NEUROPATHOLOGICAL CHANGES IN THE PDAPP TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE

P. Pasbakhsh*[1], M. Mehdizadeh[2] and N. Omidi[2]

1) Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

2) Department of Anatomy, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

Abstract- Alzheimer's disease (AD) is a uniquely human disorder. Although the pathogenesis of AD is not fully understood, growing evidence indicates that the deposition of beta-amyloid (Aβ) and the local reactions of various cell types to this protein play major roles in the development of the disease. In the present study transgenic mice expressing mutant amyloid precursor protein (APP) has been used. These mice exhibit selective neuronal death in the brain regions that are most affected in AD, suggesting that amyloid plaque formation is directly involved in AD neurons loss. Brains from 12 transgenic animals and 12 age-matched non transgenic littermate controls (1 and 2 years old) were examined histopathologically. One year old transgenic animals (n=6) exhibit deposits of human Aβ in the hippocampus, corpus callosum and cerebral cortex. By 2 years of age, a great number of diffuse and mature plaques were present in the cortex and hippocampus, and subcortical regions like thalamus and striatum. Another major finding was reduction of cholinergic cells in the medial septum, striatum and diagonal band of Broca. The present data are consistent with the hypothesis that the neuropathology begins in the cerebral cortex and hippocampus before spreading in a retrograde fashion to subcortical regions.

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Key words: Alzheimer's disease, immunohistochemistry, medial septal nucleus, diagonal band of Broca

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects a large proportion of elderly people. Although genetic factors seem to strongly contribute to disease susceptibility, only a small number of cases are caused by dominant mutations (1). To date, all such mutations alter processing of the amyloid precursor protein (APP), leading to changes in the production or fibrillization of amyloid-β (Aβ), the major constituent of amyloid plaques in AD brain (1-3).

Aβ peptide, derived from the APP, seems to have a central role in the neuropathology of AD (4, 5), which is characterized by formation of senile plaques and neurofibrillary tangles, and loss of neurons (4, 5). Amyloid peptide plaques, one of the two diagnostic brain lesions observed in Alzheimer's original patient, are microscopic foci of extracellular amyloid deposition. These plaques and associated axonal and dendritic injury generally can be found in large numbers in the limbic and association cortices (3). Such plaques contain extracellular deposits of Aβ that occur principally in a filamentous form, as star-shaped masses of amyloid fibrils. Dystrophic neuritis occurs both within this amyloid deposit and immediately surrounding it.

The pathology of AD involves production and deposition of Aβ in senile plaques in an insoluble form (amyloid). The amyloid deposits could be directly toxic to cells or neurodegeneration could involve amyloid-related activation of glia and

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*** Corresponding Author:**
P. Pasbakhsh, Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 66112356
Fax: +98 21 66419072
E-mail: pasbakhsh@hotmail.com

associated inflammatory responses (6). Evidence that Aβ immunization also reduces cognitive dysfunction in murine models of AD would support the hypothesis that abnormal Aβ processing is essential to the pathogenesis of AD, and would encourage the development of other strategies directed at the amyloid cascade (7).

Besides the extracellular deposition of Aβ, the AD brain is characterized by intracellular neurofibrillary tangles and profound changes in the cholinergic system (8, 9). The major cholinergic innervation to the cerebral cortex originates from the nucleus basalis of Meynert (NBM), together with the horizontal limb of the diagonal band of Broca (HDBB). Cholinergic innervation to hippocampus is mainly provided by the medial septum (MS) and the vertical limb of the diagonal band of Broca (VDBB) (10, 11). In AD brain, a profound loss of these cholinergic basal forebrain neurons has been reported (12, 13). In both neocortex and hippocampus of AD brain, a loss of cholinergic fibers and terminals, decrease in cholinergic receptors and/or signal transduction and significant reductions in choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) enzyme activities have been reported (12-18).

The relationship between cerebral amyloid deposition and cholinergic depletion in AD remains poorly understood (4, 19, 11). This neuron loss may be the result of Aβ neurotoxicity to the cholinergic terminals followed by retrograde degeneration. Alternatively, degeneration of cholinergic basal forebrain neurons may be the primary lesion with subsequent loss of cortical cholinergic innervation. It has also been reported that AChE accelerates the assembly of Aβ into insoluble amyloid fibrils (20, 5). Accordingly, dysfunction of the cholinergic system may influence cerebral amyloidosis. Vice versa, it has been demonstrated that Aβ is neurotoxic to cholinergic neurons and that low concentrations of Aβ can directly inhibit cholinergic signaling (21, 22). Thus, increased Aβ levels may contribute physiologically and/or pathologically to the cholinergic changes in AD brain.

Several transgenic mice models over expressing APP and its clinical mutants have been generated which recapitulate aspects of AD. A transgenic mouse model of AD has been developed that expresses high levels of human mutant APP (23, 24). The transgenic animals were generated using a platelet-derived growth factor-β promoter driving a human APP (h APP) minigene encoding the APP717 V6F mutation associated with familial AD (25). Between 6-9 months of age heterozygous PDAPP mice exhibit thioflavin-S-positive Aβ deposits, neurotic plaques, synapse loss, astrocytosis and microgliosis in the cortex and hippocampus. The magnitude of the cortical neuropathology increases after 9 months of age. The Aβ-containing plaques are directly associated with reactive gliosis and dystrophic neuritis, suggesting that the plaques may induce neurodegenerative changes in animals over 9-months of age; however, there is no evidence of neurofibrillary tangles or widespread neuronal loss. Many neurons in the brain regions typically affected in AD, including entorhinal cortex, hippocampus, parahippocampal gyrus, amygdala, frontal, temporal, parietal and occipital association cortices, and certain subcortical nuclei projecting to these regions, contain large, non membrane bound bundles of abnormal fibers that occupy much of the perinuclear cytoplasm (26). The most notable feature of these transgenic mice is their Alzheimer like neuropathology, which includes extracellular Aβ deposition, dystrophic neuritic component, gliosis and loss of synaptic density with regional specificity resembling that of AD. Based on the limited sampling to date, plaque density appears to increase with age in these transgenic mice, as it does in humans (26). In AD, there is degeneration of cortical neurons (27) and of subcortical neurons that project to the cortex (28). Several studies have found evidence of cholinergic nerve terminal abnormalities in AD mouse models (4, 6, 29-31) but they have not found clear evidence of cholinergic cell degeneration.

In the present experiment we have studied these mouse models to evaluate cholinergic alternations that result from, or lead to, cerebral amyloidosis. For this purpose we have used biochemical and morphological techniques to assess cholinergic changes in neocortex and basal forebrain of PDAPP transgenic mice and moreover, to test the hypothesis that cortical cholinergic depletion has an effect on amyloid plaque formation.

MATERIALS AND METHODS

Transgenic mice

Games *et al.* have produced a transgenic mouse model of AD that expresses high level of human mutant APP with valine at residue 717 substituted by phenylalanine (23). Homozygous mice were derived over 8-10 generations from hybrid backgrounds representing combinations of C57BL/6 + DBA + Swiss Webster strains. A total of 12 homozygous animals were compared with 12 wild-type mice from the same hybrid strain at 1-year and 2-years of age. All experiments on animals were performed in accordance with UK legal requirements.

Histology and immunohistochemistry

Male mice were deeply anesthetized with Nembutal (120 mg/kg, i.p.) and perfused with saline followed by 10% neutral buffered formalin. Forty μm thick coronal sections were cut on a freezing microtome. For immunocytochemical staining the following antibodies were used: 1) human-specific monoclonal Aβ antibodies which identifies human Aβ in transgenic mice (antibody 3D6, Elan pharmaceutical, Inc); and 2) polyclonal goat antibody against human ChAT (AB144P; Chemicon International, Inc.).

The sections were processed for immunocytochemistry using the ABC method with free-floating sections. In brief, sections were washed in phosphate buffered saline (PBS), treated with 1% hydrogen peroxide in PBS, and blocked in 5% normal goat serum or normal horse serum in 0.3% Triton X-100 in PBS (PBST). On day 1, sections were incubated with diluted primary antibodies (1: 1,000 anti-Aβ, and 1:200 anti-ChAT) with 1% normal serum in PBST, overnight at room temperature. On day 2, the sections were incubated with secondary antibodies (1.5 μg/ml either biotinylated goat anti-rabbit IgG, or horse anti-mouse IgG, Vector Labs, Burlingame, CA) for 30 minutes, and with an avidion/biotin/peroxidase reagent (1: 250 dilution; ABC Elite, Vector Labs) for 1 hour. Sections were reacted in an acetate buffer (pH 6.0) containing 0.035% diaminobenzidine tetrahydrochloride, 2.5% nickel ammonium sulfate, and 0.001% hydrogen peroxide for 5-10 minutes.

All incubations were done on a shaker table, at room temperature and with 3×10 minute washes in PBS. Sections were mounted on subbed slides, dehydrated, and cover slipped. To be certain of the specificity of the immunoreactivity, the primary antibody step was omitted in the staining procedure. This procedure blocked specific immunostaining with both antibodies. The immunohistochemical staining procedure was run on tissue sections from both transgenic and wild-type controls at the same time in order to control for antibody concentration, reaction times, *etc.*, since these variables influence staining intensity.

Morphometry

To count the basal forebrain cholinergic cell profiles, Neurolucida software was used (MicroBrightField, Inc.) with a × 40 microscope objective. Outlines were drawn at low power (2.5 ×) around the striatum, the MS nucleus, VDDB and HDBB. Cell profiles that measured > 10 μm in diameter were not counted within the confines of each of the four nuclei. Evenly spaced sections were counted for a total of 9-11 sections/brain, and more sections were generally present in the control mice vs. PDAPP mice throughout the rostral-caudal extent of each nucleus. Cell profile counting began rostrally at the origin of the VDBB, and stopped at the level of the decussation of anterior commissure. The long axis of cholinergic somata was measured at 400 × magnification in 30-60 cells in each brain. The total number of cell profiles within each of the cholinergic nuclei was estimated using Abercrombie's correction factor for split cell counting error (Abercrombie, 1946). The person performing the counts was blind to the animal's experimental condition.

Statistical analysis

For the statistical analysis, we used Chi square and Fisher's exact tests and *P* values were computed by SPSS software, version 11. *P* value < 0.05 was considered as significant.

RESULTS

Amyloid peptide plaques were found in large numbers in the limbic and association cortices in sections stained with antibody against human Aβ. We also found that Aβ-containing neuritic plaques accumulate with age in the PDAPP mouse (Fig. 1). By 12-months of age, diffuse and mature plaques were present in the cortex and hippocampus but no plaque in subcortical regions such as the thalamus or striatum was found (Fig. 1. A). Compared to the 1-year old mouse, at 2 years of age there were many more mature and diffuse plaques in the cerebral cortex and hippocampus (Fig. 1. B). A lower number of compacted plaques were also found in subcortical regions such as the caudate-putamen (CPU) and in white matter regions.

To view cholinergic changes as well, tissues were stained for both ChAT (black reaction) and Aβ (brown reaction). Cholinergic disruption in neocortex of aged PDAPP mice was most evident around plaques. As shown in figures 1. C and D, immunostaining for Aβ revealed numerous compact amyloid plaques and diffuse amyloid (arrow heads) in neocortex of 12 and 24-month old PDAPP mice. Fibers frequently grew toward the amyloid but then formed loops or sharply turned around to grow away from the amyloid.

The basal forebrain cholinergic neurons that project to cortical and hippocampal regions reside in the MS nucleus, and in the VDBB and HDBB and CPU. Figure 2 (A, B and C) illustrates sections within the basal forebrain complex (MS/VDBB/HDBB) and CPU. Representative sections immunostained with an antibody against choline acetyltransferase. There was a reduction in the number of basal forebrain cholinergic neurons in the 2-year old PDAPP mice. Cell profile counts were taken from sections in control and PDAPP mice in the combined MS and VDBB, and from the HDBB and CPU. In the PDAPP vs. control mice there were 17% fewer neurons in the HDBB (Student's $t = 2.31$, $P = 0.038$), and 14% fewer neurons in the MS + VDBB and CPU (Student's $t = 2.89$, $P = 0.016$). In the four regions combined, there were 15% fewer neurons in the PDAPP vs. control mice (Student's $t = 2.83$, $P = 0.018$). Cell size in the HDBB and MS + VDBB and CPU, as measured by the long-axis of the somata, was not significantly different in the two groups. ANOVA for ChAT-positive neuron number were calculated for the MS/VDBB/HDBB and CPU separately in the PDAPP and control mice (Fig. 2. D).

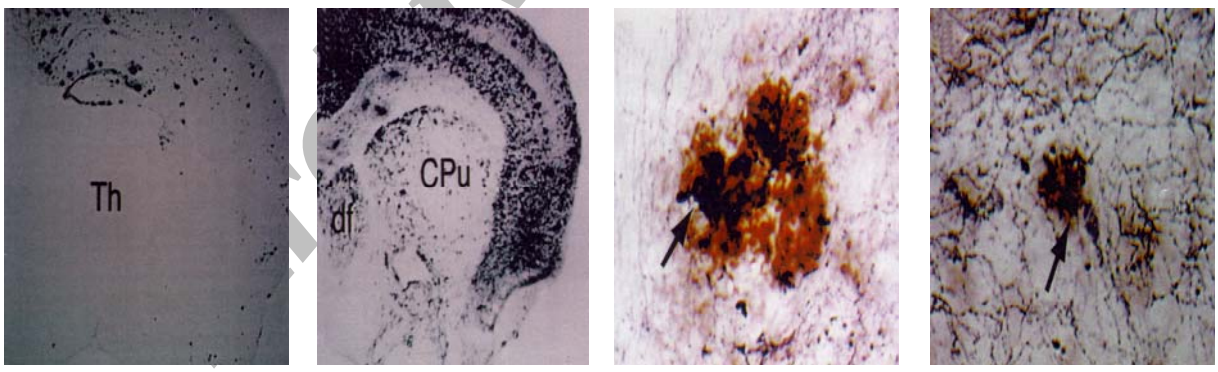
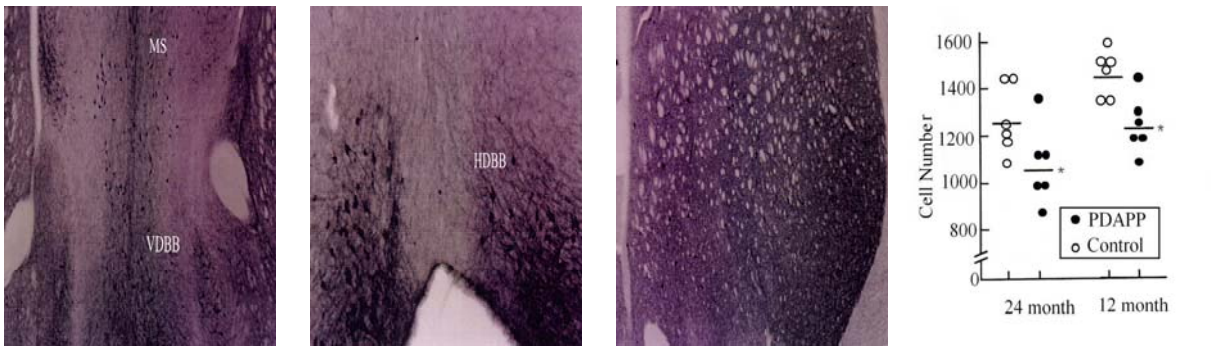


Fig. 1. Amyloid-β (Aβ) containing plaques accumulate with age in the transgenic mice over expressing mutant human amyloid precursor protein (PDAPP). Brain sections were stained with an antibody against human Aβ. There are very few Aβ-containing plaques in the 1-year old PDAPP mouse (A). At 2 years of age, there are many mature and diffuse plaques in the cerebral cortex, hippocampus and caudate-putamen and in white matter regions (B). Mature neuritic plaques in a 2-year-old mouse are covered with markedly swollen ChAT containing varicosities (arrow, C). Neuritic plaques in 1-year-old PDAPP mouse, a diffuse plaque (arrow) can be seen surrounded by numerous cholinergic fibers with nerve terminal varicosities (D). The plaque is located in the hippocampus. Section stained only for ChAT. CPu, caudate-putamen, df, dorsal fornix; DE, dentated gyrus; Th, thalamus. Scale bar, 300 μm in A and B and 15 μm in C and D.



A **B** **C** **D**

Fig. 2. There is age related change in the number of basal forebrain cholinergic neurons in the PDAPP mouse. Basal forebrain cholinergic neurons were examined in the medial septal (MS) nucleus and in the vertical limb of the diagonal band of Broca (VDBB) and horizontal limb of the diagonal band of Broca (HDBB) (A, B) and in caudate-putamen (CPU) (C). The sections are immunostained with an antibody against choline acetyltransferase; scale bar, 300 μm. Fig. 2. D shows comparison of cell counts taken from sections in control and PDAPP mice in the combined MS and VDBB and CPU in the 1-year (young) and 2-year old PDAPP mice (aged) vs. age-matched controls. Data represent total profile counts for individual animals in the four groups, and represent the estimated total number of cells after correction for split cell count error. Lines represent the mean values for the group and indicate that the difference was statistically significant vs. control animals at 2-years of age ($P< 0.01$). In PDAPP vs. control mice, there was a difference of around 15% in the number of somata in MS + VDBB + CPU.

DISCUSSION

The demonstration that Aβ and APP C-terminal fragments containing Aβ are neurotoxic in cell culture gave rise to the hypothesis that Aβ may be a primary cause of neuronal degeneration in AD (32, 33). The neurotoxicity of Aβ is dependent on its aggregated state (33, 31, 34). The cholinergic pathology in the PDAPP mouse is similar to that in AD post-mortem brain where there are marked decreases in the density of cholinergic nerve terminals and ChAT enzyme activity (11), and the cholinergic deficits are positively correlated with the level of cognitive impairment in AD patients (14).

Deposition of amyloid is a hallmark lesion of AD, and genetic analysis has demonstrated that Aβ is central to AD pathogenesis (35, 36). Similarly, depletion of the cholinergic system is a robust finding in AD and correlates with cognitive impairment (9, 37, 26). Yet, the link between cerebral amyloidosis and the cholinergic deficit remains poorly understood. The present study was undertaken to investigate alternations in the cholinergic system in the PDAPP mouse model of Alzheimer's disease. The mice develop amyloid plaques and cerebrovascular amyloid deposition throughout the neocortex and hippocampus, with only modest amyloid deposition in the basal forebrain (38, 39). Individual amyloid deposits in PDAPP mice are also morphologically similar to those in AD brain and include congophilic amyloid cores, amyloid associated dystrophic neuritis, astrocytosis, and micro gliosis (40).

Results of the present study reveal a robust decrease in cholinergic fiber length with distorted and dystrophic cholinergic fibers surrounding the amyloid, very similar to that in AD brain (11). Our results suggest that the cortical cholinergic deficit in PDAPP mice is locally induced by Aβ deposition. These results are also consistent with earlier observations that retrograde degeneration in the NBM only occurs after more severe cortical tissue damage (41).

Correlative analysis of ChAT and AChE enzyme activities and amyloid load in AD brain has revealed conflicting findings. Some studies have found negative correlations (9, 42, 43, 44) whereas others have found no relation (11, 45, 46). In AD and in mice, a significant amount of ChAT and AChE staining is associated with dystrophic neuritis surrounding amyloid plaques (47, 31). The present data indicate that the cholinergic nerve terminal degeneration occur around the plaques and can precede Aβ plaque deposition. Behavioral impairments (48), synaptic transmission deficits (49), and loss of cortical nerve terminal markers (50) precede the formation of neuritic plaques in mouse models of AD.

At 2-year of age, there is a very high density of Aβ containing neuritic plaques in the cortex but only low density in the striatum of the homozygous PDAPP mice. At the same point of time there is a reduction in cholinergic nerve terminals around the plaque at cortex and reduction in ChAT enzyme-activity in striatum. In this study, the density of cholinergic nerve terminals in the cortex was reduced by over 60% in the 2-year old PDAPP mouse, and the reduction in the number of basal forebrain cholinergic somata that innervate this cortical region was only 15%. Cholinergic cell degeneration appears to be due to a retrograde mechanism in AD as well. It has been hypothesized that cholinergic depletion in AD contributes to cerebral amyloidosis. This hypothesis is based on the observation that protein kinase C through muscarinic receptor binding stimulates the non amyloidgenic pathway of APP processing by increasing soluble APP production and reducing Aβ generation (51-53). Thus the loss of the cholinergic innervation may lead to increased production of Aβ and amyloid deposition. *In vivo* support for altered APP processing has been provided in both NBM-lesioned rats and in rats after muscarinic agonist treatment (1, 54).

In conclusion, our results suggest that the homozygous PDAPP mouse exhibits cholinergic degenerative pathology similar to that observed in AD, and it has been speculated that AD neuropathology begins in the cerebral cortex and spreads to subcortical nuclei via the axonal projections to the cortex. The subcortical cell degeneration has been proposed to occur via retrograde transport of a toxin, such as Aβ.

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