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SIGNIFICANCE OF INTRACELLULAR Ab42 ACCUMULATION IN ALZHEIMER'S DISEASE

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TABLE OF CONTENTS

1. ABSTRACT

Aβ plays a pivotal role in the pathogenesis of Alzheimer's disease (AD), but it is still obscure how it causes AD. We have established transgenic mice carrying wild-type or familial Alzheimer's disease (FAD) mutant-type presenilin 1 (PS1). In these mice, the number of cortical and hippocampal neurons decreased along with age in mutant mice. In addition, the old mutant mice showed a significant increase of dark neurons by silver staining and the number of neurons with intracellular Aβ42 by immunohistochemistry. Our extended study also showed a significant increase of intracellular Aβ42-positive neurons in isolated cases of AD as well as in PS1 mutant FAD cases. These neurons frequently showed apoptotic staining. However, coincidence of apoptotic markers and intraneuronal neurofibrillary tangles (NFT) was insignificant. Notably intraneuronal Aβ42-labeling was frequently seen in a case of AD showing cotton-wool type senile plaques with a few NFT positive neurons and dystrophic neurites. These results indicate that intraneuronal deposition of Aβ42 is important in the pathogenesis of AD.

2. INTRODUCTION

Pathology of Alzheimer's disease (AD) is characterized by senile plaques (SPs), neurofibrillary tangles (NFTs) and neuronal loss. Although SPs are seen in aged non-demented individuals, they are otherwise specific to AD brain, and the extent is much more severe in AD. In AD brain, SPs appear earlier than NFTs, and NFTs are seen in number of other neurological disorders. Familial Alzheimer's disease (FAD) genes, amyloid precursor protein (APP) and presenilin1 (PS1) and 2 (PS2), cause abnormal Aβ production. These findings suggest that SPs are the most important clues to unleash pathomechanisms of AD (1). Since SPs are formed by extracellular deposits of β amyloid with or without reactive glial cells and neurites containing NFTs, attention has been focused mostly on extracellular Aβ. Indeed, extracellularly applied Aβ was shown toxic to neurons in vitro (2) and in vivo (3), but to obtain this result non-physiological doses were required. Moreover, it has been shown that the extent of SPs does not correlate well with the severity of AD (4). In our previous report, we have found that presenilin1 (PS1) transgenic mice carrying FAD mutations show age-related neuronal loss with intracellular Aβ42 deposits without SP formation (5). We extended the study and found that apoptotic neurons frequently showed intracellular Aβ42 labeling in AD brain (6). Here, we re-emphasize the significance of intracellular Aβ42 accumulation by adding a new observation in AD with cotton-wool type SPs.

3. MATERIALS AND METHODS

3.1. PS1 transgenic mice and tissue processing

Establishment of PS1 transgenic mice is reported previously (5). Briefly, male mice carrying human PS1 controlled by the PDGF promoter were produced, and

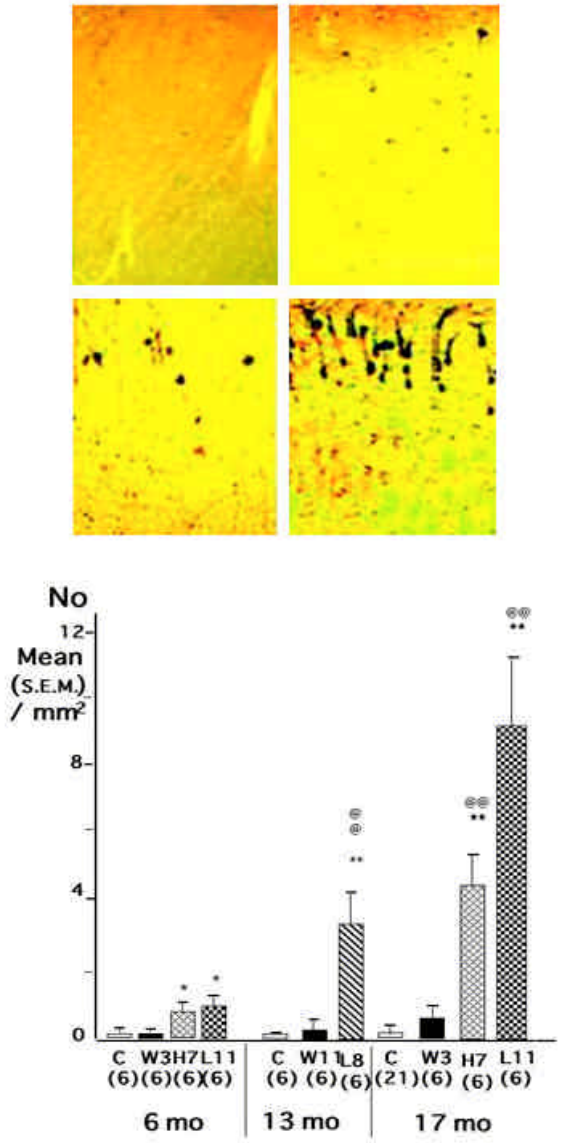

Figure 1. Dark neurons were more frequent in aged mutant mice. Left panel, silver staining for dark neurons. Left, 6 months old; right, 17 months old; upper, W#3; lower, L/V#11. Right panel, quantitation of dark neurons. *, p<0.05 and **, p<0.01 compared to control; @@, p<0.01 compared to wild-type.

offspring were expanded by in vitro fertilization with the FVB/N genetic background. We generated two lines of wild-type mice (W#3, W#11), two lines of L286V mutant mice (L/V#8, L/V#11), and one line of H163R mutant mice (H/R#7). Animal experiments followed our institute guidelines, and animals were kept in an SPF condition. Expression levels of PS1 mRNAs were almost equal by the Northern blot analysis, and those of the functional proteins, N-terminal and C-terminal fragments were also the same except for W#11 which showed a less amount of the protein on Western blotting.

Mice were perfused with 4% paraformaldehyde, and the brains were removed 24 hrs after the perfusion fixation. The brains were further fixed in 4% paraformaldehyde (PF) for a week, embedded in paraffin, and 5 μm section were used for H. E. staining, TUNEL staining (Trevigen, Gaithersburg, Maryland), Hoechst 33342 (Molecular Probes, Engene, OR) staining and dark neuron staining (7). For immunohistochemistry, the fixed brains were immersed in 20% sucrose in 0.01 M PBS, and 30 μm frozen sections were stained immunohistochemically by the free floating method as described previously (8). Primary antibodies used were rabbit polyclonal antibodies specific for Aβx-40 or Aβx-42 (9), for synthetic Aβ1-28 (10), for APP695 (Affiniti, Mamhead, UK), and for GFAP (Biomeda, Foster, California). For Aβ staining, tissues were treated briefly with formic acid.

For quantitative studies of surviving neurons, 5 μm-thick paraffin sections were stained using the Nissl method, and all neurons with diameters greater than 6 μm were counted in three consecutive areas and three different sections of the frontal cortex from 3-5 animals using a 20×objective lens and the image analyzer. CA1 hippocampal neurons with diameters greater than 8 μm were counted similarly.

3.2. Human brain studies

Brain specimens from 7 sporadic AD cases (mean age of death, 71.1±5.2 years), 2 FAD cases with PS1 mutation H163R or G384A (age of death, 59 and 48), a familial case of AD with cotton-wool type SPs (Age of death, 40s), 2 cases with progressive supranuclear palsy (PSP, age of death 58 and 79), and 8 control cases without known neurological disorders (mean age of death, 70.1±4.0 years) were used. Postmortem intervals ranged from 1.5 to 6 hrs.

Tissues were processed similarly as above, and used for immunohistochemistry. Additional antibodies used were 3D6 (11), 4G8 (Senetek), AT8 (Innogenic), SM139 (Sternberger), MAB377 (Chemicon), and others (6). To demonstrate intracellular Aβ by the immunofluorescent method, autofluorescence due to lipofuscin was eliminated by blocking with 0.3% Sudan black B (12). Quantitative studies were done similarly with a slight modification (6).

4. RESULTS

Our PS1 transgenic mice did not show SPs in the brain even at 24 months old. However, the surviving neuron counts were significantly lower in the frontal cortex and CA1 region of the hippocampus of mutant nice. The number of dark neurons increased in the cerebral cortex along with age and the counts were significantly higher in the mutant mice (Figure 1). When semiserial sections were stained by dark neuron staining and TUNEL staining or Hoechst 33342 staining, some but not all dark neurons were positive for the apoptotic markers. Immunofluorescence staining of the transgenic mice showed intraneuronal deposits of Aβ42 (Figure 2), and the Aβ42-positive neurons were significantly higher in number in mutant mice. Intracellular Aβ40-positive neurons were rare and

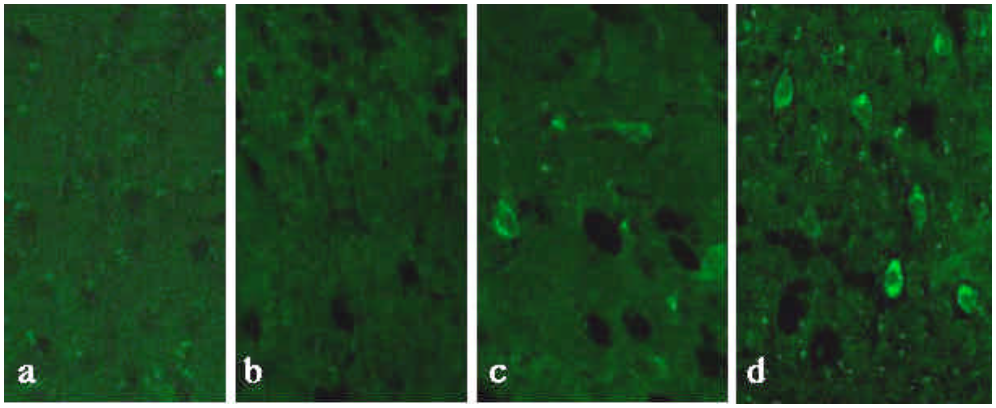

Figure 2. Immunofluorescent staining of intraneuronal Aβ in mutant transgenic mice at the age of 24 months. a, adsorbed anti-Aβx-40; b, adsorbed anti-Aβx-42; c, Aβx-40; d, Aβx-42.

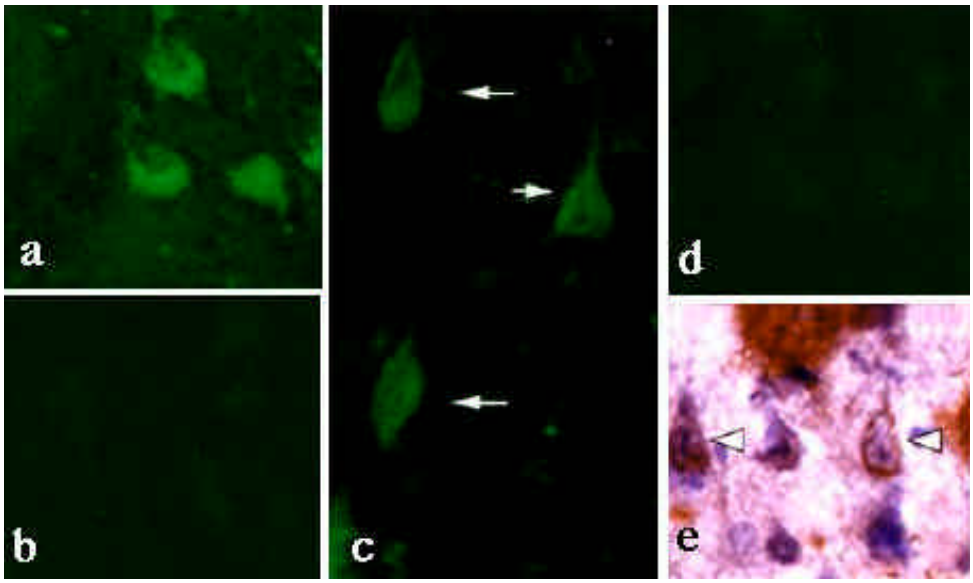

Figure 3. Immunohistochemical staining of intraneuronal Aβx-42 in AD brain.a, autofluorescence due to lipofuscin; b, autoflurescence was eliminated by Sudan black B staining; c, Aβx-42 staining; d, adsorbed anti-Aβx-42; e, horse raddish peroxidase-labeled anti-Aβx-42 staining.

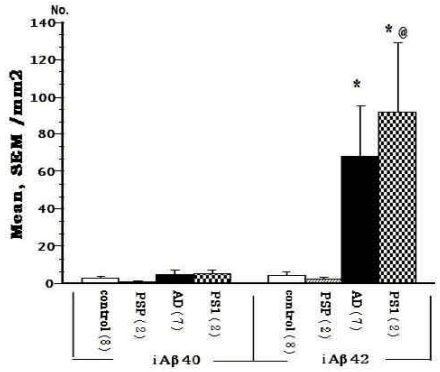

Figure 4. Quantitation of intracellular Aβ40- (iAβ40) and Aβ42-bearing (iAβ42) neurons. PSP, progressive supranuclear palsy; AD, isolated cases of AD; PS1, PS1 mutant FAD cases; number of patients in parentheses; *, $p<0.005$ compared to control and PSP; @, $p<0.05$ compared to AD.

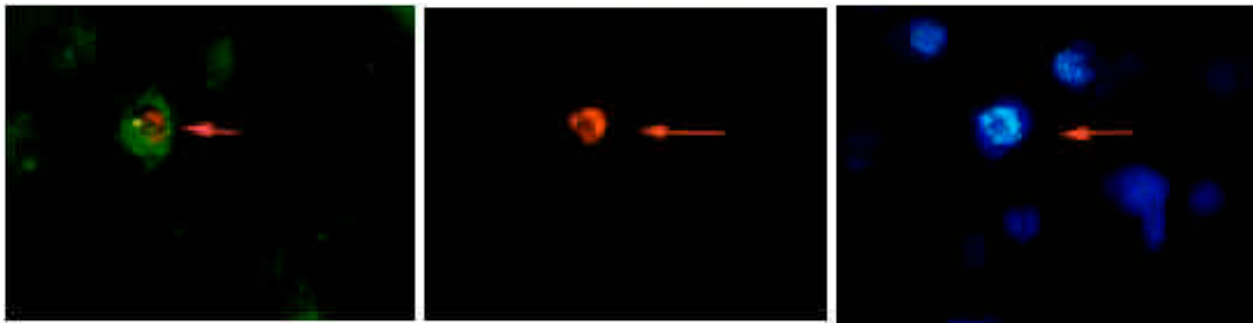

Figure 5. Triple labeling shows a neuron bearing intracellular Aβ42 (left) is positive for TUNEL (middle) and Hoechst 33342 (right) staining.

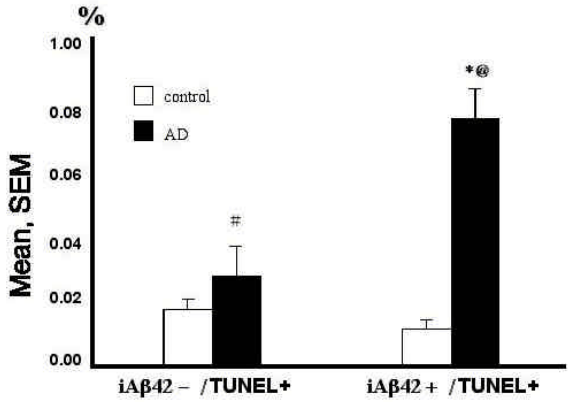

Figure 6. Quantitation of TUNEL-positive neurons with or without intracellular Aβ42 (iAβ 42). iAβ42-positive and TUNEL-positive neurons are significantly higher in AD brain. #, $p<0.01$ compared to $iA\beta42^{-}/TUNEL^{+}$ in control; *, $p<0.001$ compared to $iA\beta42^{+}/TUNEL^{+}$ in control; @, $p<0.01$ compared to $iA\beta42^{-}/TUNEL^{+}$ in AD.

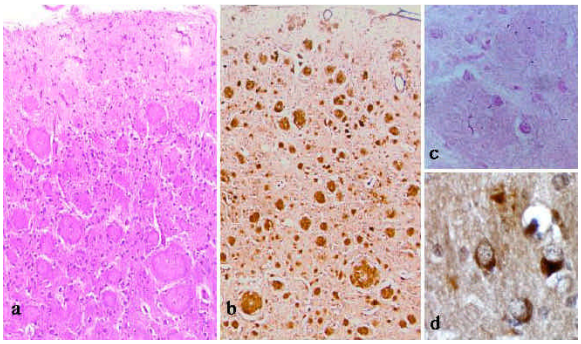

Figure 7. Histopathology of an AD case with cotton-wool type senile plaques. In this case, numerous cotton-wool type plaques are seen. NFTs-bearing neurons and dystrophic neurites are very a few, but there are many neurons containing intraneuronal Aβ42. a, H. E. staining; b, Aβx-42 staining; c, Gallyas staining; d, Aβx-42 staining.

insignificant. The intraneuronal Aβ42 was also shown by the horse radish peroxidase method, and the staining was negative with adsorbed antibodies. Since most of neurons were positive for amyloid precursor protein (APP) staining, the Aβ42 staining was not due to the crossreactivity to APP.

In human brain, the number of neurons was significantly decreased in AD. Immunofluorescent microscopy showed strong autofluorescence caused by lipofuscin granules, which was eliminated completely by treatment with Sudan black B. In this condition, intracellular Aβ42 was observed (Figure 3).

Double immunohistochemical labeling with antibodies to Aβ42 and APP or neurofillaments showed colocalization of the staining, suggesting the stained cells were neurons. However, APP single positive neurons were far more frequent, and Aβ42 did not colocalize with GFAP staining. Intracellular Aβ42 was occasionally colocalized with intraneuronal NFTs in AD brain, and intracellular Aβ42 was entirely negative in PSP brain. Aβ40-positive neurons were also rare in human materials. Quantitation of neurons containing intracellular Aβ40 and Aβ42 demonstrated a significant increase of Aβ42-bearing neurons in isolated cases with AD as well as in cases with FAD mutant PS1, but Aβ40-bearing neurons were insignificant (Figure 4).

Double or triple labeling showed that some intracellular Aβ42-positive neurons were positive for apoptosis markers such as TUNEL and Hoechest 33342 (Figure 5). The number of intracellular Aβ42 and TUNEL double positive neurons was significantly higher in AD cases (Figure 6). Intracellular NFT and TUNEL double positive neurons were much less than those with intracellular Aβ42 and TUNEL double positive neurons.

In a case with cotton-wool type plaques, huge SPs were easily recognized by H. E. staining (Figure 7a) and Aβ42 was mainly deposited in the plaques (Figure 7b). Intraneuronal NFTs-bearing neurons were only a few, and dystrophic neurites were rarely observed by Gallyas staining (Figure 7c). However, intraneuronal Aβ42-bearing neurons were frequently observed (Figure 7d). In these neurons, staining with 3D6 was also positive (not shown), suggesting that the Aβ contains the N-terminal portion.

5. DISCUSSION

There are several lines of PS1 transgenic mice (13, 14), but none has reported significant loss of neurons. In PS1 knock-in mice, neurons showed susceptibility to apoptotic stimuli (15, 16). Our PS1 mutant mice showed a significant decrease of survived neurons in the cerebral cortex and CA1 region of the hippocampus. This difference may be due to the difference in the genetic background. Our mice had a FVB background, but most of other sources had a B6 background. Transgenic mice carrying the FVB background were more prone to show neuronal death in other transgenics (17).

As a reflect of accelerated neuronal death, we could see increased numbers of dark neurons, and some of the dark neurons seemed to be positive for apoptotic markers. The dark neurons were observed in head trauma, stress, electric shock, hypoglycemia and others (18). Therefore, we think the dark neurons are at a certain stage of neurodegeneration. We must be aware that dark neurons are produced as an artifact, but it can be avoided by taking brain samples out 24 hrs after perfusion.

In order to see the cause of accelerated neuronal death, we examined the mice brain immunohistochemically. It is of interest to see increased munbers of neurons with intracellular Aβ42 deposits in aged mutant mice without extracellular deposits of Aβ. These observations suggest that intraneuronally accumulated Aβ42 may elicit the cascade of neuronal death. This was confirmed in the brain of isolated cases of AD. The number of neurons containing intracellular Aβ42 deposits were much higher in patients with PS1 mutation. Intraneuronal Aβ42 deposits were also reported by others (19, 20). Our double or triple immunolabeling showed that some of the neurons carrying intraneuronal Aβ42 deposits were positive for apoptotic markers. Our quantitative study showed a significant increase of intracellular Aβ42-positive and TUNEL-positive neurons in AD brain. It is interesting to know that the number of TUNEL-positive neurons without Aβ42 deposits was also increased in AD brain, suggesting that some other mechanisms are also involved in neuronal loss. We saw intracellular NFTs-positive and TUNEL-positive neurons, but the number was much less than thou with intracellular Aβ42.

In an AD case showing cotton-wool type plaques, intranuronal Aβ42 deposits were observed frequently. Although a large amount of Aβ was deposited extracellularly, intracellular NFTs and dystrophic neurites were a few. Therefore, intracellular Aβ42 deposits in this case seems to be more important at least than intracellular NFTs. FAD cases showing cotton-wool type plaques show spastic paraparesis and progressive dementia, and they were found carrying PS1 mutations (21, 22). Since only paraffin sections on slide glasses were available in our case, we could not identify the mutation.

It is not well known where the Aβ42 is produced and where it is deposited in subcellular compartments. Some investigators demonstrated that Aβ42 is produced and present in the endoplasmic reticulum (23, 24). Recently, it was demonstrated that Aβ is produced in a membrane compartment of a cholesterol and glycosphingolipid rich domain, named detergent-insoluble glycosphingolibid rich domain (DIG) or a raft (25, 26). In an animal model of Niemann-Pick disease type C, there were numerous intracellular vesicles with accumlation of cholesterol and Aβ (27). In the raft, a GM1 ganglioside, cholesterol, and Aβ complex is demonstrated to work as a seed of Aβ aggregation (28, 29). We are not sure the structure of intraneuronal Aβ42 deposits, but our preliminary electron microscopic examination showed that the intracellular Aβ42 is at least not fibrillar. Although the precise location of Aβ production and the mechanism of eliciting neuronal death are not known yet, intraneuronal deposits of Aβ42 seems to be important in the pathogenesis of AD.

6. ACKNOWLEDGMENTS

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