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A Potential Role for Apoptosis in Neurodegeneration and Alzheimer's Disease

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Abstract

Previous studies have shown that β-amyloid (Aβ) peptides are neurotoxic. Recent data suggest that neurons undergoing Aβ-induced cell death exhibit characteristics that correspond to the classical features of apoptosis, suggesting that these cells may initiate a program of cell death. This chapter explores the criteria and precautions that must be applied to evaluate mechanisms of cell death in vitro and in vivo, discusses the evidence supporting an apoptotic mechanism of cell death in response to Aβ in cultured neurons, and describes potential correlations for these findings in the Alzheimer's disease brain. In addition, cellular signaling pathways that may be associated with apoptosis in response to Aβ are examined, and support for apoptosis as a mechanism of cell death for other neurodegeneration-inducing stimuli (e.g., oxidative injury) is described. The connection of multiple stimuli that induce neuronal cell death to an apoptotic mechanism suggests that apoptosis could play a central role in neurodegeneration in the brain.

Key Words: Programmed cell death; immediate early gene; proto-oncogene; c-jun; c-fos, plaques; neurofibrillary tangles; DNA fragmentation; oxidative injury.

Introduction

Neuronal loss is a prominent feature of aging and Alzheimer's disease (AD) (Terry et al., 1981; Coleman and Flood, 1983; Mountjoy et al., 1983; Mann et al., 1985). This cell loss is a function of adverse conditions in the intracellular and extracellular environment of the AD brain, and specific stimuli that can promote cell death. In order to understand and control neuronal loss it is necessary to identify the specific stimuli that affect neuronal cells in AD, and relate these stimuli to the intrinsic properties of individual cell types. A better understanding of these features is essential for developing protective strategies and therapeutic interventions for this disease. The first step in this process is to identify the specific stimuli that contribute to neuronal damage and cell death in AD.

In addition to neuronal degeneration and the formation of neurofibrillary tangles (NFTs),

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the pathological hallmarks of Alzheimer's disease (AD) include the accumulation of β-amyloid (Aβ) in extracellular deposits called plaques. The Aβ deposited in plaques is a 42–43 amino acid protein (Glenner and Wong, 1984; Masters et al., 1985a; Prelli et al., 1988; Gowing et al., 1994) derived from the cleavage of the amyloid precursor protein (βAPP) (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). Until recently, plaques were considered to be the end result of neurodegenerative processes in AD, however, it is increasingly clear that plaques are active structures that exist as multiple types and exhibit a progression of changes (Cotman et al., 1991; Cummings et al., 1993a,b). Consequently, we and others have suggested that the accumulation of Aβ in AD is a pivotal event in the progression of pathology, and that Aβ might be one of the stimuli that contribute to AD pathology (Cotman et al., in press).

Both a gene dosage effect involving βAPP and the overproduction and/or abnormal processing of βAPP have been suggested as the cause of Aβ accumulation in AD (Goldgaber et al., 1987; Kang et al., 1987; Tanzi et al., 1987; Frangione, 1989; Neve et al., 1990), and mutations of the APP gene have been linked to some types of familial Alzheimer's disease (FAD) (Chartier et al., 1991; Goode et al., 1991; Murrell et al., 1991; Mullan et al., 1992b). However, the identification of several alternate gene loci involved in FAD, including one on chromosome 14 (Mullan et al., 1992a; Schellenberg et al., 1992, 1993; St. George-Hyslop, 1992; Van et al., 1992; Nechiporuk et al., 1993), make it unlikely that there is a direct connection between all forms of FAD and mutation of the βAPP gene *per se*. Additionally, the apparently normal secretion of soluble Aβ peptides has been reported by several groups (Haass et al., 1992; Shoji et al., 1992; Busciglio et al., 1993; Vigo et al., 1993), suggesting a pathway for the deposition and regulation of Aβ that does not require the misprocessing of APP.

Furthermore, a strong role has been identified for at least one other gene in nonfamilial AD cases, apolipoprotein E (Apo E) (Diedrich et al., 1991; Namba et al., 1991; Corder et al., 1993; Mayeux et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993a,b). Apo E has been shown to bind Aβ in vitro (Strittmatter et al., 1993b; Wisniewski et al., 1993) and the Apo E4 allele, a risk factor for the development of AD pathology, promotes the formation of Aβ fibrils in solution. The Apo E3 allele does not promote Aβ fibril formation as effectively (Wisniewski et al., 1994). In addition, Apo E4 is associated with an increased degree of Aβ deposition in brain (Rebeck et al., 1993; Schmechel et al., 1993). These findings suggest that there may be multiple sources of genetic risk for AD and multiple pathways that can contribute to AD pathology, and imply that Aβ may be a common denominator for some of these pathways.

The focus of this review is to examine the mechanisms that can cause neuronal damage and cell loss in AD, drawing on recent work with Aβ as a stimulus for these events in vitro. We and others have shown that Aβ induces cell death via apoptosis in cultured neurons. We will discuss the characteristics of apoptosis in these cells, the events initiated by Aβ in vitro, and compare these in vitro observations to the properties of neurons vulnerable to neurodegeneration in the AD brain. Finally, we will explore other insults that can lead to the initiation of an apoptotic pathway of cell death in neuronal cells, such as oxidative injury. These data are a part of a growing body of evidence supporting the hypothesis that apoptosis may be a fundamental mechanism of cell loss in AD. It is hoped that a better understanding of the biological effects of Aβ, and the mechanisms that mediate these effects, will lead to new strategies for therapy in the future.

Ab Toxicity

Initial investigations into the action of Aβ in vitro revealed that Aβ 25–35, 1–40, and 1–42 stimulate process outgrowth and enhance survival over short time intervals in cultured hippocampal neurons (Whitson et al., 1989; Yankner et al., 1990). Concurrent studies para-

doxically showed that these peptides could also induce neurodegeneration in culture (Yankner et al., 1989, 1990; Pike et al., 1991a,b; Behl et al., 1992; Takadera et al., 1993), suggesting that Aβ peptides were capable of exerting multiple bioactivities, i.e., enhancing growth or inducing toxicity. Further studies have clarified this issue, demonstrating that the in vitro activity of Aβ peptides is dependent on the assembly state of these peptides (Pike et al., 1991a,b, 1993; Busciglio et al., 1992; Mattson et al., 1993b).

Aβ deposits in the AD brain frequently demonstrate positive staining for Congo red and thioflavine S, suggesting an aggregated state, and Aβ isolated from AD brains shows patterns characteristic of aggregation on reducing gels (Masters et al., 1985a; Selkoe et al., 1986). Similarly, after the incubation of synthetic Aβ 1–42 peptides for several days in vitro, sheet-like structures are visible at the light microscopic level and an altered electrophoresis profile is evident on reducing gels, indicating the formation of insoluble aggregates (Pike et al., 1991b; Burdick et al., 1992). These aggregated Aβ peptides demonstrate positive Congo red and thioflavine S staining similar to that observed in the AD brain (Hilbich et al., 1991; Burdick et al., 1992). Critically, Aβ peptides that exhibit aggregation as assessed by electrophoresis or sedimentation assays demonstrate toxicity in cultured neurons, whereas Aβ peptides that do not exhibit an aggregated state by these measures do not exhibit toxicity (Pike et al., 1991a, 1992). In addition, peptide components of Aβ that do not contain the hydrophobic-rich end of Aβ, such as amino acids 1–28, do not form stable aggregates (Burdick et al., 1992; Pike et al., 1993) and are not neurotoxic (Fig. 1) (Pike et al., 1993). These studies have shown that Aβ neurotoxicity in vitro is correlated with assembly into an aggregated state, and suggest that this state is reflective of that present in congo- or thioflavine-positive accumulations of Aβ in the AD brain.

In correspondence with this suggestion, an in vivo correlate for Aβ toxicity in vitro has been observed in some studies. Injection of aggregated Aβ peptides, or plaque cores isolated from AD tissue, into rat brain in these studies results in the induction of some mark-

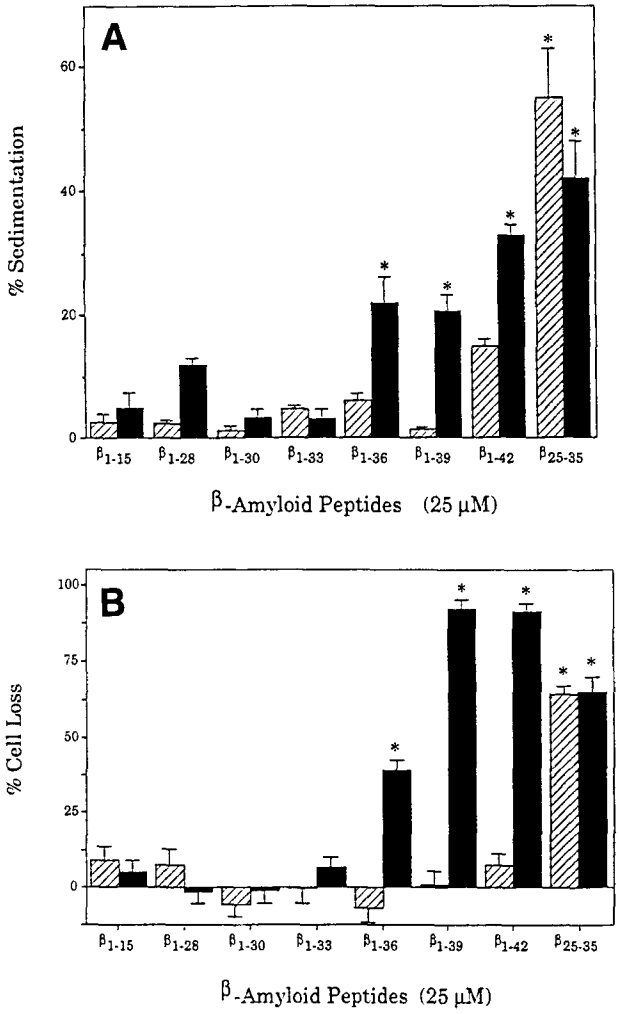

Fig. 1. **(A)** Percentage of sedimentation as a measure of the degree of aggregation under culture-like conditions for the Aβ peptides tested increasing sedimentation reflects an increasing degree of aggregation. Hatched bars represent newly solubilized peptides whereas solid bars represent aged peptides (peptides allowed to aggregate for several days before experimental use). Each bar is the mean of 3–4 observations. $*p < 0.05$, one-way ANOVA. **(B)** Percent cell loss for the peptides shown in (A). Short-term hippocampal cultures were treated with peptides for 24 h and cell survival assessed by trypan blue exclusion. $*p < 0.05$ relative to untreated controls, nested ANOVA. Note the correspondence between peptides that exhibit aggregation in (A) and neurotoxicity in (B).

ers of AD-type pathology and neurodegeneration (Frantschy et al., 1991; Emre et al., 1992; Kowall et al., 1992). These observations further suggest that Aβ might serve as a stimulus leading to altered growth and/or neuronal degeneration in AD; however, it will be essential to determine the conformational state of the injected peptides to fully evaluate these issues of Aβ toxicity in future in vivo studies.

Interestingly, other peptides that can assemble into a β-pleated sheet conformation, such as the prion protein, also mediate effects similar to those of Aβ in cultured neurons (Forloni et al., 1993a; Selvaggini et al., 1993; Tagliavini et al., 1993; DeGioia et al., 1994). However, not all β-folded peptides trigger neurodegeneration. For example, amylin 20–29 forms a β-pleated secondary structure but does not induce neuronal dystrophy or cell death (Pike et al., 1993). In contrast, aggregated full-length amylin (1–37) does induce toxicity, both in cultured neurons (May et al., 1993), and in cultured pancreatic islet cells (Lorenzo et al., 1994). Furthermore, amylin-mediated cell death in islet cells occurs via an apoptotic pathway (Lorenzo et al., 1994). Investigation of Aβ variants and other peptides such as these may lead to additional clues to the mechanism of action of Aβ on cells, both in vitro and in the AD brain.

Parallels Between In Vitro Models of Ab Action and the AD Brain

To understand the stimuli and conditions that contribute to neuronal dysfunction and cell death in AD, it is essential to make comparisons between in vitro paradigms and observations in vivo. There are a number of cases where this strategy has already been beneficial in assessing the validity of in vitro models. For example, in parallel with classical AD pathology, Aβ induces the formation of dystrophic neurites in cultured neurons (Pike et al., 1992). Observations similar to these have recently been reported by a second group (Fraser et al., 1994). Similarly, reductions in glucose metabolism have been suggested to contribute to neurodegeneration in AD (Haxby and Rapoport, 1986; McGeer et al., 1986; Hoyer et al., 1988; Beal et al., 1993; Goto et al., 1993), and Aβ has been shown to exacerbate neurodegeneration in cultured neurons when glucose levels are reduced (Copani et al., 1991). Finally, previous studies have shown that GABAergic cells are resistant to neurodegeneration in AD (Spillane et al., 1977; Rossor et al., 1982; Smith et al., 1983; Mountjoy et al., 1984; Lowe et al., 1988). In correspondence with these findings, GABAergic cells have recently been shown to be resistant to Aβ toxicity in vitro (Pike et al., 1993).

Perhaps even more interestingly, observations in cultured neurons treated with Aβ have been paralleled in vivo. For example, Aβ has previously been shown to increase the susceptibility of neurons to excitotoxicity in both rodent (Koh et al., 1990) and human (Mattson et al., 1992) neuronal cultures, and these observations have recently been confirmed in the rodent brain (Dornan et al., 1993). This series of parallels between in vitro and in vivo studies supports the hypothesis that Aβ plays a role in AD pathology. Furthermore, these studies imply that Aβ can interact with other cellular insults or risk factors and may exacerbate other pathological mechanisms in AD.

Identification of the importance of Aβ assembly state for the biological activity of this peptide, and the potential for interaction of Aβ with other brain insults, has opened up several areas of investigation. While these data resolve the controversy over the toxicity of Aβ in vitro and support the idea that Aβ deposits may be a central aspect of AD pathology, they also bring into focus the question of the mechanism of Aβ toxicity in these studies. This question has several levels. Is Aβ directly or indirectly neurotoxic? By what cell death pathway do cells exposed to Aβ die? Do cellular signaling pathways play a role in the response to Aβ, and if so, what aspects of the response to Aβ do they mediate? Can the mechanism of Aβ-induced cell death be modulated or controlled?

What Is the Pathway of Ab-Mediated Toxicity: Apoptosis vs Necrosis

The first issue we have attempted to address is the pathway of cell death in response to Aβ. There are two basic pathways or mechanisms of cell death: necrosis and apoptosis. Necrosis is characterized by dilation of the endoplasmic reticulum and mitochondria, and rapid loss of plasma membrane integrity. As a result of cell lysis, there is a characteristic pattern of inflammation and a secondary cycle of damage to surrounding tissues. During this process the cellular DNA is randomly degraded, resulting in a diffuse smear of DNA fragments that is observable following gel electrophoresis.

Apoptosis is classically defined on the basis of morphological criteria (Kerr et al., 1972; Wyllie et al., 1980). According to these criteria, apoptosis is characterized by cell surface protuberances (blebs), chromatin condensation, and nuclear shrinkage (pyknosis), followed by fragmentation of the nucleus (karonexis) into multiple bodies. In addition, there is dispersion of the polyribosomes and cell shrinkage, however, in contrast to necrosis, the endoplasmic reticulum remains relatively intact and the mitochondria appear normal and unswollen (Wyllie et al., 1980; Arends and Wyllie, 1991). Importantly, plasma membrane integrity is maintained until late in the course of apoptosis and the membrane-bound cellular remains are removed by macrophages, thus minimizing inflammation (Wyllie et al., 1980).

In addition to these morphological events, there may be an activation of endogenous endonucleases resulting in the cleavage of cellular DNA into oligonucleosome-length fragments, observable as a ladder following agarose gel electrophoresis (Wyllie et al., 1980). Owing to the controlled nature of this process, it has been suggested that apoptosis is a mechanism that is well-suited to the elimination of old, damaged, or "unwanted" cells (Wyllie et al., 1980), and correspondingly, that apoptosis may serve a protective role in disease (Bursch et al., 1992). Alternatively, we have suggested that apoptosis may in fact participate in chronic disease conditions such as AD (Loo et al., 1993).

Necrosis has been suggested to reflect "passive" cellular degeneration as a result of traumatic injury, and circumstances in which an insult causes an acute loss of cellular regulation and function (Kerr and Harmon, 1991). Apoptosis has a different set of biological implications, in that the cell is thought to actively participate in its degeneration in a pathway of gene-directed cellular suicide. Thus, a cell may initiate an apoptotic program of cell death in response to a specific signal in the form of a toxin or subacute insult, or via a homeostatic cellular signaling pathway, even in adult tissues (Ijiri and Potten, 1983; Bursch et al., 1986, 1992; McConkey et al., 1989c, 1992; Kerr et al., 1991; Oberhammer et al., 1991, 1992). This distinction between apoptosis and necrosis is critical in regard to the potential for the development of clinical treatments. Specifically, the controlled nature of the apoptotic pathway may allow for intervention in, or interruption of, the progression of pathology. Conversely, in the case of necrosis, once the threshold for cell death is passed, the cellular damage is likely to be irrevocable, and the inflammation associated with the necrotic pathway is likely to involve other nearby cells. As a result, the potential for intervention may be different from, and potentially limited compared to, necrosis-related cell loss.

Criteria for the Identification of Apoptosis as a Mechanism of Ab-Induced Cell Death

As discussed earlier, apoptosis is defined on the basis of strict morphological and biochemical criteria. Thus, to identify apoptosis in primary neuronal cultures in response to Aβ one must show changes in morphology at the light and electron microscopic level consistent with cell shrinkage, nuclear condensation, and the formation of apoptotic bodies or blebs. Con-

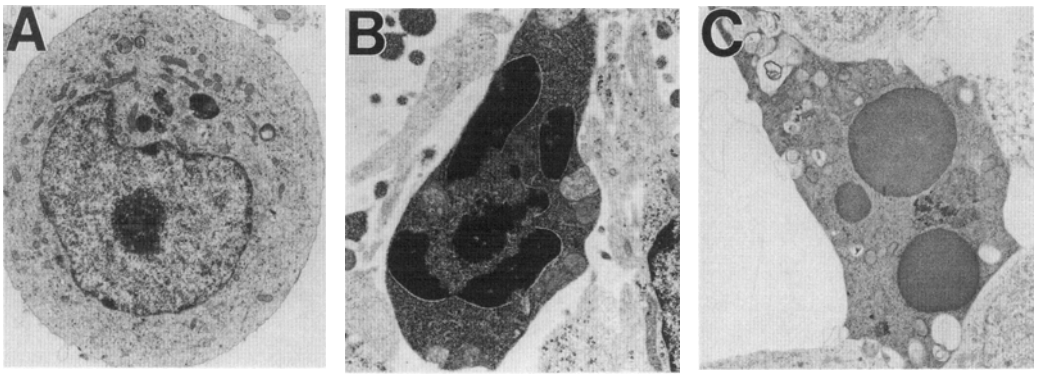

Fig. 2. Transmission electron micrograph of cultured hippocampal neurons following treatment with the following peptides. **(A)** Neuron treated with an inactive scrambled Aβ peptide. **(B)** and **(C)** Neurons treated with Aβ. In correspondence with the characteristics of apoptosis, Aβ-treated neurons exhibit compact patches of condensed nuclear chromatin and polyribosomal dispersion, with no apparent plasma membrane disruption or organelle breakdown.

versely, morphological features suggestive of necrosis, such as the swelling of cell organelles, should not be evident. In addition, biochemical evidence of apoptosis, such as DNA fragmentation, would strongly support an apoptotic mechanism of cell death.

Identification of Ab-Induced Apoptosis In Vitro

Previous work has demonstrated a strict correlation between lactate dehydrogenase (LDH) release and glutamate-mediated necrotic degeneration (Koh and Choi, 1987). A clue that the type of cell death initiated by Aβ peptides may be apoptotic rather than necrotic was the observation that LDH release measured 24 h after treatment with Aβ did not correlate with the extent of morphological degeneration observed at this timepoint, indicating that plasma membrane integrity was still preserved. In addition, neurons exposed to Aβ degenerate asynchronously over a time course of 24–48 h and exhibit small, condensed, irregularly shaped cell bodies (Loo et al., 1993). These features, late membrane lysis, asynchronous degeneration, and cell shrinkage, are hallmarks of apoptosis.

More stringent criteria for the morphological confirmation of apoptosis include the identification of surface blebbing in scanning electron microscopy (SEM) and nuclear condensation in transmission electron microscopy (TEM). Scanning and transmission electron micrographs clearly reveal ultrastructural changes consistent with apoptosis in Aβ treated cultured neurons (Figs. 2 and 3) (Loo et al., 1993; Watt et al., 1994).

As previously mentioned, a definitive biochemical feature of many cell types undergoing apoptosis is the degradation of DNA into oligonucleosome-length fragments (Wyllie et al., 1980). DNA isolated from neurons treated with Aβ for 24 h exhibits a ladder of oligonucleosome-length fragments of DNA (Fig. 4). Similar to young cells, mature cortical neurons exposed to Aβ also undergo DNA fragmentation. Importantly, DNA fragmentation is not a nonspecific consequence of neuronal injury, since neurons exposed to the calcium ionophore A23187 show only random DNA degradation. Taken together, these morphological and biochemical data provide strong evidence for the hypothesis that Aβ can induce primary neurons to undergo apoptosis.

Comparable findings have been described for cortical cultures by Forloni et al. (1993b), however, Behl et al. (1994a) have recently reported that Aβ-induced cell death is necrotic

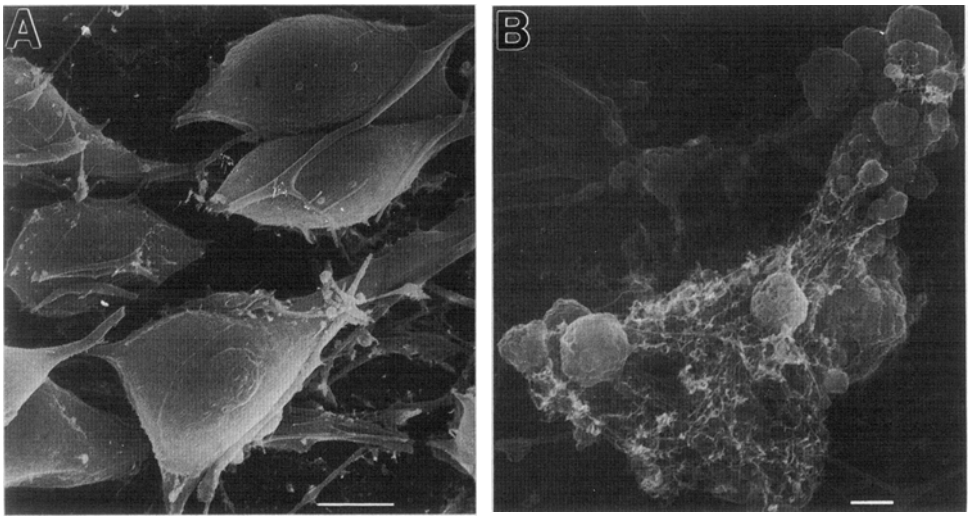

Fig. 3. Scanning electron micrograph of cultured hippocampal neurons following treatment with the following peptides. **(A)** Neurons in untreated cultures display normal ultrastructure. **(B)** Following 24-h exposure to Aβ peptides, neurons appear to be enmeshed in Aβ fibrils and exhibit severe membrane blebbing. Shrunken neurons with few remaining attached blebs and membrane-bound pyknotic nuclei lacking cytoplasm are also present.

rather than apoptotic. Since it is well known that differences between culture systems may result in profound differences in cellular response, it appears likely that the apparent discontinuity between these studies will be resolved on this basis. In this regard, the issue of culture environment is of particular significance for the study of cell death mechanisms. Previous studies have shown that stimuli that induce apoptosis at low doses can induce necrosis at higher concentrations (McConkey et al., 1989b; Lennon et al., 1991; Kunimoto, 1994). Consequently, differences in stimulus dose and the health of cultured cells can be expected to similarly influence the induction of necrosis vs apoptosis, since these factors will impact the relative magnitude of a toxic stimulus within a given cell system. This consideration is further complicated by the fact that cultured cells also undergo a phenomenon termed secondary necrosis, in which cells that have proceeded through apoptosis lyse and exhibit the signs of necrosis at later timepoints. This process is owing to a lack of phagocytic cells for the removal of membrane-bound apoptotic bodies and cellular debris in most culture systems. As a result, the time at which cell death is evaluated in cultured cells may also be an important consideration in assessing cell death mechanisms.

Other Aspects of Aβ-Induced Apoptosis

Calcium has been suggested to play an important role in apoptosis and has been linked to the regulation of cellular signaling pathways associated with apoptosis, cytoskeletal breakdown, and endonucleases involved in DNA fragmentation in some, but not all, systems that undergo this process (McConkey et al., 1988, 1989b; Koike et al., 1989; Orrenius et al., 1989; Alnemri and Litwack, 1990; Johnson et al., 1992; Lennon et al., 1992; McCabe et al., 1992). Calcium has also been proposed to contribute to neuronal cell death induced by Aβ (Mattson et al., 1992, 1993a), and recent reports suggest that Aβ may create a cation channel capable of permitting calcium entry in liposomes (Arispe et al., 1993a,b). These data raise the possibility that calcium may play a role in Aβ-induced apoptosis.

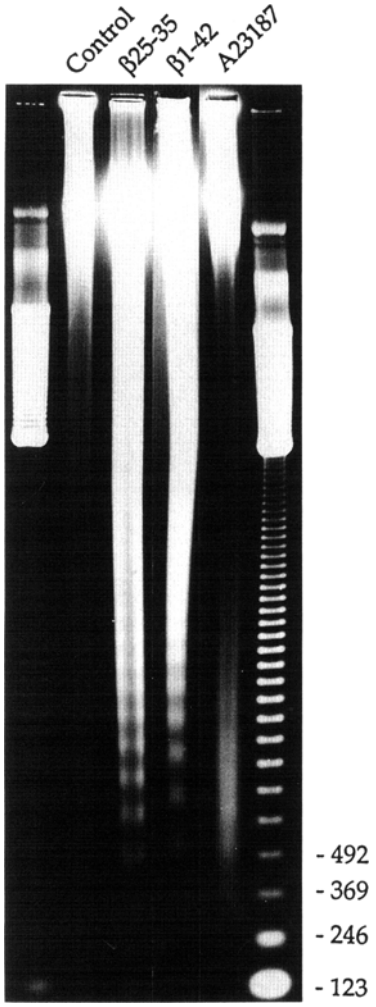

Fig. 4. Ab 1–42 and Aβ 25–35 induce primary CNS neurons to undergo DNA degradation into oligonucleosome-length fragments, characteristic of apoptosis. Cultures exposed to the calcium ionophore A23187 (1 μ*M*) generate a diffuse smear of randomly degraded DNA characteristic of necrosis.

Interestingly, the involvement of calcium in apoptosis in other systems appears to be stimulus specific. For example, exposure of thymocytes to concanavalin A causes a rise in intracellular calcium similar to that produced by glucocorticoids, but without the associated induction of DNA fragmentation and apoptosis (McConkey et al., 1989a). In addition, it is increasingly clear that cellular signaling systems, such as the cAMP and protein kinase C pathways, play specific roles in controlling apoptosis (Kizaki et al., 1989; McConkey et al., 1989a, 1990, 1993; Ojeda et al., 1990; Knox et al., 1992; Deckwerth and Johnson, 1993). In this light it is interesting to speculate that an Aβ-mediated rise in intracellular calcium could in part reflect the activation of specific signaling pathways as a step in the initiation of apoptosis, rather than simply a deregulation of calcium homeostasis in these cells. Correspondingly, the effects of Aβ may be mediated in part by these cellular signaling pathways.

Potential Markers for the Initiation of Apoptosis

Apoptosis has been shown to be dependent on protein synthesis in many systems, including neuronal cells, suggesting that the initiation of an apoptotic pathway of cell death requires the activation of specific intracellular signaling pathways and cell death genes in these cases (Wyllie et al., 1984; Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990; Pittman et al., 1993). Such genes would presumably mediate the controlled destruction of the cell, possibly regulating DNA fragmentation or maintaining membrane integrity as a part of the apoptotic process.

At present there is little information available on gene induction during apoptosis in primary neurons. There has, however, been progress on identifying candidate genes in other systems, providing a starting point for the evaluation of an Aβ-mediated cell death program. One group of genes that has been associated with apoptosis in a number of paradigms are the immediate early genes (IEGs).

IEGs in Apoptosis in Other Systems

The protein products of some IEGs, e.g., *c-jun* and *c-fos*, are capable of forming dimers and acting as transcriptional regulatory proteins (Angel et al., 1988; Rauscher et al., 1988; Abate

et al., 1989; Sonnenberg et al., 1989). The cooperative interaction of different sets of IEG protein products allows the regulation of individual IEGs by specific second messenger systems and stimuli to interact and produce a specialized, or combinatorial response. IEGs have been shown to play an important role in this regard in neurons (Sheng and Greenberg, 1990; Morgan and Curran, 1991). Furthermore, this combinatorial system allows IEGs to act as cellular third messengers, potentially regulating multiple processes, such as proliferation, differentiation, and apoptosis, within a single cell.

A number of IEGs have been implicated in apoptosis, including *c-fos* and several members of the *c-jun* IEG family. Evidence suggesting a role for Jun and Fos proteins in apoptosis has been obtained in lymphoid cell lines (Gunji et al., 1991; Kharbanada et al., 1991; Rubin et al., 1991, 1992; Bhalla et al., 1992; Brach et al., 1992; Colotta et al., 1992; Bullock et al., 1993), rat thymocytes (Grassilli et al., 1991; Sikora et al., 1993a,b), rat ventral prostate (Buttyan et al., 1988), and mouse mammary epithelial cells (Marti et al., 1994). Additionally, similar findings have been reported in the rat brain following status epilepticus and hypoxic-ischemic brain injury (Dragunow et al., 1993, 1994), although there is some controversy regarding the mechanism of delayed neuronal cell death in this model (Kiessling et al., 1991; Deshpande et al., 1992). Interestingly, although *c-jun*, *c-fos*, and AP-1 activity are induced in thymocytes in response to stimuli that induce proliferation and entry into the cell cycle (e.g., concanavalin A), and stimuli that induce apoptosis (e.g., dexamethasone), the kinetics and magnitude of IEG induction in response to these stimuli differs. These experiments illustrate the specificity of the IEG response associated with apoptosis in these cells, and the potential for these genes to regulate multiple processes within the same cell (Grasselli et al., 1992; Sikora et al., 1993b).

Although *c-jun*- and *c-fos*-related genes appear to play a role in apoptosis in some systems, it is clear that the cellular mechanism of apoptosis differs among cell types, and that these IEGs do not necessarily play the same role in all systems (Walker et al., 1993; Goldstone and Lavin, 1994). Furthermore, a variety of additional immediate early and other genes have been linked to the initiation or regulation of mammalian apoptosis; these include *c-myc*, *bcl-2*, *bcl-x*, *bax*, RP-8, p53, p35, *nur77*, cyclin D1, and interleukin 1-β converting enzyme (Nunez et al., 1990; Owens et al., 1991; Bissonnette et al., 1992; Evan et al.,1992; Garcia et al., 1992; Shaw et al, 1992; Shi et al., 1992; Boise et al., 1993; Hockenberry et al., 1993; Kane et al., 1993; Miura et al., 1993; Oltvai et al., 1993; Rabizadeh et al., 1993; Yonish et al., 1993; Yuan et al., 1993; Freeman et al., 1994; Hermeking et al., 1994; Liu et al., 1994; Miyashita et al., 1994). The number and diversity of these cell death-related genes illustrates the complexity of the interactions controlling the process of apoptosis at the gene level (Smith et al., 1994). We have approached this issue by examining the possibility that the rapid induction and transcriptional regulatory capability of IEGs may provide an early marker for the signal transduction pathways initiated during apoptosis.

IEGs in an In Vitro Model of Aβ Toxicity

To test the hypothesis that IEGs may be a marker for Aβ-mediated apoptosis in AD, we examined the expression of these genes in an vitro model of Aβ toxicity. We observed increased immunoreactivity for Jun-related proteins in both young and mature primary cultures of hippocampal neurons treated with Aβ (Fig. 5) (Anderson et al., submitted). Increased Jun expression was an early event in response to Aβ, and Jun immunoreactivity remained elevated for prolonged periods after Aβ exposure. A less consistent and more transient increase in immunoreactivity for Fos-related proteins was also noted. Additionally, after 24 h of Aβ treatment many Jun-positive cells failed to exclude the vital dye trypan blue, suggesting that cells expressing Jun proteins may have undergone cell death.

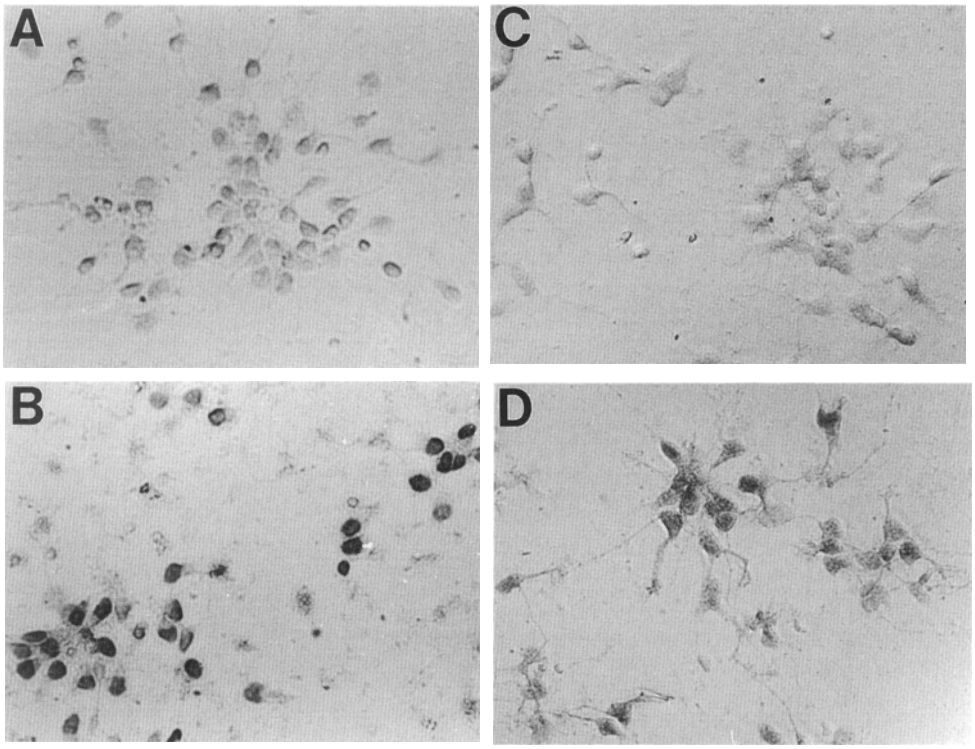

Fig. 5. Immunostaining for Jun and Fos proteins in short-term cultures of hippocampal neurons. Cells were treated with Aβ peptides or an equivalent volume of media exchanged (controls) and fixed at 2 or 4 h following treatment. 2-h control cells **(A)** and 2-h Aβ-treated cells **(B)** stained for Fos-related proteins. 4-h control cells **(C)** and 4-h Aβ-treated cells **(D)** stained for Jun-related proteins.

Jun Proteins Are Not Induced in Cells That Are Resistant to Aβ-Mediated Apoptosis

One test of the hypothesis that the expression of Jun proteins is an early marker for events associated with cell death in response to Aβ is to determine if the expression of these proteins is restricted to cells that undergo Aβ-mediated apoptosis. Previous studies of AD have shown that GABAergic neurons appear relatively resistant to loss in AD (Spillane et al., 1977; Rossor et al., 1982; Smith et al., 1983; Mountjoy et al., 1984; Lowe et al., 1988). Consistent with these findings, GABA-immunoreactive neurons in culture exhibit a relative resistance to Aβ-induced cell death (Pike and Cotman, 1993). Interestingly, GABA-positive cells do not exhibit an increase in immunoreactivity for Jun proteins in response to Aβ, suggesting that Aβ does not initiate the same signaling pathways in GABAergic cells as in other cells (Fig. 6). This may be owing to differences in the calcium buffering capacity of GABAergic cells, or the signaling pathways and mechanisms regulating IEG expression within these cells. These data suggest the IEG induction in these cultures is a specific and cell-type dependent response to Aβ, and may be related to the initiation of a cell death pathway or other processes associated with cell death.

Identification of Apoptosis as a Mechanism of Death In Vivo

To support the hypothesis that Aβ-mediated apoptosis is a mechanism of cell death in AD it will be critical to identify apoptosis in the post-mortem AD brain. This task may prove to be a technically difficult one. Previous work in *Caenorhabditis elegans* has shown that a pro-

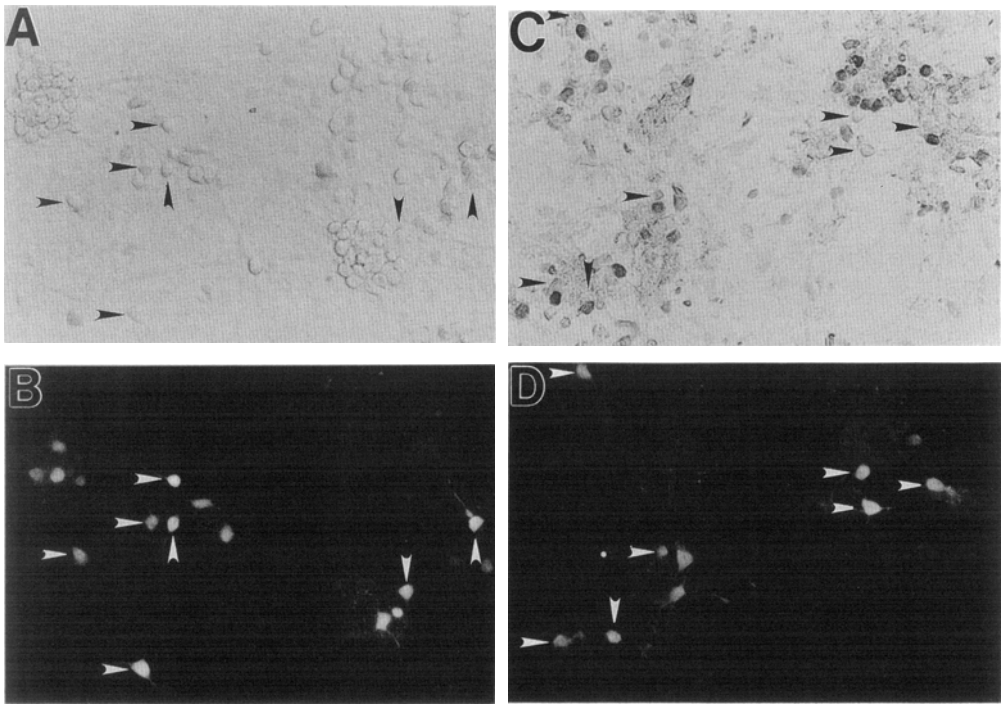

Fig. 6. Absence of induction of Jun proteins by Aβ in GABA-immunoreactive neurons in mature hippocampal neurons. Cultures were treated for 36 h with Aβ 25–35 before fixation and immunostaining. **(A)** Jun immunostaining in control cultures. **(B)** GABA immunostaining in same field as (A). **(C)** Jun immunostaining in Aβ-treated cultures. **(D)** GABA immunostaining in same field as (C). Arrowheads indicate GABA-positive, Jun-negative cells.

gram of cell death can be completed within as little as 1 h (Ellis et al., 1991). The rapidity of these events makes the identification of apoptosis in vivo difficult, even in tissue where it is known to occur (Bursch et al., 1992; Raff et al., 1993). This limitation will only be compounded in a progressive degenerative disorder such as AD, in which degeneration may take place over a course of years.

Consequently, one might expect only a relatively small, asynchronous population of neurons to undergo apoptosis at any given time. These factors suggest a paucity of cells actively in the process of undergoing apoptosis and, as a result, may preclude the use of gross biochemical markers, such as gel electrophoresis for DNA fragmentation, as a viable option in the detection of apoptosis in vivo. Similarly, it may be difficult to detect apoptotic alterations in cellular ultrastructure using electron microscopy. Finally, additional factors relating to the restrictions of working with human postmortem tissue may also result in some limitations for the detection of cells undergoing apoptosis in AD. For example, ischemia has been suggested to initiate apoptosis in the rat brain (Goto et al., 1990; Shigeno et al., 1990, 1991; Dessi et al., 1992; Heron et al., 1993). Although several studies suggest that apoptosis is not the mechanism for delayed neuronal death following ischemic insult (Kiessling et al., 1991; Deshpande et al., 1992), it will be essential to carefully consider and control for the factors of postmortem delay and associated ischemic insult in studies of cell death in the AD brain.

Despite these limitations, several markers can be suggested for the identification of apoptosis in in vivo. First, if the expression of the IEGs Jun and Fos in response to Aβ is reflective of early events in the initiation of

apoptosis, the expression of these proteins may be similarly altered in AD. Second, although biochemical markers of DNA fragmentation do not appear to be a viable option, a histological marker of this process is available. Although the data obtained with this technique must be interpreted cautiously, since this marker does not give a specific indication of apoptotic vs necrotic DNA fragmentation, it can be used to provide an indication of the events occurring in vivo, as we discuss later. Finally, as in the case of the identification of apoptosis in vitro, the definitive criterion for the identification of apoptosis in the AD brain is the observation of morphological features consistent with the classical definition of apoptosis. To this end, by staining with a DNA dye, such as bisbenzimide, it may be possible to detect evidence of nuclear chromatin condensation and alterations in nuclear morphology characteristic of apoptosis at the light microscopy level.

IEGs in the AD Brain

If IEGs are involved in neuronal cell death program in AD, one would predict an intensification of immunoreactivity for IEGs and the colocalization of IEGs and neuronal pathology in some cells. We have recently reported on Jun- and Fos-related protein immunoreactivity in AD and control brains (Anderson et al., 1994). In this study, an intensification of both Jun and Fos immunoreactivity was observed in AD cases as compared to controls. This intensification appeared to reflect an increase in both the number of stained cells and the intensity of staining in individual cells. Double labeling experiments for Jun or Fos proteins and a marker for neuronal pathology, paired helical filament-1 (PHF-1), revealed a colocalization of Jun and Fos immunoreactivity with neuronal pathology in a subset of cells in the AD cases (Fig. 7).

Both Jun and Fos-positive processes and cytoplasmic staining were detected in this study. Intensification of c-Fos immunoreactivity in AD, and the distribution of immunoreactivity in neuronal processes, has been reported previously (Zhang et al., 1992). The observation of cytoplasmic staining is counter-intuitive in light of the definition of Jun and Fos family members as nuclear proteins. This distribution of Jun and Fos immunoreactivity in cellular compartments uncharacteristic of their in vivo localization may represent postmortem artifact (*see*, for example, Schwab and Geddes, 1994). However, it is of interest to note that Smeyne et al. (1993) have recently described the accumulation of the nuclear protein c-Fos in the cytoplasm of fibroblasts undergoing apoptosis in vitro, and a similar accumulation of Fos protein has been observed in cortical neurons following ischemia (Uemura et al., 1991). These observations suggest that these IEGs may play a role in degeneration in some neurons. Alternatively, it has been suggested that there is an overactivation of signaling pathways in the AD brain (Saitoh et al., 1993).

In addition to these observations, both Jun and Fos proteins were detected in colocalization with GFAP-positive astrocytes surrounding thioflavine-positive plaques (Fig. 8). Thus, the expression of Jun and Fos in the AD brain is not restricted to cells that reveal classical markers of pathology such as NFTs. The expression of Jun and Fos proteins in these cells likely indicates the initiation of several distinct signaling pathways, which may mediate different cellular functions in different cell types in response to Aβ or Aβ-induced injury. Consequently, these data reinforce the idea that it is essential to evaluate a complete picture of AD pathology using multiple markers for changes in cellular function and the initiation of pathological mechanisms.

Evidence for Chromatin Condensation and Nuclear Fragmentation in AD Neurons

As we have discussed, histological markers may provide one of the few means available to examine the presence of apoptotic nuclei in the AD brain. We have detected neurons that exhibit DNA degradation using a histological technique in which the 3'-OH termini of DNA strand breaks generated during DNA fragmentation are labeled with a biotin or digoxigenin tagged

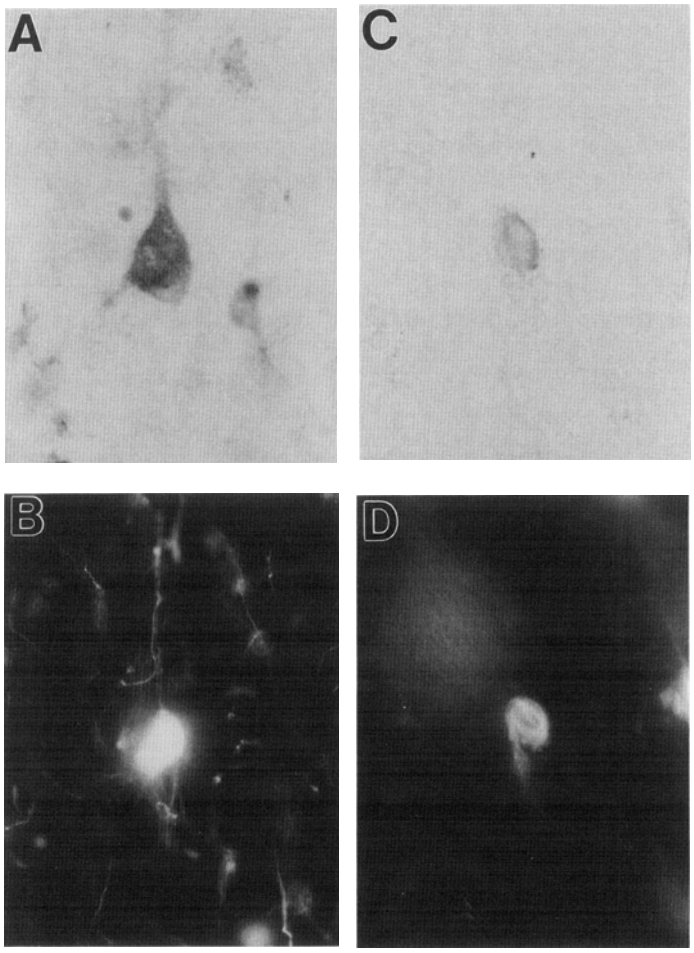

Fig. 7. Colocalization of Jun- and Fos-related immunoreactivity with PHF-1 in AD brain tissue. **(A)** Jun immunostaining in AD brain. **(B)** PHF-1 immunostaining in same field shown in (A). **(C)** Fos immunostaining in AD brain. **(D)** PHF-1 immunostaining in same field shown in (C).

dNTP using the enzyme terminal deoxynucleotidyl transferase (TdT) (Su et al., in press). Cells undergoing apoptosis or necrosis have large numbers of 3'-OH termini, conversely, normal cells have very low numbers of such terminal DNA strand breaks. Although, as we have noted, this technique has not been proven to be specific for cells that are undergoing apoptosis, we have observed several features of this labeling that are suggestive of an apoptotic process in AD. First, in tissues with short postmortem delay (<3 h) few cells exhibiting TdT labeling are apparent in control cases, whereas TdT-labeled cells are easily detectable in AD cases. Importantly, TdT labeling is generally visible in cellular patterns that appear to reflect the chromatin condensation and margination of chromatin characteristic of apoptosis (Fig. 9). Interestingly, a similar report on the detection of apoptosis following epileptic brain damage in human tissue has recently been published (Pollard et al., 1994).

Other Neuronal Apoptosis-Inducing Stimuli

If apoptosis serves as a protective and/or pathological mechanism in aging and disease,

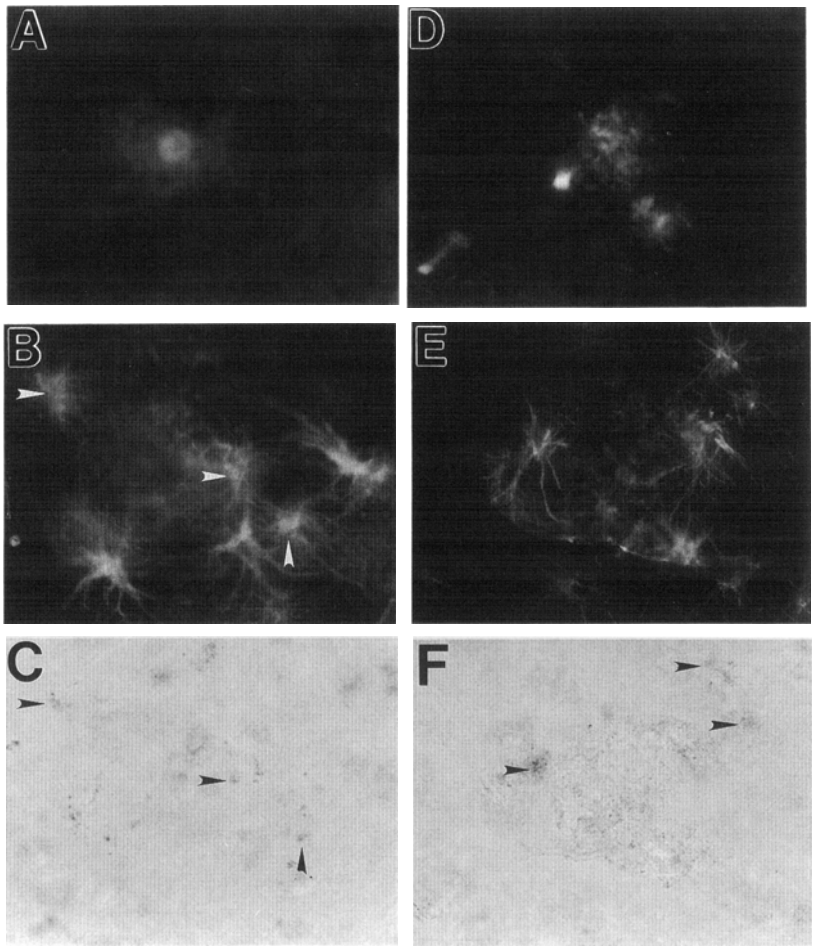

Fig. 8. Colocalization of Jun- and Fos-related immunoreactivity with GFAP-positive astrocytes surrounding thioflavine-positive plaques in AD brain tissue. Thioflavine **(A)**, GFAP **(B)**, and Jun **(C)** staining of a representative plaque exhibiting Jun immunoreactive astrocytes in an AD case. (A), (B), and (C) show the same field. Thioflavine **(D)**, GFAP **(E)**, and Fos **(F)** staining of a representative plaque exhibiting Fos immunoreactive astrocytes in an AD case. (D), (E) and (F) show the same field. Arrowheads indicate GFAP-positive/Jun-positive (A–C) or GFAP-positive/Fos-positive (D–F) cells.

it is important to examine other stimuli that can trigger this cellular response. Oxidative damage and stress have long been suggested to be contributing factors to a wide variety of degenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, and increases in indicators of oxidative injury with aging are well documented (Halliwell, 1989; Ames et al., 1993; Olanow, 1993). In this context, it has been suggested that there is not only an overall increase in oxidative damage to proteins in AD, but an increase in oxidative damage to specific proteins, e.g., glutamine synthetase (Smith et al., 1991). In addition, immunoreactivity for superoxide dismutase and catalase is colocalized with a subset of both NFTs and plaques in AD tissue, supporting a role for oxidative injury in AD pathology (Pappolla et al., 1992). Furthermore, Aβ itself has been suggested to cause oxidative damage to neurons (Behl et al., 1994b; Hensley et al., 1994).

Interestingly, data from a variety of systems and paradigms suggest a relationship between

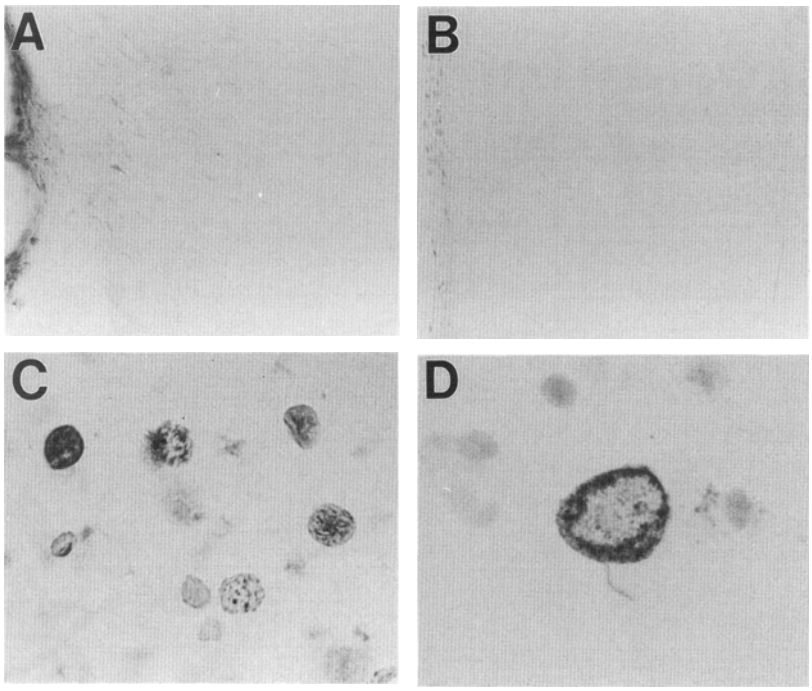

Fig. 9. TdT-labeling of DNA strand breaks in control and AD cases. **(A)** Low-power photomicrograph of biopsy tissue labeled for 3'-OH DNA termini using TdT, postmortem delay of 0 h. Note the lack of TdT-positive nuclei. **(B)** Low-power photomicrograph of a control case, postmortem delay of 3 h. Again, there are few nuclei exhibiting DNA strand breaks. **(C)** High-power photomicrograph of an AD case exhibiting four different patterns of TdT-positive nuclei. Note the frequency of these nuclei in comparison to the controls. Postmortem delay of 6.5 h. **(D)** High-power photomicrograph of a single TdT-positive nucleus from a different AD case. Note the apparent condensation and margination of the chromatin labeled for 3'-OH termini. Postmortem delay of 4 h.

oxidative mechanisms and apoptosis (Hockenberry et al., 1993; Kane et al., 1993; Malorni et al., 1993; Zhong et al., 1993). Additionally, neurons cultured in a high oxygen atmosphere (Enokido and Hatanaka, 1993), or depleted of glutathione (Ratan et al., 1994), have been shown to undergo apoptosis. To investigate the possibility that direct exposure to reactive oxygen species can trigger neuronal cell death via apoptosis, neurons were treated with hydrogen peroxide (H_2O_2). Exposure of neurons to H_2O_2 for periods as brief as 5 min was sufficient to cause cell death. Cell death progressed over a time course of several hours, and neurons displayed morphological features characteristic of apoptosis at the light microscopic level, including cell shrinkage. Furthermore, cells treated with H_2O_2 exhibited DNA degradation into oligonucleosome-length fragments, similar to observations for Aβ (Whittemore et al., 1994).

These investigations illustrate that both Aβ and oxidative injury can serve as apoptotic stimuli in vitro, and suggest that these stimuli could interact during aging, contributing to neuronal cell loss in vivo. Interestingly, studies in other systems have revealed that *c-jun* is selectively induced in association with DNA fragmentation in response to reactive oxygen species, such as H_2O_2 (Manome et al., 1993a,b), however, it is unclear whether *c-jun* is able to effectively transactivate AP-1 in these systems (Abate et al., 1990; Bannister et al., 1991). Despite the need to clarify this issue, it is possible to speculate that there may be some similarities between the cellular signaling pathways associated with Aβ-mediated and H_2O_2-mediated cell death.

Features of Apoptosis in Response to Aβ and Oxidative Injury in Primary Neurons

In order to study the mechanism of apoptosis in a system it is important to identify the characteristics of cell death and define criteria for this cell death pathway in that system. We have described a number of features of apoptosis in hippocampal neurons treated with Aβ and H_2O_2, and suggest several characteristics that may be associated with the specific pathway of apoptosis initiated in these cases. These include:

1. Delayed cell lysis;
2. Maintenance of mitochondrial integrity;
3. Polyribosomal dispersion;
4. Membrane blebbing;
5. Chromatin condensation;
6. DNA cleavage into oligonucleosome-length fragments; and
7. Inhibition of DNA fragmentation and cell death by endonuclease inhibitors.

These features may also include the induction of a specific set of genes that are associated with or participate in apoptosis. Future studies will help to clarify this issue, definitively identify specific apoptosis-related genes, and define the role of these genes.

Although our observations are in close correspondence with the classical features of apoptosis, it is increasingly apparent that apoptosis is a complex mechanism that can proceed via multiple different pathways depending on the type and state of a cell (Chuang et al., 1994; Martin et al., 1994). For example, although the cleavage of DNA into oligonucleosome-length fragments is typically thought to be associated with apoptosis (Kerr and Harmon, 1991), this event is not critical to the apoptotic program in all cells (Monti et al., 1992; Oberhammer et al., 1992; Zakeri et al., 1993). Similarly, apoptosis is dependent on macromolecular synthesis in many (Kerr and Harmon, 1991), but not all, cells that exhibit the other characteristics of this process (Duke et al., 1983; Martin et al., 1990; Waring, 1990; Batistatou and Greene, 1991; Cotter et al., 1992; Kruman et al., 1992; Lennon et al., 1992). Finally, calcium influx has been reported to be critical to the initiation of DNA fragmentation in many cells (Kerr and Harmon, 1991), however, there are now a number of cases in which this process has been suggested to be calcium independent (Alnemri and Litwack, 1990; Bansal et al., 1990; Kure et al., 1991; Lennon et al., 1992; Whyte et al., 1993; Chuang et al., 1994). These points illustrate the necessity to consider and evaluate multiple markers and features of apoptosis in studying the mechanism of cell death in a particular system or in response to a particular stimulus.

In addition to differences in the apoptotic program initiated in individual cell types, the factors that could influence the "decision" of a cell to undergo apoptosis vs necrosis must also be considered. For apoptosis to be a useful mechanism for controlled, "physiological," cell death in a population of cells that does not undergo replacement, cells must have at least two things: a threshold for the initiation of this process, and a system of checks and balances to regulate it. Correspondingly, there may be a variety of initial factors that determine whether a cell will die via necrosis or apoptosis in response to an adverse stimulus.

It may turn out that necrosis is generally associated with trauma, head injury, and other sources of acute insult to the nervous system, whereas apoptosis may be associated with milder, long-term, cumulative, or synergistic insults—e.g., loss of neurotrophic support, Aβ accumulation, or oxidative damage (Lennon et al., 1991). In this context, healthy cells may be more capable of regulating apoptosis and initiating compensatory mechanisms in response to cellular stress or injury, thus raising the threshold for the initiation of apoptosis. Conversely, unhealthy cells may be incapable of mobilizing the energy and cellular systems required to carry out a program of apoptosis, and may in fact be more vulnerable to necrosis. The principles defining the relationship of extrinsic stimuli (conditions in the extracellular environment, adverse stimuli, and so on) to

intrinsic cellular characteristics (vulnerability to insult, condition or health of the cell, particular signaling pathways initiated in response to Aβ) are just beginning to be identified. Once the factors regulating the initiation of apoptosis and features characteristic of the program of apoptosis initiated by a particular stimulus in a particular cell type are identified, it may be possible to define strategies to raise the threshold for cell death, and delay neurodegeneration.

Conclusion

As discussed in this work, recent evidence suggests that apoptosis may be a primary mechanism in the degeneration of neurons in age-related diseases such as Alzheimer's disease (AD). These studies indicate that β-amyloid (Aβ) that is assembled into an aggregated state can initiate an apoptotic cell death program in cultured neurons. This cell death program is rapid, cell-specific, and may involve the activation of cell death proteins as has been described in other systems. In fact, we have observed the expression of immediate early genes (IEGs) in response to Aβ, and found a selective association of IEG expression with cell populations that are vulnerable to Aβ toxicity, suggesting that IEGs may play a role in Aβ-induced apoptosis. Interestingly, age-related events other than Aβ accumulation, such as hydrogen peroxide (H_2O_2)-mediated oxidative injury, can also induce apoptosis. Future in vitro experiments will help define the stimuli, second messenger pathways, and regulatory systems participating in the activation and regulation of Aβ- and H_2O_2-induced cell death.

Although studies *in situ* are still at an early stage, data supporting a role for apoptosis in AD is accumulating. For example, a corollary to the Aβ-mediated induction of Jun and Fos proteins in cultured neurons is apparent in AD tissue. In addition, some PHF-1 immunoreactive cells also display immunoreactivity for Jun and Fos proteins, suggesting a relationship between IEG expression and pathology in some instances. Furthermore, apoptotic nuclear changes similar to those identified in vitro are also apparent in the AD brain. These observations are consistent with the hypothesis that apoptosis may be a mechanism for the removal of damaged cells in AD. Insight into the intrinsic properties that result in the resistance of neuronal subtypes (such as GABAergic cells) to Aβ-mediated apoptosis in vitro, and to degeneration in the AD brain, may reveal new information regarding the mechanism of cell death and potential interventions in the progression of AD pathology.

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