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DNA methylase and demethylase activities are modulated by one-carbon metabolism in Alzheimer's disease models☆,☆☆

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Abstract

Late-onset Alzheimer's disease seems to be a multi-factorial disease with both genetic and non-genetic, environmental, possible causes. Recently, epigenomics is achieving a major role in Alzheimer's research due to its involvement in different molecular pathways leading to neurodegeneration. Among the different epigenetic modifications, DNA methylation is one of the most relevant to the disease. We previously demonstrated that presenilin1 (*PSEN1*), a gene involved in amyloidogenesis, is modulated by DNA methylation in neuroblastoma cells and Alzheimer's mice in an experimental model of nutritionally altered one-carbon metabolism. This alteration, obtained by nutritional deficiency of B vitamins (folate, B12 and B6) hampered *S*-adenosylmethionine (SAM)-dependent methylation reactions. The aim of the present paper was to investigate the regulation of DNA methylation machinery in response to hypomethylating (B vitamin deficiency) and hypermethylating (SAM supplementation) alterations of the one-carbon metabolism. We found that DNA methylases (DNMT1, 3a and 3b) and a putative demethylase (MBD2) were differently modulated, in line with the previously observed changes of *PSEN1* methylation pattern in the same experimental conditions.

Keywords: DNA methylation; DNA demethylase; Alzheimer's Disease; One-carbon metabolism; S-adenosylmethionine; B vitamin deficiency

1. Introduction

Many human diseases present a complex pattern of different interconnected pathogenic, causative cofactors. In the recent years, a great attention was given to epigenetic factors and, among these, to DNA methylation in particular. It appears evident that DNA methylation could be, at least in part, at the basis of several human pathologies [1,2].

In previous studies we demonstrated that alterations of methylation metabolism were associated with the overexpression of presenilin1 (*PSEN1*), a gene involved in the production of amyloid β peptide whose deposits in intraneuronal spaces are one of the key features of Alzheimer's disease (AD) [3–6]. Our data are supported by other studies that evidenced how DNA methylation is prone to changes in AD and in aging in relation to time, brain region and genes [7–11]. These studies also stressed the observation that DNA methylation may undergo dynamic regulation also in a differentiated tissue. Moreover, a recent study evidenced that different factors involved in methylation maintenance are decreased in neurons in AD cases [12]. These data showed for the first time that homeostasis of methylation machinery (besides DNA methylation pattern) in AD brains, in areas more prone to AD features, could be altered in the direction of a methylation decrement.

DNA methyltransferases (DNMTs) use *S*-adenosylmethionine (SAM, which is the main methyl donor in eukaryotes) as substrate in a reaction that transfers the methyl group to a cytosine on DNA leading to *S*-adenosylhomocysteine (SAH) production. This molecule is a competitive inhibitor of DNMTs; for this reason, SAM/SAH ratio is considered an indicator of methylation potential of a biological system. Normally, SAH is quickly hydrolyzed to homocysteine (HCY) and adenosine; even if SAH synthesis would be thermodynamically favored, hydrolysis can go on since both adenosine and HCY are rapidly converted. HCY, in particular, can be remethylated to methionine (using vitamin B12 and folate as cofactors) or *trans*-sulfurated to cystathionine (using vitamin B6 as cofactor) [13,14]. The clearest evidence of one-carbon metabolism involvement with late-onset AD is the association of the disease with

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hyperhomocysteinemia, low B vitamins and impaired methylation [15–17]. On the basis of these data, we set up our nutritionally based protocol of altered one-carbon metabolism. The protocol, applied both to neuroblastoma SK-N-BE cell line and to TgCRND8 mice, consisted of a deficiency of folate, vitamin B12 and vitamin B6 [4,5] to hamper HCY transformation. Through this expedient, we were able to obtain HCY and SAH accumulation and SAM/SAH ratio impairment, with consequent decreased methylation ability.

In order to clarify the mechanistic insights of methylation-dependent PSEN1 up-regulation in our experimental system, we decided to study the regulation of the most known DNA methyltransferases (DNMT1, 3A and 3B) and one of the putative DNA demethylase (MBD2) [18,19]. DNMT1 is considered the maintenance DNA methyltransferase; it methylates CpG moieties with high specificity, with preference for hemimethylated DNA and in a processive reaction [20]. On the contrary, DNMT3a and 3b show no preference for hemimethylated DNA being, for this reason, indicated as *de novo* DNA methyltransferases [21]. Interestingly, DNMT3a and 3b have shown significant methylation activity on non-CpG sites [22–24]. Whereas DNA methyltransferases are well characterized on their biochemical and biological features, characterization of an enzyme able to actively demethylate DNA still has some aspects that deserve further clarification. Different enzymes with possible DNA demethylase activity were identified: 5-methylcytosine-DNA glycosylase, Gadd45a, MBD2, MBD2b, MBD3 and MBD4 [25–31]. It was also recently demonstrated that DNMTs could be associated with demethylase activity [28,32,33]; finally, it was recently demonstrated that MeCP2 phosphorylation can mediate demethylation/methylation of specific CpG sites [34]. In the present article, we decided to investigate MBD2 since it is well characterized and its association with one-carbon metabolism has already been evidenced [29–31]. Szyf et al. hypothesized that MBD2 can bind to methylated CpGs on DNA and remove the methyl group in presence of H_2O, leading to the formation of non-methylated CpG-bearing DNA (with no cytosine excision) and methanol [29]. We must underline that other authors disputed these findings and that the same authors do not exclude the possibility that MBD2 can interact with other proteins in the active demethylation reaction [19]. They also demonstrated that active DNA demethylation dependent on MBD2 activity was inhibited by SAM [31]. This indication, together with the knowledge that SAM and SAH availability plays a fundamental role in DNA methylation reactions [35], lead us to study the influence of one-carbon metabolism alterations on DNA methyltransferases and demethylase activity in our AD models.

2. Methods and materials

2.1. Cell cultures

Neuroblastoma SK-N-BE human cell line was maintained in F14 medium with 10% foetal calf serum (FCS) and shifted to complete differentiation medium (control medium, with 1% FCS plus 10 μM retinoic acid) or to differentiation medium deficient of folate, vitamin B12 and vitamin B6 (B deficient). B vitamin deficiency does not affect cell proliferation/differentiation as previously reported [3,4]. SAM 100 μM (*S*-adenosylmethionine disulphate *p*-toluensulfonate) was obtained by Gnosis (Desio, MI, Italy) and was added to media according to the experimental design. Cultures were re-fed every second day and stopped after 72 (methylation assays) or 96 h (gene and protein expression analyses); times indicated refer to medium shift as Day 0. Experiments were repeated at least three times.

2.2. Mice and diets

TgCRND8 (carrying human mutated APP gene) and wild-type 129Sv mice were maintained and assigned to control or B vitamin deficient diet as previously described [6]. SAM was administered to appropriate experimental groups (both in control and B vitamin deficient diet) by gavage-needle force-feeding at dosage of 400 μg/day (administrated as 800 μg every other day to limit animal stress). Analyses were carried out on a total of 48 animals; six (three females and three males) mice per group were fed for 60 days after weaning with either control or deficient diet, with or without SAM. Brains were collected and homogenized as previously described [5]; for bisulphite assay, two brain homogenates were grouped in pool for each experiment repetition.

All the experiments were performed in such a way as to sacrifice the minimum number of animals required and were approved by the author's institution according to guidelines of Italian Ministry of Health (D.L. 92/116).

2.3. Real-time polymerase chain reaction analysis

RNA was extracted from homogenized brain with the RNeasy Lipid Tissue mini kit (Qiagen, Milano Italy); 1 μg of total RNA was used for cDNA synthesis, with 50 pmol of random examers and 50 U of transcriptor reverse transcriptase (Roche) at 50°C for 1 h, as indicated by the manufacturer. 1 μg of total cDNA was used for each real-time reaction; analyses were performed in triplicate for each sample as previously described [5] with an annealing temperature of 62°C. cDNA levels were standardized by normalizing them to the β-actin control and presented as the fold increase (ratio of the experimental gene value/actin gene value) over the control sample. Oligonucleotides used as primers in polymerase chain reaction (PCR) reactions were previously described (human β-actin [4]; human DNMT1, DNMT3a, DNMT3b [36]; human MBD2 [37]; mouse β-actin [5]; mouse DNMT1, DNMT3a, DNMT3b [38]) except for mouse MBD2 that were designed with the help of Primer3 software (http://frodo.wi.mit.edu/): MMMBD2L1 (5′-ACCTGGGAAATGCTGTTGAC-3′, forward) and MMMBD2R1 (5′-TGGCAATGTTGTGTTCAGGT-3′, reverse), giving an amplicon of 146 bp. Expression levels of interest genes were also normalized to other two reference genes, GAPDH and 18S, giving similar results (data not shown).

Expression of 5-MCDG was analyzed by semi-quantitative standard reverse transcriptase-PCR and ethidium bromide agarose gel electrophoresis using primers and conditions previously described [39].

2.4. Western blotting

Cultured cells and homogenized brains were lysed with 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.2 % Nonidet P-40, 1 % 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM EDTA, pheylmethylsulfonyl fluoride (PMSF, 200 μM), leupeptin (1 μM), pepstatin A (1 μM) and calpain inhibitor I (5 μM); 20–40 μg of protein extracts were run on 12% sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis, then blotted onto nitro-cellulose (Bio-Rad, Hercules, CA, USA). Western blot signals were acquired and analyzed by a Fluor-S densitometer and the Quantity One software (Bio-Rad); optical densities (O.D.) from at least three different experiments were calculated for each sample and normalized with the corresponding 14,3,3β and β-actin (not shown) signals O.D.; the O.D. ratios were then compared and expressed as the average fold increase, with 1 as the control (Tg or wild type, Diet A) value.

Primary antibodies characteristics are summarized in Table 1; secondary antibodies came from an ECL Western blotting reagents kit (GE Healthcare Europe); all the chemicals were from Sigma (St. Louis, MO, USA).

2.5. Preparation of DNA templates for methyltransferase/demethylase assays

DNA substrate for methyltransferase and demethylase assay was synthesized by PCR amplification of human and murine sequences of PSEN1 5′-flanking region previously analyzed by bisulphite method [6]. Human DNA was amplified using HSPS1BisF2 and HSPS1BisR2 primer pair, giving a fragment of 762 bp; mouse DNA was amplified by MMPS1BisF1 and MMPS1BisR2 primer pair, giving a fragment of 723 bp. Features of primers and of amplified fragments were previously described [6].

DNA from SK-N-BE cells and mice brain were extracted by classical phenol method. PCR amplifications were carried out on 50 ng of genomic DNA in a PTC-100 thermal cycle (MJ Research) performing 40 cycles (94°C×1 min, 62°C×1 min, 68°C×1.30 min), using a PlatinumTaq HiFi DNA polymerase (Invitrogen). Amplified fragments from four PCR replicates were run on 1.2% agarose gel; specific bands were removed from the gel and purified using a QiaQuick Gel Extraction Kit (Qiagen). DNA was quantified by A_{260}/A_{280} spectrophotometer analysis; sequence specificity of the amplified fragments was assessed through DNA sequencing (in service by PRIMM).

Twenty-five micrograms of DNA was incubated with 60 U of SssI methylase (New England Biolabs), SAM 3.2 mM, 50 mM EDTA for 4 h at 37°C. SAH was removed with Microcon 10 concentrator (Millipore), and the reaction was repeated with fresh 40 U of SssI and 3.2 mM SAM for further 4 h. DNA was recovered by phenol:chloroform:

Table 1
Characteristics of antibodies used for Western blotting

Protein	Antibody		Manufacturer	Band size	Dilution
DNMT1	sc-70981	Monoclonal	Santa Cruz Biotechnology	184 kDa	1:200
DNMT3a	sc-20703	Polyclonal	Santa Cruz, CA, USA	100–130 kDa	1:100
DNMT3b	sc-81252	Monoclonal		97 kDa	1:100
MBD2	sc-9397	Polyclonal		47 kDa	1:100
14-3-3β	sc-629	Polyclonal		30 kDa	1:200

All antibodies recognized both human and murine epitopes.