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The epigenetic effects of amyloid-β_{1-40} on global DNA and neprilysin genes in murine cerebral endothelial cells

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ABSTRACT

Amyloid-β (Aβ) is the core component of senile plaques, which are the pathological markers for Alzheimer's disease and cerebral amyloid angiopathy. DNA methylation/demethylation plays a crucial role in gene regulation and could also be responsible for presentation of senescence. Oxidative stress, which may be induced by Aβ, is thought to be an important contributor of DNA hyper-methylation; however, contradicting this is the fact that global DNA hypo-methylation has been found in aging brains. It therefore remains largely unknown as to whether Aβ does in fact cause DNA methylation/demethylation. Neprilysin (NEP) is one of the enzymes responsible for Aβ degradation, with its expression decreasing in both Alzheimer and aging brains. Using high-performance liquid chromatography (HPLC), we explore whether Aβ is responsible for alteration of the global DNA methylation status on a murine cerebral endothelial cells model, and also use methylation-specific PCR (MSPCR) to examine whether DNA methylation status is altered on the NEP promoter region. We find that Aβ reduces global DNA methylation whilst increasing NEP DNA methylation and further suppressing the NEP expression in mRNA and protein levels. Our results support that Aβ induces epigenetic effects, implying that DNA methylation may be part of a vicious cycle involving the reduction in NEP expression along with a resultant increase in Aβ accumulation, and that Aβ may induce global DNA hypo-methylation.

Abnormal deposition of amyloid beta (Aβ) in brain parenchyma and cerebral vessels is the most significant and important pathological hallmark of both Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA) [1,2]; and it is well known that Aβ induces neuronal, endothelial and glial cell death through the initiation of multiple apoptosis pathways [3,4].

Exposure of endothelial cells to Aβ induces both oxidative stress and endothelial damage [5], whilst the accumulation of Aβ peptide is also common in senescent brains, although little is known about the significance of this phenomenon [6]. The role of oxidative stress in accelerating senescence has already been well-documented, with this effect coming mainly as a result of reactive oxygen species inducing both protein and DNA damage [7]. We therefore speculate that Aβ plays an important role in senescence.

DNA methylation/demethylation is one of the mechanisms operating in the epigenetic regulation of gene expression, with gene expression being suppressed or silenced by the methylation of cytosine in CpG islands on the individual gene promoter region, a phenomenon which occurs mainly as a result of interference in the binding of the transcription factors to their consensus elements. The majority of the CpG islands are unmethylated in the promoter region of actively expressed genes [8].

However, DNA hyper-methylation is observed in certain specific genes and in a variety of conditions which have a strong association with oxidative stress, such as cancer, ischemia-reperfusion and senescence [9]. In contrast, global DNA, referring to whole genome hypo-methylation, occurs along with aging; thus, global DNA methylation status may well be a biomarker of senescence [10].

Aβ accumulation has been shown to be determined, in part, by the activities of several enzymes associated with degradation,

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including neprilysin (NEP), which belongs to a family of zinc metalloproteinases [11]. Over-expression of NEP is found to significantly reduce the amyloid burden in AD mice [12], whilst a significant reduction in NEP expression has been found in CAA, senescent and AD brains [13]. The latter could be a phenomenon attributed to low somatostatin levels in senescence [14].

However, the NEP promoter region contains CpG islands, which are recognized 'hot spots' for DNA methylation. There could, therefore, be an additional mechanism leading to NEP down-regulated by NEP promoter DNA hyper-methylation. This could be the result of Aβ-related oxidative stress. Furthermore, the reduction in NEP expression could induce further Aβ accumulation, or the phenotype of AD and senescence.

In this study, we explored whether Aβ is responsible for altering global DNA methylation status and increasing DNA methylation on the NEP promoter, and then examine the consequences of NEP DNA methylation in its mRNA and protein levels.

Materials and methods

Murine cerebral endothelial cell (CEC) culture. Murine CECs were prepared and characterized as previously described [15]. Murine CECs of 4–15 passages were used in the present study.

Amyloid-β_{1-40} preparation and treatment. Synthetic $A\beta_{1-40}$ peptides were purchased from Biosource International (Camarillo, CA, USA), with stock solutions of 10 mg/mL being prepared by dissolving the lyophilized $A\beta_{1-40}$ peptides in 0.1 per cent (v/v) trifluoroacetic acid (TFA) in de-ionized water. After incubating for two hours at 25 °C, the peptide stock solutions were first diluted in phosphate buffered saline (PBS, 136.7 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2) to a concentration of 500 μM, and then rotated at 60 revolutions per minute for five days at 55 °C before being further used in cell culture experiments. The CECs were treated with $A\beta_{1-40}$ at different concentration levels under the serum-free medium for 48 h.

5-Aza-2'-deoxycytidine (AzaC). In order to inhibit genomic DNA methylation, the CECs were treated with 10 μM 5-AzaC (Sigma, St. Louis) for 48 h, both with and without a combination of $A\beta_{1-40}$ at 25 μM.

Sample preparation of global DNA mehtylation assay by high performance liquid chromatography (HPLC). DNA was extracted by the routine method. 50 μg DNA was dissolved with 100 μL of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) with RNase A (Worthington) and RNase T1 (Roche) at the final concentrations of 100 U/mL and 1000 U/mL, respectively. The resultant solution was incubated at 37 °C for 3 h, then, an equal volume of phenol/chloroform/isoamyl alcohol (25:25:1, pH 8.0) was added and mixed thoroughly.

After centrifugation, the top aqueous layer was transferred into a new centrifuge tube and the volume of the solution was measured. DNA was precipitated by sodium acetate and 95% (v/v) ethanol. After incubation at −20 °C for 30 min, DNA pellets were recovered by centrifugation, disposal of the supernatant, washing twice with 70 per cent ethanol, and the removal of ethanol by air-drying.

The air-dried DNA samples were resuspended in a deoxyribonuclease I (DNase I, GIBCO-BRL, Gaithersburg, MD) digestion buffer (10 mM Tris–HCl, pH 7.2, 0.1 mM EDTA, 4 mM magnesium chloride) to form a solution with a final concentration of DNase I of 15,000 U/mL, and then incubated in a 37 °C water bath for 24 h.

Following the addition of two volumes of 30 mM sodium acetate, zinc sulfate (final concentration of 1 mM), and four units of nuclease P1 (US Biological), the DNA sample solution was again incubated in a 37 °C water bath for a further 48 hours. Finally, 1/10 volume of 10× alkaline phosphatase buffer (10 mM Tris–HCl, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 50% glycerol) and 36 U of alkaline phosphatase were added to the DNA solution and incubated overnight at 37 °C.

Global DNA methylation assay by HPLC, conditions of HPLC. The HPLC (Perkin-Elmer) was equipped with a diode array detector (Perkin-Elmer 235 C) and a biocompatible binary pump (Perkin-Elmer 250). All eluents were filtrated by mixed cellulose ester membrane (0.2 μm) and were degassed prior to use. A 20 μl sample of DNA sample was injected onto a ZORBAX 300SB-C18 column (Agilent Technologies) which had been previously equilibrated with the mobile phase buffer (50 mM diammonium orthophosphate and 50 mM orthophosphoric acid, pH 4.1) and eluted with various elution profiles at room temperature. The mobile phase was run at 1 mL per minute, with the concentration of deoxyribonucleotides being assessed by UV spectrophotometry at 275 nm [16].

Methylation-specific PCR (MSPCR) and unmethylation-specific PCR (UMSPCR) assays. In order to confirm that methylation had indeed occurred in the NEP promoter region, we performed MSPCR [17] which detected the methylation status of the CpG islands in the promoter region. Genomic DNA was modified by $NaHSO_4$ for converting all of the unmethylated (but not the methylated) cytosines to uracil, and subsequently amplified with different sets of primers specific to methylated vis-à-vis unmethylated DNA. Briefly, 2 μg of genomic DNA was denatured by NaOH and then modified by 3 M $NaHSO_4$ for 16 hours with the CpGenome™ DNA Modification Kit (Intergen, Purchase, NY, USA).

PCR was performed using 2 μl aliquots of the above DNA preparations as templates. The primer sequences of the NEP promoter region are: 5'-GAA CTC CGA ACG AAT AAA CG-3' (sense) and 5'-ATT TAG GGA ATT GTT TTC GC-3' (antisense) for the methylated template with a predicted size of 212-bp; and 5'-TTT TGG TTT TGT TTT TTT TTG TG-3' (sense) and 5'-TCC CAA CCA ATA AAC ACA CCA A-3' (antisense) for the unmethylated template with a predicted size of 230-bp.

PCR amplification comprised of 35 cycles (94 °C, 53 °C, and 72 °C each for a one-minute period) after the initial Taq activation step (95 °C for ten minutes). Each PCR product (15 μl) was then loaded onto 1% agarose gels stained with ethidium bromide and visualized under UV light then quantified using the NIH Image J 1.37 software.

RNA isolation for reverse transcription (RT-PCR) and real time PCR. Total RNA was isolated with an RNeasy Kit (QIAGEN). After RT, real-time PCR was performed by a TaqMan EZ RT-PCR core kit (Applied Biosystems) in an ABI-7000 sequence detector (Applied Biosystems) according to the manufacturer's protocol. The specific forward primer, reverse primer and TaqMan probe were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) as follows: 5'-CAT CAC GTC TGA GTA CAC ACT TCA GA-3' (sense), 5'-AGG ACG GCT GCC TGC TC-3' (antisense), and 5'-FAM-CGA ATC GTC TAC ATT TC-FAM-3' (probe). The mRNA of β-actin was also examined as an internal reference in RT-PCR. The amount of NEP mRNA was normalized to the β-actin to obtain the relative threshold cycle (ΔC_t) and then related to the ΔC_t of the controls to obtain the relative expression level ($\Delta\Delta C_t$) of NEP.

Western blot analysis of NEP. The total membrane protein proportion of CECs was extracted by Mem-Per Eukaryotic Membrane Extraction Reagent Kit (No. 89826, PIERCE, Rockford, IL, USA). The samples were loaded into an 8 per cent SDS–PAGE and fractionated for 1½ h at 150 V. The proteins were electroblotted onto a BioTrace PVDF membrane (P/N 66543, Pall, Pensacola, FL, USA) followed by incubation in a blocking buffer.

The membrane was again incubated with an anti-neprilysin rabbit IgG antibody (1:1000; AB5458, Chemicon-Millipore, Temecula, CA, USA). A horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (1:1000; ab6721, abcam, Cambridge, UK) with an ECL chemiluminescent substrate (Amersham,

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GE Healthare, Buckinghamshire, UK) was used to visualize the NEP-specific bands. Mouse β-actin protein was selected as the internal control protein with an anti-β-actin mouse IgG antibody (1:500; MAB1501, Chemicon-Millipore, Temecula, CA, USA) and an HRP-conjugated goat anti-mouse secondary antibody (1:5000; BioNova, Taiwan).

Statistical analysis. All of the data are expressed as means ± SD derived from triplicates of at least three separate experiments. The comparison between the experimental groups and controls is based upon the two-tailed Student's *t*-test. A *p*-value of <0.05 is considered to be statistically significant.

Results

Aβ$_{1-40}$-induced global DNA hypo-methylation

The concentrations of cytosine (C) and methyl-cytosine (met-C) were detected by HPLC, with Met-C/C ratios being calculated for every sample. The relative folds of the met-C/C ratios from the samples of CECs treated with Aβ$_{1-40}$ (5 μM or 25 μM for 48 h) to those of the controls are shown in Fig. 1. The relative folds of the met-C/C ratios were reduced to 0.8565 ± 0.1477 and 0.6663 ± 0.0201 for the samples treated with 5 μM and 25 μM of Aβ$_{1-40}$, respectively. It indicates that Aβ$_{1-40}$ at 25 μM significantly reduced the status of global DNA methylation, $p < 0.05$ (Fig. 1).

Aβ$_{1-40}$-induced NEP gene promoter hyper-methylation

DNA of CECs treated with Aβ$_{1-40}$ of various concentrations (0, 5, 10, 25, and 50 μM) was then subjected to MSPCR and UMSPCR of the NEP promoter region.

The ratios of MSPCR and UMSPCR products (MSPCR/UMSPCR), as measured by the densitometer, were subsequently determined to reveal the status of DNA methylation on the NEP promoter region. DNA methylation was found to be significantly increased after treatment of Aβ$_{1-40}$ at concentrations above 10 μM. The maximal effects occurred at a concentration of approximately 25 μM (Fig. 2).

It was further established that DNA methylation on the NEP promoter region could be reversed by methyl-transferase inhibitor, aza-C (Fig. 3).

Aβ$_{1-40}$-suppressed NEP mRNA expression reversed by methyl-transferase inhibitor, Aza-C

NEP mRNA was measured by real-time PCR, with those CECs treated with methyl-transferase inhibitor, aza-C serving as the negative control. The $\Delta\Delta C_t$ of the CECs treated with Aβ$_{1-40}$ at 25 μM was 4.864 ± 0.672, whilst the $\Delta\Delta C_t$ for CECs treated with aza-C at 10 μM was −0.399 ± 2.242 and the $\Delta\Delta C_t$ for CECs treated with both was 2.684 ± 1.837. These results indicate that Aβ$_{1-40}$ at 25 μM resulted in the suppression of NEP mRNA expression, and that aza-C at 10 μM reversed the effects, thereby implying that the suppression of NEP mRNA expression by Aβ$_{1-40}$ at 25 μM is, in part, attributable to DNA methylation (Table 1).

Aβ$_{1-40}$-suppressed NEP protein expression reversed by methyl-transferase inhibitor

Measurement of the NEP protein was undertaken by Western blotting to confirm the consequences of the hyper-methylation of the NEP gene promoter induced by Aβ$_{1-40}$, with those CECs treated by methyl-transferase inhibitor, Aza-C, serving as the negative control. The results are compatible with the expression of NEP mRNA, with Aβ$_{1-40}$ being found to suppress the protein expression of NEP and this effect being reversed by Aza-C (Fig. 4).

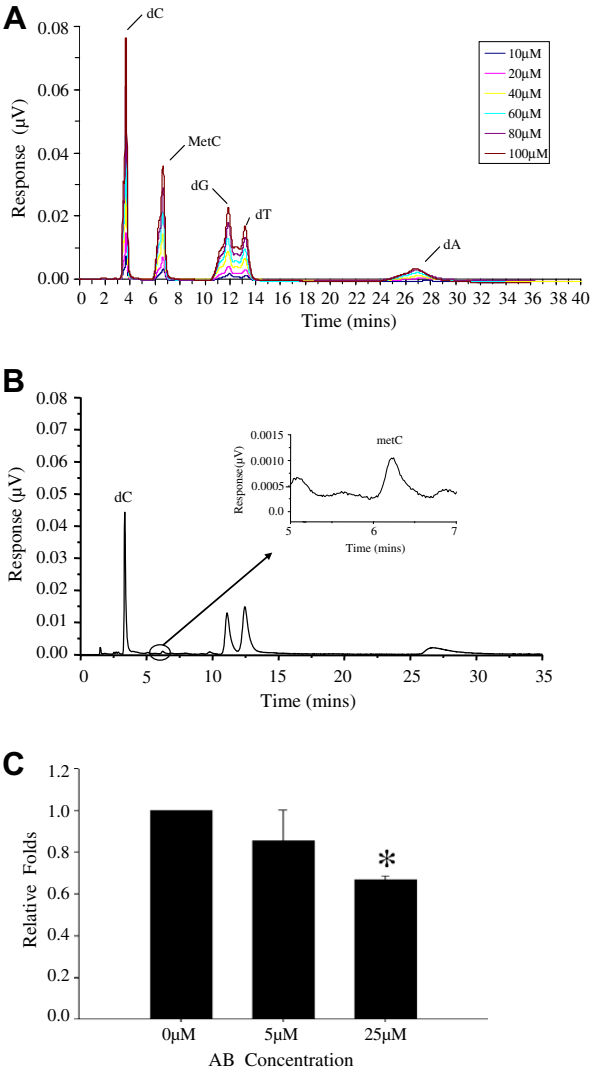

Fig. 1. (A) HPLC results of standard samples consisting of dC, metC, dG, dT, and dA at different concentration levels. (B) HPLC analysis of a representative sample of DNA extracted from CECs treated with Aβ$_{1-40}$ 25 μM for 48 h. (C) HPLC analysis of changes in global DNA methylation for DNA extracted from CECs treated with Aβ$_{1-40}$ at indicated concentrations. As compared to the controls with no Aβ$_{1-40}$ treatment (0.6663 ± 0.0201 vs. 1), significant reductions in global DNA methylation are found in CECs treated with 25 μM, but not 5 μM Aβ$_{1-40}$. The data represent three sets of experiments, * in Fig. 1c indicates $p < 0.05$.

Discussion

The epigenetic effects of Aβ on the brain remain unclear; therefore, in this study we tested the changes in global DNA methylation in CECs both with and without the treatment of Aβ by HPLC, with our results revealing that Aβ at higher concentrations induces global DNA hypo-methylation. There is clearly a need for further investigation of the cause of Aβ-induced global DNA hypo-methylation; however, the phenomenon indicates that Aβ could induce epigenetic changes similar to those involved in normal aging, since global or whole genomic DNA hypo-methylation has been documented in the aging brain [10].

One of the consequences of DNA hypo-methylation may be the enhancement of the formation of senile plaques in AD. Hypo-methylation in the promoter region, which results in the over-expression of amyloid precursor protein (APP), presenilin1 (PS1), and beta-secretase (BACE), may be associated with the occurrence of AD [18]. *S*-Adenosylmethionine, a methyl group donor, may be

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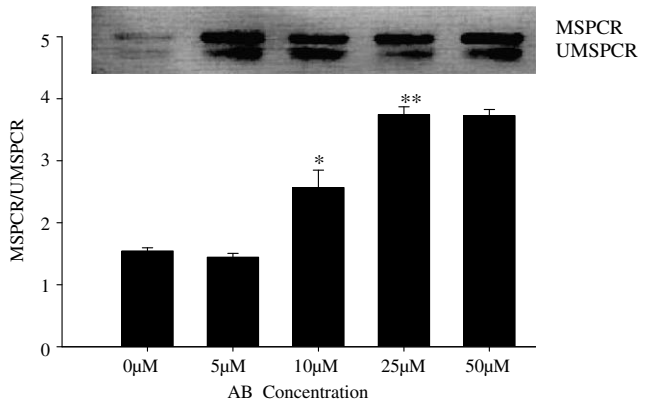

Fig. 2. Changes in CpG island methylation on the NEP promoter region in CECs treated with $A\beta_{1-40}$ at different concentrations are assessed by MSPCR and UMSPCR. There are significant increases in the ratios between MSPCR and UMSPCR products (MSPCR/UMSPCR) in the samples treated with $A\beta_{1-40}$ at concentrations above 10 μM. The data represent three sets of experiments, * indicates that the difference between the control was significant ($p < 0.01$).

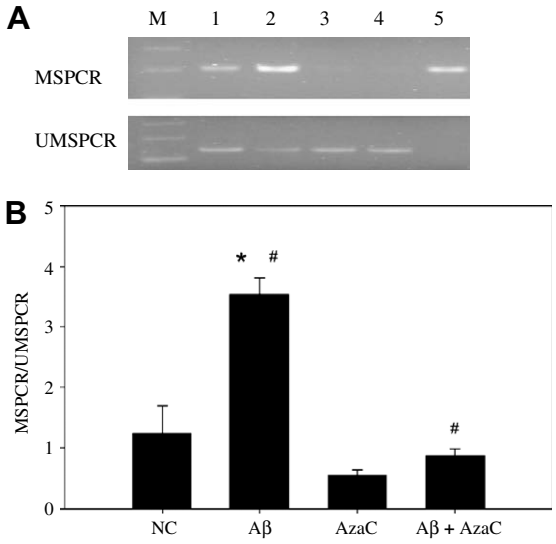

Fig. 3. (A) The changes in CpG island methylation in the NEP promoter region upon exposure to $A\beta_{1-40}$ at 25 μM, with and without AzaC at 10 μM are assessed by MSPCR and UMSPCR. *M* refers to the molecular weight marker (100 bp). Lane 1 refers to the negative controls; lane 2 refers to $A\beta_{1-40}$ treatment at 25 μM for 48 h; lane 3 refers to AzaC treatment at 10 μM for 48 h, lane 4 refers to $A\beta_{1-40}$ treatment at 25 μM plus AzaC at 10 μM for 48 h, lane 5 refers to the positive controls in which DNA was treated with methylase prior to MSPCR and UMSPCR. The amount of MSPCR product was increased with a concurrent reduction in the UMSPCR with $A\beta_{1-40}$ treatment, whereas the DNA hyper-methylation induced by $A\beta_{1-40}$ could be reversed by AzaC. This is a representative result of the independent experiments. (B) Three separate experiments of changes in CpG island methylation in the NEP promoter region upon exposure to $A\beta_{1-40}$ at 25 μM with or without AzaC at 10 μM treatment. The figure illustrates the compiled data from three separate experiments for each paradigm. * indicates that the difference between the control, or # Aβ treatment alone, was significant at $p = 0.011$ and 0.003.

Table 1
Real-time RT-PCR assay for neprilysin mRNA.

		C_t	ΔC_t	$\Delta\Delta C_t$
$A\beta_{1-40}$	Neprilysin	29.166 ± 4.314	6.727 ± 3.043	4.864 ± 0.672**
	β-actin	22.439 ± 1.215		
AzaC	Neprilysin	27.004 ± 2.921	1.464 ± 3.024	−0.399 ± 2.242
	β-actin	25.541 ± 2.953		
A + A	Neprilysin	27.623 ± 3.826	4.547 ± 0.912	2.684 ± 1.837
	β-actin	23.076 ± 3.040		
Control	Neprilysin	26.218 ± 2.092	1.863 ± 2.748	
	β-actin	24.350 ± 1.468		

$A\beta_{1-40}$ = amyloid-β_{1-40}, 25 μM; AzaC = 10 μM; A + A = Amyloid-β_{1-40}, 25 μM and AzaC, 10 μM.

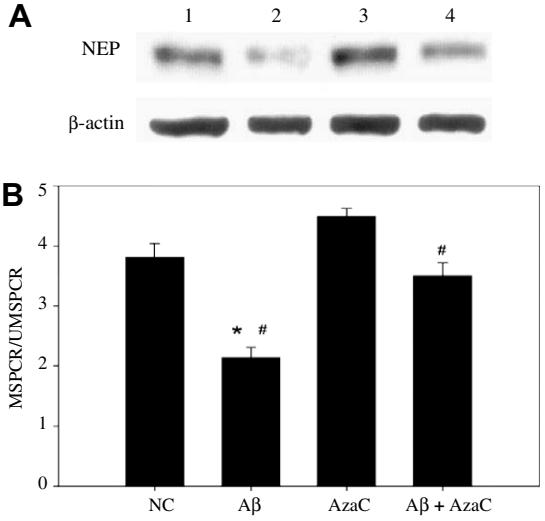

Fig. 4. (A) NEP protein levels were examined by Western blotting, with β-actin protein serving as an internal control lane 1 refers to the controls, lane 2 refers to $A\beta_{1-40}$ treatment at 25 μM for 48 h, lane 3 refers to AzaC treatment at 10 μM for 48 h, lane 4 refers to $A\beta_{1-40}$ treatment at 25 μM plus AzaC at 10 μM for 48 h. Although the NEP protein level was suppressed by $A\beta_{1-40}$, this could be reversed by the DNA methylation inhibitor, AzaC. This is a representative result of the independent experiments. (B) Three separate experiments of changes in NEP protein levels upon exposure to $A\beta_{1-40}$ at 25 μM with or without AzaC at 10 μM treatment. The figure illustrates the compiled data from three separate experiments for each paradigm.* indicates that the respective difference between the control, or # Aβ treatment alone, was significant at $p = 0.001$ and <0.001.

capable of preventing or stopping Aβ accumulation in transgenic AD mice [19], this therefore raises the possibility that Aβ, through its epigenetic effects, could be one of the accelerators of aging and senile plaque formation.

A reduction in NEP expression has been associated with AD and aging brains, and possibly CAA brains [20]. NEP insufficiency has been found to potentially increase the accumulation of Aβ in AD animals [21]. DNA hyper-methylation on the NEP gene promoter has been detected in both prostate cancers and hepatocellular carcinoma [22,14]. Using MS-PCR and UMS-PCR, in this study we have proven that Aβ was responsible for inducing DNA hyper-methylation on the NEP gene promoter. The real-time PCR and Western blotting subsequently revealed that Aβ suppressed both NEP mRNA and protein expression, with the reduction of both levels potentially being reversed by the DNA methylase inhibitor, AzaC. These findings support the hypothesis that Aβ suppresses NEP expression by an increase in DNA methylation on the promoter region. A reduction in NEP expression reduces the Aβ clearance and probably elevates Aβ accumulation. This could be part of a vicious cycle which may well play an important role in the pathophysiology of AD.

The phenomenon of global DNA hypo-methylation simultaneously accompanied by hyper-methylation of the DNA promoter on certain specific genes has been found in a few types of cancers [23]. Aging, or senescence, is associated with a slowing-down of DNA replication, which depends on the number of active replications and the rate of DNA chain elongation [24]. In the model of cellular aging, global DNA hypo-methylation relating to the down-regulation of methyltransferase, Dnmt1, and hyper-

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methylation of specific genes relating to the up-regulation of methyltransferase, Dnmt3b, could be responsible for the inactivation of general replication and a subsequent reduction in DNA replication capacity. This implies that there may be simultaneous occurrence of global DNA hypo-methylation and some specific gene hyper-methylation in senescence.

In summary, this study finds that, similar to the epigenetic pattern of aging, Aβ causes global DNA hypo-methylation and NEP hyper-methylation, which consequently suppresses its expression in mRNA and protein levels. These findings imply that Aβ may be involved in the formation of Aβ itself, and can therefore probably switch the epigenetic situation into an aging pattern.

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