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PRESENILIN 2 OVEREXPRESSION IS ASSOCIATED WITH APOPTOSIS IN NEURO2A CELLS

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Abstract

Presenilin 1 (PS1) and PS2 are evolutionarily conserved transmembrane proteins of the aspartyl protease family. Initially, they were reported to be associated with the early onset of familial, early-onset Alzheimer's disease. PS1 has been implicated in several crucial brain functions including developmental processes, synaptic plasticity, and processing of various molecules, while PS2 has been poorly studied and is considered to be a compensatory partner of PS1. Certain controversial reports have suggested that PS2 has a role in apoptosis, though the underlying mechanism is not clear. To ascertain the role of PS2 in apoptosis, mouse neuroblastoma cells (Neuro2a) were transfected with a cDNA construct encoding full length mouse PS2 and analyzed for viability, expression of PS1, PS2, Bax and p53, Bax protein, and status of chromatin condensation. Our results showed reduced viability, condensed chromatin and higher expression of Bax at mRNA and protein levels, but no change in the expression of p53 and PS1 in PS2-overexpressing Neuro2a cells. Thus, it is evident that PS2, independent of PS1, is associated with apoptosis via a Bax-mediated pathway. These findings might help in the understanding of the involvement of PS2 in apoptosis and its associated brain disorders.

Keywords

• Chromatin condensation • Gene expression • *p53* • Presenilin

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Introduction

Presenilin 1 (PS1) and PS2 are highly conserved hydrophobic multi-pass transmembrane proteins of the aspartyl protease family. They were initially identified as candidate genes involved in familial, early-onset Alzheimer's disease (EOAD) [1]. To date, more than 240 mutations in *PS1* and at least 47 mutations in *PS2* have been identified [2]. PS1 and PS2 both form the catalytic core of an aspartyl protease called γ-secretase complex along with the proteins nicastrin, Aph-1, and Pen-2. They cleave a large number of substrates including Notch, N-cadherin, ephrin-B2, ErbB4, and amyloid precursor protein (APP). Several studies have shown that mutations in PS lead to an aberrant cleavage of amyloid precursor protein, causing AD [3].

Besides their role in AD pathology, PS1 and PS2 are involved in several functions such as brain development [4-6], synaptic plasticity, memory [7], protein trafficking, cell adhesion, and calcium homeostasis [8]. *PS1* is mapped to mouse chromosome 12 at 37.0 cM [9] and codes for 3.0 kb transcript which makes ~50-55 kDa protein of 467 amino acids [10]. On the other hand, PS2 is located on mouse chromosome 1 at position 101.0 cM and codes for 2.3 kb mRNA in the brain. It makes ~50-55 kDa protein of 448 amino acids [11]. Full length PS proteins are highly unstable and undergo endoproteolysis to produce a ~32 kDa N-terminal fragment and a ~20 kDa C-terminal fragment [12].

Despite sharing many sequence similarities, PS1 is well characterized while PS2 is poorly studied in relation to calcium homeostasis [13], regulation of acetylcholinesterase activity [14], aging [15-16], and other functions of the brain. PS2 is suggested to be a compensatory partner of PS1 in various signaling pathways, including the notch developmental cascade [17].

There are contradictory reports showing the involvement of *PS2* overexpression in apoptosis using different mammalian cell lines [18-20] as well as discrepancies in the underlying pathway. Some of the studies worth mentioning suggest that *PS2* gene overexpression caused apoptosis [21], while its depletion protected against apoptotic cell death induced by trophic factor withdrawal or amyloid b (Ab) [22]. Conversely, Gamliel *et al*. [18] reported that overexpressed *PS2* does not induce apoptosis in Neura2a, CHO, and HEK293T cells. In regard to mechanisms, Passer *et al*. [23] showed that Bcl-X(L) interacts with PS1 and PS2 proteins, influencing mitochondrial-dependent apoptosis. Others considered that the activation of p53 signaling was the major effector contributing to PS2-induced apoptosis [24, 20]. These controversies prompted us to investigate whether *PS2* overexpression causes apoptosis in mouse neuroblastoma (Neuro2a) cells and the underlying pathway. We analyzed viability, *PS1*, *PS2*, *Bax* and *p53* mRNA expression, and the status of chromatin condensation in *PS2*-overexpressing Neuro2a cells. We showed reduced viability, condensed chromatin and higher expression of *Bax* in *PS2*-

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overexpressing Neuro2a cells. However, no change was observed in the expression of *p53* and *PS1* in *PS2*-overexpressing Neuro2a cells.

Materials and methods

Cell culture

The mouse neuroblastoma Neuro2a cell line was obtained from the National Centre for Cell Science in Pune, India. The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA), supplemented with 10% fetal calf serum and 5% CO_2, and incubated in a humidified incubator at 37°C. One batch of cells was grown using only transfection reagent (Control). To examine PS2 function, the cells were transfected with pcDNA3.1 (Vector) and pcDNA3-PS2cDNA (Overexpressed) using jetPRIME™ DNA and siRNA transfection reagent (Hi-media), according to the manufacturers' instructions. After 48 h, cells from the three groups were washed and harvested in phosphate-buffered saline (PBS).

Cell viability

The viability of Neuro2a cells was determined by measuring their capacity to exclude the vital dye trypan blue. In brief, the control, vector and *PS2*-overexpressed Neuro2a cells were washed, gently scraped and centrifuged. The pellet was resuspended in 500 µl of culture medium containing 0.1% trypan blue, loaded onto a hemocytometer, and examined under a light microscope. Then the total, viable, and dead cells were counted and represented as % cell viability.

Acridine orange/ethidium bromide (AO/EB) staining

Procedures were followed as described previously by Ribble *et al.* [25]. Briefly, the cells were harvested in PBS-ethylenediaminetetraacetic acid (PBS-EDTA) medium, pelleted by centrifugation at 1,000 g for 5 min, and washed with 1 ml of cold PBS. The cell pellets were then re-suspended in 25 µl cold PBS and 2 µl AO/EB dye mix was added. The stained cell suspension (10 µl) was placed on a clean microscope slide and covered with a coverslip. The cells were viewed under a microscope with an excitation filter of 480/30 nm.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from the control, vector, and *PS2*-overexpressed Neuro2a cells using a TRI Reagent™ kit (Sigma-Aldrich, St. Louis, MO, USA) in compliance with the manufacturer's instruction. It was estimated by measuring absorbance at 260 nm. RNA samples with $A_{260/280} \geq 1.8$ were used thereafter. Total RNA from different groups was resolved on a 1.2% agarose formaldehyde gel and the integrity of the RNA was checked by ethidium bromide staining of 18S and 28S rRNA.

For RT-PCR, the cDNA was synthesized from the RNA of Neuro2a cells of different experimental groups. The primer sequence and PCR conditions are given in Table 1. The PCR products were resolved on 2% agarose gel. The signals were scanned and analyzed using Alpha-EaseFC software (Alpha Innotech Corp, San Leandro, CA, USA).

Immunoblotting

The proteins were extracted by solubilization buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P40, 0.5% deoxycholic acid, 1% sodium dodecyl sulphate (SDS), and 1 mM phenylmethylsulphonyl fluoride, PMSF] for 15 min at 4°C. The sample was then centrifuged for 10 min at 12,000 g and the supernatant was saved. The amount of protein in the membrane-enriched preparation was estimated by the bicinchoninic acid method. The protein (40 µg) was denatured, resolved by 15% Tris-glycine SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% (w/v) nonfat milk prepared in PBS for 2 h and incubated overnight with primary antibodies (rabbit anti-Bax 1:1000, and mouse anti-β-actin horseradish peroxidase, HRP, conjugate, 1:10,000). After washing it in 0.1% phosphate buffered saline with Tween 20 (PBST) twice, the membrane was incubated with HRP-conjugated secondary antibodies (goat-anti-rabbit, dilution 1:2000 for PS1 and rabbit-anti-mouse, dilution 1:2000 for PS2) obtained from Bangalore Genei (Bengaluru, Karnataka, India), washed in 0.1% PBST twice, and detected by enhanced chemiluminescence (ECL).

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by a *post hoc* test of Tukey's method through Jandel Scientific SigmaPlot for Windows (standard version 2.0). The values were reported as mean (± SD), and P values less than 0.05 were considered as significant. For RT-PCR, the signal intensity for PS1, PS2, Bax and p53 message was normalized against the signal intensity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and represented as a histogram with the mean (± SD) of three values calculated as Relative Density Value, RDV (integrated density value, IDV, of PS1/GAPDH, PS2/GAPDH, Bax/GAPDH and p53/GAPDH). For Immunoblotting, the signal intensities for Bax were normalized against the signal intensity of ß-actin and represented as a histogram with a mean of three values, calculated as RDV (IDV of Bax/b-actin).

Results

Overexpression of *PS2* in Neuro2a cells shows reduced viability, upregulated Bax, and condensation of chromatin

In order to determine the involvement of PS2 in apoptosis, we checked cell viability, mRNA levels of *PS1*, *PS2*, the apoptotic markers Bax and p53, chromatin condensation in control, vector, and *PS2*-overexpressing mouse Neuro2a cells. To analyze cell viability using trypan blue assay, values from the vector were considered to be 100%. The assay showed reduced viability (80.8%, $P < 0.05$) in the overexpressing cells but no significant change in control as compared to vector (Fig. 1).

In RT-PCR analysis, primers detected 289 nucleotide fragments of *PS1* mRNA, 493 nucleotide fragments of *PS2* mRNA, 344 nucleotide fragments of *BAX* mRNA and 275 nucleotide fragments of *P53* mRNA. To analyze the expression of *PS1*, *PS2*, *BAX* and *P53*, values from the vector were considered to be 100%. RT-PCR analysis showed overexpression

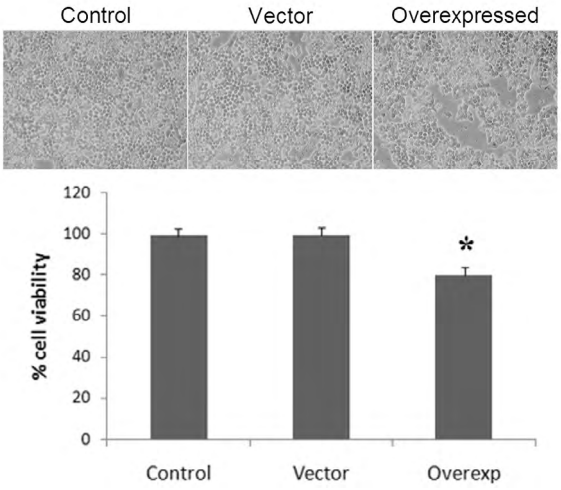

Fig. 1. A. Neuro2a cells as observed by inverted bright field microscope. "Control" represents the cells with only transfection reagent, "vector" the cells transfected with the vector pcDNA3.1 without insert, and "overexpressed" the cells transfected with mouse PS2 cDNA in vector pcDNA3. B. Histogram representing % cell viability using trypan blue assay in Neuro2a cells from three independent experiments. * denotes significant differences ($P < 0.05$) in *PS2*-overexpressed cells as compared to control and vector.

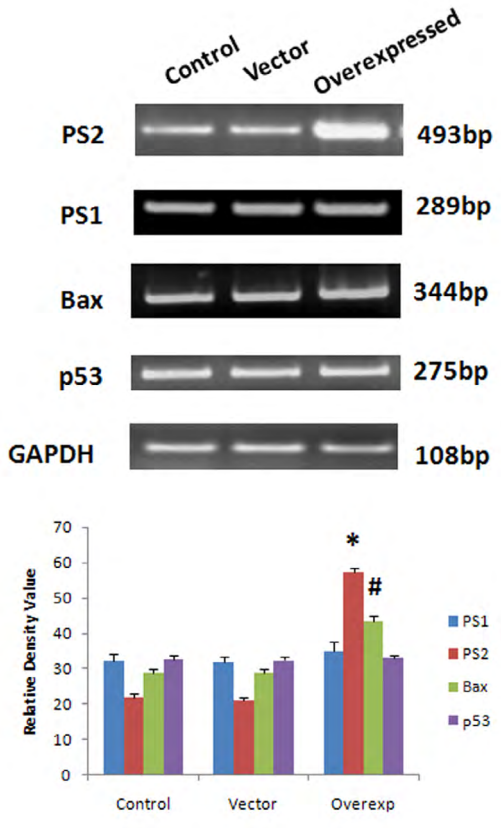

Fig. 2. RT-PCR analysis showing *PS2* (493 nucleotide fragment), *PS1* (289 nucleotide fragment), *Bax* (344 nucleotide fragment), *p53* (275 nucleotide fragment) mRNA and internal control GAPDH (108 nucleotide fragment) in Control, Vector and *PS2*-overexpressed Neuro2a cells. The band intensity was quantified densitometrically, normalized against GAPDH and plotted as histogram representing Relative Density Value (RDV) from three independent experiments (± SD). * and # denote significant differences ($P < 0.05$) in *PS2*-overexpressed cells as compared to control and vector.

of *PS2* (305%, $P < 0.05$) and upregulation of *Bax* mRNA expression (151%, $P < 0.05$) but no significant change in *PS1* and *p53* mRNA expression in mouse Neuro2a cells overexpressing *PS2* as compared to the vector. Also, no significant change was observed in the control as compared to the vector (Fig. 2).

In immunoblot analysis, the 22 kDa band of Bax protein and the 42 kDa band of β-actin were detected. To analyze the expression of Bax protein, values from the vector were considered to be 100%. Immunoblot analysis revealed that Bax protein expression increased significantly (119.5%, $P < 0.05$) in mouse Neuro2a cells overexpressing *PS2* as compared to the vector. No significant change was observed in the control as compared to the vector (Fig. 3).

To understand the status of chromatin condensation, we used the acridine orange/ethidium bromide staining method. The mouse Neuro2a cells overexpressing *PS2* showed high chromatin condensation but no significant change in the control as compared to the vector (Fig. 4). Thus the data showed that overexpression of *PS2* causes apoptosis via upregulation of *Bax* without any change in *p53* expression, and leads to chromatin condensation. Interestingly, this function of PS2 is independent of PS1.

Discussion

Apoptosis is a well-defined program, which plays a significant role in the physiological processes of development, differentiation, regulation, and function of multicellular organisms. Hence, the dysregulation of the apoptotic program may result in several pathological conditions such as cancer, neurodegenerative and ischemic disorders. This program is tightly regulated by the coordinated expression of several genes [26]. This study deals with alterations in the expression of the *PS2* gene and its role in apoptosis. We have overexpressed *PS2* in mouse Neuro2a cells and used different approaches to check apoptosis. *PS2* overexpression resulted in reduced cell viability, higher chromatin condensation, and upregulation of the Bax apoptotic marker without any change in the expression of *p53*. These results are partially supported by earlier

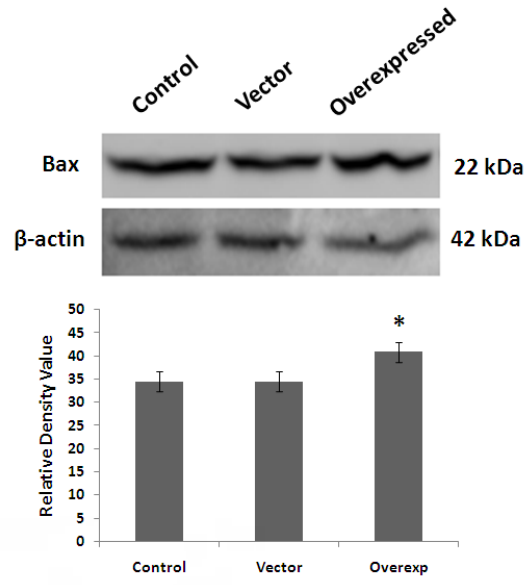

Fig. 3. Immunoblot analysis showing Bax (22 kDa) and internal control β-actin (42 kDa) in the cerebral cortex of mice during development. Histogram represents Relative Density Value, RDV (integrated density value, IDV, of Co-IP Notch-1 NICD/β-actin, input Notch-1/β-actin and PS1/β-actin) from three independent experiments (± SD).

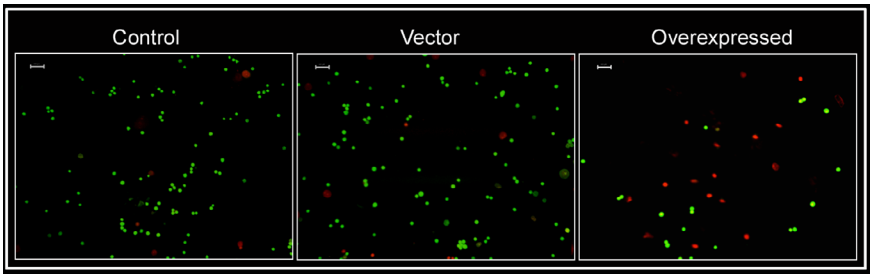

Fig. 4. Status of chromatin condensation as observed by acridine orange/ethidium bromide staining of Control, Vector and *PS2*-overexpressed Neuro2a cells.

studies showing a prominent pro-apoptotic role of *PS2* overexpression in mammalian cell lines [19, 27]. Our data provide insight into PS2-mediated apoptosis via a Bax-dependent and p53-independent pathway. Interestingly, the high level of chromatin condensation observed can be correlated with an upregulation of Bax, which activates caspases. This leads to increased chromatin condensation and cellular apoptosis [28]. Moreover, Bax-mediated p53-independent apoptotic activation of caspase 3 has also been reported in several studies [29, 30]. We also found that in Neuro2a cells, *PS1* expression is unaffected by *PS2* overexpression, suggesting its independent expression as recently shown in mouse embryonic fibroblasts and adult mouse brain by Frånberg *et al*. [31].

Thus, it may be suggested that the induction of *PS2* expression leads to a cytoplasmic influx of Ca^{2+} from the endoplasmic reticulum [32], which in turn upregulates *Bax* expression [33]. This eventually activates the caspase-mediated apoptotic cascade. PS2-induced upregulation of *Bax* can also be correlated with neurodegeneration, as *Bax* expression is high in Alzheimer's disease (AD) [34]. However, further investigations are required to check *Bax* expression in *PS2* mutation-associated EOAD. The distinct role of PS2 in Bax-mediated apoptosis might provide a novel insight into its involvement in several disorders resulting from abnormal or excessive apoptosis, including schizophrenia, mental retardation, cerebral palsy, stroke, neurodegenerative disorders such as AD, Parkinson's disease, and Huntington's disease. Further studies on *PS2*-overexpressing or knockout mice are warranted in the understanding of the novel functions of PS2, particularly in relation to brain disorders.

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