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Alzheimer Disease: DNA Fragmentation Indicates Increased Neuronal Vulnerability, but not Apoptosis

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Abstract. Although nerve cell loss is prominent in certain brain regions in Alzheimer disease (AD), it is currently unresolved how these cells die. Recent studies unanimously agree that there are more neurons displaying DNA fragmentation in AD compared with normal controls. However, controversy remains as to whether cell death is mediated by apoptosis or necrosis. We addressed this question by comparing AD lesions with those from cases with pontosubicular neuron necrosis (PSNN), a human pathological condition with unequivocal neuronal apoptosis, with regard to cell and nuclear morphology, immunohistochemistry, and in situ tailing. Immunohistochemistry was performed for an array of proteins with presumptive roles in the apoptotic process or the protection thereof, i.e. a recently described apoptosis-specific protein (ASP), the transcription factor c-Jun, Bcl-2, and various stress proteins: alpha B-Crystallin, heat shock protein (HSP) 27, HSP 65, HSP 70, HSP 90, and ubiquitin. Apoptotic neurons in PSNN displayed chromatin condensation, nuclear fragmentation, and cytoplasmic condensation. They were labeled with the in situ tailing technique and stained for the ASP. Despite the large numbers of cells with DNA fragmentation identified in the hippocampus of AD brains, only exceptional cells displayed the morphological characteristics of apoptosis or labeled for the ASP. We suggest that the increased rate of neuronal DNA fragmentation in AD patients indicates a higher susceptibility of the cells to metabolic disturbances compared with normal controls. The large number of cells with DNA fragmentation most likely reflects metabolic disturbances in the premortem period, and cell destruction is mediated through necrosis rather than apoptosis.

Key Words: Alzheimer disease; Apoptosis; Apoptosis-specific protein; c-Jun; DNA fragmentation; Neuronal cell death; Pontosubicular neuron necrosis.

INTRODUCTION

Neuron loss exceeding 50% in certain brain areas is characteristic of the degenerative process in Alzheimer disease (AD) (1, 2). The extent of neuronal and synaptic loss correlates better with the severity of clinical disease than the hallmark neuropathological lesions, amyloid plaques, and neurofibrillary tangles (3, 4). Despite intense investigation, little is known about the mode and mechanisms of neuronal cell death in AD. An at least 2-fold increase in DNA strand breaks has been detected in AD whole brain tissue (5), and current histochemical techniques using a terminal deoxynucleotidyl transferase (TdT) –based incorporation of nucleotides at free 3-OH ends allow the detection of fragmented DNA at the single cell level (6, 7). Several investigators have reported quite unanimously on an increased incidence of cells with DNA fragmentation in AD and several other neurodegenerative diseases (8–16). This has been widely suggested as evidence for apoptotic cell death (8, 11–15).

Various lines of evidence point towards a role for apoptosis in the neurodegenerative process of AD (17, 18). Familial AD-associated mutations of presenilin 1 and 2 sensitize transfected cells to various apoptosis-inducing stimuli, and mutants of amyloid precursor protein (APP) induce DNA fragmentation in neurons (19–23). Fetal cortical neurons from cases of Down syndrome apoptose rapidly in culture with a concomitant increase in reactive oxygen species and lipid peroxidation (24). Elevated levels of the death-promoting protein Bax have been described in human neurons in response to βA4 (25) in vitro and in AD neurons in vivo (26). The anti-apoptotic protein Bcl-2 has been reported to be upregulated in cells with DNA fragmentation and downregulated in tangle-bearing cells (27). C-Jun with a putative role in transcriptional regulation of apoptosis-associated genes (28–31) has been colocalized to AD neurons with DNA fragmentation (32). However, the term "apoptosis" clearly implies a well-defined cellular morphology, mainly chromatin condensation, nuclear fragmentation, and cytoplasmic condensation (33), whereas the majority of neurons in AD with DNA fragmentation lack clear morphological features of apoptosis (9, 10, 16).

Recently, Grand et al reported the detection of an apoptosis-specific protein (ASP) in various human and rat cell lines by means of a crossreacting antibody originally raised against an N-terminal (73–87) amino acid sequence of v-Jun (c-Jun/AP-1, Ab-2) (34). The predominant localization of the ASP in the cytoplasm, its relative insolubility, and its appearance rather late in the process of apoptosis suggest that it forms part of the modified cytoskeleton in apoptotic cells (34). We applied this

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TABLE 1
Specifications of Primary Antibodies Used for Immunohistochemistry

Antigen	Antigen source/ specificity	Antibody	Clone	Dilution	Pre-treatment	Source
Immediate early gene–encoded proteins:						
c-Jun/AP-1	Residues 209–225 of v-Jun; C-terminal	Rabbit PAB (Ab-1)		1:100	MW	Oncogene, Cambridge, Mass
Apoptosis-related proteins:						
c-Jun/AP-1 (ASP)	Residues 73–87 of v-Jun; N-terminal	Rabbit PAB (Ab-2)		1:100	MW	Oncogene
Bcl-2	Human Bcl-2, residues 41–54	Mouse MAB	124	1:50	MW	Dako, Glostrup, Denmark
Heat shock proteins:						
Alpha B-crystallin	Purified 23K human protein	Rabbit PAB		1:700	None	Hans van Noort, Leiden, The Netherlands
HSP 27	Breast tumor cell line	Mouse MAB	G3.1	1:1000	MW	Stressgen, Victoria, Canada
HSP 65	Mycobacterium	Mouse MAB	ML30	1:100	None	J. Ivanyi, London, UK
HSP 70	HSC/HSP 70 from HeLa cells	Mouse MAB	C92F3A-5	1:200	MW	Stressgen
HSP 90	HSP 90 from water mold	Mouse MAB	AC88	1:1000	MW	Stressgen
PHF/ubiquitin	Paired helical filaments	Mouse MAB	3.39	1:20000	None	See reference 39
Ubiquitin	Human ubiquitin	Mouse MAB	FPM1	1:50	MW	Novocastra, Newcastle upon Tyne, UK

AP: activating protein; ASP: apoptosis-specific protein; PAB: polyclonal antibody; MAB: monoclonal antibody; MW: microwave; HSP: heat shock protein; HSC: heat shock cognate; PHF: paired helical filament.

marker for apoptosis in concert with antibodies against other apoptosis-associated antigens to search for evidence for neuronal apoptosis in AD. In parallel, we studied the expression of these antigens in pontosubicular neuron necrosis (PSNN), a brain disorder predominantly affecting premature infants and most likely caused by a combination of anoxia, hypercapnia, and hypoglycemia, which reveals abundant neuronal apoptosis in human autopsy tissue (35, 36).

MATERIALS AND METHODS

Tissue

Brain tissue from 12 cases with clinically diagnosed and neuropathologically confirmed AD, 10 age-matched controls, and 3 cases with PSNN was obtained at autopsy, fixed in buffered formalin, and routinely embedded in paraffin. All AD cases fulfilled the quantitative neuropathological criteria for the diagnosis of AD according to Khatchaturian (37) and CERAD (38). For immunohistochemistry and in situ tailing, temporal lobe sections including temporal isocortex, hippocampus, and entorhinal cortex were used. In addition, we used midpontine sections of PSNN.

Immunohistochemistry

We used primary antibodies against apoptosis-related antigens and heat shock proteins. Table 1 lists their characteristics and the dilutions at which they were applied. Immunohistochemistry was performed as follows: deparaffinized sections were microwaved for 15 minutes (min) in citrate buffer (pH 6.0) and allowed to cool to room temperature. Anti-c-Jun/AP-1 (Ab-2), anti-alpha B-Crystallin, anti-paired helical filament/ubiquitin, and anti-Hsp 65 were also used without microwaving. Endogenous peroxidase was blocked by treatment with 0.2% H_2O_2-methanol followed by incubation with 10% fetal calf serum (FCS) in phoshate buffered saline (PBS) for 10 min. Primary antibodies were diluted as indicated in 10% FCS and permitted to bind overnight at 4°C. Sections without primary antibody were similarly processed to control for binding of the secondary antibody. Biotinylated anti-mouse or anti-rabbit antibodies (Amersham, Arlington Heights, Ill) were then applied at a dilution of 1:200 in 3% human serum for 1 hour (h) followed by Avidin-Peroxidase (Sigma, St. Louis, Mo) 1:100, also for 1 h at room temperature. We used 3-amino-9-ethylcarbazole (AEC, Sigma) as the chromogenic substrate. Alternatively, an alkaline phosphatase/anti-alkaline phosphatase system (Dako) employing Fast Red TR Salt (Sigma F1500) as chromogen was used for visualization of the primary antibodies. Sections were lightly counterstained with hematoxylin.

Histochemical Detection of DNA Fragmentation (In Situ Tailing [IST])

Terminal deoxynucleotidyl transferase (TdT) –mediated incorporation of digoxigenin-labeled nucleotides was used to detect DNA fragmentation as described by Gold et al (7). Briefly,

deparaffinized sections were pretreated with 5% Proteinase K (Sigma) in PBS for 15 min at 37°C followed by incubation with TdT labeling mix (10 μl 5× tailing buffer, 1 μl digoxigenin-labeled deoxynucleotides, 2 μl cobalt chloride [25 mmol], 12 U terminal transferase, dH_2O added to a total volume of 50 μl) for 1 h at 37°C. Alkaline phosphatase–conjugated anti-digoxigenin Fab-fragments (1:500) were applied for 1 h; NBT/BCIP (4-nitrobluetetrazoliumchloride/5-bromo-4-chloro-3-indolylphosphate) was used for visualization of the complex (all reagents were purchased from Boehringer-Mannheim, Mannheim, Germany). This was followed by light hematoxylin counterstaining and coverslipping.

RESULTS

Morphology of Neurons with DNA Fragmentation in PSNN, AD, and Controls

In PSNN, apoptotic neurons were mainly present in the pons and in the dentate gyrus, CA 1, and subiculum regions of the hippocampus. In midpontine sections, a mean number of 106.3 (n = 2) apoptotic neurons per square millimeter was observed predominantly in the ventral part of the pons. Criteria for apoptosis were a highly condensed and mostly fragmented nucleus, and a shrunken cell body with condensed cytoplasm (Fig. 1A). More than 80% of neuronal apoptotic nuclei were stained using the in situ tailing assay (Fig. 1B). Neurons not conforming to the apoptosis criteria were rarely labeled. No apoptotic cells other than neurons were present.

In AD, 30.3 cells/mm² in the CA 4 region (number of cases examined, n = 10), 9.2 cells/mm² in the CA 1 region (n = 9), and 13.4 cells/mm² in the subiculum and entorhinal cortex (n = 11) were labeled for DNA fragmentation. Of these cells, an average of 32% in CA 4 could be identified as neurons based on morphological criteria, 25.6% in CA 1, and 38.1% in the subicular/entorhinal region. In general, labeled neurons revealed a loose and finely granular chromatin structure and did not show the above mentioned clear morphological criteria of apoptosis, i.e. chromatin condensation, margination of chromatin at the nuclear membrane, nuclear fragmentation, and cell shrinkage (Fig. 1H). However, exceptional labeled neurons displayed a reduction in cell size or clumping of chromatin, although a detailed analysis of nuclear structure was precluded by the intense labeling.

In contrast, in age matched controls, only 3.2 cells/mm² in CA 4 (n = 9), 1.1 cells/mm² in CA 1 (n = 9), and 0.7 cells/mm² in the subicular/entorhinal region (n = 8) were positive for in situ tailing. On average, 4.4% of the labeled cell population in CA 4, 4.6% in CA 1, and 4.6% in subiculum/entorhinal cortex were neurons. No morphological alterations reminiscent of apoptosis could be detected in the set of cells labeled for DNA fragmentation.

Detection of the Apoptosis-specific Protein in Apoptotic Neurons in PSNN, AD, and Controls

Performing immunohistochemistry for the recently reported "apoptosis-specific protein" (ASP), we observed a very high labeling efficiency for apoptotic neurons in PSNN. Morphometric analysis of pontine sections from 2 cases revealed labeling percentages for apoptotic neurons of 80.5% and 90.1%, respectively. Nonapoptotic neurons remained unstained (Fig. 1C).

In AD brains, neuronal labeling for the ASP was only very rarely detected. In a total of 12 AD brains, in which at least 3 hippocampal sections were screened for immunolabeling for ASP, only 2 strongly labeled neurons could be identified, one of them clearly displaying chromatin condensation at the nuclear membrane and cell body shrinkage, which are highly suggestive of an ongoing apoptotic process (Fig. 1I). In hippocampal sections of controls, no staining of neurons for the ASP was noted.

Expression of Bcl-2 in PSNN, AD, and Controls

In PSNN, Bcl-2 expression was restricted to single, large neurons. It was absent in the ventral pontine nuclei, where the majority of apoptotic neurons were observed. In a hippocampal section of PSNN, mild Bcl-2 reactivity was found in layer II of the temporal cortex, but was completely absent in the dentate gyrus, subiculum, and CA 1 regions, which are prone to apoptosis in this disease.

In CA 1 and subicular/entorhinal regions, similar numbers of pyramidal neurons were labeled with equal intensity in AD and control brains. Leucocytes and ependymal cells were labeled in all cases of PSNN, AD, and controls (Fig. 1E), and the relative labeling intensity exceeded that observed in neurons. Both in AD and in age-matched controls, dentate gyrus and CA 4 neurons did not exhibit Bcl-2 immunoreactivity.

Expression of c-Jun in PSNN, AD, and Controls

In PSNN, c-Jun expression was observed in nonapoptotic and occasionally in apoptotic neurons in the pontine nuclei and throughout the hippocampus (Fig. 1D). No association between c-Jun immunoreactivity and apoptotic activity of defined pontine areas could be established. Labeling was confined to the cytoplasm.

In AD and controls, pronounced c-Jun immunoreactivity was observed throughout the hippocampal subfields. Most neurons localized in the CA 4, CA 3, CA 2, and CA 1 subfields, the subicular/entorhinal region, and nearly all dentate gyrus neurons showed distinct labeling for c-Jun (Fig. 1G). No difference between AD and control tissue regarding localization, number of cells labeled, and intensity of immunostaining was noted. The observed staining pattern was also exclusively cytoplasmic.

TABLE 2
Summary of Clinicopathological Data and Immunohistochemical Results

Case	Age	Sex	Cause of death	PMT (h)	IST+ cells/mm²	Apoptotic cells/mm²	Labeling of Hippocampal Neurons for ASP (cells/mm²)	Bcl-2	c-Jun
AD1	65	F	Respiratory failure	34	24.1	0	0	−	±
AD2	79	F	Decubital ulcers	27	55.9	0	0	+++	+++
AD3	77	F	n/a	36	22.7	0	0	++	+++
AD4	81	M	Cardiac arrest	40	11.0	0.07	0.07	−	+++
AD5	70	F	Decubital ulcers	37	n/a	0	0	++	+++
AD6	79	M	Cardiopulmonary arrest	16	8.1	0	0	±	±
AD7	87	F	Decubital ulcers	50	6.1	0	0	±	+++
AD8	75	M	Pneumonia	45	14.3	0	0	−	−
AD9	76	M	Pneumonia	28	11.4	0	0	+	+++
AD10	86	F	Myocardial infarction	59	15.8	0	0	++	+++
AD11	87	F	n/a	72	13.3	0.06	0.06	+	+++
AD12	60	F	Sepsis	10	10.3	0	0	+	+++
CO1	57	M	Pneumonia	34	2.4	0	0	++	+++
CO2	58	F	Sepsis	27	1.5	0	0	±	n/a
CO3	73	M	Myocardial infarction	6	0.4	0	0	±	+++
CO4	70	M	Respiratory failure	44	1.5	0	0	+++	+++
CO5	75	M	Wernicke's syndrome	16	n/a	0	0	++	±
CO6	71	F	Cardiac arrest	18	1.8	0	0	n/a	++
CO7	65	M	Cardiac arrest	6	2.4	0	0	++	+++
CO8	84	M	Cardiopulmonary arrest	10	0.8	0	0	+++	+++
CO9	77	M	n/a	11	0.0	0	0	±	n/a
CO10	46	M	Cardiopulmonary arrest	7	0.0	0	0	++	+++
PSNN1	38th week of gestation	F	Disturbed placental maturity	24	98.6	112	118	±	++
PSNN2	39th week of gestation	F	Placental infarction	24	68	94	94	n/a	+++
PSNN3	1 month	M	Vitium cordis	24	25	53.7	21.5	−	+++

IST+ cells comprise neurons and glial cells. Regions analyzed for IST, ASP, Bcl-2, c-Jun, and apoptotic cells include CA 4, CA 3, CA 2, CA 1, subiculum, entorhinal cortex, and the adjacent temporal cortex. Results for PSNN1 and PSNN2 were obtained on pontine sections, PSNN3 analysis was performed on hippocampal sections. Semiquantitative scores represent subjective assessments of the numbers of labeled neurons, as follows: −: no neurons labeled; ±: few neurons labeled, +: up to 25% of neurons labeled; ++: 25–50% of neurons labeled; +++: over 50% of neurons labeled. Abbreviations used: PMT: postmortem time; h: hours; IST: in situ tailing; ASP: apoptosis specific protein; AD: Alzheimer disease patient; CO: control subject; F: female; M: male; n/a: data not available.

TABLE 3
Summary of In situ Tailing and Immunohistochemical Results in PSNN, AD, and CO

Method/antibody applied	PSNN		AD	CO
	Apoptotic neurons	Nonapoptotic neurons		
In situ Tailing	++	+	+	±
Anti-ASP	++	−	+/−	−
Anti-c-Jun	+	++	++	++
Anti-Bcl-2	−	±	++	++
Anti-alpha B-Crystallin	±	+	±	±
Anti-HSP 27	−	−	±	±
Anti-HSP 65	±	++	++	++
Anti-HSP 70	+	++	±	±
Anti-HSP 90	+	++	++	++
Anti-PHF/Ubiquitin	−	−	++ (tangles)	± (tangles)
Anti-Ubiquitin	+	++	++ (tangles)	± (tangles)

Scores represent subjective assessments of numbers of labeled cells, as follows: −: no labeling observed; ±: exceptional cells labeled; +: few cells labeled; ++: many cells labeled. Anti-alpha B-Crystallin, anti-HSP 27, and anti-HSP 70 immunolabeled single chromatolytic cells. Only 2 apoptotic neurons were observed in sections of AD and none in controls. Abbreviations used: PSNN: patients with pontosubicular neuron necrosis; AD: Alzheimer disease patients; CO: control subjects; ASP: apoptosis specific protein; HSP: heat shock protein; PHF: paired helical filament.

Expression of Alpha B-Crystallin, HSP 27, HSP 65, HSP 70, HSP 90, Ubiquitin, and PHF/Ubiquitin in PSNN, AD, and Controls

Except for HSP 27 and PHF/ubiquitin, all heat shock proteins were detected in pontine neurons of PSNN. As with c-Jun, we were unable to localize HSP immunoreactivity to areas mainly affected by apoptotic neuronal cell death. Labeling efficiencies for apoptotic cells applying the various HSP antibodies were between 15% and 30%, similar to or below those for morphologically normal neurons (Fig. 1F).

In AD and controls, the typical granular mitochondrial staining pattern was observed in both neurons and glial cells with the antibody against HSP 65. Immunostaining for HSP 90 revealed a large proportion of neuronal cells, as well as astrocytes with pronounced depiction of neuronal processes. Expression of both proteins was not related to AD neuropathology, and similar staining patterns were observed in AD and controls. Neuronal immunostaining for alpha B-Crystallin and HSP 27 was restricted to a few ballooned, chromatolytic temporal cortex neurons in AD and controls, and no neuronal labeling was found in the hippocampal subfields and entorhinal cortex. HSP 70 immunoreactivity also highlighted single chromatolytic temporal cortex neurons and additionally revealed multiple extracellular granular structures, especially in CA 4 and CA 1 regions in AD. Ubiquitin and PHF/ubiquitin immunoreactivity was predominantly found in AD neurofibrillary tangles and dystrophic neurites, with a few tangles detected in some controls (data not shown).

DISCUSSION

AD is a devastating neurodegenerative disorder that affects 15–25% of individuals in the steadily rising age group above 80. In recent years, it became increasingly clear that nerve cell loss and reduction in synaptic connectivity are the major neuropathological correlates of dementia (3, 4, 40). To conceive potential therapeutic targets, it is of major importance to clarify the mechanisms leading to nerve cell death (41).

Of the 2 main broad concepts of cell death, i.e. programmed cell death (apoptosis) and passive cell death (necrosis), apoptosis has recently gained much attention in AD research. This is mainly based on the discovery

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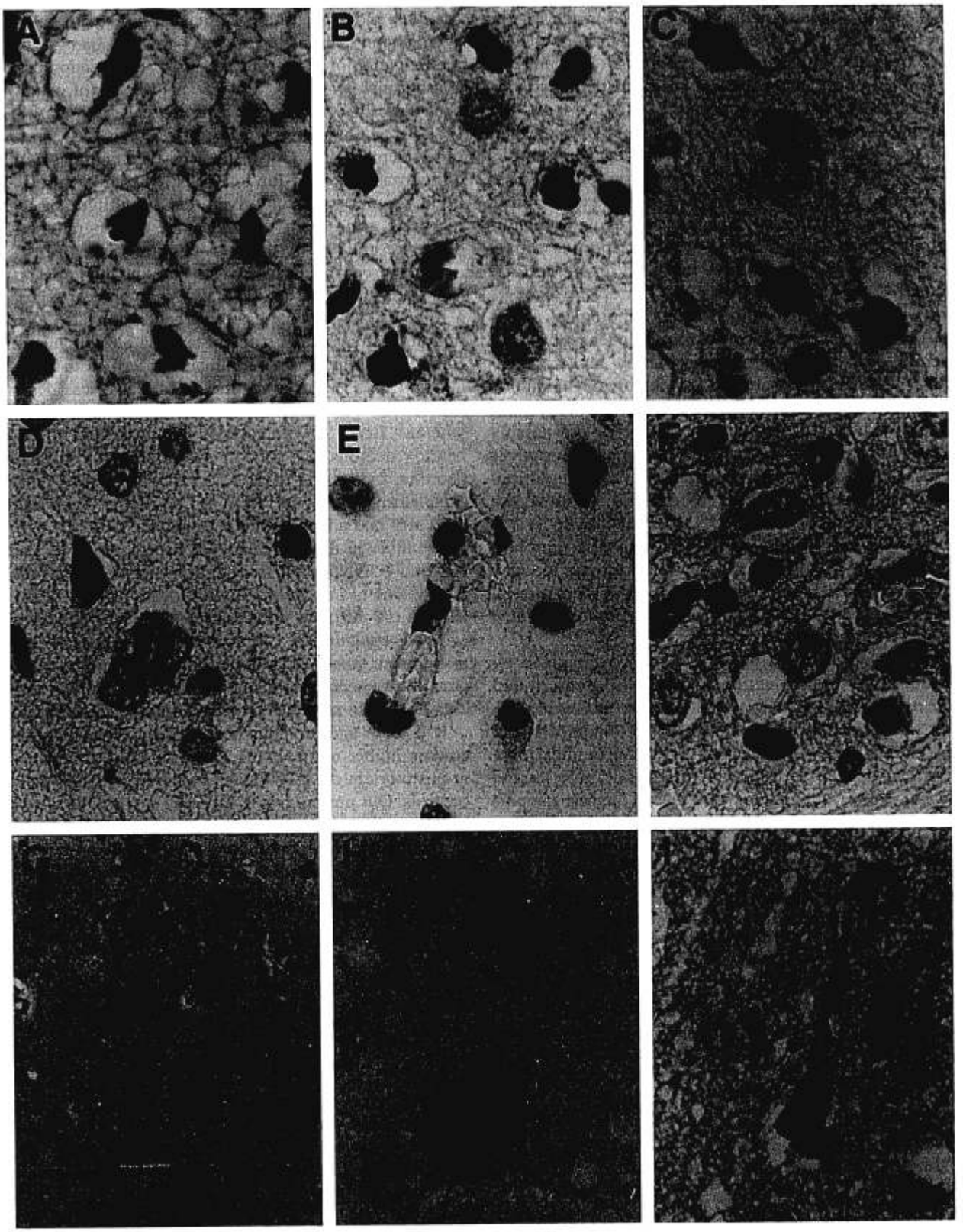
Fig. 1. A–F: Pontosubicular neuron necrosis. A: Multiple neurons displaying chromatin condensation, nuclear fragmentation, and cell body shrinkage typical of apoptosis; H&E staining. B: IST+ apoptotic neurons. C: Apoptotic neurons immunostained for the apoptosis-specific protein (ASP); A–C: ×925. D: Generalized cytoplasmic labeling of nonapoptotic neurons for c-Jun. E: Immunolocalization of Bcl-2 in a leucocyte; the apoptotic neuron remains unstained. F: Immunolabeling for HSP 70 detects few apoptotic cells; the majority remain unlabeled; D–F: ×760. G–H: Alzheimer disease: G: Generalized cytoplasmic immunoreactivity for c-Jun in CA 1 neurons; ×370. H: Representative IST+ cells in AD; morphologically normal nuclei exhibit labeling for DNA fragmentation. I: An exceptional AD neuron immunolabeled for the ASP and demonstrating condensation of chromatin at the nuclear membrane typical for cells undergoing apoptosis; temporal cortex; H–I: ×1180.



of AD-linked genes shown to be involved in the regulation of programmed cell death (19–23). Further evidence comes from the study of Down syndrome (24) and from the application of histochemical methods demonstrating DNA fragmentation in brain tissue (8–11, 13, 14, 16, 42). A deranged balance of pro- and antiapoptotic proteins reported in AD as well as other neurodegenerative diseases (25–27, 43) also supports a role for some form of programmed cell death. Recently, aberrant expression of cyclin-dependent kinases and other cell cycle regulators that may initiate apoptotic cell death by abortive activation of the cell cycle machinery has been reported in AD patients (44–47).

Apoptosis is strictly defined by morphological alterations in dying cells. Cytoplasmic condensation, cell shrinkage, and condensation, margination, and fragmentation of the chromatin are the key features of apoptotic cells as seen by light and electron microscopy (33). It has proven difficult to unequivocally identify the presumably small number of neurons in AD fulfilling the apoptosis criteria. Although proper assessment of nuclear fine structure may be hindered by the colorimetric substrate, the majority of cells labeled for DNA fragmentation in AD as well as in controls do not exhibit apoptotic morphology (9, 10, 16). We therefore sought an alternative way to investigate the contribution of apoptosis to neuronal cell death in AD neurons by examining apoptosis-related proteins.

Various studies claim that the protein recognized by the c-Jun/AP-1 (Ab-2) antibody is not the transcription factor c-Jun itself, but a protein found to be highly expressed in apoptotic cells; hence the term "apoptosis-specific protein" (34, 48). Our immunohistochemical data on glioblastoma, hippocampus from kainic acid treated rats, and acute inflammatory leukoencephalitis support this view (data not shown). The predominant localization of the ASP in the cytoplasm, its relative insolubility, and its appearance rather late in the process of apoptosis suggest that it may be a constituent of the modified cytoskeleton in apoptotic cells (34). However, its marked expression in apoptotic as opposed to necrotic cells and its strong association with cardinal features of apoptosis, namely cytoplasmic and chromatin condensation, make it an ideal marker for late-stage apoptosis. This property has recently been exploited in a study on the role of apoptosis in atherosclerosis (49). In our study, we detected 2 neurons stained for the ASP in a total of 36 hippocampal sections from AD patients, and no such staining was observed in controls. By contrast, apoptotic neurons in PSNN showed massive expression of the ASP.

C-Jun transcription factor is induced in response to various cellular stressors leading to degeneration and cell death as well as survival and regeneration. The eventual cell fate seems to depend on the specific homo- or heterodimerization of c-Jun with members of the Jun and Fos families and various other transcription factors (30). In sympathetic neurons, c-Jun was shown to be essential for the induction of apoptosis, and its antagonization prevented neuronal death (28, 29). Elevated levels of c-Jun have been described in various neurological diseases, i.e. AD (32, 50, 51), amyotrophic lateral sclerosis (52), and multiple sclerosis (53). Our results show that similar numbers of neurons express c-Jun in PSNN, AD, and controls. This finding is not surprising given that a multitude of cellular reactions aside from the induction of programmed cell death are initiated by this transcription factor (30).

A disturbed balance between the antiapoptotic protein Bcl-2 and the proapoptotic Bax was hypothesized to play a role in βA4-mediated neurodegeneration (25). Various immunohistochemical studies reported an upregulation of Bcl-2 in AD brains, predominantly ascribed to extensive gliosis (54, 55). An upregulation of Bcl-2 was described associated with neuronal DNA fragmentation in AD, whereas Bcl-2 downregulation was detected in tangle-bearing neurons (27, 56). In our material, no differential neuronal expression of Bcl-2 in AD vs controls could be established. However, forming homo- or heterodimers with pro- and antiapoptotic proteins, the Bcl-2 family may well influence the propensity of neurons to cell death in AD and PSNN. Apart from Bcl-2, diverse stress proteins are capable of influencing the life-death balance in in vitro models (57–60). We were unable to detect a selective up- or downregulation of stress proteins in degenerating neurons in our material by immunohistochemistry, although quantitative techniques may be warranted to further elucidate the role of stress proteins in neurodegeneration.

It is now agreed that vulnerable brain regions in AD contain higher numbers of cells with DNA fragmentation in postmortem autopsy tissue compared with age-matched controls. Our present results strongly support the view that, with very few exceptions, these cells with DNA fragmentation do not die by apoptosis, as defined by morphological criteria and the expression of apoptosis-associated antigens. The consistent differences between AD patients and controls obtained in various independent studies (8–10, 13, 14, 16) make it unlikely that the DNA fragmentation observed is due to technical artifacts. Recently, postmortem times (PMTs) longer than 6.5 h were reported to increase the number of cells labeled for DNA fragmentation (32). We did not find such a correlation in our material in a previous study (9), and our findings are thus in accordance with many other reports (8, 11, 12, 15, 16, 61). We cannot formally exclude, however, that vulnerable AD neurons are more susceptible to longer PMTs, as suggested by some investigators (10, 32). We used the paradigm of PSNN to control for variables like PMT and fixation time to show that neurons can display the full spectrum of apoptotic changes including the expression of apoptosis-related proteins, and that these alterations can be readily detected in human autopsy tissue. The question thus arises

as to the role of neuronal DNA fragmentation in the pathogenesis of AD.

The cells labeled by in situ tailing could represent a population of neurons with in vivo DNA damage. Ongoing DNA repair processes or a general deficit of DNA repair can cause increased numbers of DNA strand breaks. Indeed, an accumulation of DNA damage has been described in AD patients before (5, 62–64). Morphological techniques for the evaluation of DNA fragmentation are, however, very insensitive, in general only label cells with massive and irreversible DNA damage, and cannot be expected to identify cells with deficits in DNA repair.

We therefore prefer the alternative explanation, namely that the cells with DNA fragmentation in AD are indeed dying cells, although not presenting with the classical features of apoptosis. Since their number, however, is too high for a disease with slow progression over many years, we assume that the final trigger for death in these cells mainly occurs during the terminal period of the patient's life (9). A synchronous induction and progression of cell death is supported by the morphological uniformity of the in situ tailing positive cells. This view implies that neurons in AD differ from those of controls in their susceptibility to death signals, which may result from an accumulation of DNA damage, as suggested previously (5, 62, 63), from as yet unknown metabolic disturbances (65–68), or from modifications in the intracellular control mechanisms of cell life and death. Minor additional noxious stimuli, such as preterminal hypoxia, or changes in the cellular microenvironment may be sufficient to kill these exquisitely vulnerable AD neurons.

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