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Apoptosis in the aged dog brain

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Abstract Apoptosis similar to that seen in Alzheimer's disease patients was found in the brain of aged dogs by the TUNEL method of detecting in situ DNA fragmentation. Apoptosis was observed in both neurons and glial cells, and was morphologically characterized by round and swollen cytoplasm and aggregated nuclear chromatin, although these changes were slight. Neurons and astrocytes in the gray matter and oligodendrocytes in the white matter were affected. The number of ApopTag-positive brain cells increased slightly with age, but was not correlated to the number of senile plaques. A good correlation between the number of ApopTag-positive cells and the dementia index was clearly found. The present study indicates that brain cell apoptosis could account for dementia in aged dogs and suggested that aged dogs may be useful as a simplified animal model for Alzheimer's disease in man.

Key words Apoptosis · Dementia · Dogs · Senile plaques · TUNEL method

Introduction

Senile plaques of both diffuse and amyloid types, as seen in patients with Alzheimer's disease (AD) [3, 17, 19] and amyloid angiopathy have been found in the brains of aged dogs (more than 9 years old) [31]. However, neurofibrillary tangles (NFT), which may directly account for dementia in AD, have not been observed in the brains of aged dogs [32, 37] or other aged animals [10, 21, 22, 24, 25, 28, 33], except for sheep [23] and the Asiatic brown bear [5].

Neuronal cell loss is another histopathological hallmark of human dementia conditions [14, 30]. Recently, apoptotic neuronal cell death was detected in the brains of AD patients [6, 14, 29]. However, only a weak correlation between cell death and NFT and no relation between cell death and senile plaques were reported. This suggests the possibility that apoptotic neuronal cell death, and not senile plaques or NFT, is responsible for AD dementia. Moreover, amyloid β protein (Aβ), which is a major component of mature senile plaques, and amyloid angiopathy have been shown to be toxic in primary neuronal cultures [4, 7, 15, 16, 18, 35, 40] and PC12 cells [1, 2, 13, 39], and this neuronal cell death is likely to occur, at least in part, via apoptosis in vitro [36].

Therefore, we decided to investigate apoptosis in the brains of aged dogs. Very recently, a dementia index for dogs was proposed by Uchino et al. [34]. In the present study, we found brain cell apoptosis in aged dogs and examined the correlation between apoptosis and clinical dementia.

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Materials and methods

General neuropathology

Fifty-five canine brains obtained at autopsy were used in this study. Thirteen dogs, aged 13–24 years, had been clinically evaluated for dementia before death using the index proposed by Uchino et al. [34] (Tables 1, 2); the remaining 42 dogs, aged from 1 month to 18 years old, were not evaluated for dementia. The postmortem time in the study ranged from 1 to 24 h. The brains were fixed in 10% neutral buffered formalin and routinely embedded in paraffin. Sections, 6 μm thick, from the mid part of the brain including the cerebral cortex, medulla, hippocampus and thalamus were stained with hematoxylin and eosin, Congo red and periodic acid methenamine silver (PAM) staining.

Table 1 Criteria for evaluation of dementia in dogs

Items	Score
1. Appetite	
A: Normal	1
B: Abnormal, with diarrhea	2
C: Abnormal, without diarrhea	5
2. Life rhythm	
A: Normal (daytime = active, night = rest and sleep)	1
B: Day and night = mostly rest and sleep	3
C: Day = rest and sleep, night = prowl	5
3. Walking	
A: Normal	1
B: Trudging	3
C: Abnormal, one direction including circling	5
4. Excretion	
A: Normal	1
B: Incontinence	2
C: Cannot stop (always excreting)	3
5. Feeling	
A: Normal	1
B: Failing of hearing sense	2
C: Hypersensitivity of smell	3
6. Posture	
A: Normal	1
B: Head and tail down	3
C: Abnormal	7
7. Barking	
A: Normal	1
B: Monotonous and loud	3
C: Barking throughout night or at unusual object	7
8. Emotional expression	
A: Normal	1
B: Decrease of body language	3
C: Loss of body language	5
9. Relationship	
A: Normal	1
B: Loss of relationship with humans or other animals	3
C: Complete loss of relationship with owner	5
10. Situational judgment	
A: Normal	1
B: Abnormal (+)	3
C: Abnormal (++)	5
Total score of the items (dementia index);	< 21 = Normal
	21–29 = Predementia
	> 29 = Dementia

In situ detection of apoptosis

To detect apoptosis in the brain sections, a modified TUNEL method [9] was applied (ApopTag™ In Situ Apoptosis Detection Kit; Oncor, Gaithersburg, Md.). Briefly, brain sections were digested with proteinase K (20 µg/ml; Sigma, St. Louis, Mo.), then incubated with digoxigenin-labeled dUTP in the presence of terminal deoxynucleotide transferase (TdT). Sections were further incubated with peroxidase-conjugated anti-digoxigenin antibody. The reaction products were visualized with 3, 3′-diaminobenzidine tetrahydrochloride (Sigma). As a negative control, distilled water or phosphate-buffered saline was substituted for the TdT solution.

In addition, to clarify the effect of postmortem time on DNA degradation, the brain samples from three cases were kept under autolytic conditions either at room temperature for 36, 45 or 61 h, or at 37° C for 2, 4 or 17 h. To examine the effects of prolonged fixation, fresh brain tissues were fixed in formalin solution for 2, 4, 6, 8 or 12 weeks. The tissues were stained by the TUNEL method and the numbers of positively labeled cells were counted.

Double-labeling immunohistochemistry

To identify the type of cells that were ApopTag-positive, immunohistochemistry was performed in some brain tissues that were also investigated using the TUNEL method. The primary antibodies used were anti-neurofilament 200 (Sigma) specific for neurons, anti-glial fibrillary acidic protein (GFAP; Dako, Carpinteria, Calif.) for astrocytes, and anti-2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase; Sigma) for oligodendrocytes. After the secondary antibody reaction using biotinylated goat anti-rabbit IgG, the avidin-biotin alkaline phosphatase complex reaction (Vectastain, Vector, Burlingame, Calif.) and red-color visualization were performed.

Quantitative evaluation and statistical analysis

Senile plaques were counted on PAM-stained transverse sections from the mid part of the brain, including the parietal to temporal lobes, hippocampus and thalamus. The ApopTag-positive cells were counted on the ApopTag-stained sections and the numbers found in four distinct regions (cortex, medulla, hippocampus and thalamus) were expressed separately. The correlations between ApopTag-positive cells and age, senile plaques or dementia index were expressed as correlation coefficients (r^2).

Results

Effect of postmortem delay and fixation time on the ApopTag method

Fresh brain samples were maintained at room temperature (20–25° C) for 36, 45 or 61 h. All the samples were fixed in buffered formalin and embedded in paraffin under the same conditions. The numbers of ApopTag-positive cells did not differ among the samples. Other fresh tissue samples were placed in an incubator (37° C) for 2, 4 or 17 h. The number of ApopTag-positive cells was increased by 4- and 17-h incubation. Therefore, the postmortem time influenced the results of the ApopTag method at elevated temperature, but not at room temperature. Prolonged fixation in 10% neutral buffered formalin did not influence the number of ApopTag-positive cells.

Morphological features of ApopTag-positive cells

ApopTag-positive cells were present in both gray and white matter. Morphologically, positive cells in the gray matter, most of which were neurons, were swollen and were larger than unlabeled cells, but the changes were slight. The neuropil surrounding the positive cells appeared spongy. Positive nuclei were larger and rounder

Table 2 Dogs evaluated for dementia index (*M* male, *F* female, *SF* spayed female)

Case no.	Age (years)	Sex	Demential index	Senile plaques* (plaques/mm²)	ApopTag-positive cells (cells/mm²)
1	16	SF	50	2.44	357.16
2	15	F	47	9.76	135.33
3	16	F	44	1.13	193.48
4	16	M	41	1.76	14.01
5	14	M	40	1.24	85.32
6	17	F	38	1.91	79.58
7	15	SF	38	1.22	218.63
8	15	SF	37	0.91	0.28
9	15	M	37	1.16	6.36
10	13	M	30	4.41	0.34
11	24	M	29	0.07	13.73
12	16	F	28	7.24	0.55
13	15	F	27	2.90	0.66

* All types of senile plaques were counted

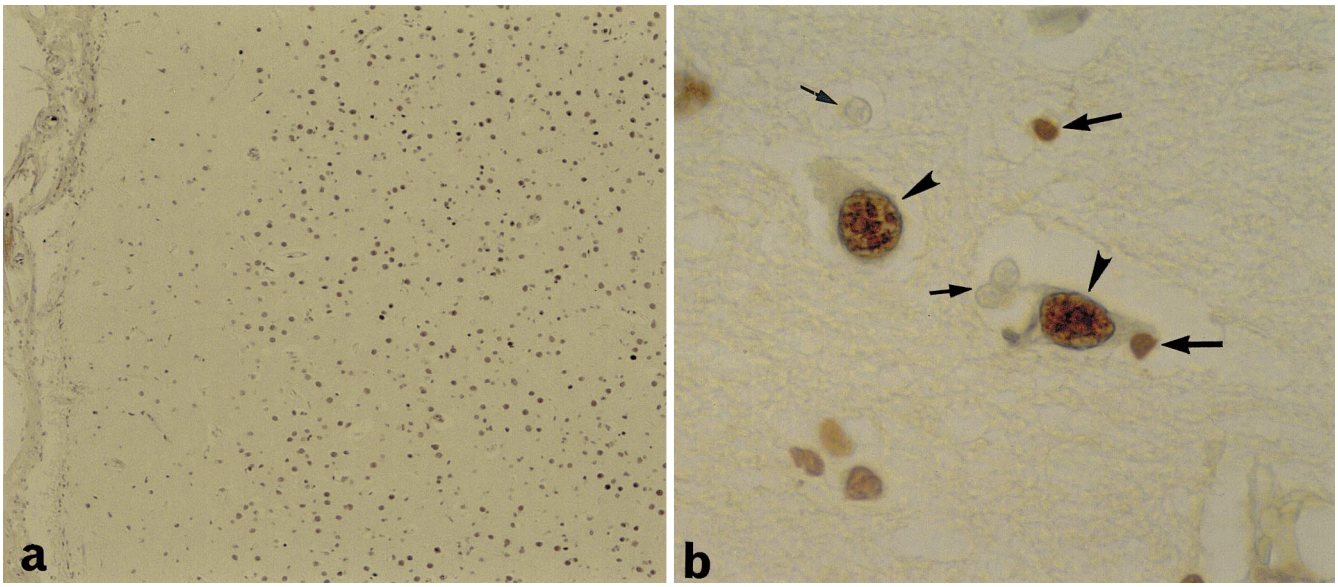

Fig. 1a, b Identification of brain cell apoptosis by the TUNEL method. **a** Distribution of ApopTag-positive cells in the gray matter of a 15-year-old dog. Counterstained with methyl green. **b** Morphology of ApopTag-positive neurons in the cortex of a 17-year-old dog. The nuclei of the cells are slightly swollen (*arrowheads*). Surrounding ApopTag-positive (*small arrows*) and negative cells are glial cells (*large arrows*). Counterstained with methyl green. **a** × 50, **b** × 800

than negative nuclei and contained more aggregated chromatin. The stainability of the positive nuclei varied from strong to faint (Fig. 1a, b). Apoptotic bodies or chromatin margination, which are the typical morphological characteristics of apoptosis, were not observed. Positive nuclei were usually found in neurons rich in lipofuscin. Positive nuclei of glial cells, most of which were present in the white matter, were also characterized by a dense staining pattern and chromatin margination. The nuclei of pial cells were also labeled in all cases, but the staining, although homogeneous, was weak.

Immunohistochemical identification of ApopTag-positive cells

The type of cells that was ApopTag positive was identified by combined immunohistochemistry using antibodies that recognize neurons, astrocytes or oligodendrocytes. ApopTag-positive cells in the gray matter including the cortex, hippocampus and thalamus comprised neurons showing immunoreactivity for neurofilament 200 (Fig. 2a) and astrocytes immunoreactive for GFAP (Fig. 2b). Cells positive for both ApopTag and GFAP tended to be close to ApopTag-positive neurons. Most ApopTag-positive cells present in the subcortical zone and white matter were immunoreactive for CNPase specific for oligodendrocytes (Fig. 2c).

Incidence of ApopTag-positive cells in comparison to age and senile plaques

The numbers of ApopTag-positive cells in the 55 canine brains were plotted against age. The correlation coefficient

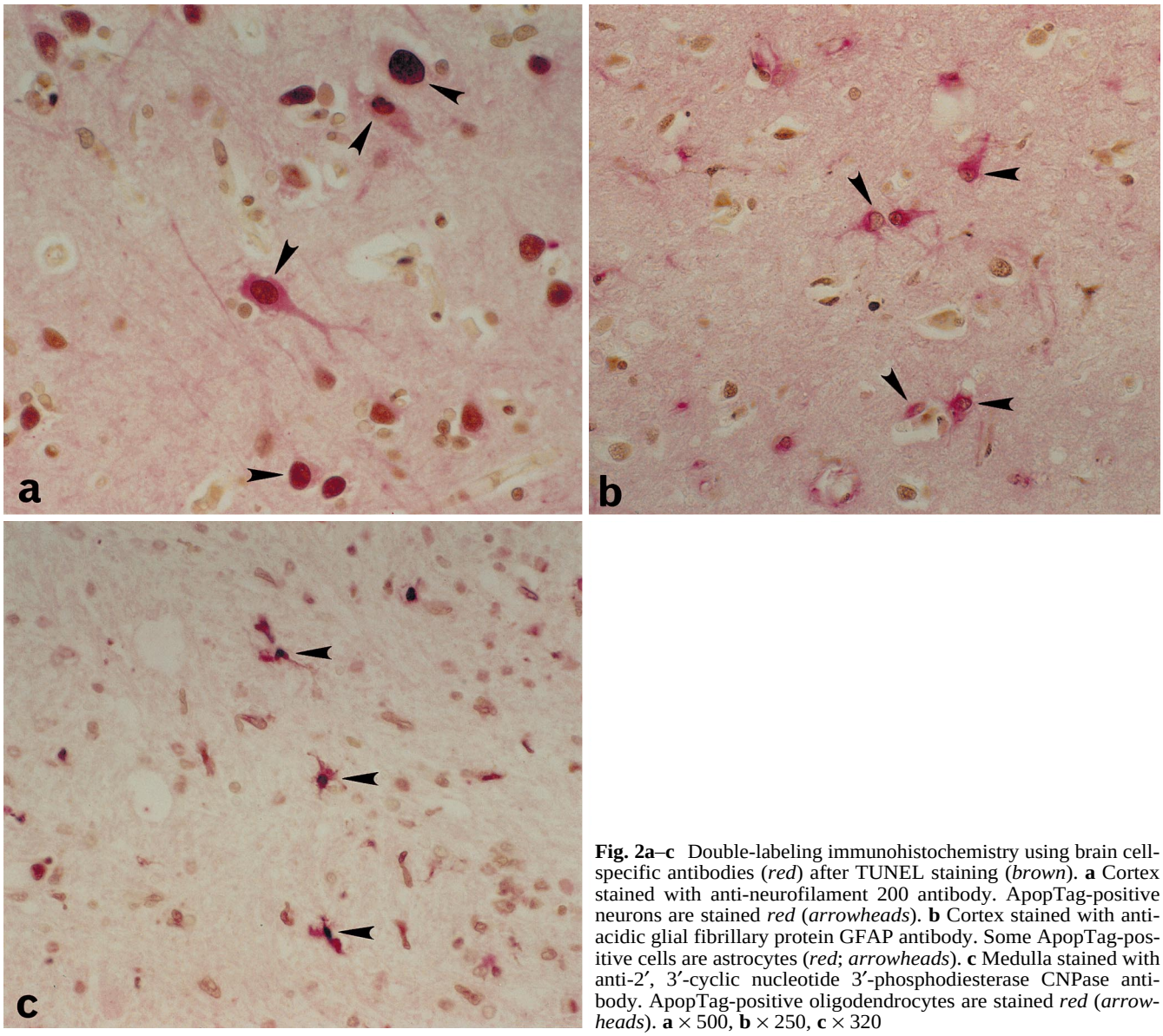

Fig. 2a–c Double-labeling immunohistochemistry using brain cell-specific antibodies (*red*) after TUNEL staining (*brown*). **a** Cortex stained with anti-neurofilament 200 antibody. ApopTag-positive neurons are stained *red* (*arrowheads*). **b** Cortex stained with anti-acidic glial fibrillary protein GFAP antibody. Some ApopTag-positive cells are astrocytes (*red*; *arrowheads*). **c** Medulla stained with anti-2′, 3′-cyclic nucleotide 3′-phosphodiesterase CNPase antibody. ApopTag-positive oligodendrocytes are stained *red* (*arrowheads*). **a** × 500, **b** × 250, **c** × 320

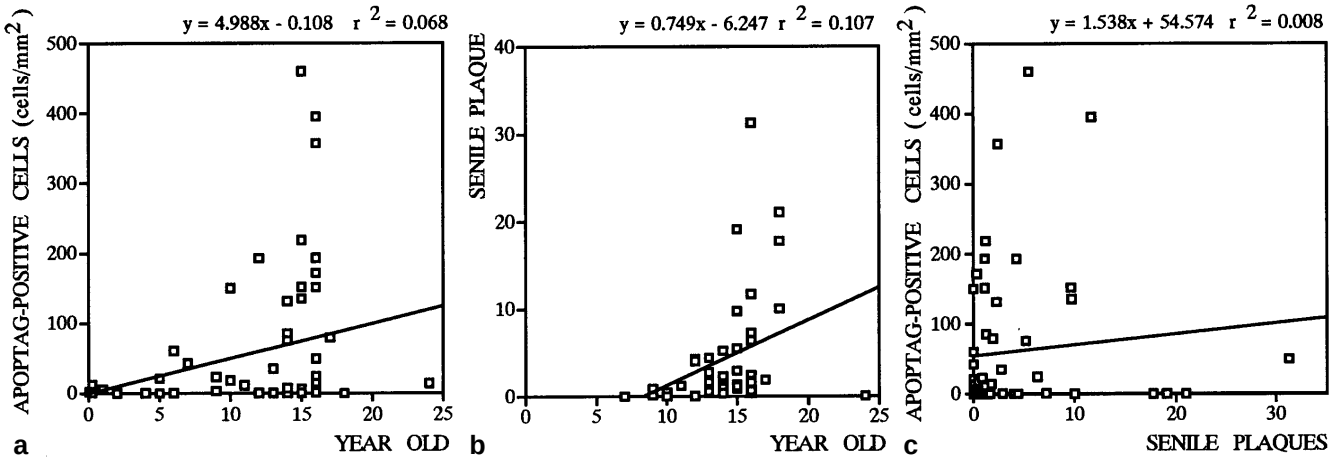

Fig. 3a–c Apoptosis in the aged dog brain. **a** Correlation between ApopTag-positive cell number and age. **b** Correlation between senile plaques and age. **c** Correlation between ApopTag-positive cells and senile plaques

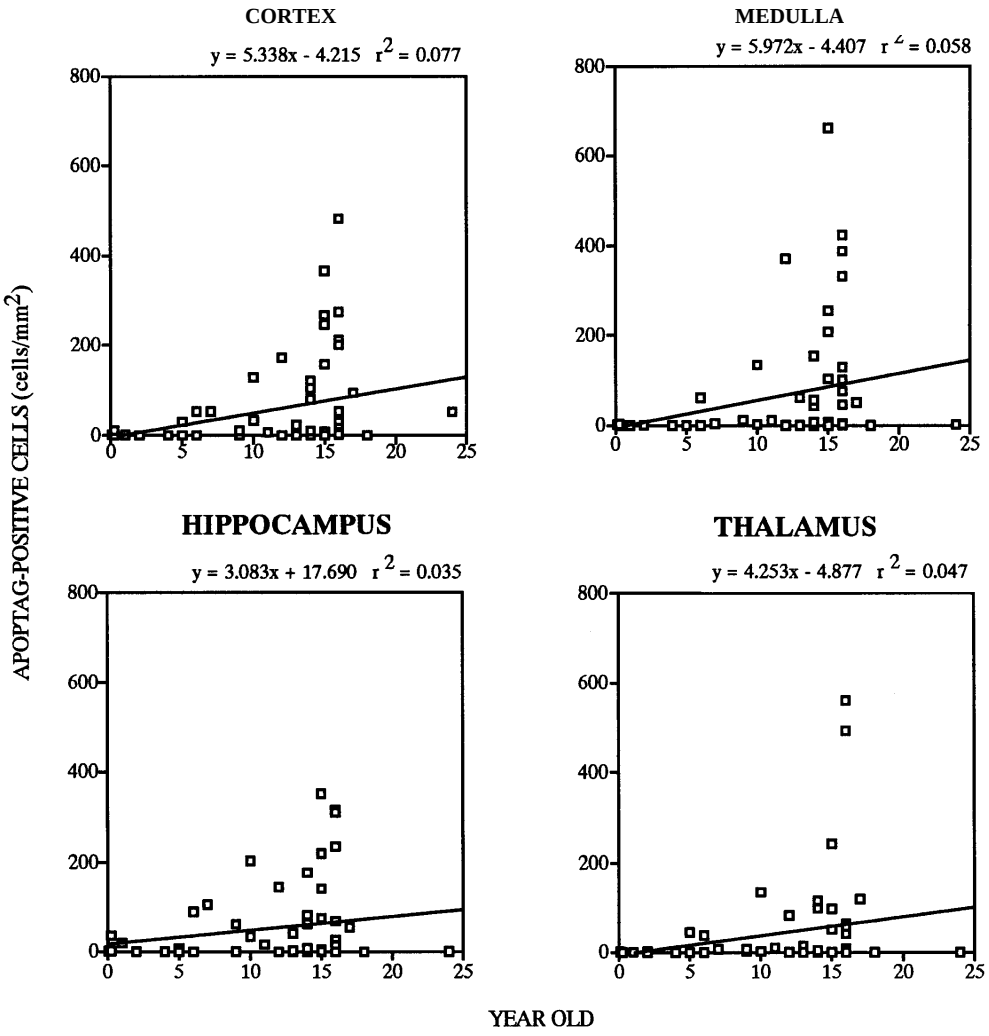

Fig. 4 Correlation of ApopTag-positive cells and age in distinct brain regions; cortex, medulla, hippocampus and thalamus

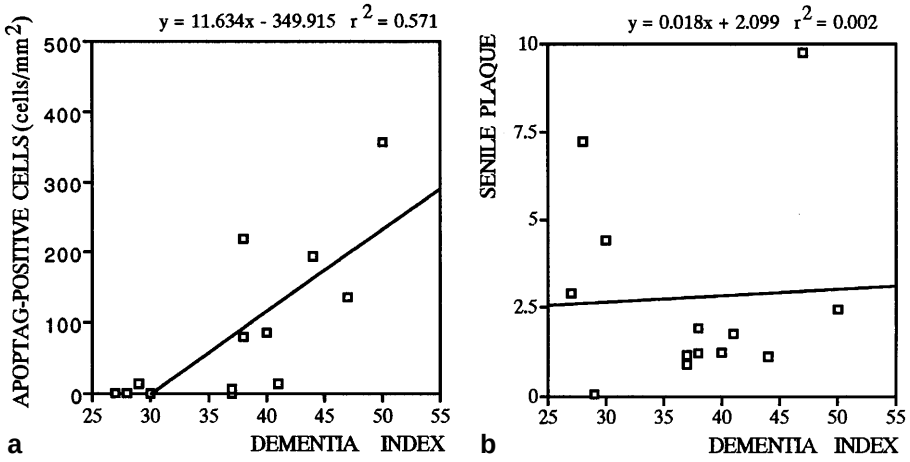

Fig. 5a, b ApopTag-positive cells in 13 dogs evaluated for dementia index. **a** Correlation between ApopTag-positive cells and dementia index. **b** Correlation between dementia index and senile plaques

(r^2) was 0.068, indicating a weak correlation between the factors, but the number of ApopTag-positive cells tended to increase with age (Fig. 3a). The relation between the number of senile plaques and age was moderate (r^2 = 0.107; Fig. 3b). The numbers of senile plaques and of ApopTag-positive cells showed no correlation (r^2 = 0.008; Fig. 3c).

The numbers of ApopTag-positive cells in the distinct brain regions also increased with age (Fig. 4). The highest correlation was found in the cortex (r^2 = 0.077), followed by medulla (r^2 = 0.058), thalamus (r^2 = 0.047) and hippocampus (r^2 = 0.035).

Incidence of ApopTag-positive cells
and senile plaques in comparison to dementia index

In the 13 aged dogs that had been clinically evaluated for dementia index, significant correlations between ApopTag-positive cells and dementia index (r^2 = 0.571) were detected (Fig. 5a), but no relationship was observed between senile plaques and the index (Fig. 5b).

Discussion

Previous studies on normal and pathological tissues have shown that the ApopTag technique sometimes labels degenerating nuclei induced by autolysis, tissue fixation or processing [11, 12, 26, 36]. In this study, we demonstrated that postmortem time, if the samples were kept at room temperature, did not influence ApopTag labeling, as shown previously in experimental studies using tissues from Alzheimer's, Huntington's and Parkinson's disease patients [6, 14, 29]. Additionally, prolonged fixation in 10% neutral buffered formalin did not increase DNA fragmentation. However, unusual autolytic conditions such as maintaining tissue samples at a high temperature (37° C) led to an increase in the number of ApopTag-positive brain cells. Specimens should, therefore, be placed in a refrigerator or kept at room temperature before necropsy or fixation. Samples which have been left at high temperatures should be excluded from studies of brain cell apoptosis.

The morphological characteristics of ApopTag-positive cells in this study were mild shrinkage of the cytoplasm and slightly enlarged nuclei, as described previously in AD brain [29]. We found no apoptotic bodies, which are considered to be the final morphological stage of apoptosis, in any of the present cases. The staining pattern of ApopTag-positive nuclei in the present study suggested that they had undergone the early stages of apoptosis, which can be detected by the highly sensitive ApopTag technique. At the early stage of apoptosis, DNA can be fragmented but the cell morphology still remains normal. Thus, although the morphological changes that we observed were slight, the function of the ApopTag-positive cells could be markedly reduced, a situation which could be called functional apoptosis.

The nuclei of the pial cells were also positively labeled, but the staining pattern of these cells differed from those of the positively labeled brain cells, suggesting that these reactions might have been artifactual. Dragunow et al. [6] also suggested that cell death in the meninges may reflect a normal quick turnover or a postmortem artifact. Therefore, these cells were not counted in this study.

In this study, senile plaques were detected in animals older than 9 years as reported in our previous investigation [31], and the number of plaques tended to increase with age. However, a population of dogs, even after 10 years of age, which developed fewer plaques was observed. In the present study using ApopTag-positive cell counting, similar age-related increases in ApopTag-positive cell numbers were observed, although there were exceptions. Nevertheless, the numbers of ApopTag-positive cells and senile plaques were not related. These results suggest that the two age-related changes (apoptosis and senile plaques) could be independent events. We were able to demonstrate a correlation between the dementia index and ApopTag-positive cells, suggesting that apoptosis in the brain, but not senile plaques, is responsible for dementia symptoms in aged dogs.

In the present study, glial cells as well as neuronal cells underwent apoptotic alterations, indicating the involvement of glial cell loss in canine dementia. In particular, medullary cells, most of which were oligodendrocytes, were severely affected in older dogs. Additionally, a considerable number of astrocytes in the cortex also showed such alterations. These observations suggest that a wide variety of brain cells participate in the pathological changes responsible for dementia symptoms.

In man, similar brain cell apoptosis has been observed in AD patients but not in non-AD age-matched controls [14, 29]. The nuclei both of NFT-bearing and non-NFT-bearing cells in the AD brain were ApopTag labeled. The numbers of ApopTag-positive cells and NFT showed a positive, although weak, correlation, but those of the positive cells and senile plaques were not related. NFT do not represent a histopathological alteration that is specific for AD, being also observed in a variety of neurodegenerative disorders [8, 27, 38]. Accordingly, such neuronal cell apoptosis would be more important for the dementia condition. Interestingly, no NFT have been reported previously in aged dogs, nor were they observed in the present cases. Therefore, at least in dogs, brain cell apoptosis appears to be a unique histopathological condition accounting for dementia. The reason why NFT are not observed in animals, including dogs, is controversial and will be discussed elsewhere.

In the present study, we also investigated the brains of very old (18- and 24-year-old) dogs. They showed no apoptosis and the dementia index for the 24-year-old dog was in the non-dementia category (unfortunately the dementia indices of the 18-year-old dogs were not determined). This could indicate the presence of a "supernormal" aged population [20], also in dogs. Most dogs die before reaching 18 years of age, although a few are still active at that age. These animals might belong to a genetically determined special population, providing valuable information with which to clarify the mechanism of senile dementia.

The pathomorphological advantage of investigating the brain of aged dogs is that they do not show NFT. Thus, the brains of aged dogs represent an excellent model for human AD or Alzheimer-type senile dementia as a simplified tool with which to probe these complicated disease conditions.

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