Gene expression of Alzheimer-associated presenilin-2 in the frontal cortex of Alzheimer and aged control brain

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Abstract The presenilin-2 (PS2) gene expression pattern in Alzheimer's disease (AD) and control brains was examined using nonradioactive in situ hybridization. Message for PS2 was primarily detectable in neurons, particularly in somal cytoplasm. Intense staining signal was most commonly found in large pyramidal neurons, whereas moderate or faint staining was usually present in smaller neurons. The pattern of PS2 gene expression exhibited a laminar distribution profile in the frontal cortex. A small subset of tangle-bearing neurons exhibited PS2 hybridization signal in AD. PS2 mRNA expression appeared correlated to a high degree with lipofuscin autofluorescence in a large subset of neurons.

Key words: Presenilin-2; Gene expression; Alzheimer's disease; Chromosome 1; Neuropathology

1. Introduction

Alzheimer's disease, the leading cause of dementia in the elderly, is characterized by several striking histopathological features including abundant senile plaques, neurofibrillary tangles, and extensive neuronal death localized primarily to vulnerable brain regions, such as the cerebral cortex and hippocampus, of affected individuals. The etiology of this disease is not well understood, however, genetic components contribute to a significant percentage of cases, particularly in familial Alzheimer's disease (FAD). Several genes have been implicated in the pathogenesis of affected individuals, including apolipoprotein E (ApoE) on chromosome 19 [1,2], amyloid precursor protein gene (APP) on chromosome 21 [3], presenilin-1 (PS1/S182) on chromosome 14 [4], and presenilin-2 (PS2/STM-2) on chromosome 1 [5,6]. The allele of ApoE is associated with AD in a major proportion of cases with late onset (>60 years of age [1,2]). The latter three genes (APP, PS1 and PS2) are associated with FAD with early onset (<60 years of age [3–6]). Mutations in APP account for a small portion of FAD cases, whereas mutations in PS1 and PS2 genes have been found in a majority of FAD cases. Thus far, at least 22 missense mutations have been identified in the PS1 gene [4] and two missense mutations have been described in the PS2 gene in early-onset FAD [5,6]. Although the primary structure of presenilins suggests that they are integral membrane proteins, their physiological functions remain presently unknown. Presenilins showed a significant homology with *C. elegans* protein *Spe* 4, which is involved in receptor localization and protein processing [4–6]. Therefore, it has been suggested that presenilin may participate in APP trafficking or processing and ultimately lead to the abnormal production of β-amyloid, which accumulates in senile plaques and degenerating neurons in AD [4–6].

Recently, a partial cDNA ALG-3, which is a mouse homologue of PS2, has been shown to rescue a T cell hybridoma from T receptor- and Fas-induced apoptosis [7]. It is unclear, however, whether the full length PS2 cDNA has a similar or inverse function, because it is not known whether this partial cDNA ALG-3 conferring resistance to cell death is translated into a polypeptide. Indeed, neuronal death is a prominent feature of AD and several lines of evidence suggest that apoptosis occurs in AD [8–10]. Thus, it is possible that presenilins may be associated with neuronal cell death.

Immunohistochemical evidence has shown that PS-1 protein is detectable within neuritic plaques in sporadic AD [11], suggesting a role for PS1 protein in the development of AD pathology. To date, no data regarding the expression pattern of PS2 and its correlation with the pathology of AD are available. Therefore, the characterization of expression pattern of PS2 in AD brain is prerequisite to understanding the role of PS2 in sporadic AD. In the present study, nonradioactive in situ hybridization was used in combination with immunohistological techniques to examine PS2 mRNA expression and correlation with classical pathological lesions in sporadic AD and aged control brain.

2. Materials and methods

2.1. cDNA cloning and probe preparation

To obtain a human PS2 cDNA, 1 μl of aliquot from the human brain cDNA library (Stratagene) was amplified by polymerase chain reaction (PCR) with the following primers [6]: forward GCCAAGAATTCGTGGTGCTTCCAGAGGCA (containing the *Eco*RI restriction site) and reverse GCAGCTCTAGAAAATTCCCTGCAGCTTGCA (containing the *Xba*I restriction site). PCR was performed under the following conditions: 94°C, 1 min; 60°C, 1 min; and 72°C, 1 min for 30 cycles with the Pwo DNA polymerase (Boehringer Mannheim). PCR products were digested by *Eco*RI and *Xba*I and ligated to a pcDNA3 vector (Invitrogen). The positive insert was sequenced on double strands. For in situ hybridization, digoxigenin-labeled antisense and sense riboprobes were prepared using a Genius Kit (Boehringer Mannheim). Briefly, *Eco*RI- or *Xba*I-linearized PS2 cDNA in a pcDNA3 vector was transcribed in vitro with either SP6 or T7 RNA polymerase to generate antisense and sense probes. After transcription, template DNA was digested by RNase-free DNase and the digoxigenin-labeled transcripts were precipitated using ethanol.

2.2. Preparation of tissue

Six cases of clinically and neuropathologically defined AD (average age 74 years, average postmortem delay 4.5 h) and four age-matched control cases (average age 77 years, average postmortem delay 5 h) were used in this study. Frozen brain tissue from frontal cortex was fixed in 4% paraformaldehyde in 100 mM Tris buffer (pH 7.4) for 1–2 h and stored in 20% sucrose in 100 mM Tris buffer overnight until the tissue sunk. Fixed tissue was sectioned on a cryostat (30 μm) and

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sections were collected in Tris buffer (100 mM, pH 7.4) and digested with proteinase K (Boehringer Mannheim) at a final concentration of 1 μg/ml in 10 mM Tris, pH 8.0 at 37°C for 20 min. This reaction was stopped using 100 mM glycine in 100 mM Tris buffer for 30 s, followed by a 4% paraformaldehyde for 10 min. Sections were washed in 100 mM Tris buffer for subsequent hybridization.

2.3. In situ hybridization

The in situ hybridization in free-floating slices was essentially conducted according to the procedure described previously with slight modification [12–14]. Briefly, sections were hybridized overnight with the antisense or sense probe at a final concentration of approximate 100 ng/ml in hybridization solution containing 50% formamide, 5×SSC, 2×Denhardt's solution (USB), 10% dextran sulfate, 0.1% SDS, 10 mM Tris and 100 μg/ml salmon sperm DNA (Sigma) at 48°C. The sections were then washed in 2×SSC for 5 min and STE (500 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA) for 1 min at room temperature, followed by treatment with 20 μg/ml RNase A (Boehringer Mannheim) in STE buffer for 30 min at 37°C. Subsequently, sections were washed in 2×SSC and 1×SSC for 15 min each at room temperature and in 0.1×SSC for 15 min at 60°C. Subsequent immunological detection was performed according to the manufacturer's instruction (Boehringer Mannheim), sections were washed in buffer 1(100 mM Tris, pH 7.5, 150 mM NaCl) for 1 min and incubated in buffer 2 (1% blocking solution) for 20 min, followed by the addition of anti-digoxigenin antibody at a 1:500 dilution with buffer 2 and incubated for 1 h at room temperature. The sections were washed in buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 20 min and were incubated with NBT/BCIP (dilution 1:50) in buffer 3 overnight in the dark. The color reaction was stopped in the water.

2.4. Immunohistochemistry

To determine whether PS2 gene expression had any correlation with pathological lesions in AD, double-labeling technique for PS2 gene expression and immunoreactivity was employed. Briefly, subsequent to in situ hybridization by the above methods, sections were subject to immunostaining by the following antibodies. Two well-characterized monoclonal antibodies, AT8 (Innogenetics, Belgium, 1:5000) and PHF-1 (kindly provided by Dr. S. Greenberg; 1:200), were used to determine whether PS2 mRNA expression neurons were colocalized with neurofibrillary tangles (NFTs). Both antibodies recognize abnormal phosphorylation of tau protein without showing crossreactivity with normal tau (for review see [15]). The AT8 antibody stains intracellular NFTs as well as pretangle neurons, whereas the PHF-1 antibody stains both intracellular NFTs and extracellular NFTs. Additionally, a polyclonal antibody (β42) directed against residues 1–42 of the β-amyloid protein (1:200) [16] was used to detect PS2 mRNA expression within plaques. After first labeling for PS2 mRNA, the sections were then double-labeled with AT8 and PHF-1 or βA4 antibodies. Labeling for the second antigen was detected using FITC-conjugated IgG (green fluorescence, Sigma).

3. Results

3.1. Distribution of PS2 mRNA in AD and control brain

In situ hybridization using the digoxigenin-labeled probes revealed a dense hybridization signal in the sections hybridized with the antisense probes, whereas no significant hybridization signal was observed in the sections hybridized with sense probes. Figs. 1 and 2 show representative microscopic views of sections from the frontal cortex stained with antisense and sense probes. Robust levels of PS2 gene expression were found in the gray matter of AD and control brain. Furthermore, various degrees of PS2 hybridization signal were expressed maximally in neurons within the frontal cortex. Very little hybridization signal was detectable in white matter. The pattern of PS2 gene expression in sections stained with antisense probe exhibited a laminar distribution profile. Layer I of frontal cortex appeared almost devoid of significant hybridization signal. Layers II, III, and V exhibited intense hybridization signal (Fig. 1). Interestingly, large pyramidal neurons and their neurites localized in layer III and V usually displayed the most intense hybridization signal compared with neighboring cells, which exhibited relatively weak staining (Fig. 3). PS2 hybridization signal was typically observed in the peripheral region of the soma, suggesting that it was predominantly located in neurons based on the morphological characteristics of the stained cells (Fig. 3). The expression difference of PS2 in AD and control brain was also compared. In most cases examined, no significant difference in PS2 gene expression was apparent between AD and control brain, but PS2 mRNA expression in one control case was lighter than that in the AD cases examined, and in one AD case, the staining signal was stronger than that in the four control cases. This did not appear related to postmortem interval or any other obvious features of the tissues.

3.2. PS2 gene expression in neurons correlates with neuropathology

Sections hybridized with digoxigenin-labeled PS2 probes were subsequently subjected to immunostaining with AT8/PHF-1 antibodies. Double-labeling for PS2 gene expression and AT8/PHF-1 immunoreactivity revealed that a small subset of neurons expressing PS2 gene colocalized with paired helical filament (PHF) formation (Fig. 4a,b). PHF was not detected in most PS2 expression neurons. Similarly, double-labeling for PS2 gene expression and β-amyloid protein immunoreactivity demonstrated that no significant changes in PS2 expression were found within plaques, although intense PS2 hybridization signal was occasionally observed within a few plaques (data not shown). Interestingly, a significant subset of neurons expressing PS2 were associated with lipofuscin-related autofluorescence (Fig. 5a,b) and lipofuscin-bearing neurons were significantly colocalized with PS2 labeled-neurons in control and AD brain. This was evident in many if not to majority of PS2 labeled neurons.

4. Discussion

In situ hybridization with digoxigenin-labeled probes offers many advantages over the use of radioactive probes. First, the signal obtained with digoxigenin-labeled probes provides high cellular and morphological resolution with lower levels of background. Second, subsequent to in situ hybridization with digoxigenin-labeled probes, sections can be double-labeled for further analysis, quantification, and comparison. In the present study, the expression patterns of mRNA for human PS2 from frontal cortex of AD and aged control brain by in situ hybridization analysis revealed several findings. First, message for PS2 showed a laminar distribution in cerebral cortex and was primarily detectable in neurons. This result is consistent with previous reports from normal human temporal lobes for PS2 expression visualized by radioactive hybridization [17] and is also comparable with PS1 expression pattern in murine brain observed using non-radioactive hybridization and immunohistochemistry [14,18]. Second, stronger hybridization signal was found in large neurons while mild or weak signal appeared in small neurons. It is well-established that the frontal cerebral cortex, one of the most vulnerable regions in AD, exhibited specific patterns of neuropathology in specific layers of cortex. NFTs are predominantly present in the cell bodies of neurons of layers III and V, although there

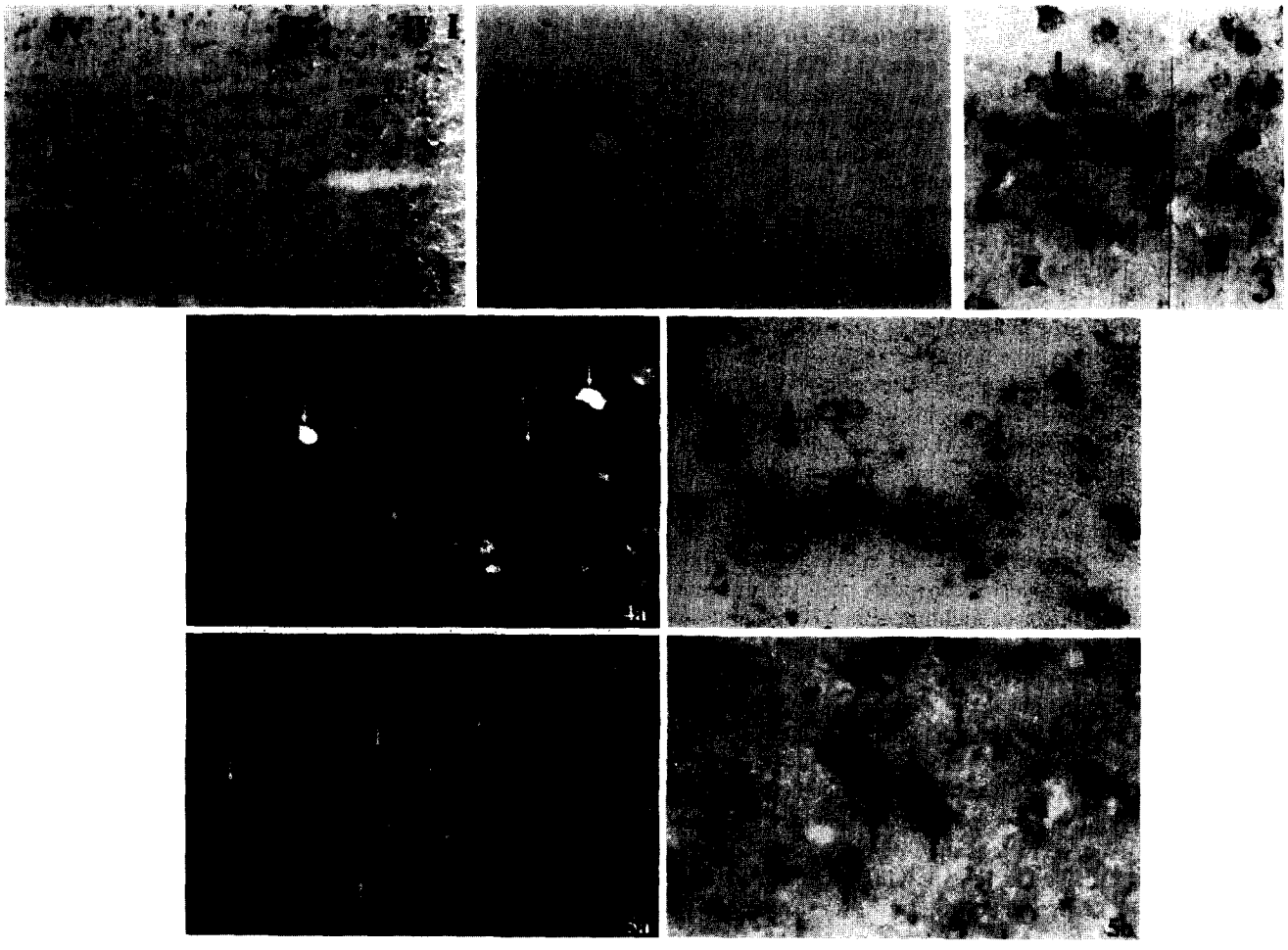

Fig. 1. In situ hybridization of PS2 to frontal cortex of AD using digoxigenin-labeled antisense riboprobes, the pattern of PS2 gene expression exhibited a laminar distribution profile mostly in neurons (×100).

Fig. 2. Digoxigenin-labeled sense riboprobes for PS2 showed no significant hybridization signal in frontal cortex (×100).

Fig. 3. High magnification of PS2 gene expression in neurons. Large pyramidal neurons and their neurites usually displayed intense hybridization signal (arrows) compared with neighboring cells which exhibited weak staining (arrowheads) (×400).

Fig. 4. Double-labeling for PS2 gene expression and AT8/PHF-1 in AD, a small subset of PS2-labeled neurons (black arrow, b) were colocalized with paired helical filament formation (white arrow, a) (×400).

Fig. 5. PS2-expressing neurons (black arrow, b) were associated with lipofuscin-related autofluorescence (white arrow, b) (×400).

are striking regional differences in the proportion of NFTs in these two layers [19,20] and neuritic plaques predominantly affect layers II and III [20]. In addition, large neurons in neocortical and hippocampal areas are particularly vulnerable to cell death or NFT formation [21], suggesting that either there are factors present in some neurons which protect them from neurodegeneration or that susceptible neuronal populations have some unique properties that lead to neurodegeneration. Our results showing higher PS2 expression in large neurons and within neuronal laminar layers (II, III and V) of cerebral cortex suggests that PS2 may participate in events that lead to neurodegeneration in AD via presently unknown pathways. It was recently reported that a partial cDNA ALG-3, which is a mouse homologue of PS2, rescues T cell hybridoma from T cell receptor- and Fas-induced apoptosis [7]. Furthermore, programmed cell death occurs during neuronal development, aging and in the AD brain [8–10]. Our previous immunohistochemical evidence showed that apoptotic changes are present in AD brain and the number of neuronal nuclei displaying the distinct morphology of apoptosis is much greater in AD than in aged control brain [8,9]. In the present study, no significant difference in PS2 mRNA expression in AD and control brain was observed. It seems that PS2 mRNA levels in AD do not correlate with DNA damage. Our explanations for this result are as follows. First, eventual development of sporadic pathology may result from the other alterations, such as the generation of specific variant transcripts and truncated transcripts of PS2 gene or its abnormal protein expression, rather than PS2 mRNA level changes. Indeed, previous reports have demonstrated that specific splice variants and truncates of PS1 transcripts were found in sporadic cases of AD [22]. Second, relative PS2 mRNA level may be influenced by postmortem which displays varying degrees of RNA degradation. This is unlikely however, since postmor-

tem delays are short and matched across groups. Third, other AD susceptibility genes may contribute to AD. To define the exact correlation between DNA damage and PS2 expression, double staining for PS2 and DNA damage is needed.

Our results suggest that there is no simple relationship between PS2 expression and tangle and plaque formation. Sequence comparison of presenilins and *Spe* 4 has lead to the hypothesis that presenilins may have some role in β-amyloid trafficking or processing. There is some evidence supporting this hypothesis. For example, a fibroblast cell line isolated from a patient with PS1 defect produced abnormally high β-amyloid [23] and PS1 protein appeared in AD neuritic plaques [11]. Indeed β-amyloid is able to induce apoptosis in primary culture neurons [24]. The pathological function(s) of PS2 and its relationship with β-amyloid remains to be determined. PS-2 as suggested by homology to ALG-3 may play a role in neuronal vulnerability to various stimuli that can initiate neuronal damage and eventually program cell death.

Lipofuscin accumulation has been considered to be age-related, morphological marker of nervous system aging. Lipofuscin autofluorescent granules were widely observed in brain regions such as cerebral cortex and hippocampus in aging rat and human, including AD tissue [25,26]. It has been suggested that lipofuscin deposits represent the accumulation of the peroxidative action of free radicals on membranes. A significant colocalization of PS2 mRNA expression and lipofuscin-related autofluorescence in a large subset of neurons from cerebral cortex of AD and aged control brain suggests that PS2 may have some relationship with lipofuscin deposits via some as yet unknown pathway such as lipid peroxidation of neuronal membrane. Their precise relationships between PS2 and lipofuscin deposits needs to be further investigated.

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