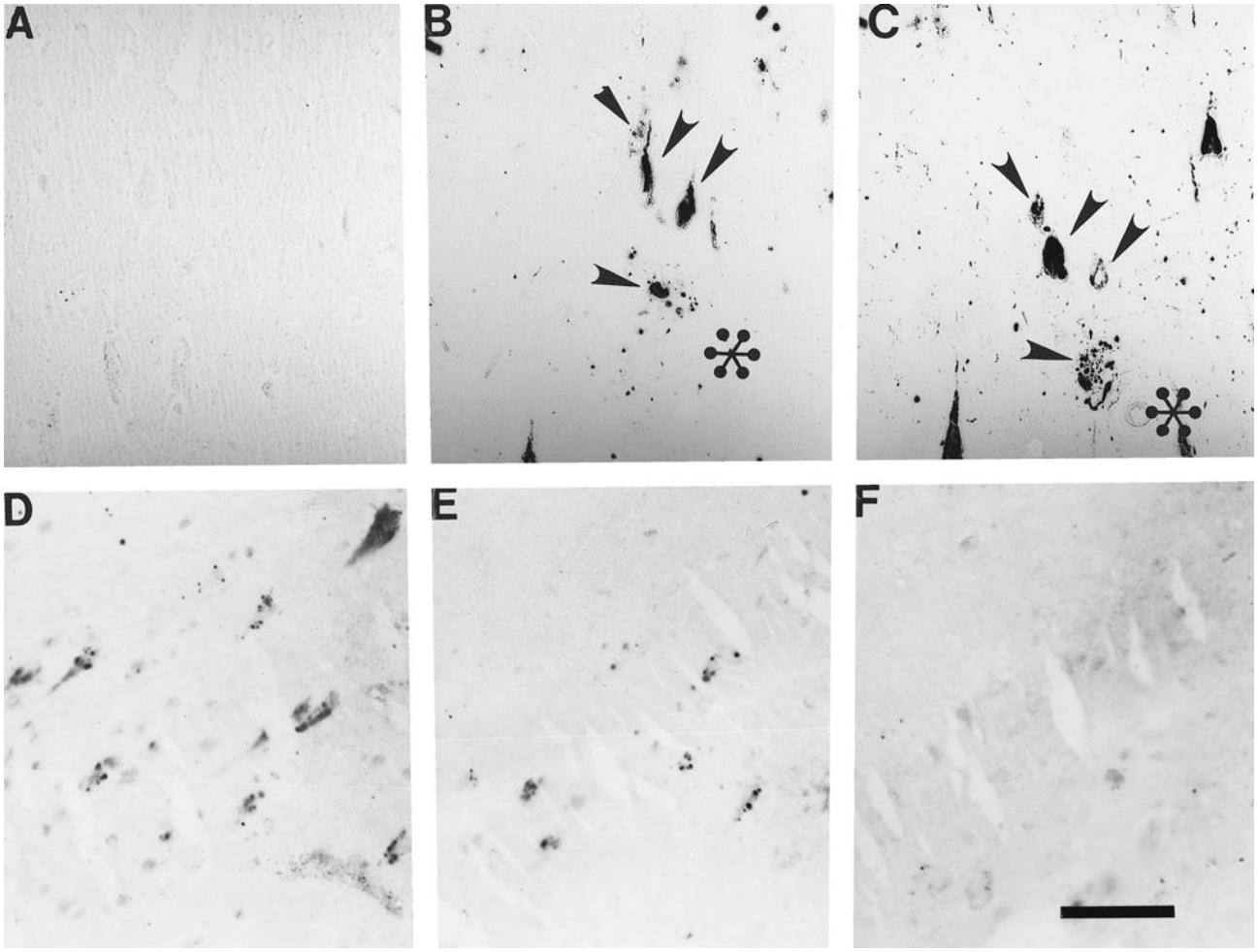

Fig. 3. Immunocytochemical localization of phospho-p38 in neurons of a hippocampal section from an AD patient (B). Phospho-p38 staining is barely detected in hippocampus in control cases (A). Phospho-p38 staining (B) was essentially identical to that of p38 (C), most of the same neurofibrillary tangles (arrowheads) and senile plaques are labeled by both p38 and phospho-p38. Asterisk indicates landmark blood vessel in adjacent sections, arrowhead shows the overlap of the staining. D, E, F show the effect of alkaline phosphatase treatment on phospho-p38 staining, D: no alkaline phosphatase treatment; E, F: alkaline phosphatase treated for 1 h or 2 days respectively. Scale bar: 100 μm.

after 2–3 days treatment (Fig. 3F). These latter data indicate that the phosphate groups on the p38 found in NFT/SP are more readily accessible than those found on p38 in granulovacuolar degeneration.

Immunoblot analysis revealed a major anti-p38 immunoreactive band with an approximate molecular weight of 38,000 in the AD and controls. However, the band was much weaker in the control brain homogenates (Fig. 4A). The statistical analysis of the quantification result, which is normalized by HO-2, shows that p38 increase by 50% in AD compared with control ($p = 0.078$) (Fig. 4B). Likewise, in immunoprecipitation experiments, the recovery of p38 from AD brain homogenates was significantly higher than that seen in age-matched controls (Fig. 6A). These results not only demonstrate the specificity of p38 antibody, but also are consistent with p38 elevation in AD brain compared with controls.

p38 has been shown to phosphorylate the tau protein in vitro (6, 7), the major component of PHF in NFT (21). The complete overlap of AT8 and p38 staining indicates that p38 may phosphorylate tau in vivo as well. Therefore, we explored whether phosphorylated tau is physically associated with the kinase. In a dot blot assay using PHF preparations from control and AD cases, p38 antibody showed reactivity with only the AD samples (Fig. 5), a staining pattern similar to that of PHF-1, an established antibody against PHF-tau that served as a positive control. p53 antibody, which was used as a negative control, showed reactivity in neither control nor AD samples (results not shown). This result suggests that p38 protein may co-purify with PHF. To confirm this result, we used PHF-1 to immunoblot p38 precipitates of grey matter homogenates from 4 AD and 4 control cases. PHF-1 or TG3 detected a typical AD-specific pattern of PHF-derived

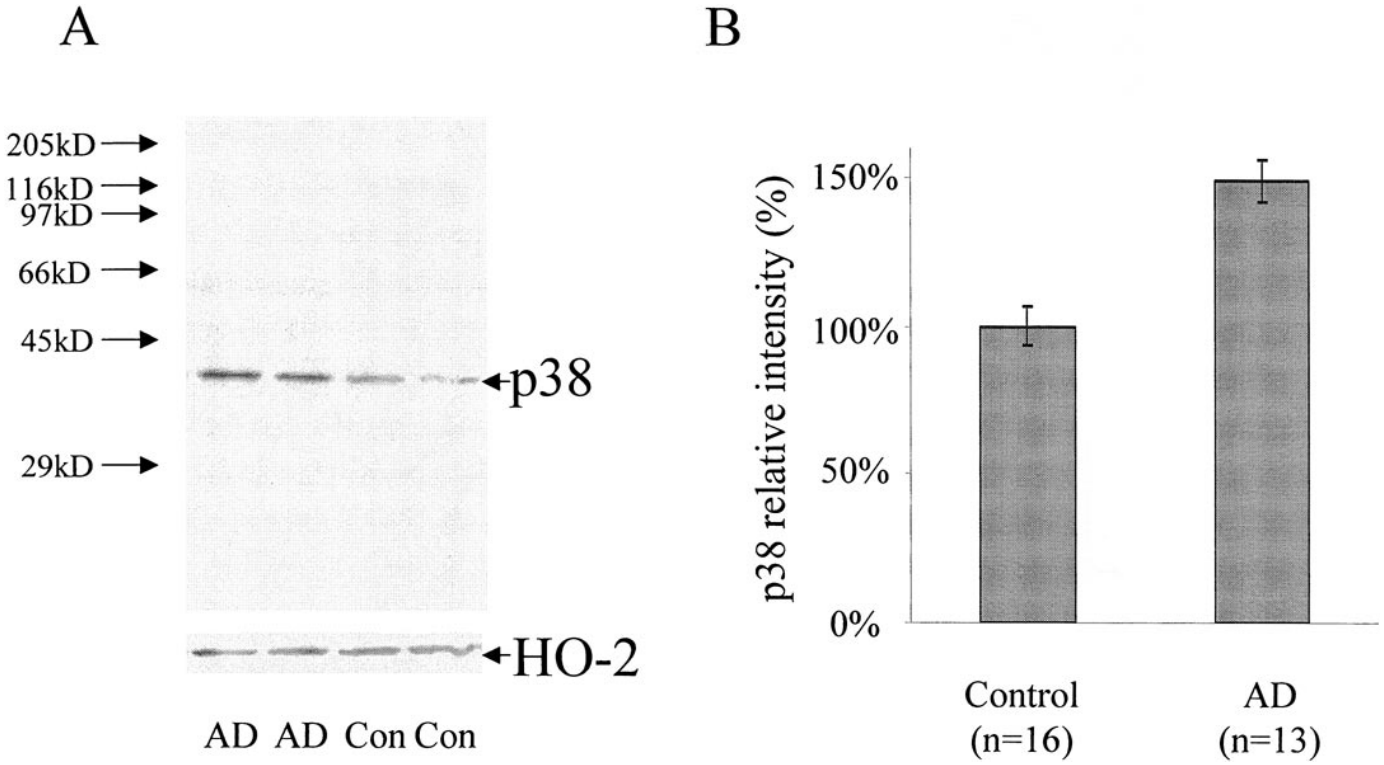

Fig. 4. A: Representative results of immunoblots of cortical gray matter, homogenized in lysis buffer and probed with antisera against p38, show a strong band at the expected molecular weight of 38 kDa in AD (AD) and weaker in control (Con) samples. B: Quantification, which is normalized by HO-2 blot, of p38 immunoblots results shows a great increase of p38 intensity in AD ($p = 0.078$). Result is shown as Ave±SEM

protein in p38 precipitates from AD but not in control homogenates (Fig. 6B), suggesting that PHF-tau co-immunoprecipitates with p38. As a control against nonspecific co-sedimentation (Fig. 6C, D), we immunoprecipitated with Ras antibody and were unable to detect PHF-1 immunoreactive material.

DISCUSSION

In this study, we demonstrated a considerable alteration in the level and distribution of p38 and phospho-p38 kinase immunoreactivity in susceptible neuronal populations of AD brain compared with age-matched controls. In AD, pronounced p38 and phospho-p38 immunoreactivity is seen localized to neurofibrillary tangles, senile plaque neurites, granulovacuolar degeneration and neuropil threads, and near complete overlap with phosphorylated tau. These data not only indicate that almost every pathological lesion contains increased p38, but also that p38 may be involved in the phosphorylation of tau in vivo (22). Indeed, p38 is capable of phosphorylating tau protein in vitro in a manner similar to the phosphorylation of PHF-tau (6, 7). The in vivo association of p38 in nearly all neurons containing phosphorylated tau implicates p38 in the phosphorylation of tau in vivo in AD. This is further supported by the co-immunoprecipitation of PHF-tau with p38 and the co-purification of p38 with PHF. Since AT8 is an early marker for phosphorylated tau and given the potential role for p38 to phosphorylated tau in vivo, the complete overlap between p38 and AT8 staining strongly suggests that the elevation and activation of p38 is a proximal event in the pathogenesis of AD. Given that cytoskeletal reorganization is a cardinal feature in AD, it is interesting to note that the activation of the p38 MAPK/HSP27 pathway may also play a central role in modulating the response of microfilaments to oxidative stress (23–25).

Oxidative damage is a key feature in the AD brain. As would be expected, there is an induction of specific antioxidant systems such as superoxide dismutase, several heat shock proteins, and HO-1 (17, 26–28). The localization of these proteins to AD pathology suggests that there may be a generalized mechanism of induction of these proteins. The activation of the p38 pathway in AD, which is demonstrated by the findings presented here, provides such a mechanism since it is firmly established that the p38 pathway is primarily activated by a variety of cellular stresses, including oxidative stress and, upon activation, can induce the expression of specific gene

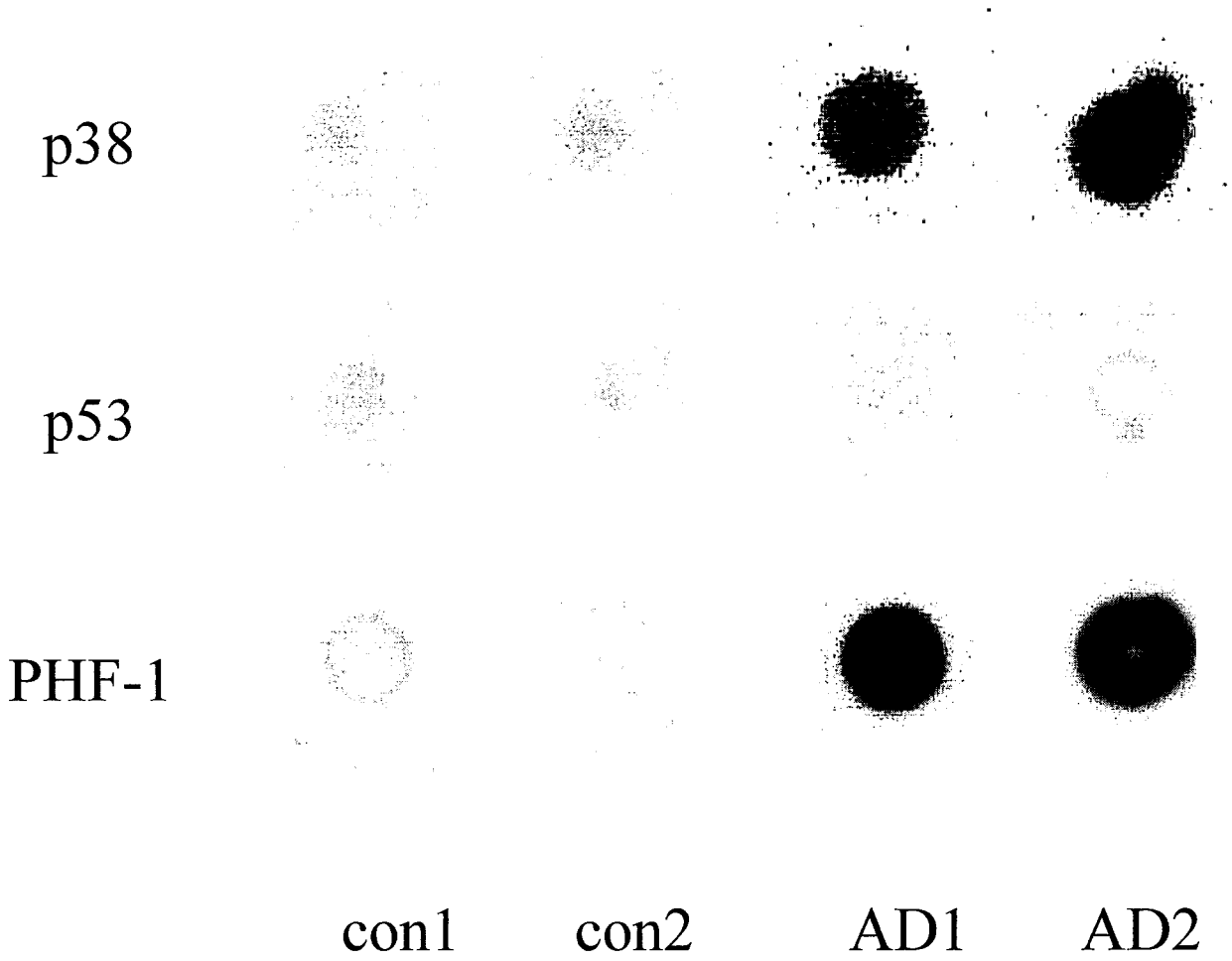

Fig. 5. Dot blot of PHF, purified from AD patients (AD1, AD2) and control cases (con1, con2) and probed with antisera against p38, show strong immunoreactivity for PHF from AD but not from control. PHF-1 (positive control) and antisera against p53 (negative control) are also shown.

products including HSP27 and HO-1 (27, 28). The Purkinje cells, in which there was no difference between AD and control, may reflect the similar protective response to oxidative stress in aging, but not AD pathology, since Purkinje cells demonstrate high oxidative stress in all aged individuals and consequent oxidant damage (29). Since MAPK is activated by phosphorylation, the elevated protein level of p38 demonstrated in this study, and the fact that the majority of p38 is phosphorylated, likely indicates an effort by the neurons, in the face of oxidative stress, to induce the protective effect of the p38 pathway.

It has been shown that oxidative stress is associated not only with the onset and effector phases of apoptosis, but also with stimulated proliferation. A number of studies emphasize the induction of proliferation by some levels of oxidants (30, 31) and the addition of antioxidants to cells can have anti-proliferative effects (32). Thus, the concepts of redox biochemistry and oxidative stress are emerging as potentially central players in the regulation of the cell cycle. It is interesting to note that oxidative stress and an attempted re-emergence of the cell cycle phenotype are both intimately associated with susceptible neurons in AD (33, 34). Although p38 appears to mediate a stress response that is often associated with subsequent cell death, some recent reports (35–40) have suggested protective roles for p38 kinase. As the major kinase in oxidative response and in the promotion of cell hypertrophy and proliferation, p38 may provide an important link between oxidative stress and the re-entry into the cell cycle observed in AD. p38 is activated by bFGF treatment, and this activation is specifically required for the proliferative response to bFGF (41). bFGF is likewise elevated in AD and therefore may also induce cell cycle re-entry (34). Notably, p38 can independently reverse the Rb-mediated repression of E2F1, which is necessary for the expression of many genes that are required for cell cycle progression (10). The activation and cooperation of p38 and ERK, a protein that has also been shown by us

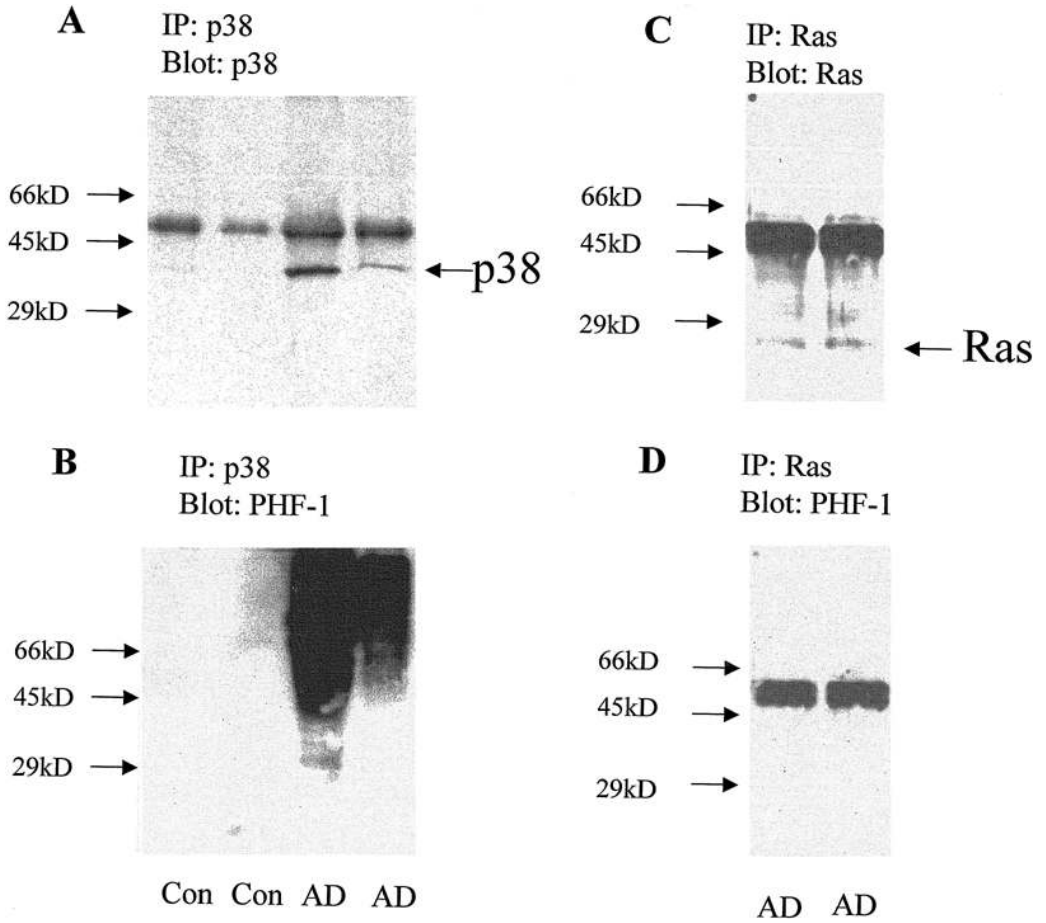

Fig. 6. p38 immunoprecipitates from 2 control (Con) and 2 AD (AD) cases were immunoblotted. The amount of p38 recovered from cases of AD is higher than from control cases (A). The band at 50 kD in (A) represents the heavy chain of rabbit IgG. Replicate blots probed with the PHF-1 antibody show that PHF-tau co-precipitates with p38 in AD cases (B). Similar results were also seen with TG-3 (data not shown). Data shown are representative of 4 control and 4 AD cases. As a control, no PHF-1 immunoreactivity are detected in Ras precipitates (D), in which Ras immunoreactivity is demonstrated (C). The blot used in (C) is directly probed with PHF-1 (D) without stripping, thus the heavy chain that appeared in (C) still exists.

and others to be activated in AD (42), is required for G-CSF-induced hemopoietic cell proliferation (40). Taken together, the activation of p38 kinase pathway in susceptible neurons in AD suggests that p38 probably plays an important role in mediating cell cycle re-entry and tau-phosphorylation when these cells are challenged by oxidative stress (43).

In conclusion, the co-localization of p38 and phospho-tau in AD demonstrated here by immunocytochemistry and immunoprecipitation indicates a key role for p38 in the pathogenesis of AD. Given the physiological role of p38, we suspect that this kinase is a key element linking tau phosphorylation, cell cycle re-entry, and oxidative stress in the disease process.

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