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Commentary

Detection of Actin Cleavage in Alzheimer's Disease

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Alzheimer's disease (AD) is defined by the classical pathological hallmarks of senile plaques and neurofibrillary tangles (NFTs). Whether these are a cause or a consequence of the disease is still under some controversy, but the difficulty in understanding the relationship between these evident pathological alterations in AD brains has propelled interest in other possible mechanisms for AD. It is highly suspected that cognitive decline is linked to neuronal cell death. Growing interest in apoptosis as a molecular basis for neurodegenerative diseases has not escaped attention in the Alzheimer's field of research. The manuscript of Yang et al[1] in this issue describes a novel approach to the detection of apoptosis-related events in AD. To place this study in perspective, a brief review of other findings relating to apoptosis and AD is provided.

Neuronal Loss and Alzheimer's Disease

It has been known for some time that AD pathology encompasses selective neuronal synaptic loss and cell death (reviewed in Ref. 2). Recently, the technique of stereological quantitation has enabled Hyman and colleagues to identify neuronal loss in the entorhinal cortex as a very early event of Alzheimer's disease, one that increases with severity of illness.[3] In contrast, neuronal loss in the superior temporal sulcus of AD patients occurs as a later event in the progression of the disease and increases with the duration of the disease and with the severity of cognitive impairment. Although the NFTs also increase with the duration and severity of disease, neuronal loss is markedly enhanced and does not correlate with the presence of senile plaques.[4] In contrast, neuronal loss is not detected in nondemented aged individuals.[3,4] Therefore, in some selective areas of the brain, neuronal loss precedes strong clinical manifestation of the disease. The initial symptoms of cognitive deficit suggest neuronal dysfunction, and the progressive loss of these functions indicates that neuronal death is apoptotic rather than necrotic. The hypothesis that apoptotic neuronal cell death occurs in Alzheimer's or any other neurodegenerative disease is particularly attractive as apoptosis can be prevented by general inhibitors in many situations.

Apoptosis and Alzheimer's Disease

The evidence for apoptosis in AD is varied and sometimes controversial. The absence of techniques to detect apoptosis in live tissue leaves us with ambiguous data. Postmortem tissue presents many problems. First, artifacts can arise with increasing length of retrieval of postmortem brains. The standard *in situ* method of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) used for the detection of DNA fragmentation in apoptotic cells is particularly sensitive and will also detect DNA fragmentation occurring during necrosis especially in long-term postmortem interval tissues. Even in the best preserved tissues, it is likely that most apoptotic cells will undergo phagocytosis and present negative results when tested for DNA fragmentation. Second, no one has yet investigated the molecular mechanisms of human neuronal apoptosis or cell death. We assume that the mechanisms gleaned from human neuroblastoma cell types or from rodent primary cultures will be the same in human adult brains. It is likely that the human neuron, which is terminally differentiated at an early age yet long lived, has a special adaptive response to an apoptotic stimuli or insult. Therefore, a challenged neuron may not display characteristic apoptotic morphological events in the earlier phases of apoptosis and may avoid detection by standard methods. In addition, the apoptotic program may be different depending on the neuronal subtypes, and some neuronal subsets may have increased protection against apoptosis. This assumption is supported by the fact that only subsets of neurons are affected in neurodegenerative disorders. Therefore, the presence of one marker for apoptosis may not necessarily indicate neuronal apoptosis. The apoptotic program may also change depending on the insult, especially in the initial and pre-commitment stage of apoptosis. In addition, the proteins expressed in the initial phases of apoptosis may

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well be expressed in other situations not involving cell death.

There is strong *in vitro* support for the role of apoptosis in AD. Mutations in the three genes linked to AD, amyloid precursor protein, presenilin I, and presenilin II, induce apoptosis in transfected cultured cells.[5–8] In addition, presenilin II was shown to be a substrate for the apoptotic effector protein, caspase-3, resulting in alternate carboxyl-terminal cleavage and the production of an insoluble carboxyl-terminal fragment of presenilin II.[9] The amyloid β-peptide, increased in both sporadic and familial AD associated with either amyloid precursor protein, presenilin I, or presenilin II mutations, induces neuronal cell death through an apoptotic mechanism.[10–20] In human primary neuron cultures, amyloid β-peptide toxicity weakens the anti-apoptotic protection of the neurons, and a second insult is required to induce cell death.[21]

However, it has been more difficult to assess the presence of apoptotic neurons *in vivo*. Using tools that are available and known to identify apoptosis in cell cultures, many individuals have tested the apoptotic hypothesis in AD. The initiation of cell death normally includes immediate-early gene expression. The transcriptional factors, c-jun and c-fos, have been intensively studied for their role in neuronal apoptosis, and c-jun seems likely to induce neuronal cell death.[22,23] Reports show increased c-jun and c-fos expression in the brains of AD patients as compared with age-matched controls.[24,25] However, it is known that immediate-early genes are also expressed during neuronal cell activation and can be influenced by various drugs.[26,27] Therefore, in the absence of additional markers of neuronal cell death, the expression of c-jun and c-fos in neurons of AD does not produce conclusive evidence of apoptosis. There is evidence that genes that are normally responsible for cell cycle traverse are re-expressed in terminally differentiated apoptotic neurons. Cyclin D1 is particularly studied in neurons and has been shown to increase in primary neurons undergoing apoptosis.[28,29] In AD but not in normal controls, the MPM-2, an antibody to mitotic phosphoepitopes, and cdc2 and cyclin B1 kinase antibodies, stain NFTs, neuritic processes, and neurons.[30,31]

After the initiation of apoptotic cell death, the progression to apoptosis will depend on the expression of the regulators of cell death. A group of proteins known as the bcl-2 and bcl-2 related proteins have been identified that either promote or protect against apoptosis.[32] The expression of these proteins in human adult brains could control the response to the initial insult. These are interesting candidates to explain the age and central nervous system (CNS) dependence of AD. For example, bcl-2 expression decreases with age in the CNS but not in the peripheral nervous system.[33] Bcl-2 expression in neuronal culture systems protects against a wide variety of insults.[34–37] On the other hand, other members of this family, such as bax protein, promote cell death.[38] In AD, bcl-2 and bax are increased in neurons except in NFT-positive cells.[39,40] Others have observed increased bcl-2 levels in glial cells.[41]

Irreversible commitment to apoptosis occurs with the activation of one or a group of mammalian cysteine proteases called caspases.[42] Caspases are expressed as proenzymes and are proteolytically activated. Caspases cleave carboxyl-terminal to an aspartic acid and generally recognize four amino acid substrate sites as their target. These proteins can be subdivided in three classes based on their specific substrates.[43] Caspase-3, also known as apopain, CPP32, or YAMA is particularly important for developmental neuronal cell death.[44,45] Caspase-3 null mice have increased numbers of neurons that normally would be eliminated during development.[46] The fact that other cell types are not affected in the caspase-3 null mice indicates that caspase-3 can be targeted to prevent cell death in neurons without causing increased proliferation of the neighboring cell types such as astrocytes or microglia, a situation that would result in a detrimental treatment for neurodegenerative diseases. Caspase-3 has many cellular substrates: poly(ADP-ribose) polymerase (PARP),[44,45] catalytic subunit of the DNA-dependent protein kinase,[47] 70-kd subunit of the U1 small ribonucleoprotein,[48] and actin.[49–51] At this stage, the cell undergoes a severe morphological change consisting of DNA fragmentation, nuclei condensation, and nuclear breakdown.

Actin and Apoptosis

Recently, many studies have concentrated on actin as a substrate of active caspase. Actin is a substrate of caspase-1 (interleukin-1β-converting enzyme) and caspase-3 (YAMA, apopain, or CPP32).[49–51] The full-length 45-kd actin contains two caspase substrate-specific sites at amino acids 11 and 244 and can be cleaved into 41-kd, 30-kd, and 15-kd fragments. Cleavage of the actin is, however, minimal, and most of the time, down-regulation of the level of full-length actin cannot be detected even in the presence of ongoing proteolytic cleavage of actin. In human neutrophils, others have found that actin cleavage could not be inhibited completely with caspase inhibitors but were with acetyl-leucine-leucine-norleucinal, a proteasome and calpain inhibitor. These studies suggest that the activation of other proteases in apoptotic cells may be responsible for the cleavage of actin.[52] The effect of caspases on actin cleavage could very well be cell type specific. For example, some cell lines do not produce *in vivo* actin-cleavable activity.[50,53] Gelsolin, an actin-regulatory protein, can prevent actin cleavage, but this activity is indirect as it is shown that gelsolin actually inhibits caspase-3 activation and apoptosis in some cells.[54] Gelsolin itself is reported as a substrate of caspase-3 in neutrophils, suggesting that it may be responsible for morphological alterations observed during apoptosis.[55]

Proteolytic cleavage of actin precedes DNA fragmentation, raising an important question as to its role in the apopotic process of the cell. Actin maintains morphological integrity, and it is assumed that cleavage will result in a weakened cytoskeleton. However, the full-length 45-kd protein is still very abundant in cells where actin fragments are detected, even if in some apoptotic cells actin mRNA itself is reduced and precedes actin cleavage and

disruption of the cytoskeleton.[56] Most interesting is the role of actin in the inhibition of DNAse I, the enzyme responsible for DNA fragmentation in apoptotic cells (reviewed in Ref. 57). Kalayar and colleagues have elegantly demonstrated that the proteolytic fragments of actin have lost DNAse I inhibitory activity.[58]

In their manuscript, Yang et al[1] look at actin breakdown as a tool to determine earlier neuronal apoptosis in AD. They raised an antibody specific to the five amino acids of the carboxy-terminus of the 30- to 32-kd actin fragment. On Western blots, their antibody, named fractin, detects the fragment in apoptotic differentiated human neuroblastoma SY5Y cells, as well as that cleaved *in vitro*. The activity is specific for apoptotic cells and is not seen in necrotic SY5Y cells despite considerable DNA breakdown induced by H_2O_2. The actin fragment is detected early in apoptotic SY5Y and likely precedes DNA fragmentation. The caspase-3 inhibitor DEVD, but not the caspase-1 and -4 inhibitor YVAD, inhibits the actin cleavage. Therefore, this tool appears highly specific in apoptotic neuron-like cells.

When used for the study of apoptosis in AD, fractin immunoreactivity is detected only in tissues obtained from AD patients. Two early-onset cases show more reactivity than four late-onset cases, but all six cases are positive for fractin immunoreactivity. In contrast, in five normal cases ranging in age from 46 to 93 years, fractin immunoreactivity was not detected except for limited immunoreactivity in a clinically normal 64-year-old with high plaque numbers. Although TUNEL and fractin staining overlap, fractin appears to be more restricted than TUNEL staining, which can be artificially high in long-interval postmortem tissue. Therefore, fractin staining has a distinct advantage over the detection of DNA fragmentation in the study of neurodegenerative processes.

It is interesting that the fractin immunoreactivity is detected in areas containing abundant amounts of senile plaques. Although neuronal loss in AD does not correlate well with plaque density,[34] these results suggest that the neurons that are present in this area are not all normal and may be undergoing apoptosis. Surprisingly, the immunoreactivity is found both in neurons and microglia. In neurons, the immunoreactivity is not restricted to cell soma but is also seen at a distance in neurites, providing an additional tool to detect abnormal cell processes that are not obvious under classical pathological examination. The authors provide logical and interesting explanations for the presence of fractin staining in microglia.

It is still not clear that all cells immunopositive to fractin will undergo apoptosis in the brain. We must be careful in the interpretation of these results and remain aware that human neuron apoptosis in adult brains remains unexplored. It is possible that human neurons have developed a highly efficient system to control neuronal apoptosis, especially as these cells are nonrenewable, and the organism suffers irreversible damage upon their death. Whereas apoptosis of dividing cells or developmental neuronal apoptosis may occur fairly rapidly within days, human adult neurons may undergo this process for months or even years by activating different backup systems to prevent neuronal loss, at least up to a point. However, if fractin does identify apoptosis-related events, as seen in the neuroblastoma cell line, this antibody provides a distinct advantage over endpoint markers of apoptosis. The development of other protein-specific caspase substrate site antibodies or even active caspase-specific antibodies will eventually help clarify some of these issues. The main problem remains the inability to study the ongoing process of neuronal dysfunction and cell death in an affected brain. Determination of the state of the neurons at the end of the disease process giving a cross-sectional view of an ongoing process will not answer our questions of whether apoptosis is primary and crucial in AD. Nevertheless, our attempts to understand the apoptotic process in neurodegenerative diseases must not be hampered. By inhibiting apoptosis in neurons at an early stage of the disease, we may have a fighting chance to return these neurons to normal function.

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