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Folic acid administration inhibits amyloid β-peptide accumulation in APP/PS1 transgenic mice

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Abstract

Alzheimer's disease (AD) is associated with malnutrition, altered one-carbon metabolism and increased hippocampal amyloid-β peptide (Aβ) accumulation. Aberrant DNA methylation may be an epigenetic mechanism that underlies AD pathogenesis. We hypothesized that folic acid acts through an epigenetic gene silencing mechanism to lower Aβ levels in the APP/PS1 transgenic mouse model of AD. APP/PS1 mice were fed either folate-deficient or control diets and gavaged daily with 120 μg/kg folic acid, 13.3 mg/kg *S*-adenosylmethionine (SAM) or both. Examination of the mice after 60 days of treatment showed that serum folate concentration increased with intake of folic acid but not SAM. Folate deficiency lowered endogenous SAM concentration, whereas neither intervention altered *S*-adenosylhomocysteine concentration. DNA methyltransferase (DNMT) activity increased with intake of folic acid raised DNMT activity in folate-deficient mice. DNA methylation rate was stimulated by folic acid in the amyloid precursor protein (APP) promoter and in the presenilin 1 (PS1) promoter. Folate deficiency elevated hippocampal APP, PS1 and Aβ protein levels, and these rises were prevented by folic acid. In conclusion, these findings are consistent with a mechanism in which folic acid increases methylation potential and DNMT activity, modifies DNA methylation and ultimately decreases APP, PS1 and Aβ protein levels.

Keywords: Alzheimer's disease; Amyloid β-peptide; DNA methylation; Folic acid; *S*-adenosylmethionine

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease resulting in progressive dementia and is a principal cause of dementia among older adults. It may be triggered, at least in part, by accumulation in the brain's gray matter of amyloid plaques that contain extracellular deposits of amyloid-β peptides (Aβ) [1]. Aβ also may be transported within neurons and cause axonal deficits there [2].

AD has a non-Mendelian etiology that eventually may be explained by epigenetic modifications acting to mediate the disease's onset and progression [3]. DNA methylation is an example of epigenetic modification for which a role in AD is beginning to be elucidated [4,5]. For instance, it has been reported that an age-specific epigenetic drift occurs in late-onset AD [6]. Further, there are DNA methylation differences between late-onset AD and cognitively normal controls in human frontal cortex [7]. In particular, the PS1 gene is a specific locus of demethylation in AD patients. Since gene expression and silencing may depend on DNA methylation in promoter regions, it is possible that aberrant DNA methylation may underlie AD pathogenesis [3]. DNA methylation is catalyzed by DNA methyltransferase (DNMT). The methyl donor for DNMT reactions is *S*-adenosylmethionine (SAM), which is the precursor of *S*-adenosylhomocysteine (SAH) [8]. SAM stimulates and SAH inhibits the transfer by DNMT of methyl groups on the cytosine residues of DNA. Folate is integral to one-carbon metabolism [i.e., the homocysteine (HCY) cycle], which is a biochemical pathway wherein SAM is an intermediate [3].

There is currently no cure for AD. However, because malnutrition is a well-established risk factor for cognitive impairment, there is a strong rationale to search for nutritional interventions for AD and to understand their molecular mechanisms of action [9–12]. In particular, preclinical research is needed to discover how nutrients modulate Aβ accumulation in AD models. A recent meta-analysis found that supplementation with combinations of folic acid and vitamin B12 had no significant effect on age-related cognitive decline (i.e., cognitive aging) in older adult participants [13]. However, the randomized controlled trial that supplemented older adults with folic acid alone (without vitamin B12) found that folic acid conferred significant protection against cognitive aging [14]. Furthermore,

Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; APP/PS1 mice, mice with APPswe/PS1ΔE9 mutations; DNMT, DNA methyltransferase; HCY, homocysteine; IOD, integrated optical density; OD, optical density; ORF, open reading frame; PBS, phosphate-buffered saline; PS1, presenilin 1; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; UTR, untranslated region.

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Chan et al. reported that a nutriceutical formulation that contained folic acid, SAM, vitamin B12, vitamin E, *N*-acety-L-cysteine and acetyl-L-carnitine holds promise for treatment of early-stage AD prior to and/or as a supplement for pharmacological approaches [15].

In the present study, we hypothesized that folic acid acts through an epigenetic gene silencing mechanism to lower Aβ levels in the APP/PS1 transgenic mouse model of AD, and exogenous SAM in mice which intake control diet had no more effect then folic acid.

2. Materials and methods

2.1. Mice and diets

The Tianjin Medical University Animal Ethics Committee approved the experimental protocols in this study (number TMUaMEC 2012016). Male mice with APPswe/PS1ΔE9 mutations (APP/PS1), backcrossed to C57Bl6/J, were obtained from the Chinese Academy of Medical Sciences Institute of Laboratory. These double transgenic mice are models of AD because they express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9), both directed to central nervous system neurons under the control of independent mouse prion protein promoter elements; consequently, young adult APP/PS1 mice have Aβ-containing neuritic plaques that are absent in age-matched wild-type mice.

After genotyping, the AD transgenic mice were maintained on the control diet until age 7 months and then were assigned in equal numbers to five groups for 60 days: (a) folate-deficient diet plus daily gavage with water (Def), (b) control diet (normal folic acid content) plus daily gavage with water (Con), (c) control diet plus daily gavage with 13.3 mg/kg SAM (Con+SAM), (d) control diet plus daily gavage with 120 μg/kg folic acid (Con+FA) and (e) control diet plus daily gavage with both 120 μg/kg folic acid and 13.3 mg/kg SAM (Con+FA+SAM).

The folate-deficient diet (containing folic acid 0.2 mg/kg diet) and the control diet (folic acid 2.1 mg/kg diet) were purchased from TestDiet (St. Louis, MO. USA). All mice received food and drinking water *ad libitum*. At the conclusion of the experiment, the mice were anesthetized by intraperitoneal injection of 7% chloral hydrate (5 ml/kg) and perfused transcardially with phosphate-buffered saline (PBS). The thorax was opened to collect ventricular blood by cardiac puncture after anesthetizing. Brains were removed, bisected in the sagittal plane and stored at −80°C. The left tissue was used for immunofluorescence staining, and the right tissue was used for other assays, as described below. Livers were also removed and stored at −80°C until analysis.

2.2. Serum folate

Serum folate levels were determined using a competitive protein-binding assay with chemiluminescent detection in an automated chemiluminescence system (Immulite 1000; Siemens, Berlin, Germany) according to the manufacturer's instructions. The automated chemiluminescence system would detect all types of folate including folic acid, dihydrofolate and tetrahydrofolate [16]. A high-folic-acid standard and a low-folic-acid standard provided in the kit were used to correct the automated chemiluminescence system. Because the examination area of this system is 1–24 ng/ml and serum folate level of mice was higher, serum samples were diluted two times by 0.9% saline.

2.3. Real-time polymerase chain reaction (PCR)

Gene expression was quantified by real-time PCR. Total RNA of brain hippocampus tissues [homogenate by a motor-driven tissue homogenizer (PT1200E; Kinematica, Lucerne, Switzerland)] was extracted using Trizol according to the instructions of the manufacturer. First-strand cDNA was synthesized from 2 μg total RNA using MMLV reverse transcriptase. The 20-μl reaction volume was incubated for 60 min at 42°C and 10 min at 70°C, and held at −20°C. Real-time PCR was performed using a LightCycler 480 SYBR Green I Master Kit (Roche, Mannheim, Germany). The 20-μl PCR mixture included 10 μl of PCR Master, 5 μl of cDNA, 1 μl of forward primer, 1 μl of reverse primer and 3 μl of water (PCR grade). The reaction mixtures were incubated at 95°C for 5 min, followed by 45 amplification cycles (denaturation, 95°C for 10 s; annealing, 59°C for APP and 63°C for PS1 for 10 s; extension, 72°C for 10 s). Primers were specific for APP (forward, GGCAACAGGAACTTTGA; reverse, CTGCTGTCGGGGAAGTTTA) and PS1 (forward, CCCCA GAGTAACTCAAG ACA; reverse, CCGGGTATAGAAGCTGACTGA). The assay was performed using the Roche LightCycler 480 sequence detector (Roche, Mannheim, Germany). The expression of each gene was normalized to β-actin (forward, AATGTGTCCGTCGTG GATCT; reverse, GGTCCTCAGTGTAGCCCAA G) in order to calculate relative levels of transcripts.

2.4. Western blot analysis

Protein expression of APP, PS1 and Aβ was assessed by Western blot analysis. The brain hippocampus tissues [homogenate by a motor-driven tissue homogenizer (PT1200E; Kinematica, Lucerne, Switzerland)] were washed with ice-cold PBS and lysed with TNE-NP40 buffer. Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel by electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk and incubated with primary antibodies [anti-APP (for detecting APP695, APP770 and APP751), 1:1000, CST (Danvers, MA, USA); anti-PS1, 1:1000, Abcam (Cambridge, UK); anti-Aβ antibody (Bam-10), 1:1000 (Sigma Aldrich; St. Louis, MO, USA)] overnight at 4°C, followed with appropriate secondary antibodies (IgG-horseradish peroxidase; Zhongshan Goldbridge Biotechnology, Beijing, China) for l h at room temperature. Proteins were detected by chemiluminescence assay and then quantified by densitometric analysis using NIH Image software (version 1.61). The intensity of each protein band was normalized to the respective β-actin band.

2.5. Immunohistochemistry

The left brains were removed and postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. The brains were cut coronally into 4-μm-thick sections with a vibratome. Free-floating sections were incubated with 4% bovine serum albumin in PBS for 1 h and then reacted with mouse monoclonal anti-Aβ antibody (Bam-10), 1:200 (Sigma Aldrich; St. Louis, MO, USA); anti-APP, 1:100 (Cell Signaling Technology, Danver, MA, USA); or anti-PS1, 1:1000, Abcam (Cambridge, UK) at 4°C overnight. The Bam-10 antibody recognizes the epitope for the N-terminus (1–12 amino acid residues) of Aβ (1–42) and Aβ (1–40) and specifically stains amyloid plaques in the brains of AD mouse models. The anti-APP antibody detects APP695, APP770 and APP751.

The sections were washed with PBS and reacted with biotinylated secondary antibodies diluted 1:200 in PBS and visualized using ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). The images were obtained with a microscope (Olympus, Tokyo, Japan), and the integrated optical density (IOD) of each was determined with Image-Pro Plus Version 6.0 image analysis software.

2.6. Pyrosequencing

Methylation levels of CpG sites in APP and PS1 promoter regions were determined by pyrosequencing using the Pyromark Q24 Reagent (QIAGEN Ltd., Crawley, UK) according to the manufacturer's protocol. Tissues were digested with 100 μg/ml proteinase K at 50°C overnight, followed by phenol/chloroform extraction and ethanol precipitation of DNA. Then, incubation of the DNA with sodium bisulfite converted unmethylated cytosine residues into uracil while leaving methylated cytosines unchanged. Real-time PCR was performed using a LightCycler 480 SYBR Green I Master Kit (Roche, Mannheim, Germany). The 25-μl PCR mixture included 12.5 μl of Pyromark PCR Master, 2.5 μl of coralload concentrate, 1 μl of DNA, 2.5 μl of forward primer, 2.5 μl of reverse primer and 4 μl of water (PCR grade). The reaction mixtures were incubated at 95°C for 15 min, followed by 45 amplification cycles (94°C for 30 s, 56°C for 30 s, 72°C for 30 s). Primers were specific for APP (forward, TGATTTGGGTTAGGGAGAGG; reverse, AACCTTAACTCCTCAACCA CATTTA) and PS1 (forward, GGGGTTGGAGTTGGTTTAA; reverse, AAACCCATCC TTTCCTACAA).

2.7. DNMT activity assay

Nuclear extracts of right hippocampal tissue were isolated using the nuclear extraction kit (Merck KGaA, Darmstadt, Germany). DNMT activity was measured using a DNMT activity/inhibition assay kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). A lot-specific standard curve was created with the DNMT1 provided in the kit. Optical density (OD) was measured on a microplate reader at 450 nm, and DNMT activity [(OD/(h·mg)]was calculated according to the following formula:

$$\text{DNMT activity (OD/h/mg)} = \frac{(\text{average sample OD} - \text{average blank OD})}{\text{protein amount(g)} * \times \text{h} * *} \times 1000$$

* Protein amount added into the reaction.
** Incubation time used for the reaction.

2.8. Methylation potential assay

SAM, SAH and the SAM:SAH ratio (i.e., methylation potential) were determined in liver and brain tissue samples that were stored at −80°C and then thawed for analysis. The liver and brain tissue samples were homogenized by a motor-driven tissue homogenizer (PT1200E; Kinematica, Lucerne, Switzerland) and kept on ice. One-hundred-milligram extracts of liver tissue or brain tissue were removed to new tube and resuspended in 300 μl 0.4 mol/L ice-cold perchloric acid. Liver cytosolic protein was obtained using a water bath sonicator (Bioruptor UCD-200, Diagenode). Homogenates were centrifuged at 20,000 *g* for 10 min at 4°C, and supernatant were collected. The supernatant of each sample was filtered through 0.45 μm (Millipore, Billerica, MA, USA) and then was loaded into a Venusil MP-C18 column (250 mm×4.6 mm, 5-μm particle; Agela Technologies, Wilmington, DE, USA) fitted with a matched guard column, run by a Waters HPLC system (Milford, MA, USA) and connected to an ultraviolet detector. Absorption of eluted compounds was monitored at λ=254 nm. A two-buffer elution system was used: mobile phase A and B; both contain 10 mmol/L ammonium formate and 4 mmol/L 1-heptanesulfonic acid (pH=4). Mobile phase B contains