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Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ

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Abstract Loss of nerve cells is a hallmark of the pathology of Alzheimer's disease (AD), yet the patterns of cell death are unknown. By analyzing DNA fragmentation in situ we found evidence for cell death not only of nerve cells but also of oligodendrocytes and microglia in AD brains. In average, 30 times more brain cells showed DNA fragmentation in AD as compared to age-matched controls. Nuclear alterations suggestive of apoptosis were rare in degenerating cells. Even though the majority of degenerating cells were not located within amyloid deposits and did not contain neurofibrillary tangles, neurons situated within areas of amyloid deposits or affected by neurofibrillary degeneration revealed a higher risk of DNA fragmentation and death than cells not exposed to these AD changes.

Key words Alzheimer's disease · Cell death · DNA fragmentation · β-Amyloid

Introduction

Besides the classical pathological lesions that characterize Alzheimer's disease (AD) – amyloid plaques and neurofibrillary tangles (NFT1) [1] – one major feature of the disease is a substantial loss of neurons and synapses in affected brain areas [9, 21, 24, 30, 33]. Indeed, recent evidence suggests that neuronal and synaptic loss represent better correlates of dementia than the classical AD markers [9, 21, 33]. A variety of different mechanisms have been suggested to contribute to neuronal death in AD, including direct toxicity of βA4 peptide [38, 39], a generalized defect in DNA repair [27, 29], defects in oxidative energy metabolism [15, 22] as well as indirect hypoxic states due to amyloid angiopathy [36]. To explain the pathogenesis of AD, it is crucial to understand, which cells die and what are the cause(s) of cell death.

In the present study we have used a recently developed molecular biological technique [11–13, 16, 35] to label dying cells in situ and have identified degenerating cells and their relationship to amyloid deposits and NFTs in AD brain tissue.

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Materials and methods

General neuropathology

The present study is based on 18 cases of clinically diagnosed and neuropathologically confirmed AD and 15 age-matched non-demented controls derived from a prospective longitudinal clinicopathological study of aging [17]. Brains were fixed in buffered formalin and tissue blocks were routinely embedded in paraffin. Sections were stained with hematoxylin/eosin, luxol fast blue for myelin and modified Bielschowsky silver impregnation. The extent of AD pathology was determined by counting plaques and NFTs in standardized regions of the frontal, parietal and temporal neocortex as well as of hippocampus and subiculum, as described in detail earlier [17].

Histochemical detection of DNA fragmentation

DNA fragmentation was visualized using the technique described by Gold et al. [12, 13]. Deparaffinized sections were pretreated with 5% proteinase K (Sigma P0390, St Louis, Mo.) for 15 min. The sections were then incubated for 1 h in a reaction mixture containing 10 µl 5 × tailing buffer, 1 µl digoxigenin DNA labeling mixture, 2 µl cobalt chloride (25 mmol), 12 U terminal transferase and the necessary volume of distilled water to give a total volume

of 50 µl. After extensive rinsing in TRIS-buffered saline the sections were treated with a 1:250 dilution of alkaline phosphatase-labeled anti-digoxigenin antibody for 1 h at room temperature. The color reaction was developed with 4-nitroblue-tetrazolium-chloride/5-bromine-4-chloride-3-indolyl-phosphate (x-phosphate). All enzymes and reagents were purchased from Boehringer (Mannheim, Germany).

The reliability of histochemical visualization of DNA fragmentation has been experimentally tested in various model systems. Brain tissue from guinea pigs and rats with autoimmune encephalomyelitis that contained high numbers of T lymphocytes with apoptosis [31] were analyzed for DNA fragmentation. In this situation, all cells with morphological features of apoptosis were labeled with the DNA fragmentation assay [12, 13]. Brain tissue from this experimental series, subjected to autolysis for up to 24 h, revealed a complete labeling of apoptotic cells in the absence of background staining of non-apoptotic cells. In addition, in the present investigation, we analyzed normal aged brain tissue with autolysis times ranging from 5.5 to 45 h. In these samples we found no increase of cells labeled in the DNA fragmentation assay in relation to post-mortem time (Fig. 1 a). Similarely, we found no correlation between the numbers of cells with DNA fragmentation and post-mortem time in AD patients (Fig. 1 a).

Immunocytochemistry

After DNA labeling, immunocytochemistry was performed for visualization of βA4-amyloid deposits and NFTs as well as for identification of cell types; DNA fragmentation was demonstrated using an alkaline phosphatase/anti-alkaline phosphatase system (Dako D651; Glostrup, Denmark). The reaction product was visualized with Fast Red TR Salt (Sigma F1500). The following primary antibodies were used: 4G8 (anti-βA4-amyloid peptide [19]), anti-paired helical filament/ubiquitin (3–39 [34]), anti-glial fibrillary acidic protein for astrocytes (Dako Z 334, Glostrup, Denmark), anti-ferritin for microglia [14] and anti-myelin oligodendroglia glycoprotein (MOG) for oligodendrocytes (8–18/C5 [4]).

Quantitative evaluation

In a first experiment DNA fragmentation was visualized in complete hemispheric sections at the level of the mid-thalamus in three AD cases. The density of cells with DNA fragmentation was determined in the following regions: parietal, cingulate, lateral, basal temporal and entorhinal cortex, hippocampus and subiculum. As the highest incidence of cells with DNA fragmentation for all cases was found in the subiculum, hippocampus and entorhinal cortex, further quantiative evaluation of cells with DNA fragmentation was performed in a standardized region of the temporal lobe including hippocampus, subiculum, entorhinal cortex and temporal isocortex by counting labeled cells in an area of 160 mm^2/case. The values were then transformed to cells/mm^2.

NFTs and amyloid deposits were evaluated in detail in adjacent serial sections. In each of the different cortical areas (temporal cortex and subiculum) we determined the number of NFTs in five contigous microscopic fields, measuring 1.4 mm^2. The values in tables and figures represent average numbers of neurons with NFT/mm^2. Amyloid deposits were determined by overlaying the sections with a stereological grid and calculating the percentage area of the subiculum and temporal cortex covered by amyloid deposits.

In addition, seven AD cases showing large numbers of cells with DNA fragmentation and a high load of amyloid deposition were selected for double staining with anti-βA4. All cells with DNA fragmentation in the subiculum were counted and their location, either within or outside amyloid deposits, was determined. The percentage area covered by the amyloid deposits was determined in the same location. The increased risk of death to cells located within an amyloid deposit was calculated by dividing the percentage of dying cells/mm^2 located within plaques by the percentage area covered by amyloid.

In a similar way six AD cases with large numbers of both cells with DNA fragmentation and NFTs were analyzed by double staining with anti-ubiquitin. In standardized regions of the subiculum the number of neurons with DNA fragmentation and the number of nerve cells with both NFTs and DNA fragmentation was determined in a total number of 2102 nerve cells. The increased risk of cell death for neurons in relation to neurofibrillary pathology was determined by dividing the percentage of dying cells in the NFT-positive cell population by the percentage of dying cells in the non-NFT-containing neuronal cell population.

Results

Neuropathological findings

The numbers of plaques and NFTs were significantly higher in AD cases compared to age-matched controls (Table 1). All AD cases but none of the controls fulfilled the quantitative neuropathological criteria for the diagnosis of AD according to Khatchaturian et al. [18] as well as the CERAD criteria [26].

Incidence of dying cells in AD cases in comparison to age-matched controls

In the brains of normal aged individuals, only a few cells with nuclear DNA fragmentation were found. The values were in the range of 0 to 0.5 cells/mm^2. The vast majority of dying cells were located in the white matter and, by immunocytochemical counter staining, could be identified as oligodendrocytes. Only exceptional nerve cells in the gray matter (0–0.03 cells/mm^2) were labeled (Table 1). There was no correlation between the number of labeled cells with age or post-/mortem time (Fig. 1 a, b).

Table 1 DNA fragmentation in the brain of Alzheimer's disease Neurons and glia cells were differentiated according to structural features; in addition glia cells were characterized by double staining with antibodies against mylin oligodendroglia glycoprotein (oligodendrocytes), GFAP (astrocytes) and ferritin (microglia)

	Alzheimer's disease	Controls
Number of patients	18	15
Mean age (years)	78 ± 2	73 ± 4
Disease duration (years)	6.4 ± 3.7	–
Amyloid deposition (% area) in temporal lobe[a]	6.8 ± 0.8*	0.7 ± 0.3
Tangles in temporal lobe[a] (cells/mm^2)	13.3 ± 2.4*	1.1 ± 0.5
DNA fragmentation (cells/mm^2):		
Total labeled cells	3.9 ± 1.3*	0.14 ± 0.04
Labeled neurons	1.1 ± 0.4*	0.02 ± 0.01
Labeled glia	2.8 ± 1.0*	0.12 ± 0.03

* $P < 0.0001$
[a] Means of temporal iso- and allocortex

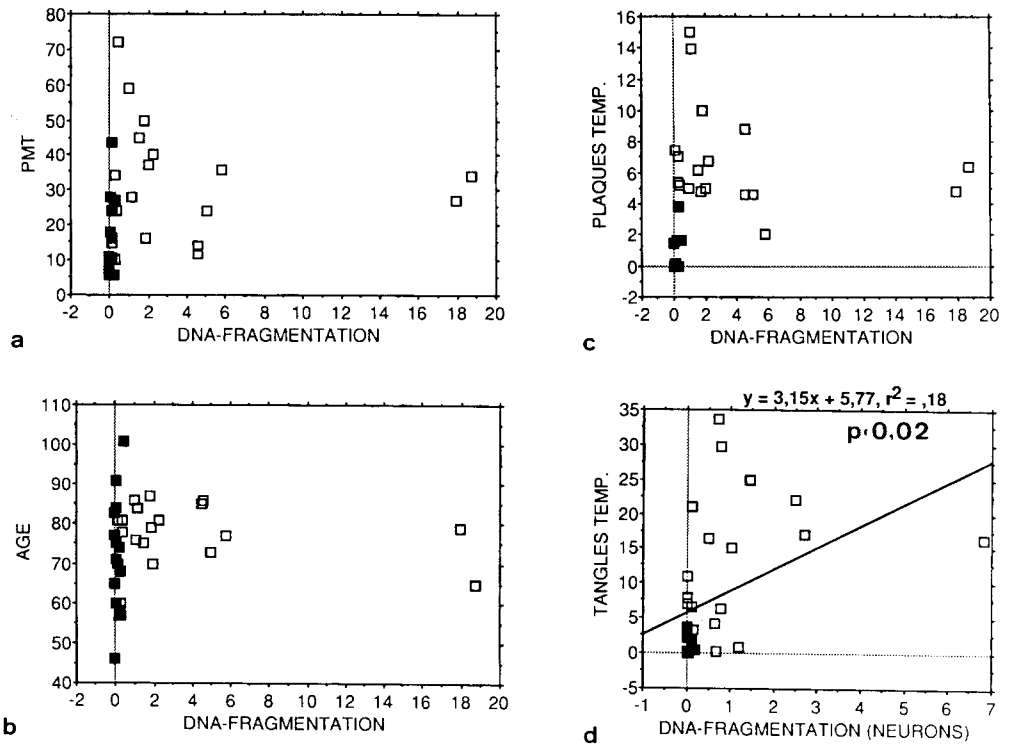

Fig. 1 Blotting of cells with DNA fragmentation against post-mortem time (PMT) (**a**, hours), age (**b**), amyloid plaques (**c**) and neurofibrillary tangles (**d**) in temporal lobe of Alzheimer's disease (AD) cases and controls. *Solid squares* represent controls (n = 15), *open squares* AD cases (n = 18). We found no correlation between the numbers of cells with DNA fragmentation with either PMT (**a**), age (**b**) or density of amyloid deposition (**c**). There was a weak but significant correlation between neurofibrillary tangles (NFTs) and nerve cells with DNA fragmentation (**d**). The values given for DNA fragmentation and NFTs represent positive cells/mm^2. The values for plaques represent the percentage area covered by βA4 peptide immunoreactivity in the tissue sections

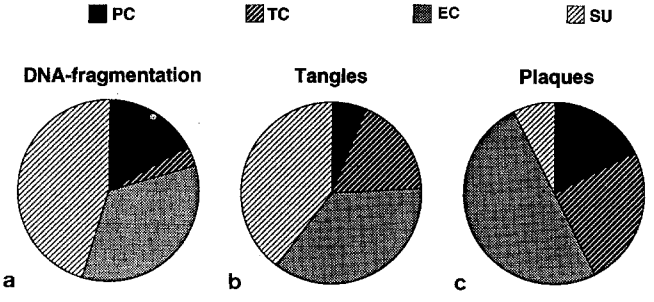

Fig. 2 a–c Distribution of cells with DNA fragmentation, NFTs and amyloid plaques in a case with severe AD pathology. The highest density of cells with DNA fragmentation (**a**) and with NFTs (**b**) was present in the entorhinal cortex and subiculum. The highest plaque density (**c**) was found in the entorhinal cortex and the temporal isocortex. (*PC* parietal cortex, *TC* temporal cortex, *EC* entorhinal cortex, *SU* subiculum)

In AD brains we found the largest numbers of degenerating cells in the subiculum, the hippocampus and the entorhinal cortex. A representative example is shown in Fig. 2. The topographical distribution of cells with DNA fragmentation correlated well with the distribution of NFTs but not with that of amyloid plaques.

Within the temporal lobe (in both the allocortex and infratemporal isocortex), cells with detectable DNA fragmentation (Fig. 3) were significantly more numerous in AD brain as compared to controls (Fig. 1, Table 1). On average, there were 25 times more labeled glia cells and more than 50 times more labeled neurons in AD temporal lobe compared to controls (Table 1). Similar to controls there was no correlation between the number of cells with DNA fragmentation and either the age, or the post-mortem time in AD patients (Fig. 1 a, b).

Identification of cells with DNA fragmentation

On average 28% of all degenerating cells could be identified as neurons in the temporal lobe of AD patients (Fig. 3 a–d). In addition, double-staining experiments revealed that glia cells showing DNA fragmentation were mostly immunoreactive with anti-MOG and anti-ferritin (Fig. 3 e–h) and were, thus, oligodendrocytes and microglial cells. Labeled oligodendrocytes were predominantly found in the subcortical white matter, underlying areas with severe neuronal loss (Fig. 3 f, g). In these regions some degree of myelin degradation was present, suggesting ongoing Wallerian degeneration. Degenerating microglial cells were mainly present in cortical areas (Fig. 3 h) and were frequently found in association with amyloid deposits.

Structural alterations of cells with DNA fragmentation in AD

Recent studies suggest that apoptosis may be an important pathway of cell death in AD [7, 10]. Morphologically, apoptosis is associated with a characteristic pattern of chromatin condensation [37]. Indeed, some nuclei with DNA strand breaks in AD revealed chromatin condensation and nuclear fragmentation. However, the majority of labeled nuclei did not show the classical morphological features of apoptosis (Fig. 3 a). The cytoplasm of cells

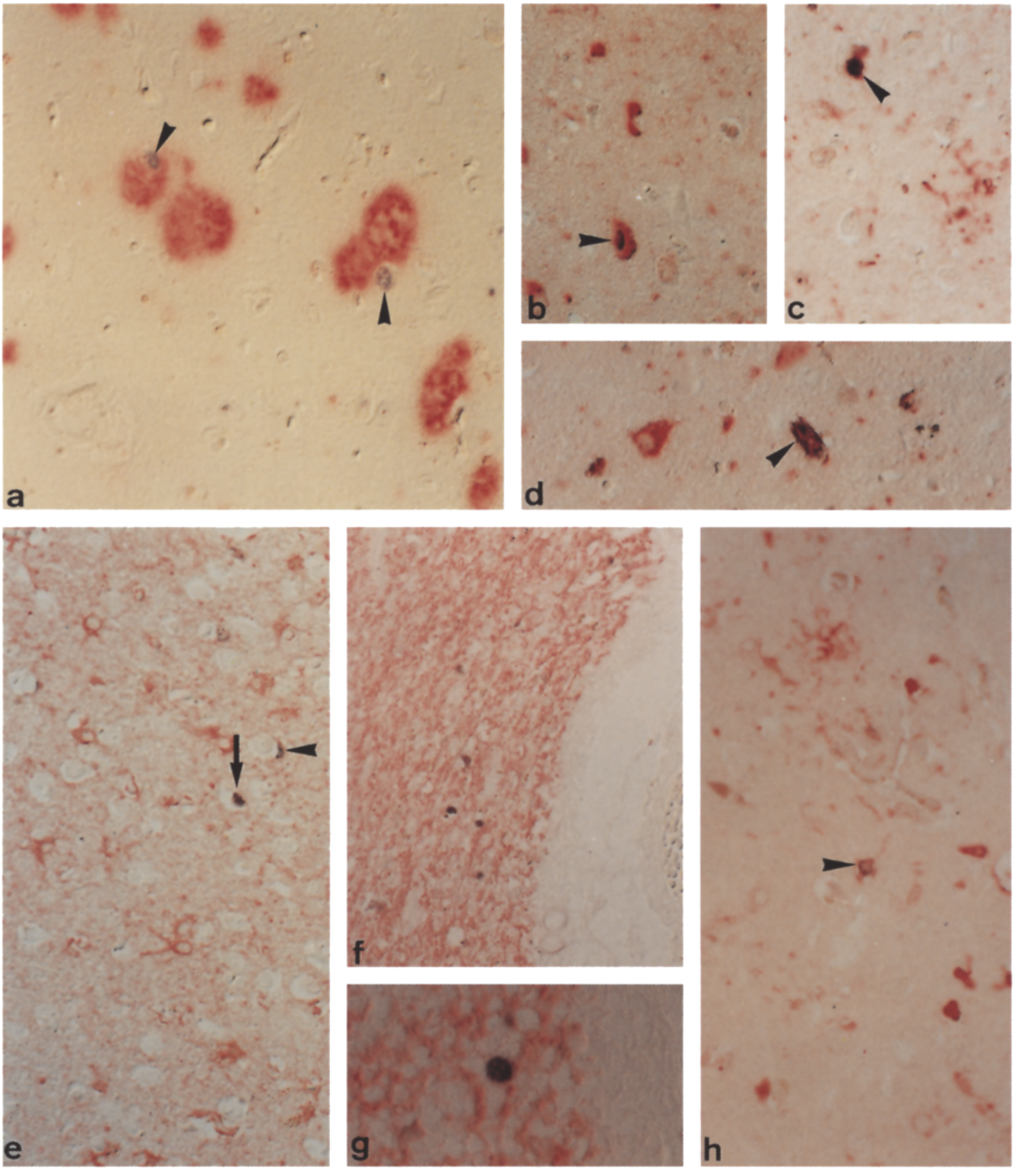

Fig. 3 a, b Labeling of DNA fragmentation in the brain with AD. **a** Double staining with 4G8 (anti-βA4 peptide), degenerating neurons *(blue, arrows)* located in amyloid plaques *(red)*. **b** Double staining with anti paired helical filaments (PHF)/ubiquitin (3–39); tangle-bearing neuron with nuclear DNA fragmentation *(arrow)*. **c** Double staining with anti-PHF/ubiquitin (3–39); tangle-bearing small neuron with nuclear DNA fragmentation *(arrow)*. **d** Double staining with anti-PHF/ubiquitin (3–39); degenerating tangle-bearing neuron with dispersed fragmented DNA after nuclear dissolution *(arrow)*. **e** Double staining with anti-glial fibrillary acidic protein (GFAP) antibody; a nerve cell *(thick arrow)* and a satellite cell *(arrowhead)* reveal DNA fragmentation; no degeneration of GFAP-positive cells. **f** Double staining with anti-myelin oligodendroglia glycoprotein (MOG); a cluster of oligodendrocytes with DNA fragmentation in the subcortical white matter. **g** Double staining with anti-MOG; oligodendrocyte in the white matter showing nuclear DNA-fragmentation. **h** Double staining with anti-ferritin as a marker of microglia; a ferritin-positive microglia cell with nuclear DNA fragmentation *(arrow)*. **a–e** × 300, **f** × 200, **g** × 600, **h** × 500

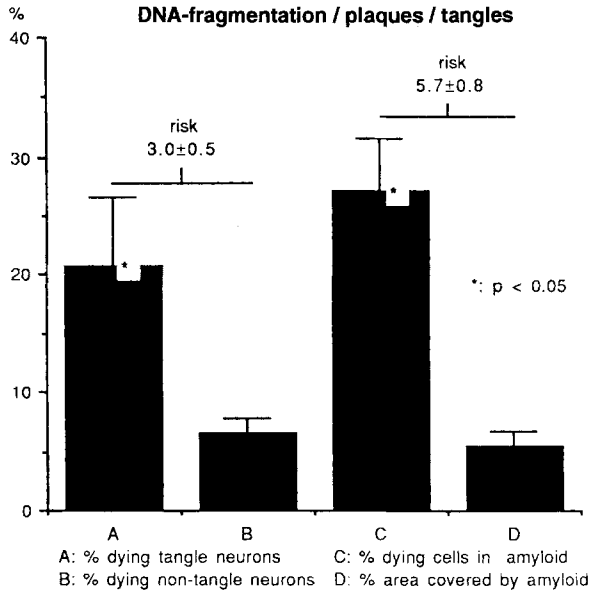

Fig. 4 Relation of neurofibrillary tangles *(A, B)* and amyloid deposition *(C, D)* with DNA fragmentation in AD

with nuclear DNA fragmentation was frequently swollen or appeared empty around the labeled nucleus (Fig. 3 e).

Relation of cell death to amyloid deposition

Since β-amyloid peptide may exert neurotoxicity in vitro [38, 39], we were interested in learning more about the relationship between amyloid deposition and cell degeneration. Neither in the AD group alone nor in the whole sample of both AD and controls could we find a significant correlation between the extent of amyloid deposition and the amount of cell death in the brain (Fig. 1 c). This was due to the fact that not all cases with a high density of amyloid deposition revealed high numbers of cells with DNA fragmentation and vice versa. Combining the detection of DNA fragmentation with immunocytochemistry for βA4-peptide in seven cases with high density of plaques and degenerating cells we found that only 13–50% (average: 27%) of the degenerating cells were located within areas of β-A4 peptide deposition (Figs. 3 a, 4). However, based on the area occupied by senile plaques, 3.6–10 (average: 5.7) times more degenerating cells were located in areas of amyloid deposition than in unaffected tissue (Fig. 4).

Relation of neuronal cell death to NFT pathology

We found a weak but significant correlation between the global number of neurons showing DNA fragmentation and the density of NFTs in the temporal lobe (subiculum and temporal cortex; Fig. 1 d). We, thus, selected for further analysis six cases with high density of nuclear DNA fragmentation as well as abundant NFTs in the subiculum. In these selected cases and areas the numbers of neurons

Table 2 Relation between neurofibrillary pathology and neuronal DNA fragmentation in the subiculum of Alzheimer's disease patients. Ghost tangles and neurons with DNA fragmentation comprised different populations. (*Ghost Tg* extracellular "ghost" tangles, *DNA Frag* neurons with nuclear DNA fragmentation)

Case	Tangles/ mm²	Ghost Tg/ mm²	DNA Frag/ mm²
A	9.6	0	3.3
B	18.2	0	3.4
C	21.0	10	6.4
D	54.0	11	23.5
E	33.0	17	41.0
F	40.0	59	52.5

with DNA fragmentation ranged from 3.3 to 52.5 cells/mm² (Table 2). We found a high correlation between NFTs and DNA fragmentation in neurons. Although extracellular "ghost tangles" only exceptionally contained remnants of fragmented DNA (Fig. 3 d), a large number of extracellular tangles were associated with a high incidence of DNS fragmentation in the remaining nerve cells (Table 2). An average of 41% of all neurons with DNA fragmentation (range: 18–66%) contained NFTs (Fig. 3 b–d). The average incidence of cell death in tangle-bearing neurons was 3.0 times higher compared to that in non-tangle-bearing nerve cells (Fig. 4).

Discussion

In the present study we found a significantly larger number of cells with DNA fragmentation in the brains of patients with AD compared to age-matched controls. The method used in this study is based on the detection of nuclear DNA strand breaks that occur in large numbers in cells undergoing necrosis or programmed cell death [11–13, 16, 35]. Previous studies on a variety of normal and pathological tissues [12, 13, 31, 35] revealed that this technique reproducibly labels degenerating cells, with a level of sensitivity below the threshold for detecting DNA alterations induced by autolysis, tissue fixation or processing. This view is based on the following observations: in the brain lesions of autoimmune encephalomyelitis this method selectively and completely labels cells which show the morphological features of apoptosis [12, 13, 31]. Similarely, in the experimental brain lesions induced by the toxicity of kainic acid neurons dying in the course of excitotoxicity are efficiently and selectively labeled by this method [12, 13]. As shown in the present study, autolysis alone does not apparently induce enough DNA damage to be detected by the assay. This is further supported by the observation that, in contrast to AD in autopsy tissue of multiple sclerosis patients, DNA fragmentation was predominately found in oligodendrocytes and T lymphocytes at the margins of actively demyelinating plaques [28]. Our data, thus, suggest that the accumulation of fragmented DNA in AD brains, previously demonstrated by biochemical techniques [27, 29], can at least in

part be explained by an increased incidence of acute cell death.

Cell loss in AD is believed to be confined to nerve cells [24]. Thus, an unexpected finding of our study was that in both AD and controls DNA fragmentation was more abundant in glia cells than in neurons. Mainly oligodendrocytes and microglia were affected. Oligodendrocytes with DNA fragmentation were predominantly found in areas with axonal damage and secondary myelin breakdown. Previously oligodendroglial loss and degeneration have been shown in experimental models of fiber tract degeneration in the central nervous system [8, 23]. Microglial cell degeneration in the plaques may be related either to direct toxic effects of the βA4 peptide or may reflect immune activation and self destruction by the release of toxic macrophage products [25].

One key question on the pathogenesis of AD relates to the mode of cell death. Recently apoptosis of nerve cells has been suggested as a major pathway of cell destruction in this disease [7, 10]. Apoptosis is a form of programmed cell death that can be induced by a variety of different signals [5]. It is mediated by activation of endonucleases which induce fragmentation of nuclear DNA into 180 base-pair fragments. This process is associated with characteristic condensation of nuclear chromatin [5, 37]. Although we found chromatin condensation in some degenerating neurons, the majority of labeled cells did not reveal the morphological patterns of apoptosis that have been defined ultrastructurally [5, 37]. This finding suggests that the majority of dying nerve cells are destroyed either by necrosis or by some form of programmed cell death other than classical apoptosis [32]. This view is supported by a recent in vitro study suggesting that βA4 peptide induces necrosis rather than apoptosis in nerve cells [3].

Amyloid plaques and NFTs are the hallmarks of AD pathology; their role in the pathogenesis of the degenerating process, however, remains unknown. Therefore, we studied the contribution of these pathological alterations to cell death in this disease. Even though the majority of degenerating cells were not located within amyloid deposits and did not bear NFTs, our data suggest that depositions of amyloid as well as accumulation of abnormal cytoskeletal elements are associated with an increased risk of death for affected neurons. Although from our data a direct causal relationship cannot be proven, in vitro studies suggest that this may at least be the case for amyloid. Our results are in line with previous studies showing that amyloid βA4 peptide increases the vulnerability of neurons to a variety of noxious stimuli, such as excitotoxicity, hypoglycemia, hypoxia and oxidative stress [2, 6, 20]. Since βA4 peptide may exert its toxic action not only on cells located directly within the amyloid deposit, but also through damage on processes of cells located outside, the contribution of amyloid deposition to cell death may be even higher than determined in this study.

Although in most AD brains the number of degenerating cells was small (less than 2 cells/mm^2), a much larger number (up to 52 cells/mm^2) of dying neurons was found in the subiculum of selected cases that also had large numbers of extracellular "ghost" tangles. This latter incidence of dying neurons is higher than what would be expected in a chronic disease with a progressive course over many years. In addition, other cases with severe AD-type pathology did not reveal such extensive cell degeneration, suggesting that ultimate cell death in AD brains requires additional precipitating factors. Thus, the rate of acute cell death in the preterminal phase of the disease might increase, probably due to synergy of the disease-specifc process with other factors, such as hypoxia and vascular or metabolic disturbances. This could occur at any time during the course of the disease but is more likely at the end stages of disease.

In conclusion, our findings suggest that both neurons and glia cells have a higher risk of death in AD than in normal aged control brains. Such an increased risk appears to be imposed in part by amyloid deposition and the accumulation of NFTs in nerve cells.

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