J. Toxicol. Pub. Health
Vol. 17, Supplement, pp. 71-77, 2001

Animal Models for Aging and Neurodegenerative Diseases: Brain Cell Apoptosis in the Dog and its Possible Mechanisms

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ABSTRACT : *The brain of the aged dog possesses senile plaques and amyloid angiopathy, which characterize Alzheimer's disease brains. We have defined the dementia condition of aged dogs and examined which mechanism(s) is responsible for the condition. A series of studies revealed that the dementia condition in aged dogs is significantly related to the number of apoptotic brain cells including both neurons and glial cells, but not to the number of senile plaques. On the other hand, 5-azacytidine (5AzC) is a cytidine analogue, and is thought to induce kinds of cell differentiation possibly through hypomethylation of genomic DNA. We have revealed neuronal apoptosis induced in 5AzC-treated fetal mice and PC12 cells. The ribosomal protein L4 (rpL4) gene is expressed prior to the apoptosis in the PC12 cell system. Therefore, the involvement of the rpL4 gene expression in age-related brain cell apoptosis in dogs may contribute to the investigation of Alzheimer's dementia.*

Key Words : *Apoptosis, 5-azacytidine, Brain, Dementia, Dog, Ribosomal protein L4*

I. INTRODUCTION

Various chemical compounds, both endogenous and exogenous, are known to be toxic to neuronal tissues. An example of endogenous neurotoxic compounds is α amyloid, accumulation of which is observed in the Alzheimer's disease (AD) brain. Transgenic mice with the β amyloid precursor protein gene have been used as animal models of the disease. However, a more suitable animal model which shows dementia conditions similar to humans is needed to elucidate a precise β amyloid toxicity. In the present study, we indicate that the aged dog is an appropriate model for Alzheimer researches and that brain cell apoptosis may be responsible for dementia condition of aged dogs.

A considerable number of genes are known to be involved in the apoptotic cascade, but genes responsible for neuronal cell apoptosis are still obscure. We, further, tried to determine the gene(s) related to neuronal cell apoptosis, using 5-azacytidine, a potent endo- and exogenous neurotoxic agent, on the PC 12 cell system.

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II. APOPTOSIS IN THE AGED DOG BRAIN

Senile plaques (SPs) as seen in the patients of AD have also been found in the brains of aged dogs (Uchida *et al.*, 1991). However, neurofibrillary tangles (NFTs), another characteristic lesion in AD, have never been observed in the brains of aged dogs (Uchida *et al.*, 1992). Neuronal cell loss, an additional histopathological hallmark in AD, is not prominent in the aged dog brains. Recently, apoptotic neuronal cell death was detected in the brains of AD patients (Dragunow *et al.*, 1995; Lassmann *et al.*, 1995; Su *et al.*, 1994). Moreover, amyloid β protein (Aβ) which is a major component of SPs has been shown to be toxic in primary neuronal cultures (Copani *et al.*, 1995; Loo *et al.*, 1993; Mattson and Goodman, 1995; Watt *et al.*, 1994; Yankner *et al.*, 1990) and PC12 cells (Behl *et al.*, 1994; Gschwind and Huber, 1995; Yankner *et al.*, 1989), and this neuronal cell death is likely to occur, at least in part, via apoptosis (Wijsman *et al.*, 1993).

Therefore, it would be worthwhile to clarify the differences of brain lesions between AD patients and aged dogs from the view point of apoptotic cell death, and to discuss the usefulness of aged dogs as a model

for AD.

Materials and Methods

Fifty-five canine brains (1 month to 24 years old) obtained at autopsy were used. The brain tissue sections were stained with periodic acid methenamine silver (PAM) for detection of SPs. To detect apoptosis on the brain sections, the TUNEL method was applied using the ApopTag *in situ* apoptosis detection kit. To identify the type of apoptotic brain cells, immunohistochemistry was performed using primary antibodies against neurofilament 200 specific for neurons, glial fibrillary acidic protein (GFAP) for astroglia, and anti-2'3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) for oligodendroglia. Thirteen (13 to 24 years old) of the 55 dogs had been clinically evaluated for dementia before death using the index (dementia index, DI) system proposed by Uchino *et al.* (Uchino *et al.*, 1995) (Table 1).

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.

Results

TUNEL-positive cells were present in both gray and white matters. Morphologically, positive cells in the gray matter, most of which were neurons, were swollen and larger than negative cells, although these changes were slight. Positive nuclei were larger and rounder than negative nuclei and contained more aggregated chromatin (Fig. 1). Apoptotic bodies or chromatin margination, which are the typical morphological characteristics of apoptosis, were not observed. Positive nuclei of glial cells, most of which were present in the white matter, were characterized by dense staining pattern and chromatin margination. No NFTs were observed in the brains examined.

TUNEL-positive cells in the gray matter including the cortex, hippocampus and thalamus were both neurons showing immunoreactivity for neurofilament 200 and astroglia immunoreactive for GFAP. Most TUNEL-positive cells present in the subcortical zone and white matter were immunoreactive for CNPase specific for oligodendroglia.

The number of TUNEL-positive cells of the 55 canine brains was plotted against age. The correlation coefficient (r^2) was 0.068, indicating a weak correlation bet-

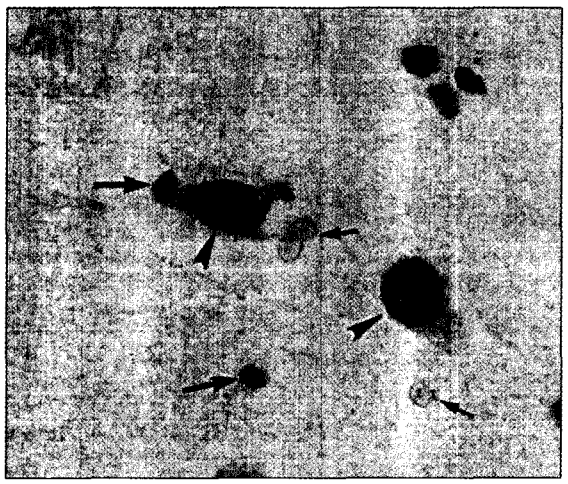

Fig. 1. Morphology of TUNEL-positive brain cells in the cortex of a 17-year-old dog. The nuclei of the positive neurons are slightly swollen (arrowheads). Surrounding TUNEL-positive (large arrows) and negative (small arrows) cells are glial cells.

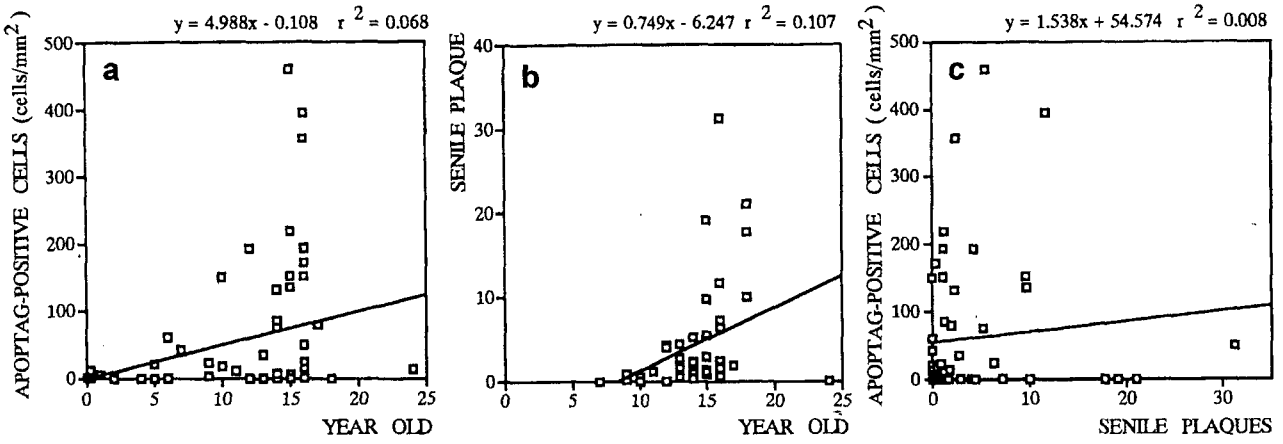

Fig. 2. Correlation between TUNEL-positive cells and age (a), between senile plaques and age (b), and between TUNEL-positive and senile plaques (c).

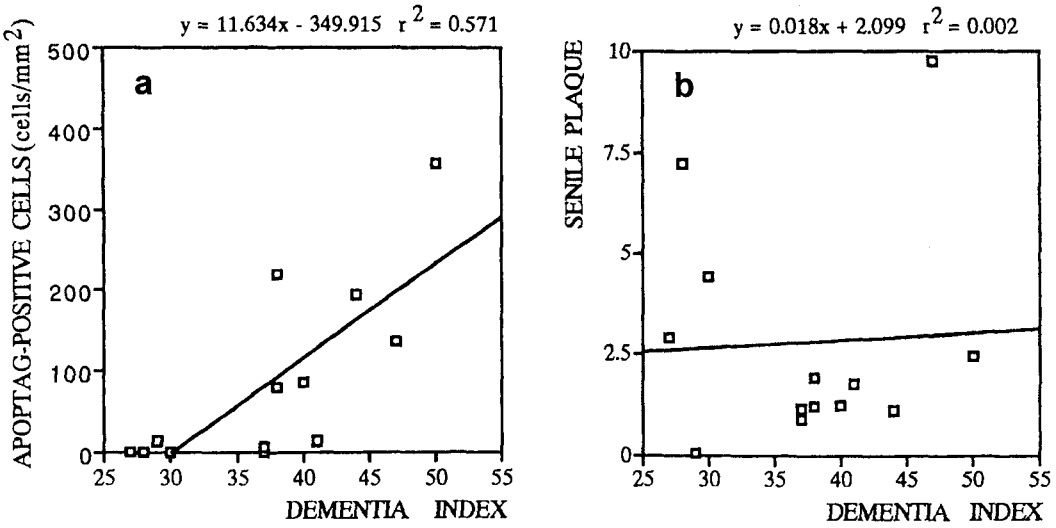

Fig. 3. Correlation between TUNEL-positive cells and dementia indices (a), and between senile plaques and dementia indices (b).

ween the factors, but the number of TUNEL-positive cells tended to increase with age (Fig. 2a). The relation between the number of SPs and age was moderate (r^2=0.107) (Fig. 2b). On the contrary, the numbers of SPs and TUNEL-positive cells showed no correlation (r^2=0.008) (Fig. 2c). The examination of 13 aged dogs, which were evaluated for dementia, revealed a significant positive correlation between the number of TUNEL-positive cells and the severity of dementia, presented as DI (Fig. 3a). However, there was no relationship between the number of SPs and DI (Fig. 3b).

Discussion

The morphological characteristics of TUNEL-positive cells in this study were mild shrinkage of the cytoplasm and slight enlargement of the nucleus. We found no apoptotic bodies, which are considered to be the final morphological stage of apoptosis, in any of the present cases. At the early stage of apoptosis, DNA could be fragmented but the cell morphology still remains normal. Thus, although the morphological changes that we observed were slight, the function of the TUNEL-positive cells would be markedly reduced. We propose to call such situation of cells as "functional apoptosis".

In the present study, glial cells as well as neuronal cells underwent apoptotic alterations. These observations suggest that a wide variety of brain cells participate in the pathological changes in aged dogs. Al-

though the numbers of SPs and apoptotic cells detected by TUNEL method tended to increase with age, the numbers of the two events were not related. The result suggests that the two age-related changes (apoptosis and SPs) could independently occur. Furthermore, brain cell apoptosis rather than SP might be more appropriate histopathological hallmark accounting for canine dementia. Conclusively, although the brain of aged dogs did not show NFT, aged dogs will be an excellent tool for clarifying the mechanism of AD dementia.

III. INCREASED EXPRESSION OF RAT RIBOSOMAL PROTEIN L4 MRNA IN 5AZC-TREATED PC12 CELLS PRIOR TO APOPTOSIS

5-Azacytidine (5AzC; C8H12N4O5, MW 244.2), a cytidine analogue, has been used as a DNA demethylating agent for promoting a certain gene expression since its molecular structure is characterized by the replacement of the 5-carbon atom of the pyrimidine ring by a nitrogen atom, rendering it incapable of accepting the methyl residue during enzymatic methylation occurring in newly synthesized DNA molecules (Jones, 1985). These events would suggest that 5AzC-incorporation instead of cytidine during DNA replication makes DNA hypomethylated and thereby activates previously methylated dormant genes (Jones, 1985, Jones and Taylor, 1980). We have conducted serial studies on 5AzC-induced neuronal apoptosis during embryogenesis (Hossain *et al.*, 1995; Hossain *et al.*, 1996) and have reported that PC12 cells derived from a rat pheochromocytoma exhibit apoptosis after 5AzC-treatment (Hossain *et al.*, 1997). The 5AzC-induced apoptotic cell death was rescued by the simultaneous treatment of a protein synthesis inhibitor, cycloheximide (Hossain *et al.*, 1997). This suggests that 5AzC would awake certain dormant genes encoding certain apoptosis-associated protein(s) through DNA hypomethylation.

We therefore tried to clone such apoptosis-related genes by a cDNA subtraction method.

Materials and Methods

After addition of 5AzC to PC12 cell culture at a concentration of 500 μg/ml, cells were collected. cDNA libraries from PC12 cells treated with or without 5AzC were prepared by a conventional cDNA subtraction method, as shown in Fig. 4. Both cDNA libraries

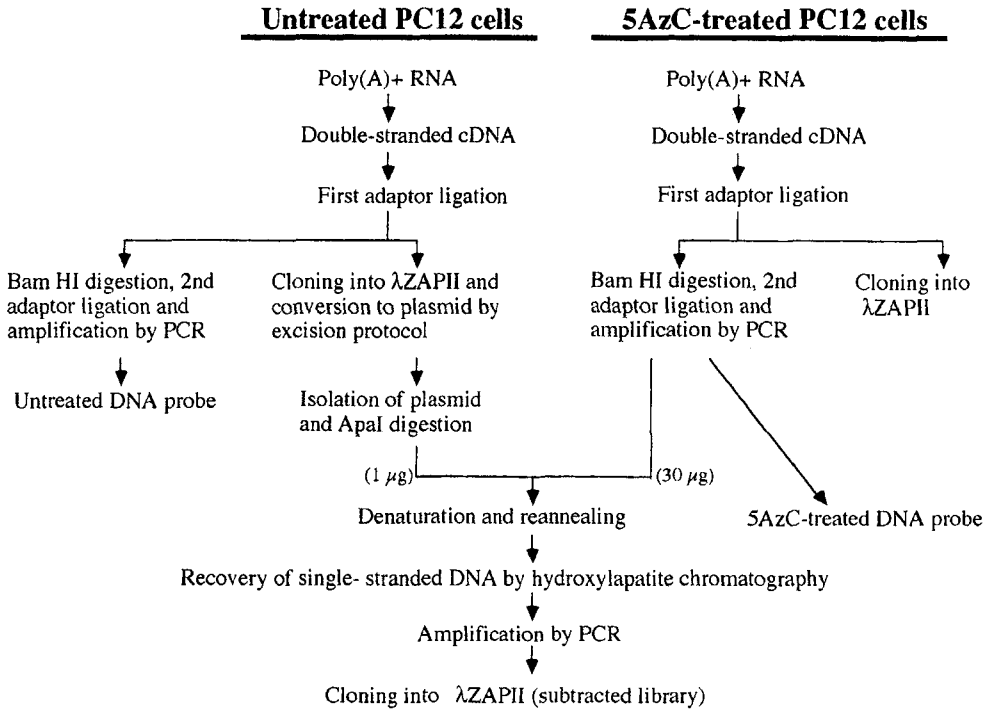

Fig. 4. Schematic outline of cDNA subtraction procedure utilized in the study.

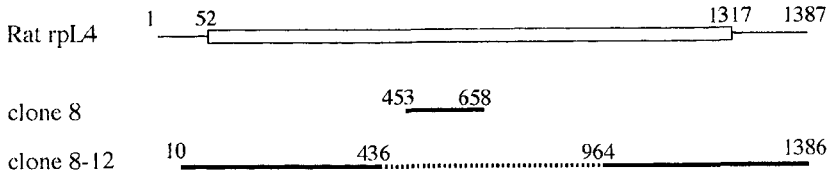

Fig. 5. Schematic diagram of rat rpL4, clone 8 and clone 8-12. The sequence of rat rpL4 gene is shown on the top. Open box indicates ORF in rat rpL4. Thick lines represent determined sequence from the present study, which is identical with rat rpL4. Dot line represents undetermined sequence.

were amplified by polymerase chain reaction (PCR) and hybridized. Unpaired single stranded DNA was amplified by PCR. Duplicate filters were prepared from the agar plate on which *E. coli* transfected with the subtracted library was cultured. The filters were differentially screened with either cDNA probe prepared from 5AzC-treated or control PC12 cells. Of 6.4×10^4 clones, 11 clones that showed a stronger hybridization signal with a 5AzC-treated probe than with a control probe were selected. After further strict selection, only one clone that certainly increased by 5AzC-treatment was obtained (clone 8), and the sequence of the clone was determined. To detect DNA fragmentation (apoptotsis) of cultured cells, the ELISA-based Cell Death Detection Kit was used.

The cDNA clone obtained from the subtraction cloning was inserted into the pCIneo vector at the downstream of CMV promoter. The sequence encoding hemagglutinin (HA) was ligated at the C terminal end of the inserts for the detection in Western analysis. COS-7 cells were transfected with the reconstructed pCIneo that underwent an insertion with a variable concentration of the clone. The transfected cells were cultured for 48 h and total DNA was collected for investigating DNA fragmentation.

Results

The northern band labeled with a probe derived from clone 8 gave much more intense signal to the lane of 5AzC-treated cells than that of control cells, indicating increased expression of clone 8 gene by 5AzC-treatment. The sequence of the gene was identical to a part of rat ribosomal protein L4 (rpL4) gene (Chan *et al.*, 1995), from nt 453 to nt 658, by searching the database provided by GenBank (Fig. 5). Further screening for 5AzC-treated cDNA library with the clone 8 as a probe resulted in the additional clone (clone 8-12) showing strong hybridization signal. The sequences of 427 bases of 5' region and 423 bases of 3' region of clone 8-12 were identical to those of nt 10 to nt 436 and nt 964 to nt 1386 of rat rpL4, respectively (Fig. 5). Thus, the clone 8-12 covers nearly full length of rat rpL4, which includes open reading frame (ORF), nt 52 to nt 1317.

5AzC-induced DNA fragmentation (apoptosis) of PC12 cells detected by ELISA was shown in Fig. 6. The amount of fragmented DNA increased by 12 hours after 5AzC exposure, but the value decreased approximately to the control level at 24 hours. Northern membranes prepared from 5AzC-treated PC12 cells were hybridized with the clone 8-12 probe. The expression of rat rpL4 increased until 6 hours after 5AzC-treatment, and this high level was kept at least until 12 hours (Fig. 7).

Dose-dependent expression of rat rpL4 protein was observed after 48 hours incubation in COS-7 cells transfected with rat rpL4 (data not shown). DNA fragmentation of the transfected COS-7 cells determined by ELISA after 48 h incubation were increased as the

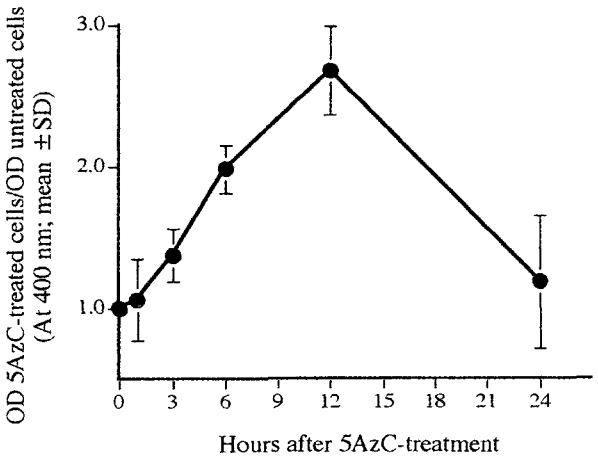

Fig. 6. DNA fragmentation (apoptosis) of PC12 cells after 5AzC-treatment measured by ELISA.

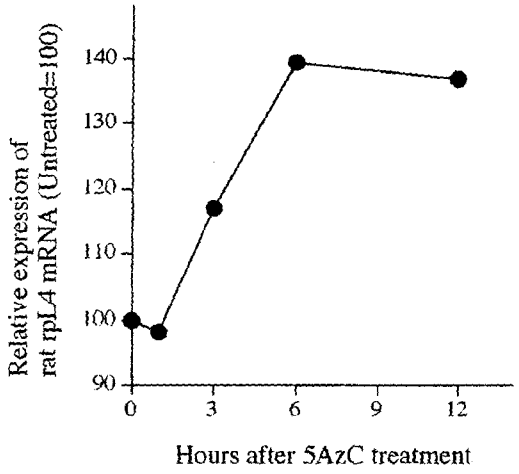

Fig. 7. Expression of rat rpL4 mRNA compensated by the that of endogenous GAPDH, resulting from Northern analysis of 5AzC-treated PC12 cells using the clone no. 8-12 as a probe.

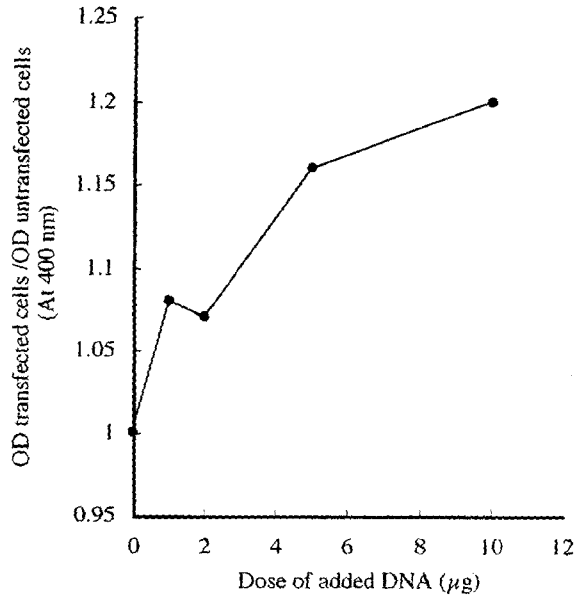

Fig. 8. DNA fragmentation (apoptosis) of the transfected COS-7 cells measured by ELISA.

amount of cDNA used for transfection (Fig. 8).

Discussion

The rat rpL4 was isolated as a gene that increased its expression by 5AzC-treatment preceding DNA fragmentation. This suggests that the rpL4 protein might be involved in an apoptotic cascade. The results of the COS-7 cell transfection also support the involvement of this gene expression in the 5AzC-induced apoptotic cascade. Ribosomal protein L4 is the forth largest molecule among the proteins composing a large ribosomal subunit (Wolfe, 1993). The promoter region of ribosomal protein genes in major higher eukaryotes is known to have an almost similar sequence including CpG-rich region (Mager, 1988), and such CpG islands are considered to be crucial for the activity of the promoter (Bird, 1986). Thus, it is likely that the expression of rat rpL4 might be epigenetically regulated through DNA methylation; that is, demethylation of rat rpL4 promoter sequence after 5AzC-incorporation may activate the expression of the gene. The mechanism through which the rpL4 protein induces apoptosis remains to be clarified.

IV. CONCLUSION

In the present study, we indicated that the dog can be used as an excellent model for researches in Alzheimer's disease. Higher intellectual level and well-known biological properties of dogs are the most advantageous characters of the model. In addition, demential conditions and brain lesions similar to Alzheimer's patients, although there are some differences, are also indicated in aged dogs in the present study. Particularly, much attention should be paid to brain cell apoptosis in aged dogs. The involvement of rpL4 gene in neuronal apoptosis was also indicated in the present study. The role of the gene product in the apoptotic process of the brain cells, especially its relation to β-amyloid toxicity, will be further examined.

Our papers (Kajikawa *et al.*, 1998; Kiatipattanasakul *et al.*, 1996) dealing with the subjects mentioned in the present study should be referential.

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