Original Paper

Amyloid Beta Peptide 1–42 Induces SH-SY5Y Cell Apoptosis via the Promotion of Meg3 Long Noncoding RNA Expression

Yongyi Huang^{a–c} Te Liu^{a, b}

^aShanghai Geriatric Institute of Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, ^bShanghai Tenth People's Hospital, Medical School, Tongji University, and ^cShanghai Topbio Co. Ltd., Shanghai, China

Key Words

Alzheimer's disease · Amyloid beta peptide 1–42 · Long noncoding RNAs · Meg3 · p53 · Apoptosis

Abstract

Background/Aim: The mechanisms and processes of amyloid beta ($A\beta$)_{1–42}/ $A\beta$ _{1–40} degeneration and deposition of neuron damage are still not clear. The long noncoding RNA (lncRNA) is one of the members of the noncoding RNA family. In this study, we aimed to investigate whether $A\beta$ _{1–42} inhibited SH-SY5Y cells in vitro through modulating Meg3 lncRNA. **Methods:** The Alzheimer's disease (AD) senile plaque cell model was generated using synthetic $A\beta$ _{1–42}-treated SH-SY5Y cells. MTT assays were used to determine the proliferation of SH-SY5Y cells. Quantitative (q)RT-PCR and Western blot analyses were used to test the expression levels of mRNA and protein. Northern blot analysis was used to confirm Meg3 lncRNA expression. **Results:** The MTT assays showed that exogenous $A\beta$ _{1–42} suppressed SH-SY5Y cells. The qRT-PCR and Western blot analyses revealed that the expression of p53 mRNA and protein was significantly increased in the AD model group, with a marked decrease in MDM2 and Ki-67 expression on day 7. Moreover, the qRT-PCR and Northern blot analyses confirmed that exogenous $A\beta$ _{1–42} promoted the expression of Meg3 lncRNA. There was a downregulation of Meg3 lncRNA expression in SH-SY5Y cells by siRNA, which could promote of the ability of MDM2 to degrade p53 protein on the ubiquitin pathway and delay SH-SY5Y apoptosis. **Conclusion:** Meg3 lncRNA is implicated as an important factor in the formation of mature $A\beta$ peptides.

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Te Liu
Shanghai Geriatric Institute of Chinese Medicine, Longhua Hospital
Shanghai University of Traditional Chinese Medicine
Shanghai 200031 (China)
E-Mail telu79@126.com

Introduction

The occurrence of Alzheimer's disease (AD) is closely related to extracellular amyloid beta ($A\beta$)_{1–42}/ $A\beta$ _{1–40} deposits and intracellular hyperphosphorylated tau protein aggregates [1, 2]. However, the mechanisms and processes of $A\beta$ _{1–42}/ $A\beta$ _{1–40} degeneration and deposition of neuron damage are still not clear. The long noncoding RNA (lncRNA) is one of the members of the noncoding RNA family. It is a long RNA molecule that contains over 200 nucleotides without the ability to encode proteins and is widely involved in the regulation of many important cellular functions, including the transcriptional regulation of gene expression, the developmental regulation of tissues and cells inside the body, the regulation of stem cell pluripotency and the reprogramming of somatic cells, and the regulation of the occurrence and development of the disease. The human maternally expressed gene 3 (Meg3) is an mRNA-like noncoding RNA with a nucleotide length of >1.6 kb [3–5]. It is a paternally imprinted gene which lies on human chromosome 14q. The Meg3 gene consists of 10 exons which form some small open reading frames by alternative splicing. However, these open reading frames do not resemble any known functional proteins or peptides. Previous studies showed that Meg3 was highly expressed in many normal human tissues, especially in the brain and pituitary gland [3–5]. Meanwhile, they have confirmed that human Meg3 is an lncRNA [3, 4]. Zhou and colleagues [3, 4] showed that Meg3 lncRNA could suppress tumor proliferation by activating p53-dependent and -independent pathways. However, the mechanism of Meg3 lncRNA in the occurrence of AD induced by $A\beta$ _{1–42} deposition is not clear.

In this study, an AD senile plaque cell model was generated using synthetic $A\beta$ _{1–42}-treated SH-SY5Y cells. We aimed to investigate whether $A\beta$ _{1–42} inhibited SH-SY5Y cells in vitro via modulation of Meg3 lncRNA.

Materials and Methods

Cell Culture and $A\beta$ _{1–42} Treatment

An AD senile plaque cell model was generated as previously described [6, 7]. The SH-SY5Y cell lines were seeded in a 6-well plate in DMEM supplemented with 10% fetal calf serum (Invitrogen, Life Technologies Corporation, Grand Island, N.Y., USA), penicillin (100 U/ml), and glutamine (0.3 mg/ml) and incubated in a humidified tissue culture incubator containing 5% CO₂ at 37°C until 80% confluent. Then, 10 µmol/l of large aggregates of synthetic $A\beta$ _{1–42} (Sigma-Aldrich, St. Louis, Mo., USA) was added to the cultures. After 24 h, the drug-containing medium was replaced with fresh normal cell medium for continued culture.

MTT Assay for Cell Proliferation

According to the previously described procedure [8], briefly, each group of SH-SY5Y cells was seeded at 2×10^3 per well in 96-well plates until 85% confluent. MTT (Sigma-Aldrich) reagent (5 mg/ml) was added to the maintenance cell medium at different time points and incubated at 37°C for an additional 4 h. The reaction was terminated with 150 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich) per well, and the cells were lysed for 15 min, after which the plates were gently shaken for 5 min. Absorbance values were determined by using the enzyme-linked immunosorbent assay reader (Model 680; Bio-Rad, Hercules, Calif., USA) at 490 nm.

RNA Extraction and Analysis by Quantitative Real-Time PCR

According to the manufacturer's protocol, total RNA from each cell was isolated with TRIzol reagent (Invitrogen). The RNA samples were treated with DNase I (Sigma-Aldrich),

quantified, and reverse transcribed into cDNA with the ReverTra Ace- α First Strand cDNA Synthesis Kit [Toyobo (Shanghai) Biotech Co., Ltd., Shanghai, China]. Quantitative real-time PCR was conducted with a RealPlex4 real-time PCR detection system from Eppendorf (Hamburg, Germany), with SYBR Green Real-Time PCR Master Mix (Toyobo) as the detection dye. Quantitative real-time PCR amplification was performed over 40 cycles with denaturation at 95°C for 15 s and annealing at 57°C for 45 s. Target cDNA was quantified with the relative quantification method. A comparative threshold cycle (Ct) was used to determine gene expression relative to a control (calibrator), and steady-state mRNA levels are reported as an n-fold difference relative to the calibrator. For each sample, the maker gene Ct values were normalized with the formula $\Delta Ct = Ct_{\text{genes}} - Ct_{18S \text{ RNA}}$. To determine relative expression levels, the following formula was used: $\Delta\Delta Ct = \Delta Ct_{\text{sample groups}} - \Delta Ct_{\text{control group}}$. The values used to plot relative expressions of markers were calculated with the expression $2^{-\Delta\Delta Ct}$. The mRNA levels were calibrated on the basis of levels of 18S rRNA. The cDNA of each gene was amplified with primers as previously described [3, 8].

Western Blot Analysis

According to the previously described procedure [6], cells were lysed using a 2× loading lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). The total amount of protein from the cultured cells was subjected to 12% SDS-PAGE and transferred onto Hybrid-PVDF membranes (Millipore, Bedford, Mass., USA). After blocking with 5% (w/v) nonfat dried milk in TBST (Beyotime), the PVDF membranes were washed 4 times (15 min each) with TBST at room temperature and incubated with primary antibody. Following extensive washing, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:1,000; Santa Cruz Technology, Santa Cruz, Calif., USA) for 1 h. After washing 4 times (15 min each) with TBST at room temperature, the immunoreactivity was visualized by enhanced chemiluminescence using the ECL kit from PerkinElmer Life Sciences (Norwalk, Conn., USA).

RNA Extraction and Northern Blot Analysis

All steps of Northern blotting were conducted according to the previously described procedure [3, 8]. For all groups, 20 μ g of good-quality total RNA was analyzed on a 7.5 M urea/12% PAA denaturing gel and transferred to a Hybond N⁺ nylon membrane (Amersham, Freiburg, Germany). Membranes were cross-linked using UV light for 30 s at 1,200 mJ/cm². Hybridization was performed with an antisense StarFire probe to detect Meg3 lncRNA fragments according to the instructions of the manufacturer [3]. After washing, the membranes were exposed for 20–40 h to Kodak XAR-5 films (Sigma-Aldrich). As a positive control, all membranes were hybridized with a human U6 snRNA probe: 5'-GCAGGGGCCATGCTA-ATCTTCTCTGTATCG-3'. Exposure times for the U6 control probe varied between 15 and 30 min.

siRNA and Cell Transfection

A siRNA-targeted Meg3 lncRNA expression plasmid was constructed as previously described [3]. SH-SY5Y cells were transfected with 0.3 μ g siRNA-Meg3 or siRNA-mock vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Statistical Analysis

Each experiment was performed as least 3 times. The data are shown as means $\pm$ SE. Differences were evaluated using Student's t test. A probability of <0.05 was considered to be statistically significant.

Fig. 1. Exogenous Aβ₁₋₄₂ influenced SH-SY5Y cell proliferation and Meg3 lncRNA expression. **a** The MTT assays showed that large aggregates of synthetic Aβ₁₋₄₂ inhibited SH-SY5Y cell proliferation in a time-dependent manner. **b** The results of qRT-PCR showed that the mRNA expression of p53 in the Aβ₁₋₄₂ treatment group was elevated markedly, while MDM2 and Ki-67 expression (normalized against 18S rRNA levels) in this group was markedly decreased compared with that in the DMSO-treated group on day 7. **c** Western blotting confirmed that expression of the BACE1, p53, and endogenous Aβ₁₋₄₂ proteins was significantly increased in the Aβ₁₋₄₂ treatment group compared with the DMSO-treated group on day 7. GAPDH was used as a loading control. **d** Northern blotting showed that the Meg3 lncRNA hybridization signals were higher in Aβ₁₋₄₂-treated cell extracts than in the DMSO-treated group. **e** The results of qRT-PCR showed that Meg3 lncRNA expression in the Aβ₁₋₄₂ treatment group was elevated markedly (normalized against U6 RNA levels) compared with that in the DMSO-treated group on day 7. ** p < 0.01, # p > 0.05 vs. DMSO-treated group (n = 3).

Table 1. Results of the MTT assays (%)

Day	Aβ ₁₋₄₂ treated	DMSO treated	Aβ ₁₋₄₂ treated, %	
			siRNA-Meg3	siRNA-mock
1	2.537 ± 0.782	2.177 ± 0.780	7.730 ± 0.746	10.433 ± 1.133
3	21.890 ± 4.062	2.780 ± 0.995	13.343 ± 2.073	25.697 ± 2.546
5	54.573 ± 3.944	4.007 ± 0.529	29.947 ± 1.442	52.720 ± 3.111
7	72.300 ± 3.632	6.640 ± 0.290	–	–
9	82.257 ± 1.732	8.497 ± 1.261	–	–

Results

Exogenous Aβ₁₋₄₂ Suppressed SH-SY5Y Cell Proliferation and Induced High Meg3 lncRNA Expression

The results of the MTT assays showed that large aggregates of synthetic Aβ₁₋₄₂ suppressed the proliferation of SH-SY5Y cells in a time-dependent manner (fig. 1a; table 1). At the same time, the results of quantitative (q)RT-PCR showed that the expression of p53 in the Aβ₁₋₄₂-

Fig. 2. Attenuation of the cytotoxicity of exogenous $A\beta_{1-42}$ by siRNA suppression of Meg3 lncRNA expression. **a** The MTT assays showed that the inhibition rates of the siRNA-Meg3-transfected SH-SY5Y cell group were decreased significantly compared with those of the siRNA-mock-transfected group on days 3 and 5 after treatment with $A\beta_{1-42}$. **b** The results of qRT-PCR showed that the mRNA expression of MDM2 and Ki-67 in the siRNA-Meg3-transfected SH-SY5Y cell group was elevated markedly, while Meg3 lncRNA and p53 expression (normalized against 18S rRNA levels) in this group was markedly decreased compared with that in the siRNA-mock-transfected group on days 3 and 5 after treatment with $A\beta_{1-42}$ on day 5. **c** Western blotting confirmed that the expression of the Ki-67 and MDM2 proteins was significantly increased in the siRNA-Meg3-transfected SH-SY5Y cell group compared with the siRNA-mock-transfected group after treatment with $A\beta_{1-42}$ on day 5. However, the expression of p53 protein was significantly decreased in the siRNA-Meg3-transfected group compared with the siRNA-mock-transfected group after treatment with $A\beta_{1-42}$ on day 5. GAPDH was used as a loading control. ** $p < 0.01$, * $p < 0.05$, # $p > 0.05$ vs. siRNA-mock group ($n = 3$).

treated group was markedly elevated compared with that in the DMSO-treated control group, while MDM2 and Ki-67 expression was decreased on day 5 of $A\beta_{1-42}$ treatment (fig. 1b). Moreover, Western blotting confirmed that expression levels of p53, $A\beta_{1-42}$, and BACE1 were significantly increased in the $A\beta_{1-42}$ -treated group compared with the control group (fig. 1c). In addition, Northern blot analysis indicated that Meg3 lncRNA hybridization signals were higher in the $A\beta_{1-42}$ -treated cell extracts than in the control group (fig. 1d). The results of qRT-PCR also showed that the expression of Meg3 lncRNA in the $A\beta_{1-42}$ -treated group was elevated markedly compared with that in the DMSO-treated control group (fig. 1e). These data indicate that exogenous $A\beta_{1-42}$ not only inhibited SH-SY5Y cell proliferation and induced the expression of amyloid precursor protein-related factors, but also promoted Meg3 lncRNA expression.

Attenuation of the Ability of $A\beta_{1-42}$ by siRNA Silencing of Meg3 lncRNA Expression

The results of the MTT assays showed that the inhibition rates of the siRNA-Meg3-transfected SH-SY5Y cell group were decreased significantly in relation to those of the siRNA-mock-transfected group on days 3 and 5 after treatment with $A\beta_{1-42}$ (fig. 2a; table 1). Moreover, the results of qRT-PCR showed that the expression of Meg3 lncRNA and p53 in the siRNA-Meg3-transfected group was markedly decreased, while MDM2 and Ki-67 expression

Fig. 3. The pathway of Meg3 lncRNA regulates MDM2 and p53 protein degradation. Ub = Ubiquitin.

was markedly elevated compared with that in the siRNA-mock-transfected group on day 5 after treatment with A β _{1–42} (fig. 2b). Meanwhile, Western blotting revealed that the expression levels of MDM2 and Ki-67, but not those of p53, were significantly increased in the siRNA-mock-transfected group on day 5 after treatment with A β _{1–42} (fig. 2c). These results indicate that the cytotoxicity of A β _{1–42} was weakened when Meg3 lncRNA and p53 protein were expressed to a lesser extent.

Discussion

AD is a complex progressive neurodegenerative disease that is characterized by an irreversible cognitive functional decline, a loss of memory, and a high degree of heterogeneity in clinical parameters. It has become the third most lethal disease in the world [1, 2]. lncRNAs are fine-tuning modifiers of nervous system developmental regulation, but knowledge regarding their control of AD is lacking [9]. In a study of the pathogenesis of AD, Faghihi et al. [10] identified an lncRNA that acted as a positive regulator of its target gene. They identified an antisense β -secretase (BACE1-AS) lncRNA which generates A β . This lncRNA increased the stability of the BACE1 mRNA, thus leading to an amplified production of A β peptides and the deleterious feed-forward cycles of disease progression [10].

In our previous study, we showed that downregulation of BACE1-AS lncRNA expression in SH-SY5Y cells by siRNA silencing resulted in attenuation of the ability of BACE1 to cleave amyloid precursor protein and delayed the induction of senile plaque formation in the AD senile plaque SH-SY5Y cell model [8]. In the present study, we demonstrated that exogenous synthetic A β _{1–42} could significantly promote the expression of Meg3 lncRNA, p53, endogenous BACE1, and A β _{1–42} in SH-SY5Y cells and significantly weakened the expression of MDM2 and Ki-67. However, p53 and Meg3 lncRNA expression was significantly reduced in SH-SY5Y cells as a result of the siRNA-mediated silencing of Meg3 lncRNA expression. Furthermore, exogenous A β _{1–42} did not stimulate the formation of endogenous A β _{1–42} in siRNA-Meg3-transfected SH-SY5Y cells.

These data indicate that inhibition of Meg3 lncRNA expression effectively inhibits the endogenous production of A β peptides. In contrast, when the expression of Meg3 lncRNA siRNA was silenced in transfected SH-SY5Y cells treated with exogenous A β peptides, the cytotoxic effects of A β were significantly reduced and these cells maintained their normal state. In addition, MDM2, one of the E3 ubiquitin ligases, is a direct p53 transcriptional target and also the most critical negative regulator of p53 [11, 12]. It can form an autoregulatory

negative feedback loop with p53 in the cell to tightly regulate the levels and activity of p53. Also, it has been shown that MDM2 is an oncogene, and it was identified as an inhibitor of DNA break repair [11, 12]. In our study, we identified exogenous A β _{1–42}-stimulated Meg3 lncRNA as a new regulator that directly repressed MDM2 to activate p53 and enhance p53 function in SH-SY5Y cells (fig. 3). Thus, Meg3 lncRNA is implicated as an important factor in the formation of mature A β peptides. The ability of MDM2 to degrade p53 protein on the ubiquitin pathway was elevated via silencing of Meg3 lncRNA expression.

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Disclosure Statement

We declare no potential conflicts of interest.

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