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DNA methylation of the 5′-untranslated region at +298 and +351 represses BACE1 expression in mouse BV-2 microglial cells

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ABSTRACT

BACE1, which cleaves the amyloid precursor protein, is the rate-limiting enzyme for β-amyloid peptide production, leading to the pathogenesis of Alzheimer's disease (AD). A high plasma level of homocysteine, acting as a potent methyltransferase inhibitor, is assumed to be a risk factor for AD onset. Using the demethylating drug 5-aza-2′-deoxycytidine (5-Aza), we tested whether and how BACE1 expression is regulated in mouse BV-2 microglial cells. 5-Aza increased both BACE1 mRNA and protein levels in a dose-dependent manner. Bisulfite-sequencing analysis revealed that two CpG sites at positions +298 and +351 in the 5′-untranslated region (5′-UTR) of the BACE1 gene were specifically demethylated in BV-2 cells treated with 5-Aza. *In silico* analysis showed that the +351 site is the STAT3/CTCF-binding site; the function of the +298 site has not been identified. To assess whether these two CpG sites play an important role in 5-Aza-induced transcriptional activation of BACE1, we constructed a BACE1 gene promoter including the 5′-UTR (−1136 to +500) fused to a CpG-free luciferase gene (pCpGL-BACE1) and its mutant pCpGL-BACE1-AA, which has substituted CG dinucleotides at the two CpG sites of pCpGL-BACE1 to AA. Promoter analysis showed a significant decrease (~30%) in the activity of pCpGL-BACE1-AA compared with that of pCpGL-BACE1. Furthermore, *in vitro* methylation of these two reporter constructs showed a complete silencing of their promoter activities. Our data demonstrate that BACE1 gene expression is regulated by DNA methylation of at least two CpG sites at positions +298 and +351 in the 5′-UTR in BV-2 microglial cells.

1. Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder that results in memory loss, dementia, and finally death. AD pathology is characterized by senile plaques and neurofibrillary tangles, which are associated with the massive loss of neurons and synapses mainly in the hippocampus and neocortex association regions. The major constituents of senile plaques are β-amyloid peptides (Aβ) of 39–43 amino acids, cleaved from amyloid precursor protein (APP). It is well-known that APP is processed by a group of secretases including α-, β-, and γ-secretase. β- and γ-Secretase generate Aβ from APP, whereas α-secretase generates a soluble product [1,2].

Two β-secretases, BACE1 and BACE2, are involved in the development of Alzheimer's disease by producing Aβ. It was reported that BACE1 was the major β-secretase for Aβ generation in neurons [3] by showing that the secretion of $A\beta_{1-40/42}$ and $A\beta_{11-40/42}$ was abolished in BACE1-knockout neurons. Several studies also demonstrated that increases in BACE1 levels and activities were detected in postmortem AD brains [4,5]. Recently, BACE1 is reported to be significantly augmented in the cerebrospinal fluid of patients with mild cognitive impairment (MCI) and may be an early biomarker of

Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid peptides; APP, amyloid precursor protein; MCI, mild cognitive impairment; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; 5-Aza, 5-aza-2′-deoxycytidine; 5′-UTR, 5′-untranslated region; pCpGL-BACE1, BACE1 gene promoter including the 5′-UTR (−1136 to +500) fused to a CpG-free luciferase gene; pCpGL-BACE1-AA, mutant pCpGL-BACE1 which has substituted CG dinucleotides at the two CpG sites of pCpGL-BACE1 to AA; pCpGL-unmetBACE1, unmethylated pCpGL-BACE1; pCpGL-metBACE1, methylated pCpGL-BACE1.

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AD [6]. Therefore, the regulation of BACE1 expression is regarded as an important initiating factor in senile plaque formation and AD development. Indeed, several BACE1 inhibitors have proceeded to preclinical or clinical trials [7].

An elevated plasma homocysteine level is generally assumed to be a risk factor for the onset of AD [8]. Using the Korean elderly population, we also reported that plasma homocysteine concentrations were higher in subjects with MCI than in normal elderly patients [9]. S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), the substrate and product of essential cellular methyltransferase reactions, respectively, are important metabolic indicators of cellular methylation status. The accumulation of homocysteine causes the accumulation of SAH, a strong DNA methyltransferase inhibitor, which reinforces DNA hypomethylation [10]. From this phenomenon, we hypothesized that hyperhomocysteinemia in MCI and AD patients might lead to DNA hypomethylation of genes associated with AD, which act as one of the main causes for sporadic AD.

In order to verify this hypothesis, we investigated whether and how DNA methylation regulated the expression of BACE1 associated with the formation of senile plaques in mouse BV-2 microglial cells by using the DNA demethylating agent, 5-aza-2′-deoxycitidine (5-Aza). Our study identified for the first time the two CpG sites in the 5′-untranslated region (5′-UTR) as the epigenetic regulatory sites responsible for BACE1 expression.

2. Materials and methods

2.1. Cell culture and drug treatments

The mouse BV-2 microglial cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Gaithersberg, MD) supplemented with 10% fetal bovine serum (FBS), 0.37% $NaHCO_3$, penicillin (100,000 unit/l) and streptomycin (100 mg/l) in attachment factor-coated culture dishes at 37 °C in 5% CO_2. When the cells reached 80% confluence, they were incubated with the indicated concentrations of 5-Aza (Sigma, St. Louis, MO) in DMEM supplemented with 5% FBS for 24 h.

2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells with or without treatment of 5-Aza using TRIzol Reagent (Invitrogen, Carlsbad, CA) as described [11], and was used for reverse transcription (RT) reactions. RT reaction was performed using 200 units of SuperScript™ III reverse transcriptase (Invitrogen) in a 20 μl reaction mixture containing 1 μg RNA for 1.5 h at 42 °C. PCR was subsequently performed in a volume of 20 μl, containing 0.5 units of Taq polymerase (Solgent, Seoul, Korea), 10 pmol of each primer, 0.25 mM dNTPs, and 2 μl of the RT sample. The amplified products were separated using a 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA). The band of the target gene was visualized using 1 mg/ml ethidium bromide under UV. The following PCR primer pairs were designed to detect each gene: BACE1-F, 5-CAC CAT CCT TCC TCA GCA AT-3; BACE1-R, 5-AAC AAA CGG ACC TTC CAC TG-3; β-actin-F, 5-ATT GCT GAC AGG ATG CAG AA-3; and β-actin-R, 5-CCG ATC CAC ACA GAG TAC TT-3.

2.3. Western blot analysis

For Western blot analysis, cells were treated with indicated concentrations of 5-Aza, washed with ice-cold DPBS, and then lysed in lysis buffer 1 [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM β-glycerophosphate, 1 mM NaF, and 1 mM Na_3VO_4, and Protease Inhibitor Cocktail™ (Roche Molecular Biochemicals, Indianapolis, IN)]. The protein concentrations were then determined with a BCA protein assay kit (Sigma). Equal quantities of protein (20 μg) were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel under reducing conditions and then electrophoretically transferred onto a nitrocellulose membrane. The blots were then probed with anti-BACE1 antibody (1:1000, AbCam, Cambridge, UK) followed by the corresponding secondary antibody and finally developed using enhanced chemiluminescence reagents (GE Healthcare, Piscataway, NJ).

2.4. Bisulfite sequencing

Genomic DNA was extracted from BV-2 cells with or without treatment of 5-Aza using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The genomic DNA (2 μg) was sheared by passage through a 30 g needle 15 times, denatured by 0.3 M NaOH for 30 min at 39 °C, and subjected to bisulfite sequencing as described [12]. Briefly, bisulfite solution (3.9 M sodium bisulfite, 0.66 mM hydroquinone, pH 5.1) was added to the denatured DNA, which was incubated at 55 °C for 16 h. The DNA was desalted using a Qiagen Quickspin column, and modification was completed by 0.3 M NaOH at 37 °C for 16 min. After the bisulfite-treated DNA was purified by a Qiagen Quickspin column, the promoter and 5′-UTR region (+68 to +446) of the BACE1 gene was amplified from the bisulfite-treated DNA by PCR and cloned into a TOPO pCR2.1 plasmid (Invitrogen) using the manufacturer's instructions. Four to seven clones obtained from each of the PCR products were randomly selected and sequenced. The primers used for PCR of bisulfite-treated DNA are as follows: BACE1-F, 5-TTT TTT TAG TTT GTT TAG GTG TTG G-3; and BACE1-R, 5-CTA AAT CTA AAT AAT AAT AAC TTC T-3.

2.5. Cloning of the BACE1 promoter

CpG-free pCpGL-basic vector (pCpGL), whose basal luciferase activity is unchanged by methylation [13], was used for cloning the BACE1 promoter region. PCR was conducted using the BACE1-F-1136 primer, the BACE1-R-ATG primer, Ex Taq polymerase (Takara, Osaka, Japan) and genomic DNA from BV-2 cells as a template. The amplified PCR product, which included the promoter region and the 5′-UTR of BACE1 gene located from −1136 to +500, was cloned into the pCpGL to generate pCpGL-BACE1. The primers used for cloning are as follows: BACE1-F, 5-GCT AAC TCG AGT AGC TGG GGC AGG TTA AAT G-3; and BACE1-R-ATG, 5-ATC GAA AGC TTA GTG AGC CCG GGC CTT GTG-3.

2.6. Mutagenesis of the BACE1 promoter

In order to substitute CG dinucleotides at positions +298 and +351 in pCpGL-BACE1 with AA dinucleotides (pCpGL-BACE1-AA), site-directed mutagenesis was performed twice for mutation of both CG sites using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The corrections were determined by DNA sequencing analyses (Macrogen Co. Ltd, Seoul, South Korea). The first primers used for mutagenesis at position +293 are as follows: BACE1-F, 5-GCT GGG TCC CCT GGA TAA CCA TCG TCG TCT C-3; and BACE1-R, 5-GAG ACG ACG ATG GTT ATC CAG GGG ACC CAG C-3. The second set of primers used for mutagenesis at position +351 are as follows: BACE1-F, 5-GCC CAC TCT CCG CAA CCTGGA CCG GGA A-3; and BACE1-R, 5-TTC CCG GTC CAG GTT GCG GAG AGT GGG C-3.