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Short Communication

Abnormal Expression of the Cell Cycle Regulators P16 and CDK4 in Alzheimer's Disease

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In this study, we demonstrate that two important regulators of the cell cycle, cyclin-dependent kinase-4 and its inhibitor p16, are increased in the brains of cases of Alzheimer's disease patients compared with age-matched controls. Both proteins are increased in the pyramidal neurons of the hippocampus, including those neurons containing neurofibrillary tangles and granulovacuolar degeneration. As p16 is not normally found in terminally differentiated neurons, it seems paradoxical that it is increased in Alzheimer's disease unless it is responding to increases in cyclin-dependent kinase-4 or other cell cycle regulators. Induction of the latter, a protein that signals re-entry and progression through the cell cycle, may itself be the consequence of a response to a growth stimulus. Re-entry into the cell cycle is likely deleterious in terminally differentiated neurons and may contribute to the biochemical abnormalities, such as oxidative stress and hyperphosphorylated τ protein, as well as the neuronal degeneration characteristic of the pathology of Alzheimer's disease. (Am J Pathol 1997, 150:1933–1939)

Association of cyclins with cyclin-dependent kinases (CDKs) results in the formation of complexes able to phosphoregulate a wide variety of substrates involved in the orderly progression through the cell cycle. Upon receiving external stimuli to divide, cells up-regulate CDKs and their cognate activating cyclins to orchestrate the numerous processes required for proliferation. Whereas progression through the cell cycle is controlled in part by the sequential synthesis and degradation of cyclins, growth arrest or terminal differentiation are controlled initially by CDK inhibitors including p16, p21, p27, and p57, which directly inhibit the cyclin-CDK complex.[1]

Transition from the G1 phase of the cell cycle and commitment to S phase/DNA replication is thought to be mediated by the association of G1 cyclin/CDK complexes such as CDK2/cyclin E and CDK4/D-type cyclins.[2] Phosphorylation of the retinoblastoma protein by CDK4/cyclin D, for example, enables activation of the E2F transcription factor and expression of genes required for DNA replication and proliferation. Active CDK4 complexes are negatively regulated by the competitive binding of the tumor suppressor gene product p16,[3] which disrupts the complex and indirectly inhibits E2F-mediated gene expression. In addition to driving cell proliferation, the expression of cyclin/CDKs have been implicated in the active process of programmed cell death in numerous *ex vivo* models,[4,5] including neuronal cells.[6,7] Cyclin D1 has been reported to be selectively induced in dying neurons,[8] and overexpression of p16 protects neuronal cells from apoptosis induced by the overexpression of cyclin-D-dependent kinase.[7]

In contrast to neurons during fetal brain development, the neuronal cells of the adult brain are terminally differentiated and do not divide. It is therefore

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significant that certain features of the pathology of Alzheimer's disease resemble events related to the transition through the cell cycle leading to the suggestion that Alzheimer's disease is a recapitulation of development.[9] For example, highly phosphorylated τ associated with the neurofibrillary tangle (NFT) lesions[10–12] is similar, if not identical, to that found during development[13–17] and correlates with cell division and differentiation[9,18] and mitosis.[19] Moreover, a p34/Cdc2-related kinase is associated with NFTs *in vivo*[20] and *in vitro* many cell cycle-related kinases including Cdc2, CDK2, CDK4, and CDK5 phosphorylate τ in a similar manner to that found *in vivo* in NFTs.[21,22] We hypothesized that the degeneration of terminally differentiated neurons in Alzheimer's disease might result from an inappropriate attempt to re-enter the cell cycle. To address this issue, we used antibodies specific for CDK4 and p16 to determine whether neurons in Alzheimer's disease showed abnormalities in the expression of cell cycle control elements.

Materials and Methods

Tissue Section Preparation

Brain tissue was obtained at postmortem from patients with histopathologically confirmed Alzheimer's disease[23] (n = 8) and non-Alzheimer age-matched controls (n = 4). Tissue was fixed for 16 hours by immersion in methacarn (methanol/chloroform/acetic acid, 6:3:1), dehydrated through graded ethanol and xylene solutions, and embedded in paraffin, and 6-μm-thick microtome sections were prepared.

Immunocytochemistry

After deparaffinization in xylene and rehydration through graded ethanol, endogenous peroxidase activity was inhibited by a 20-minute incubation in 3% H_2O_2. Nonspecific protein binding sites were blocked with 10% normal goat serum in Tris-buffered saline (50 mmol/L Tris/HCl, 150 mmol/L NaCl, pH 7.6), and immunostaining was by the peroxidase-antiperoxidase technique using 3,3′-diaminobenzidine as chromogen.[24] Antibodies to cell-cycle-related proteins p16 (clone N-20; Santa Cruz Biotechnology, Santa Cruz, CA) and CDK4 (C-22; Santa Cruz Biotechnology) were used, and adjacent tissue sections were immunostained with an antiserum to τ[25] to confirm the identity and location of neuronal abnormalities.

Both anti-p16 and anti-CDK4 are affinity-purified rabbit polyclonal antibodies raised against peptides corresponding to residues 4 to 23 of the amino terminus of human p16 protein and residues 282 to 303 of the carboxy terminus of mouse CDK4 (PSK-J3 p34) protein, respectively. Anti-p16 reacts with human p16 and shows no cross-reactivity with p15, p18, or other mitotic inhibitors, and anti-CDK4 reacts with mouse, rat, and human CDK4 but shows no cross-reactivity with other CDKs. Moreover, the specificity of these antisera for immunoblot and immunocytochemical analyses has been demonstrated by previous investigators.[26–28]

Absorption experiments were performed to verify the specificity of antibody binding. Briefly, the immunostaining protocol was repeated, except here using absorbed antisera generated by incubation of primary antisera with purified p16 or CDK4 protein (200 μg/ml) for 15 hours at 4°C. In parallel, absorptions of 1) anti-τ with p16 or CDK4 protein, 2) anti-p16 with CDK4 protein, and 3) anti-CDK4 with p16 protein were performed as controls against artifactual absorption.

Recombinant CDK4/p16 Protein Purification

CDK4 cDNA (kindly provided by E. Harlow) was cloned as a *Bam*H1 fragment into pBMS1[29] to produce GST-CDK4 fusion protein in Sf9 insect cells. A 500-ml Sf9 culture (10^6 cells/ml) in Sf-900 II SFM medium was infected at a multiplicity of infection of 3 and harvested 48 hours after infection by centrifugation at 1500 rpm in a Beckman J6-M2 centrifuge. The cells were resuspended in 100 ml of GST buffer (150 mmol/L NaCl, 16 mmol/L Na_2HPO_4, 4 mmol/L NaH_2PO_4) containing 0.1% Tween-20, 0.1 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 10 mmol/L β-glycerophosphate, and 0.1 mg/ml Pefabloc. Cells were lysed by sonication, and insoluble material was removed by centrifugation at 30,000 rpm for 30 minutes in a Beckman type 45 rotor. The Sf9 cellular lysate containing the GST fusion protein was added to 10 ml of glutathione-Sepharose beads (Pharmacia, Uppsala, Sweden). Unbound material was removed by washing with GST buffer. Bound GST-CDK4 fusion protein was eluted from the column with a buffer containing 50 mmol/L Tris/HCl (pH 8.0) and 10 mmol/L glutathione, dialyzed against 100 vol of storage buffer (50 mmol/L HEPES (pH 8), 50 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L $MgCl_2$, 25% glycerol) and stored at −80°C.

p16 cDNA was cloned as a *Bam*H1 fragment into the pGEX-2T vector (Pharmacia) for production of GST-p16 fusion protein in *Escherichia coli* DH5α cells. A 500-ml culture of DH5α cells transformed with pGEX-p16 was grown to OD_{595} of 0.4, and protein

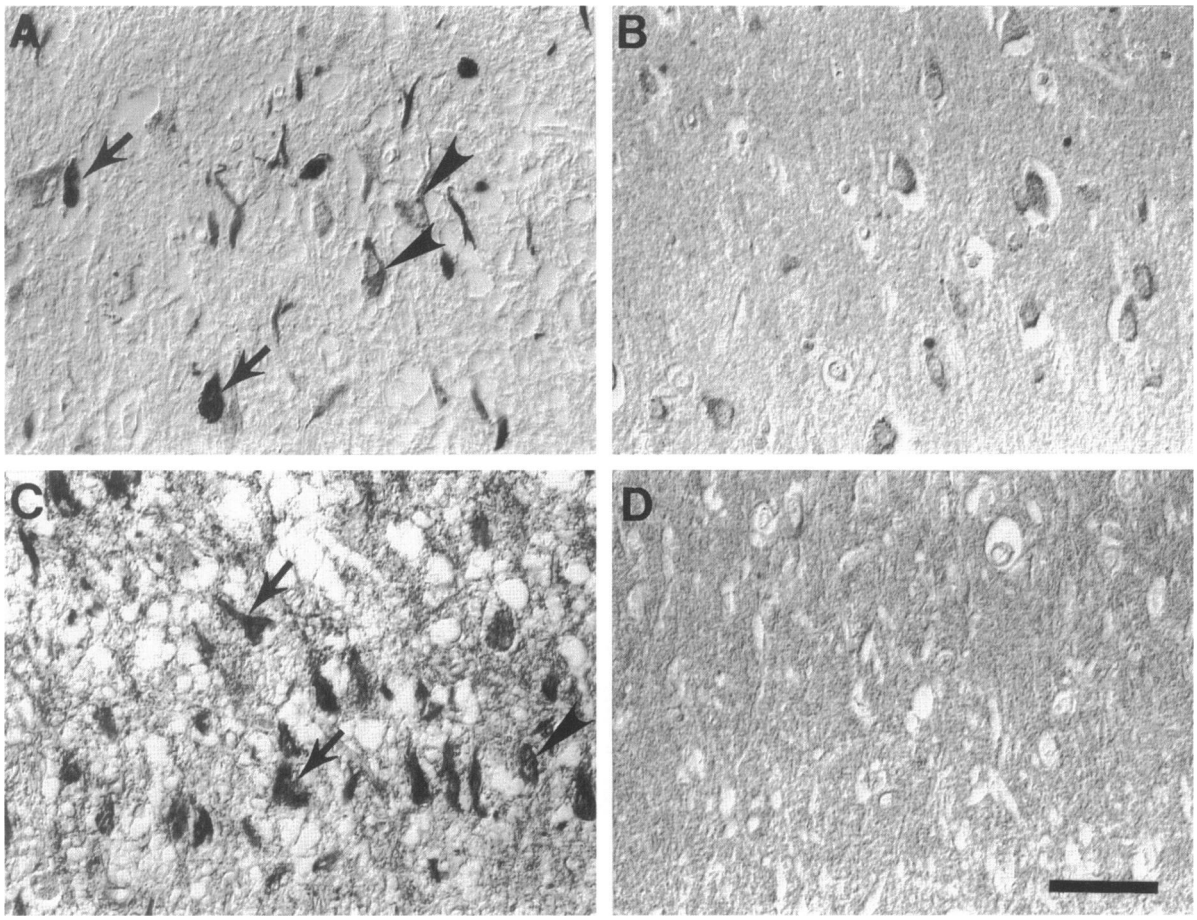

Figure 1. *Anti-P16* (**A** *and* **B**) *and CDK4* (**C** *and* **D**) *immunocytochemical labeling of Alzheimer* (**A** *and* **C**) *and age-matched control* (**B** *and* **D**) *hippocampus. Anti-p16 recognizes neurofibrillary tangles* (**arrows**) *and neurons lacking NFTs but containing granulovacuolar degeneration* (**A**, **arrowheads**). *Anti-CDK4 was also found in neurofibrillary-tangle-containing neurons but was predominantly found in extracellular NFTs* (**C**, **arrows**). *Control brain* (**B** *and* **D**) *by contrast shows only background p16 and CDK4 levels. Scale bar, 50 μm.*

expression was induced for 3 hours with 1 mmol/L isopropyl β-D-thiogalactopyranoside. GST-P16 fusion protein was purified on glutathione-Sepharose (Pharmacia) as described above.

Immunoblotting

Tissue from the gray matter of the temporal cortex of one Alzheimer and one control cases were homogenized 1:10 in 50 mmol/L Tris buffer, pH 7.6, with a Dounce homogenizer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P (Millipore, Bedford, MA) by standard procedures as previously described.[30,31] The enhanced chemiluminescence technique (Amersham, Arlington Heights, IL) was used for immunolabeling of transferred proteins.

Results

Although no specific structures were recognized by antibodies directed against p16 or CDK4 in control brain (Figure 1, B and D), in Alzheimer's disease brain, distinct pathological structures were immunolabeled (Figure 1, A and C). In both cases, NFT-containing neurons, identified morphologically by the presence of fibrils and immunocytochemically by co-localized τ staining on adjacent tissue sections, were recognized. Intraneuronal as well as extracellular NFTs were stained with p16 predominantly associated with the former and CDK4 with the latter (Figures 1, A and C and 2, A and C). Importantly, in the case of p16, pyramidal neurons not displaying overt fibrils were also stained. The staining was diffuse in the cytoplasm in addition to being strongly associated with granulovacuolar-type structures (Figures 1A and 2A). The barely detectable low dif-

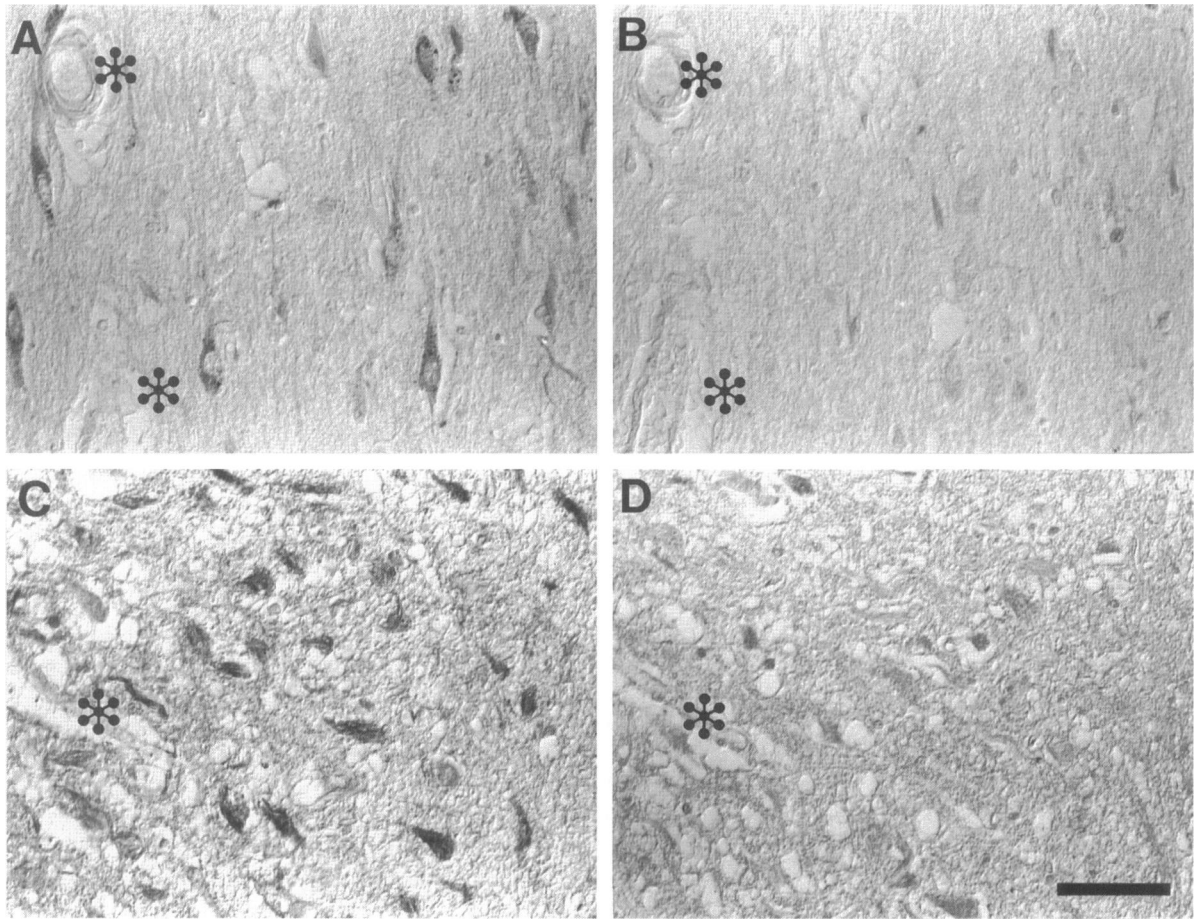

Figure 2. *The specificity of p16* (**A**) *and CDK4* (**C**) *immunoreactivity was demonstrated by absorption with purified p16* (**B**) *or CDK4* (**D**) *protein and consequent reduction in immunolabeling. *Landmark blood vessels in adjacent sections* (**A**, **B** *and* **C**, **D**). *Scale bar, 50 μm.*

fuse levels of p16 and CDK4 neuronal immunoreactivity in both Alzheimer and control brain was not immunospecific, and no other cell types were recognized with either of the antibodies (Figures 1 and 2). To confirm the specificity of p16 and CDK4 immunocytochemistry, several control experiments were performed in parallel. Absorption with the respective purified protein almost completely abolished immunostaining of the p16 antisera (Figure 2, A and B) or of the CDK4 antisera (Figure 2, C and D) whereas no blocking was observed by absorption of 1) the antiserum to τ with either p16 or CDK4 protein, 2) the antiserum to p16 with CDK4; or 3) antiserum to CDK4 with p16 (results not shown). Additionally, no specific staining was seen with rabbit antibodies against irrelevant epitopes or when either of the primary antibodies were omitted (results not shown).

The close overlap of p16 and CDK4 immunoreactive profiles seen in Alzheimer's disease with other antigenic markers such as τ led us to conduct an amino acid homology search for possible cross-reactive proteins. Using a protein sequence database (Swiss & PIR and Translated Release 97) to compare the original immunogenic peptides (see Materials and Methods), we found no homologous proteins that are known components of NFTs (including τ, ubiquitin, hsp70, MAP2, tubulin, heme oxygenase-1, or neurofilament protein (heavy, medium, and light subunits)) above a threshold of 50% using a K-Tuple of 2. Furthermore, direct sequence homology analysis using the Clustal method with a PAM250 residue weight table shows no significant regions of homology between the p16 and CDK4 peptides and these proteins.

Immunoblot analysis revealed an anti-CDK4 immunoreactive band running with an approximate molecular weight of 34,000 in the Alzheimer and control brain homogenates (result not shown). Furthermore, anti-CDK4 labeled an immunoblot preparation of recombinant GST-CDK4 fusion protein but not the GST-p16 fusion protein. However, although anti-p16 does label immunoblot preparations of GST-p16 recombinant protein, we were unable to detect any immunoreactive protein bands in either control or

Alzheimer gray matter homogenates. We suspect that this likely reflects either insufficient sensitivity of our immunoblot technique, lability of p16 in postmortem brain homogenates, or differential fractionation of the p16 protein. The last possibility is not without precedent as many protein components of the pathological lesions found in Alzheimer's disease are insoluble due to cross-linking and consequently would not be present in the homogenates used here.[32]

Discussion

In this study, we demonstrate increased p16 and CDK4 immunoreactivity in neurons in Alzheimer's disease but not in the same neuronal cell populations in control brain sections. The presence of CDK4, a cell-cycle-regulated kinase, indicates that these terminally differentiated cells may have received or intrinsically developed a stimulus to proliferate. The presence of p16, an inhibitor of CDK4-cyclin activity and thus a growth-inhibitory molecule, suggests that the neurons are responding to inappropriate growth signals. The signal for CDK4 induction is unclear, and there are three explanations that could account for this stimulation: first, it could occur due to an intracellular defect and deregulated gene expression; second, inappropriate expression of a growth factor in the local environment of the neuron may be responsible; or third, a growth-inhibitory molecule may have been lost. One possible intracellular defect affecting neurons in Alzheimer's disease is oxidative stress, which is of importance because a balance of free radicals is required in the cell cycle, and perturbations of this balance, either by increasing or decreasing free radicals, results in cell cycle abnormalities.[33–36] Interestingly, the overexpression in cells of heme oxygenase-1, a potent antioxidant that is increased in Alzheimer's disease in a manner that parallels the regional susceptibility and pathology,[30,31] results in growth arrest associated with decreased entry into S phase.[37] Whether oxidative stress and cell cycle abnormalities are dependent or coincident features *in vivo* will require further study. Candidates for the second mechanism are growth factors such as nerve growth factor (NGF), basic fibroblast growth factor (bFGF) or transforming growth factor-β1 (TGF-β), which are elevated in Alzheimer's disease brains.[38–41] It is not clear from our data whether the stimulus to proliferate in Alzheimer's disease neurons is attributable to an increase in NGF or bFGF levels. However, such a possibility may be inferred from *in vitro* studies demonstrating induction of CDK4 in PC12 cells in response to NGF and a drop in kinase activity of the CDK4/cyclin D1 complexes due to the induction of a CDK inhibitor.[42,43] Therefore, in light of our findings, the therapeutic strategy of using NGF[44] might prove deleterious to patients with Alzheimer's disease and may in fact exacerbate disease progression.

It is interesting to note that overexpression of the CDK4-activating cyclin D1 will variously lead to hyperplastic growth[45] or growth arrest in G2/M phase,[46] depending on the cell type or, in the case of neuronal cells, apoptosis.[7] This latter finding is of importance, as one might not expect to see high levels of apoptotic cells in a chronic neurodegenerative condition, bearing in mind that apoptosis generally leads to cell death within 24 hours. Our finding of increased p16 in neuronal cells could indicate that such processes are being held in check for the most part but that additional increases in CDK4 or other cell cycle regulators eventually overrides this process and leads to neuronal death. Indeed, such a scenario is supported, in light of the known biological activities of CDK4 and p16, by the present study showing that p16 is mainly associated with NFTs at their inception, yet CDK4 is predominantly found in neurons that have succumbed to pathology.

In proliferating cells, the microtubule-associated protein τ is phosphoregulated in a cell-cycle-dependent manner during mitosis.[19] Similarly, growth arrest of neuroblastoma cells before mitosis with the tubulin-depolymerizing agent nocodazole results in τ phosphoepitopes equivalent to those found in the Alzheimer's disease brain.[47] τ phosphoregulation is believed to be mediated by the brain-specific CDK-5,[48] yet other serine/threonine kinases including CDK 2, MAP kinase, and GSK-3 have been shown to phosphorylate τ in a manner similar to that found isolated from Alzheimer's disease paired helical filaments,[22] implicating cell division components in Alzheimer's disease and leading to the hypothesis that aberrant activity of CDKs is responsible for the altered phosphorylation state of τ.[18] However, as there is also an apparent induction of p16, possibly as a result of a feedback loop activated by CDK4-cyclin activity,[49] the cell division cycle cannot proceed further. Thus, τ remains hyperphosphorylated, not as a result of deregulated kinase activity but rather as a phenotype of a phospho-τ intermediate in a G2/M phase cell. Whether chronically expressed sublethal levels of CDK4 or other kinases are responsible bears further study, although such a scenario is supported by studies showing an induction of a second cell cycle inhibitor, p21, in Alzheimer's disease, which, like p16, is not present in the neurons of

control brain (A. McShea and M. A. Smith, unpublished findings) and previous investigations demonstrating proliferative markers including p105, Ki67, and mpm2 in neuronal cells in Alzheimer's disease.[47,50,51]

In conclusion, we present evidence of cell cycle dysfunction in Alzheimer's disease. The implications of this are multiple as regards neuronal degeneration and lesion formation. For the latter, cell-cycle-mediated phosphorylation of τ might be important for the formation of NFTs. Also of note is the observation that the phosphorylation and metabolism of amyloid precursor protein, the precursor of the major protein constituent of senile plaques, is also regulated in a cell-cycle-dependent manner.[52] In future studies, it will be important to determine not only the proliferative state of neuronal cells but also the phase of the cell cycle at which growth arrest occurs to further understand the significance of cell cycle abnormalities in the pathogenesis of Alzheimer's disease.

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