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Original Article

Role of apolipoprotein E in the pathogenesis of Alzheimer's disease and molecular mechanisms

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Abstract: Alzheimer's disease (AD), as one severe neurodegenerative disorder, severely threatens public health under the current aging of population. Some studies have speculated the presence of apolipoprotein E (apoE) in the occurrence and progression of AD. This study thus investigated the role of apoE in AD pathogenesis and related molecular mechanisms. Neural tissue samples from both AD model rats and normal rats were collected for quantifying apoE expression levels using RT-PCR and Western blotting. Cultured neural cells D283 were then transfected to over-express apoE or to down-regulate apoE using RNA interference approach. MTT assay and flow cytometry were applied to describe the growth and apoptosis of D283 cells. Clonal formation assay was then used to measure the ability of cell proliferation. AD rats had elevated apoE protein and mRNA levels compared to normal animals. Anti-sense siRNA of apoE inhibited the growth of D283 cells and induce their apoptosis, accompanied with lower clonal formation ability. The over-expression of apoE, on the contrary, facilitated cell growth, inhibited apoptosis, and potentiated clonal formation. ApoE expression level is closely correlated with AD. The lowering of apoE level inhibits neural cell D283 growth and clonal formation while induces cell apoptosis. Over-expression of apoE can facilitate cell growth and proliferation while inhibit apoptosis.

Keywords: Apolipoprotein E, neural cell D283, clonal formation, small interference RNA

Introduction

With the aging of population, the occurrence of Alzheimer's disease (AD) is increasing by years [1], thus severely threatening public health [2]. AD is featured with neural disorders including atrophy of cortical neurons, entanglement of neural fibers, occurrence of senile plaque, and death of hippocampal neurons [3, 4]. Clinical features of AD include retrograde memory deficits [5], cognitive dysfunction [6], and problems in language [7], logics [8], judgment [9], orientation and insights [10]. Therefore it is necessary to study the pathogenesis of AD, in order to provide useful information for disease treatment and prognostic prediction.

Current opinions believe that the pathogenesis of AD mainly exists in the deposition of beta-amyloid, tau phosphorylation and genetic mutation. Previous study has established the accumulation of beta amyloid inside senile plaques as one important reason for AD occurrence [11, 12]. The deposition of beta amyloid can form fibrous structure to inhibit the growth and apoptosis of brain neurons [13]. The phosphorylation of tau protein is also believed to be one important reason for AD pathogenesis [14]. Due to its critical function in neurogenesis and neural transmission/signal transduction, the over-phosphorylation of tau protein can cause the entanglement of neuronal fibers, leading to the atrophy of dendrites/axons, both of which may impede the intracellular transport of oxygen and nutrients [15], eventually leading to AD occurrence. Other studies have also suggested the role of genetic mutation in AD occurrence [16]. For example, the mutation of presenilin gene on chromosome 14 can led to the accumulation of beta amyloid and neuronal death [17]. Other studies have proposed the involvement of apolipoprotein E (apoE) in occurrence and progression of AD. We thus investigated the role of apoE in AD occurrence and molecular mechanisms.

As one apolipoprotein, apoE is one member of apo family (including apoE2, apoE3 and apo24)

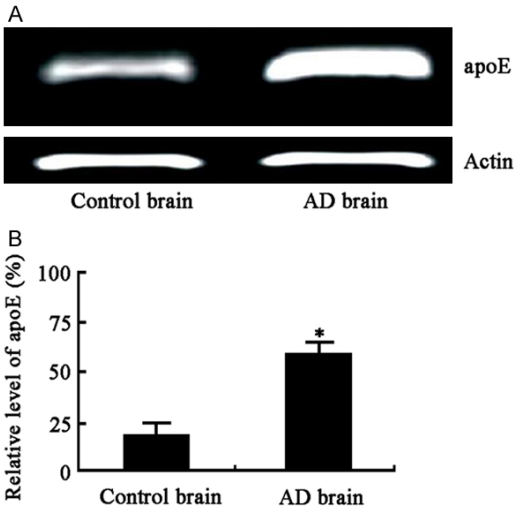

Figure 1. mRNA level of apoE. A. RT-PCR bands of apoE and actin genes from both control and AD brain. B. Relative mRNA level of apoE (N=3). *, P<0.05 compared to control group.

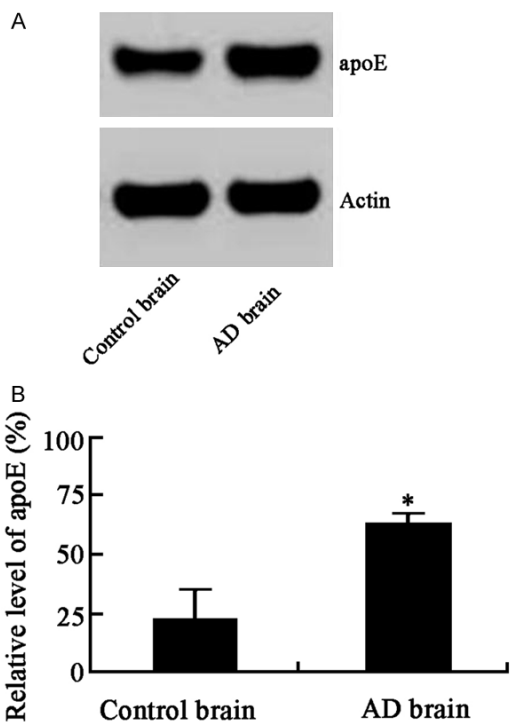

Figure 2. Protein level of apoE. A. Western blotting bands of apoE and actin genes from both control and AD brain. B. Relative protein level of apoE (N=3). *, P<0.05 compared to control group.

[18, 19]. It has been a long time since scholars believed that apoE4 may be related with AD occurrence, although direct evidence is still lacked [20]. In this study, we explored the role

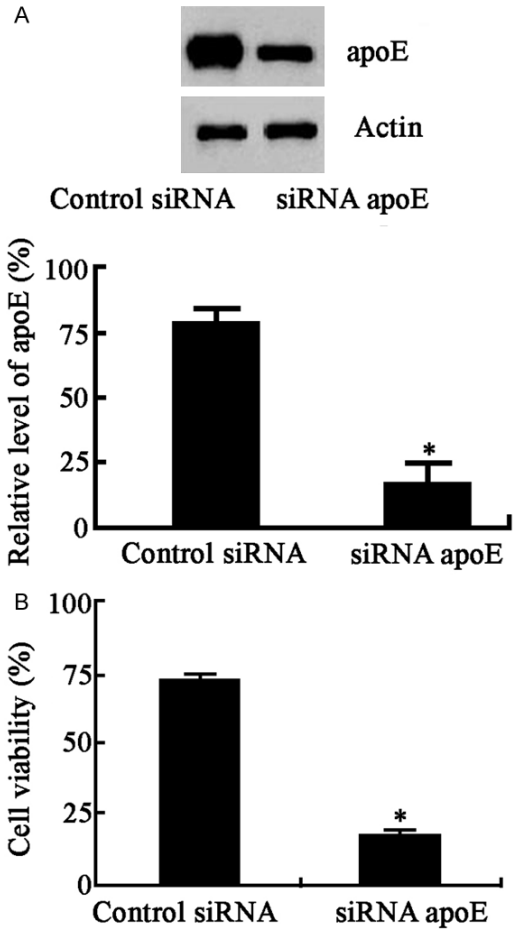

Figure 3. siRNA transfection and cell growth. A. Western blotting bands of apoE and actin proteins (top) and relative level of apoE protein (middle) (N=3). B. Relative cell viability. *, P<0.05 compared to control siRNA group.

of apoE in AD pathogenesis and related molecular mechanism, in an attempt to provide evidences for developing novel drug targets for AD treatment.

Materials and methods

Cells and reagents

Neural cells D283 were purchased from American Type Culture Collection (ATCC, US). MTT assay kit and lipofectamine 2000 kit were purchased from Dingguo Biotech (China). Flow cytometry assay kits including phophatidylserine (PS) kits, FITC-Annexin-V and caspase-3 activity kit, and caspase-3 kit were products of Baili Biotech (China). Anti-ApoE and anti-actin antibodies were provided by Sigma (US). DEME medium and bovine serum were purchased

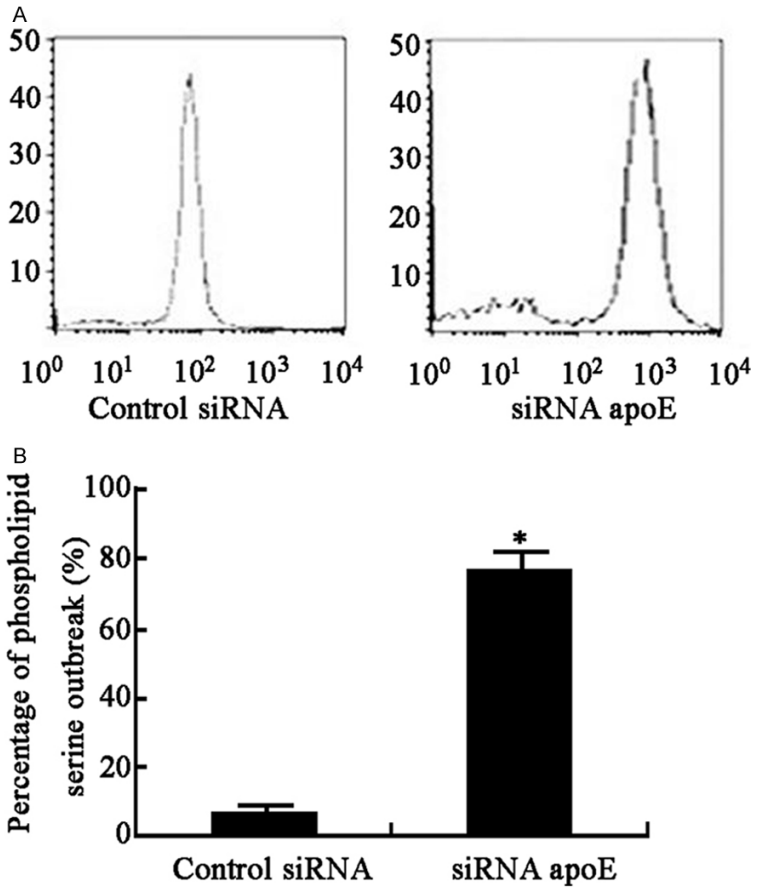

Figure 4. Phospholipid serine assay. A. Flow cytometry of phospholipid serine outbreak. B. Relative percentage of apoptotic cells (N=3). *, P<0.05 compared to control siRNA group.

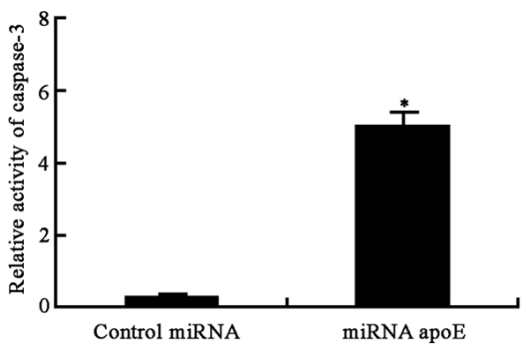

Figure 5. Caspase-3 activity of D283 cells. *, P<0.05 compared to control siRNA group.

from Beyotime (China). Anti-sense siRNA targeting apoE and control siRNA were synthesized by Gimma biotech (China).

Cell culture and transfection

D283 cells were cultured in DEME medium containing 10% fetal bovine serum in a humidified chamber at 37°C with 5% CO_2. Cells were transfected as previously documented [13] with 1 μg anti-sense siRNA targeting apoE (5'-TCCAG GCACCA AGGCA TAAGG CAGGC-3' and 5'-TGCTC CAGGA AGGCA GCACC AAGGC A-3') or controlled siRNA (5'-GCAGC ATGGC ACCAA GAAGT CCAGG C-3' and 5'-CTCCA GGAGC AGCAC TGAGC AAGGC A-3'). 24 hours after transfection, D283 cells were used in following experiments.

MTT assay

The activity of D283 cells was measured by MTT method as previously documented [14]. In brief, D283 cells at log-phase were seeded into 96-well plate (10 000 cells per well) for 24-hour incubation. 10 μL MTT reagents (10 mg/mL) were then added into each well for 6-hour incubation. 0.2 mL DMSO solutions were then added to quench the reaction. Optical density (OD) values at 492 nm were tested in a microplate reader. With reference to parallel blank control (using PBS instead of MTT reagents); relative levels of cell viability were determined.

Cell apoptosis assay

Flow cytometry and caspase activity assays were performed to detect the apoptosis of D283 cells after treatment [15]. In brief, D283 cells were centrifuged at 1 000 g for 8 min to discard the supernatants. Cells were then re-suspended in DMEM medium and were mixed with FITC-Annexin V reagents and reaction buffer (50:1:250). After 20-min dark incubation, flow cytometry was used to detect the apoptosis of all cells.

Western blotting

Using previously documented method [16], expression levels of apoE in all groups were tested using anti-apoE or anti-actin antibodies. Image J software was used to analyze the den-

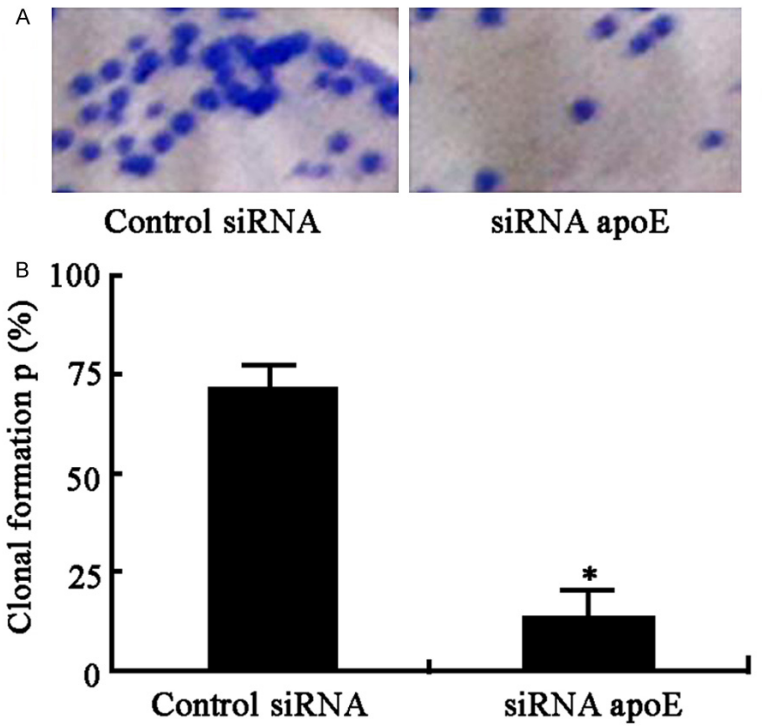

Figure 6. Clonal formation of D283 cells. A. Clonal formation images. B. Clonal formation rate. *, P<0.05 compared to control siRNA group.

sity of each blotting band. The relative expression of apoE was then determined by the ratio of density of apoE against internal reference actin.

RT-PCR

RT-PCR was performed in accordance with previous study [17] to detect mRNA level of apoE in D283 cells. Image J software was used to analyze the density of each band. The relative expression of apoE was then determined by the ratio of density of apoE against internal reference gene actin.

Caspase-3 activity

The activity of caspase-3 was measured in all D283 cells as previously reported [18].

Clonal formation assay

The clonal formation ability of D283 cells was measured using previously methods [18]. In brief, all cells were collected to detect the clonal formation ability using clonal formation assay [19].

AD model rat

AD model rats were generated by Shanghai Biotech (China), who also performed tissue collections of AD rats and control animals.

Statistical analysis

All collected data were analyzed by SPSS 17.0 software, and were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means across groups. A statistical significance was defined when P<0.05.

Results

Elevated apoE level in AD rats

Compared to healthy animals, AD rats had elevated mRNA levels of apoE (P<0.05, **Figure 1**). Protein level of apoE showed consistent patterns, as AD rats had higher level than control ones (P<0.05, **Figure 2**). These two results suggested the possible involvement of apoE in AD pathogenesis and progression.

siRNA of apoE inhibited D283 cell growth

The transfection of apoE-siRNA in D283 cells remarkably inhibited the growth of cells as compared to those cells transfected with control siRNA (P<0.01, **Figure 3**).

ApoE-siRNA induced apoptosis

Those cells transfected with apoE-siRNA had significantly outbreak of phospholipid serine (PS) (**Figure 4**) and caspase-3 activation (**Figure 5**). These data further supported that antisense RNA of apoE induced programmed cell death of D283 cells.

ApoE-siRNA inhibited clonal formation of D283 cells

As shown in **Figure 6**, the transfection of apoE-siRNA depressed the clonal formation ability of D283 cells as compared to those cells trans-

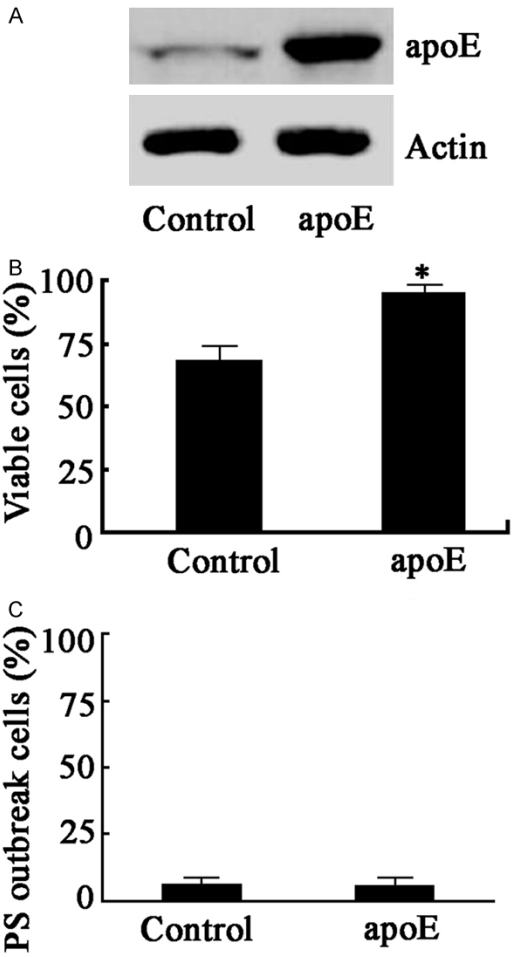

Figure 7. Cell growth by apoE overexpression. A. Western blotting showing the expression of apoE. B. Relative cell viability (N=3). C. Flow cytometry of phospholipid serine outbreak (N=3). *, P<0.05 compared to control group.

fected with control siRNA, suggesting the inhibition of clonal formation by apoE gene silencing.

ApoE over-expression stimulates D283 cell growth

As shown in **Figure 7**, the over-expression of apoE stimulated the growth of D238 cells while not inducing programmed cell death as compared to controlled cells.

Discussion

This study investigated the expression level of apoE in AD rat brain tissues and its regulatory function of cultured neural cell D283. We for the first time showed elevated mRNA and protein levels of apoE in AD rat brain tissues, suggesting the potential correlation between apoE and AD pathogenesis. We also showed the inhibition of D283 cell growth and facilitated cell apoptosis and clonal formation by silencing apoE gene expression. The over-expression of apoE obtained opposite effects. Our results also showed the regulatory function of apoE on growth and apoptosis of D283 cells on cellular levels. All these results showed the potency of apoE as the drug target against AD, as consistent with previous studies showing the neuronal apoptosis and clonal formation in AD models [20, 21].

Cell apoptosis is mainly mediated by the membrane death receptor-directed signaling pathway and intracellular mitochondrial mechanism [22]. In this study, we showed the significant outbreak of phospholipid serine in D283 cells after silencing apoE gene expression by RNA interference, suggesting the existence of cell apoptosis. We further studied cell apoptosis level by caspase activity assay, which showed the potentiated caspase-3 activity and absence of caspase-8 activation, which is one index for death receptor initiation. These results suggested that apoE-induced cell death was mediated by mitochondria, which was inconsistent with the caspase-8 regulated apoptosis in neural cells as previously reported [23]. Such inconsistency may be due to the differential cell lines used, as they had various sensitivities to external stimulus [24]. On the other hand, we speculated that apoE might directly or indirectly induced mitochondria-induced signaling pathway for cell apoptosis [25, 26]. Those proposed models, however, remain to be validated.

Certain weakness and limits, however, existed at the current study. Due to the complexity of AD model [27, 28], this study did not classify all AD animals based on the disease condition, thus making the analysis between apoE level and AD progression impossible. *In vivo* evidence is also lacked regarding the interference of apoE level to study the correlation with AD progression and potential treatment effect of apoE. Due to the limited number of sample size, our initial conclusion needs further confirmation. The data about apoE levels from different AD patients after drug treatment are also lacked, thus impeding the predictive value of apoE level in AD patients.

In summary, this study showed the elevated expression level of apoE in AD model rats, suggesting the close relationship between apoE and AD progression. We thus hypothesized apoE as one biomarker for AD. The depression of apoE level can inhibit the growth and clonal formation of cultured neural cells D283, as it can induce cell apoptosis. The over-expression of apoE level can facilitate cell growth and clonal formation while inhibit programmed cell death. Our results provide further evidences for developing novel drug targets for AD.

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Disclosure of conflict of interest

None.

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