Down-regulation amyloid β-protein 42 production by interfering with transcript of presenilin 1 gene with siRNA[1]

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ABSTRACT

AIM: To investigate the pathogenesis of $A\beta_{42}$ yielding and new drug targets as well as the possibility of RNA interference (RNAi) technique for treatment of Alzheimer disease (AD). **METHODS:** Human AD presenilin 1 (PS1) cDNA sequence was obtained from NCBI website. The three sites of RNAi action and one missense control site were selected in PS1 cDNA through online design of Ambion company. To confirm specificity of these sites, we conducted a BLAST search of the IMAGE EST library. The corresponding double-stranded DNA was used to construct pSilencer 3.1-H1 plasmid, which could transcribe small interference RNA (siRNA). Then, the pSliencer 3.1-H1 plasmids were transfected into CHO/PS1/APP cells with SuperFect transfection reagent. The cells have been transfected with the mutant PS1 and APP gene of AD. All the CHO/PS1/APP cells with pSliencer 3.1-H1 plasmids were screened out using G_{418}. Transcripts of PS1 gene in CHO/PS1/APP were measured by RT-PCR, the contents of PS1 peptide and $A\beta_{42}$ production inside CHO/PS1/APP cells were examined through Western blot and the $A\beta_{42}$ change of secretion by CHO/PS1/APP was determined with ELISA. **RESULTS:** The site 3 of PS1 mRNA was inhibited by RNAi after 2 d. The effect was more obvious with the time. The peptide corresponding to PS1 gene and $A\beta_{42}$ production in CHO/PS1/APP cells were both reduced after siRNA interfere for 3 d. $A\beta_{42}$ secretion by CHO/PS1/APP cells began to reduce on d 3, and reached the most significance on d 5. There was a time-dependent relationship between the transcript of PS1 gene and the production of $A\beta_{42}$ with RNAi action. **CONCLUSION:** PS1 is essential for γ-secretase activity. Inhibition of the PS1 can decrease the levels of $A\beta_{42}$. Some sites of PS1 mRNA, for example, the site 3, may serve as a new drug target and RNAi probably can be used for treatment of AD.

INTRODUCTION

The amyloid β-protein (Aβ) deposited in the Alzheimer disease (AD) patient's brain is synthesized as the larger β-amyloid precursor protein (βAPP). βAPP is cleaved by proteases called α- and β-secretase to secrete the derivatives as sAPP α and sAPPβ and membrane bound fragments, C-terminal fragment β (CTFβ), and C-terminal fragment γ (CTFγ), which are then cleaved within the membrane by γ-secretase to secrete the 24 or 26-residue P3 and 40 or 42-residue Aβ[1]. Genetic and biochemical evidence suggests that Aβ, particularly in its longer $A\beta_{42}$ form, plays a pivotal role in AD[2]. In addition to Aβ, γ-secretase processing also releases a cognate C-terminal fragment γ (CTFγ)[3]. CTFγ is highly conserved among members of the βAPP

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family, suggesting that it is the functional important domain of APP and may also play an important role in AD[1]. For example, CTFγ may function like the developmental regulator, Notch, that is processed by γ-secretase to release its intracellular domain (NICD), which subsequently enters the nucleus to regulate transcription[4,5]. Presenilin 1 (PS1) knockout mice and inhibitor cross-linking studies have provided convincing evidence that PS1 was an essential subunit for γ-secretase processing[6].

In order to furthermore understand βAPP processing regulated by PS1 and γ-secretase and look for new drug targets and gene therapy for AD, we used RNA interference (RNAi) technology to examine the effects of small-interfering RNA (siRNA) to PS1 on the processing of βAPP. The results showed that PS1 was suppressed and γ-secretase activity and Aβ yielding decreased. Our results are in consistent with the concept that PS1 is involved in γ-secretase activity[1,2,7].

MATERIALS AND METHODS

Cell line The cell line, CHO/PS1/APP, was Chinese hamster ovary (CHO) cells that were transfected by mutant PS1 gene and βAPP gene of AD patient and provided by Harvard Medical School, USA.

Construction of expression vector[8-10] Human PS1 cDNA sequence was obtained from NCBI website. Based on the gene sequence, we used a procedure of Ambion company online to design 3 gene sequences used for RNAi and 1 gene sequence used for missense control, which were as follows:

Gene sequence of RNAi site-1: AAGATGAGGAGCTGACATTGA; Forward DNA strand: 5′-GATCCGATGAGGAGCTGACATTGATTCAAGAGATCAATGTCA GCTCCTCATCTTTTTTGTCGACA-3′; Backward DNA strand: 5′-AGCTTGT CGACTTTTTTGATGAGGAGCTGACATTGATCTCTTGAATCAATGTCAGCTCCTCATCG-3′.

Gene sequence of RNAi site-2: AAGGATGGGCAGCTAATCTAT; Forward DNA strand: 5′-GATCCGGATGGGCAGCTAATCTATTTCAAGAGAATAGATTAGCTGCCCATCCTTTTTTGTCGACA-3′; Backward DNA strand: 5′-AGCTTGTC GACAAAAAAGGATGGGCAGCTAATCTATTCTCTTGAAATAGATTAGCTGCCCATCCG-3′.

Gene sequence of RNAi site-3: AAGGTCCACTTCGTATGCTGG; Forward DNA strand: 5′-GATCCGGTCCACTTCGTATGCTGGTTCAAGACGCCAGCATACGAAGTGGACCTTTTTTGTCGACA-3′; Backward DNA strand: 5′-AGCTTGTC GACAAAAAAGGTCCACTTCGTATGCTGGCGTCTTGAACCAGCATACGAAGTGGACCG-3′.

Gene sequence of missense control: AAGCTTCATAAGGCGCATAGC; Forward DNA strand: 5′-GATCCGCTTCATAAGGCGCATAGCTTCAAGAGAGCTATGCGCCTTATGAAGCTTTTTTGGAAGTCGACA-3′; Backward DNA strand: 5′-AGCTTGTCGACTTCCAAAAAAGCTTCATAAGGCGCATAGCTCTCTTGAAGCTATGCG CCTTATGAAGCG-3′.

To confirm above gene sequences to be unique (not consistent with other gene sequences), we conducted a BLAST (Basic Local Alignment Search Tool) search of the IMAGE EST (Expressed Sequence Tags) library at www.ncbi.nlm.nih.gov/BLAST/ and started at the 75th residue after initiating codon to search AA+N19+UU sequence or AA+N19 sequence (N19 stood for a random mRNA sequence with 19 nucleotides). The corresponding single-strand DNA sequences were synthesized by Shanghai Shenggong Bioengineering Co China. These single-strand DNA were prepared into double-strand DNA by linking reaction, then the double-strand DNA were used to construct pSliencer 3.1-H1 plasmids (Ambion Co), which were transfected to competent *Esherichia coli* $DH_{5\alpha}$ for amplification. Examining samples of pSliencer 3.1-H1 plasmids were prepared through alkaline lysis. The samples were sheared by restriction enzyme Hind III(Takara Co) and Bam HI (Takara Co) for electrophoresis and were also used for sequencing.

Cell culture and transfection CHO/PS1/APP cells were maintained in Dulbecco's minimum essential medium (Gibco) containing 10 % little bovine serum (Sijiqing Biological Co LTD, Hangzhou, China), 100 U/mL penicillin (Sigma) and100 mg/L streptomycin (Sigma). The cultures were incubated at 37 ℃ in a 5 % CO_2-humidified chamber for 5 d. The culture medium was changed every 3 d and the cells during logarithmic growth period were used for the experiments.

In detail, CHO/PS1/APP cells were seeded into poly-D-lysine (Sigma) coated 6-wells plates (Sigma) at a density of approximately 6×10^8/L, grown to 90 % confluence, and then according to the manufacturer's instructions transfected with 5 μg of pSilencer3.1-H1 plasmid through SuperFect transfection reagent (QIAGEN Co), one sort of liposome, which the CHO/PS1/APP cells were selected using G418 (Sigma) in order to successfully obtain the pure CHO/PS1/APP cells

with pSliencer 3.1-H1 plasmids. To maintain consistent transfection conditions, all the wells were adjusted to 5 μg of plasmid. After 24 h, cells and media were collected and the levels of PS1, Aβ and secreted Aβ were analyzed. The difference in transfection efficiency between the control and the Liposome control was not significant.

RT-PCR determination Transcript of PS1 gene in CHO/PS1/APP was determined using RT-PCR (from GIBCO), in order to analyze effects of siRNA on PS1 mRNA. Sequence and size of primers of PS1 gene and β-actin gene (used for control) can be seen in Tab 1. The data were processed by Gel Image Analysis System (Multi Genius model, Syngene Co).

Western blot analysis The change of $A\beta_{42}$ production in CHO/PS1/APP was examined by Western blot (Santa Cruz). The samples were obtained from direct lysis cells.

Enzyme-linked immunosorbent assays The levels of $A\beta_{42}$ secretion by CHO/PS1/APP was measured in the conditioned media samples through ELISA method (the kit from Immuno-Biological Laboratories Co LTD).

Statistical analysis The statistical analysis was performed using the SPSS 10.0 statistical package. The data were expressed as mean±SD and analysis of variance (ANOV) test was employed to determine the differences among all groups. LSD test or SNK test was used if the data were homoscedasticity, otherwise, Tamhane's method was used. $P<0.05$ was considered to be statistically significant.

RESULTS

Results of sequencing of RNAi site gene and missense control gene From the results, it can be seen that these gene sequences were all right (Fig 1).

Effect of siRNA on the transcript of PS1 gene in CHO/PS1/APP cells determined by RT-PCR The interference of siRNA to PS1mRNA in CHO/PS1/APP cells was obvious and more and more obvious with time (Tab 2, Fig 2). The interference of siRNA to PS1 in the CHO/PS1/APP cells was successful, reaching the most significance with the time.

Effect of siRNA on the contents of PS1 and $A\beta_{42}$ peptide in CHO/PS1/APP cells determined by Western blot A representative Western blot showed the relative levels of PS1 (Fig 3) and $A\beta_{42}$ (Fig 4) inside CHO/PS1/APP cells while interfering for 3 d. The two kinds of proteins were obviously reduced. On the basis of the results of the two experiments above and in combination with the result of RT-PCR, it can be found that siRNA of 'siRNA group 3' was the most effective.

Tab 1. Sequence and size of primers of PS1 gene and β-actin gene.

Gene	Sense primer	Antisense primer	Product size
PS1	5′CCGAAATCACAGCCAAGA3′	5′CATTCACAGAAGATACCAAGAC3′	414 bp
β-Actin	5′CATCTCTTGCTCGAAGTCCA3′	5′ATCATGTTTGAGACCTTCAACA3′	300 bp

Tab 2. Effect of siRNA on the transcript of PS1 gene in CHO/PS1/APP (Ratio of Absorbance value. *n*=10. Mean±SD. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* missense control.

Group	d 1	d 2	d 3	d 4	d 5
1	1.01±0.01	1.02±0.01	1.02±0.02	1.09±0.05	0.95±0.03
2	1.01±0.02	1.01±0.01	1.00±0.01	1.10±0.03	1.01±0.03
3	1.03±0.02	1.00±0.02	1.00±0.03	1.08±0.02	0.96±0.04
4	1.02±0.01	1.01±0.01	1.03±0.02	1.09±0.03	0.98±0.01
5	1.01±0.01	1.00±0.01	1.01±0.02	1.07±0.01	0.97±0.02
6	1.00±0.01	0.97±0.01	0.53±0.01$^{c\,f}$	0.47±0.02$^{c\,f}$	0.11±0.01$^{c\,f}$

The absorbance (A) value from Image Analysis; Ratio=$A_{\text{target gene}}/A_{\beta\text{-actin}}$. 1: Control; 2: Liposome control; 3: Missense control; 4: siRNA group with PS1 gene 1 interference; 5: siRNA group with PS1 gene 2 interference; 6: siRNA group with PS1 gene 3 interference.

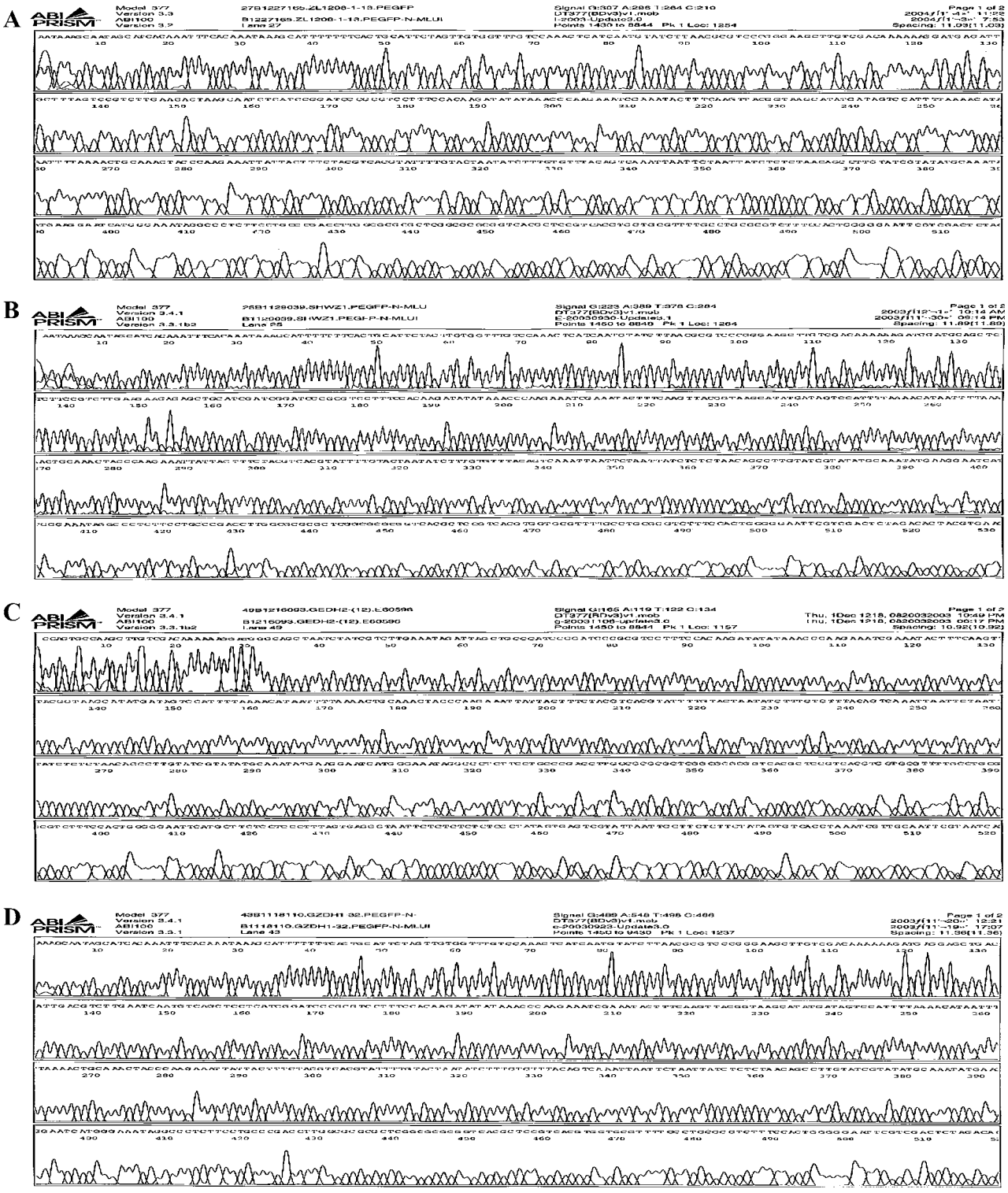

Fig 1.

Effect of siRNA on the content of $A\beta_{42}$ released by CHO/PS1/APP (ELISA assay) The $A\beta_{42}$ content secreted by CHO/PS1/APP cells did not decrease on d 1 and d 2 after RNA interference (siRNA from 'siRNA group 3'). Three days after RNA interference, $A\beta_{42}$ secretion by CHO/PS1/APP cells began to decrease; while on d 5, $A\beta_{42}$ secreted by CHO/PS1/APP decreased the most obviously (Tab 3).

DISCUSSION

Recent studies have identified PS1 as a part of γ-secretase complex. To test the role of PS1 in Aβ pro-

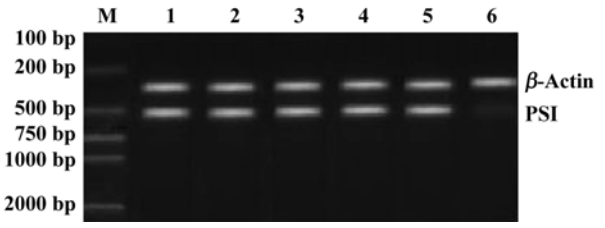

Fig 2. Effect of siRNA on mRNA transcripted by PS1 gene in CHO/PS1/APP at d 5. 1: Control; 2: Liposome control; 3: Missense control; 4: siRNA group with PS1 gene 1 interference; 5: siRNA group with PS1 gene 2 interference; 6: siRNA group with PS1 gene 3 interference; M: DNA Marker (DL2000).

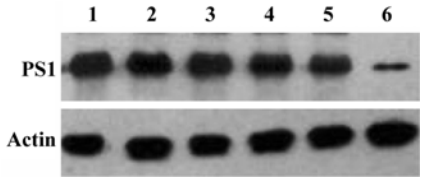

Fig 3. Effect of siRNA on the content of PS1 peptide in CHO/PS1/APP. 1: Control; 2: Liposome control; 3: Missense control; 4: siRNA group with PS1 gene 1 interference; 5: siRNA group with PS1 gene 2 interference; 6: siRNA group with PS1 gene 3 interference.

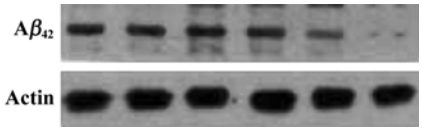

Fig 4. Effect of siRNA on the content of $A\beta_{42}$ peptide in CHO/PS1/APP. 1: Control; 2: Liposome control; 3: Missense control; 4: siRNA group with PS1 gene 1 interference; 5: siRNA group with PS1 gene 2 interference; 6: siRNA group with PS1 gene 3 interference.

duction and find out new drug sites and gene therapy for AD, the present experiment transfected the CHO/PS1/APP cells with pSilencer3.1-H1 plasmid with transcribed siRNA against target gene, in order to interfere with transcript of PS1 gene, and then determined the levels of PS1 mRNA, PS1, and Aβ peptide. Our data demonstrated that suppression of PS1 gene decreased the yielding of $A\beta_{42}$, suggesting that PS1 contributes to γ-secretase activity.

The final step of Aβ biogenesis is the processing of CTFγ by γ-secretase within the βAPP transmembrane domain. With the single exception of the Swedish βAPP mutation (K670 to N and M671 to L) all the identified changes linked to early onset familial Alzheimer disease (FAD) affect γ-secretase processing to specifically increase the levels of $A\beta_{42}$[2].

Tab 3. Effect of siRNA on content of $A\beta_{42}$ released by CHO/PS1/APP. *n*=6. Mean±SD. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* missense control.

Group	$A\beta_{42}$ contents/ng·L^{-1}
Control	1.91±0.01
Vector control	1.96±0.04
Missense control	1.94±0.01
RNAi 1 d	1.91±0.01
RNAi 2 d	1.84±0.03
RNAi 3 d	1.68±0.03^{cf}
RNAi 4 d	0.14±0.02^{cf}
RNAi 5 d	0.08±0.02^{cf}

In the last 2 years, yeast two-hybrid screens have identified several proteins that bind PS1 but their roles at least partly appear to be related to the function of PS1 other than γ-secretase activity such as regulation of apoptosis and calcium signaling[5]. The blocking expression of PS1 protein using RNAi results in a loss of γ-secretase activity.

In this study, we wondered whether suppression of PS1 could regulate γ-secretase activity in CHO/PS1/APP cells, furthermore, regulate Aβ production. The cell line was characterized by the increased γ-secretase activity and Aβ production because of transfection of APP gene and mutant PS1 gene of AD. The fact that PS1 is at least partially limiting for Aβ production suggests that γ-secretase is indeed a complex including PS1 and the complex activity can be increased by the expression reinforcement of any one of its components.

βAPP is cleaved by β-secretase to CTFβ, which is subsequently processed by γ-secretase to $A\beta_{40/42}$ and CTFγ50. A 79-residue fragment between the two cleavage sites has not yet been detected. Multiple studies indicate that γ-secretase is a large multisubunit enzyme. Based on mutagenesis and inhibitor cross-linking studies, it has been suggested that PS1 constitute the catalytically active subunit of γ-secretase. However, PS1 are not sufficient for γ-secretase activity *in vitro* and multiple subunits have been identified that are essential for generation of the active enzyme. Several of these subunits are apparently limiting for γ-secretase activity in the cell.

Since increases in $A\beta_{42}$ are consistently linked to AD pathogenesis[2], the finding that levels of PS1 can

regulate the production of this fragment makes it important candidate for evaluation in AD. The reduction in the relative $A\beta_{42}$ levels is consistent with the observed inhibition of γ-secretase with a variety of inhibitors[11], which may play an important protective function in the brain. Thus the reinforcement of γ-secretase activity may be one of reasons that cause AD. Alternatively, the increase in $A\beta_{42}$ may be a marker of the reinforcement of γ-secretase activity, which may result in the reinforcement of function of a number of γ-secretase substrates including (but not limited to) Notch.

This study is helpful to further understand the mechanisms of $A\beta_{42}$ yielding and allows us to determine if it can be served as a new drug target used for the treatment of AD. That is to say, an understanding of the nature of the PS1 and the relationship of the protein component and γ-secretase activity will significantly accelerate the discovery of a better drug (including RNAi technique) for AD treatment[12-14], which is just the ultimate goal of such study.

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