Presenilin 1 gene silencing by *S*-adenosylmethionine: a treatment for Alzheimer disease?

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Abstract Presenilin 1 (PS1) is a key factor for β-amyloid (Ab) formation in Alzheimer disease (AD). Homocysteine accumulation, frequently observed in AD patients, may be a sign of a metabolic alteration in the *S*-adenosylmethionine (SAM) cycle, which generates the overexpression of genes controlled by methylation of their promoters, when the cytosine in CpG moieties becomes unmethylated. The methylation of a gene involved in the processing of amyloid precursor protein may prevent Ab formation by silencing the gene. Here we report that SAM administration, in human neuroblastoma SK-N-SH cell cultures, downregulates PS1 gene expression and Ab production.

Key words: Alzheimer disease; β-Amyloid; DNA methylation; Presenilin 1; Homocysteine; *S*-Adenosylmethionine

1. Introduction

Many lines of evidence sustain the amyloid theory as the cause of Alzheimer disease (AD) [1,2]. The perspective of eliminating directly a portion of the neurotoxic amyloid peptide is certainly appealing. The most relevant attempt, although unsuccessful, has been anti-amyloid vaccination [3]. Although there are divergent opinions on the outcome of anti-amyloid vaccine and developments are in progress, the vaccine experiments have focused on two considerations: the first is that the target is probably correct, since vaccinated animals recovered cognition along with a lowering of free amyloid and plaques [4]; the second is that amyloid has important physiological functions [5], and as such cannot be completely eliminated [6]. We feel that a molecular intervention resulting in a restored physiological level of the enzymes involved in amyloid precursor protein (APP) processing and the prevention of β-amyloid peptide (Ab) overproduction would be a better approach.

A few papers indicated the possible role of increased homocysteine (HCY) as a risk factor in AD [7] and the decreased concentration of *S*-adenosylmethionine (SAM) measured post mortem in AD patients [8,9]. One alteration of SAM metabolism, which can modify gene expression, is the decreased uptake of folate and vitamin B12 with consequent HCY and *S*-adenosylhomocysteine (SAH) accumulation and SAM reduction [10]. Besides the lack of transformation of HCY to methionine [11], lower SAM levels could be due to methionine adenosyltransferase deficiency [12]. The lower availability of SAM could very well be related to the altered expression of genes involved in APP metabolism, finally producing the accumulation of Ab peptide. SAM is the main methyl donor and could regulate the methylation status of a gene whose product is involved in APP processing, thus preventing Ab formation by silencing the gene.

α-Secretase cleavage of APP does not produce the amyloidogenic peptides, produced, on the contrary, by β- and γ-secretase activities [13]. Many papers have contributed to the further explanation of the close connection between presenilin (PS) 1 and γ-secretase [14–16]. The physiological role of presenilins is demonstrated by the discovery that PS1 deficiency in mice was lethal [17]; this appears to be strictly connected to the activation of Notch-1, a signal transductor involved in stem cell maturation [18]. All the indications regarding PS1 as the target for AD therapy point to the silencing of this gene, but without a complete blocking [19]. Two observations have been basic for the development of this work: the gradual DNA hypomethylation in the elderly [8,9] and the accumulation of HCY in AD patients [7]. These two conditions are metabolically related, since the lack of transformation of HCY to methionine reverts the metabolism to SAH, a strong inhibitor of DNA methyltransferases, inducing DNA hypomethylation [20]. This biochemical pattern, according to the theory that many genes are expressed when the cytosines in CpG moieties of their promoters become unmethylated [21,22], could determine either the expression of unexpressed genes or the overexpression of genes normally expressed. PS1 overexpression could unbalance the processing of the Ab peptide which therefore accumulates and after several years could cause brain degeneration. We propose that the alteration of HCY and SAM metabolism could be related to the onset of AD as schematically represented in Fig. 1. We have already shown that gene expression may be repressed by remethylation [23].

2. Materials and methods

2.1. Cell culture

The SK-N-SH human neuroblastoma cell line was cultured in F14 medium with 8% foetal calf serum (growth medium, GM) or with 1% foetal calf serum and 10 μM retinoic acid (differentiation medium,

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Abbreviations: AD, Alzheimer disease; Ab, β-amyloid peptide; APP, amyloid precursor protein; PS1, presenilin 1; PS2, presenilin 2; SAM, *S*-adenosylmethionine

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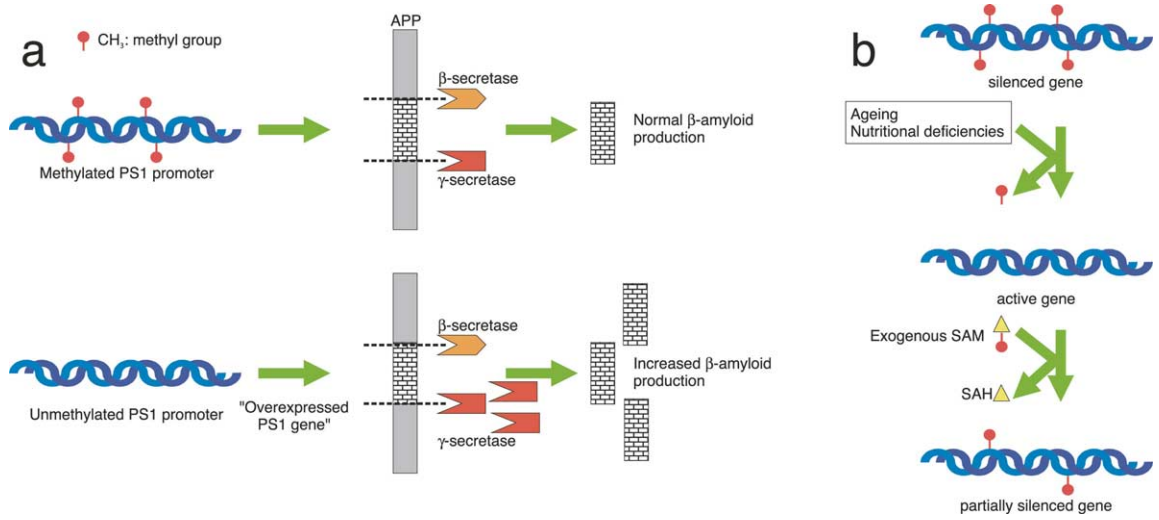

Fig. 1. Schematic representation of the relation between DNA demethylation and Ab production. a: Promoter demethylation causes the upregulation of PS1 expression and γ-secretase activity, with a consequent increase in β-amyloid production. b: DNA methylation can be reduced by ageing or nutritional deficiencies and is at least partially avoided or restored by SAM administration.

DM); 100 μM SAM was added to DM according to experimental design. Cultures were re-fed every second day; the times indicated are referred to medium shift (or change) as day 0. The experiment was repeated three times, giving similar results.

2.2. HPLC assays

Cell lysis, macromolecules precipitation and high performance liquid chromatography (HPLC) measurements were carried out as previously described [23].

2.3. Nucleic acid analysis

RNA and DNA extractions were performed by standard procedures [24]. Expression studies by reverse transcription polymerase chain reaction (RT-PCR) were performed on 1 μg of total RNA, using 50 pmol of oligo-d$(T)_{16}$ and 50 units of M-MuLV reverse transcriptase at 42°C for 1 h, as indicated by the manufacturer. PCR assays were carried out as previously described [23]; β-actin was used as a standard for the subsequent densitometric analysis. For each gene, we performed several amplification reactions with different numbers of cycles (20–40), choosing the reactions in the linear logarithmic phase for the semi-quantitative analysis [24].

DNA methylation assays were performed by multiplex *Hpa*II PCR [23,24]. Genomic DNA was treated separately with two restriction endonucleases: *Eco*RI, which has no recognition sites internal to the amplified gene region, and *Hpa*II, which has recognition sites internal to the 5′-flanking region and is methylation-sensitive (it fails to cut if the CCGG recognition sequence is methylated at any C). Intron 2, which possesses no *Hpa*II or *Eco*RI recognition sites, was used as the internal standard. *Eco*RI-digested samples were amplified to prove that the investigated regions could be amplified in all experimental conditions, thus demonstrating that the absence of a fragment was only dependent on *Hpa*II digestion. In each case, 2 μg of genomic DNA was digested overnight at 37°C with 5 units of enzyme, and then with 3 units more for an additional 6 h, in a final volume of 40 μl of appropriate buffer (Roche Diagnostics). PCR reactions were carried out as described above for the semi-quantitative analysis.

GenBank accession numbers, primers sequence and position, expected products and annealing temperatures are as follows:

1. β-actin (M10277): β-actF 5′-AAGAGAGGCATCCTCACCCT-3′ (nt 1405–1424, exon 3), β-actR 5′-TACATGGCTGGGGTGTTGAA-3′ (nt 2044–2063, exon 4); 218 bp, 58°C;
2. APP (Y00264): HSAPPP11 5′-GCTGGTGGAGACACACATGGCC-3′ (nt 1278–1299, exon 11), HSAPPM7 5′-GGATCTGAGCGGCTTTCTTGGG-3′ (nt 1480–1501, exon 12); 224 bp, 58°C;
3. PS1 (L42110): HSPS1P5 5′-ACGACCCCAGGGTAACTCCCG-3′ (nt 407–427, exon 5), HSPS1M1 5′-CTCTCTGGCCCACAGTCTCGG-3′ (nt 613–633, exon 6); 227 bp, 58°C;
4. PS2 (NM_000447): HSPS2P1 5′-GAAAGCCAGGGAGCATCATTCATTTAGCC-3′ (nt 257–285, exon 3), HSPS2M1 5′-GAAGTTCCAAGACAGTCAGCAAGAGGGTGG-3′ (nt 972–1000, exon 5); 744 bp, 62°C;
5. PS1 methylation (L76528): HSPS1P7 5′-TATAGGGGCTTTCGTCCTCAGCTCG-3′ (nt 481–505), HSPS1M11 5′-AGGTTCCTTCCAGACCAGCCG-3′ (nt 771–791), 5′-flanking, 312 bp; HSPS1P6 5′-GAAGTCCGCACGCCTCTTGTTCG-3′ (nt 1256–1278), HSPS1M12 5′-TCACCTCAATTCTCTCACCCATCCC-3′ (nt 1435–1459); internal standard, intron 2, 204 bp, 64°C.

2.4. Gel electrophoresis and analysis of PCR products

PCR products were examined by electrophoresis in 1.2% agarose gels. Each gel was acquired and analysed on a computerised densitometer (Fluor-S MultiImager, Bio-Rad). Fragment specificity was assessed by restriction analysis.

2.5. Western blot analysis

Cells were lysed with 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.2% Nonidet P-40, 1% CHAPS, 2 mM EDTA, phenylmethylsulfonyl fluoride (200 μM), leupeptin (1 μM), pepstatin A (1 μM), calpain inhibitor I (5 μM), all from Sigma. 20 μg of each protein extract was run on 6% (APP) or 12% (PS1 and PS2) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, then blotted onto nitrocellulose.

APP was detected by monoclonal antibody NCL-APP (Novocastra) recognising particularly the 110 kDa band. PS1 was detected by monoclonal antibody MAB 5232 (Chemicon) recognising a 18–20 kDa band. PS2 was detected by goat polyclonal antibody sc-1456 (Santa Cruz) recognising a 100 kDa band. 14-3-3β was detected by rabbit polyclonal antibody sc-629 (Santa Cruz) recognising a 30 kDa band.

2.6. ELISA test

Media were collected and concentrated using centrifugal filter devices (Centricon YM-3, Amicon). Ab(1–40) was analysed with Immunoassay Kit (BioSource International). All measurements were performed in duplicate.

3. Results and discussion

3.1. Rationale

The rationale of this study was to investigate which genes, involved in amyloid production, were regulated by methylation. Experiments were designed to show the regulation of gene expression, their methylation and protein synthesis after treatment with SAM. HPLC experiments in cell lysates from

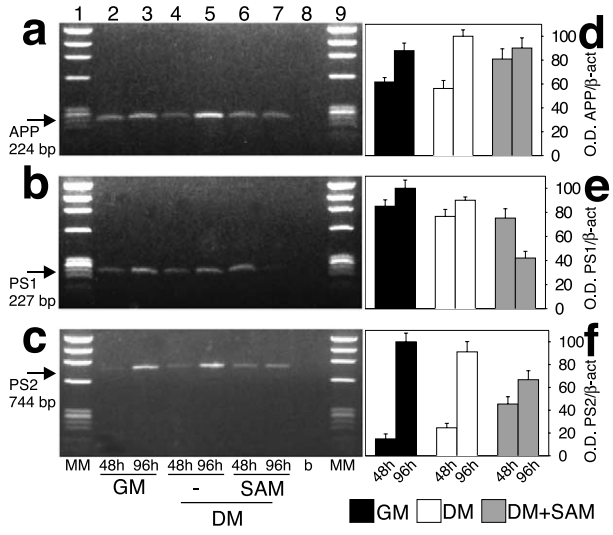

Fig. 2. SAM modulates presenilin RNA expression. Expression of APP (a), PS1 (b) and PS2 (c) after 48 and 96 h in GM (lanes 2,3), DM (lanes 4,5) and in DM in the presence of 100 μM SAM (lanes 6,7). d–f: Densitometric quantification of experiments as in a–c, respectively, obtained by optical density (O.D.) of PCR fragments normalised with O.D. of β-actin (not shown) (b: blank; MM: molecular weight marker).

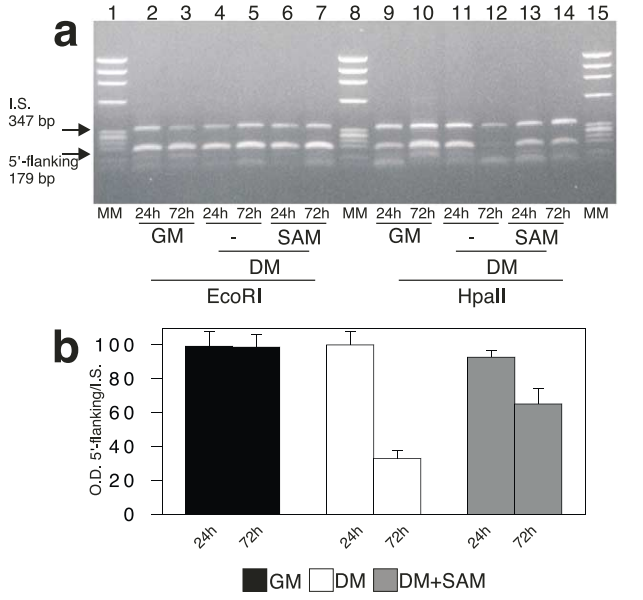

Fig. 3. SAM inhibits PS1 promoter demethylation. a: *Eco*RI panel, to the left, as positive control, and *Hpa*II panel, to the right, as methylation-sensitive experiments in GM (lanes 2,3 and 9,10), DM (lanes 4,5 and 11,12) and DM in the presence of 100 μM SAM (lanes 6,7 and 13,14). b: Densitometric quantification of O.D. of 5′-flanking PCR fragments normalised with O.D. of internal standard.

SAM-treated cultures showed a SAM uptake six times higher than controls (data not shown).

3.2. Regulation of gene expression

Gene expression was studied by RT-PCR as shown in Fig. 2 (typical RT-PCR assays as obtained from different cell experiments and amplifications). The right-hand panels show the semi-quantitative densitometric analysis of RNA expression; histogram bars are expressed as percentage ± S.E.M., with the highest value taken as 100%. APP, PS1 and PS2 showed a peak of expression at 96 h, both in GM and in DM. The expression pattern of these genes seemed to be independent of cell differentiation; only APP was slightly upregulated in DM. Presence of 100 μM SAM (lanes 6 and 7) did not alter the expression pattern of APP and PS2: the expression level was higher at 96 h. PS1 expression, in contrast (panel b, lane 7), was markedly downregulated (over 50%) at 96 h.

3.3. DNA methylation

APP and PS1 promoters have several CpG sites; we studied two of them in APP and four in PS1, with *Hpa*II/PCR. The two CCGG sites in the APP promoter (nt 3328–3331 and 3499–3502; accession number X12751) were found to be always unmethylated and three sites in the PS1 promoter (nt 612–615, 681–684 and 735–738) always methylated (data not shown). Only one site in the PS1 promoter (nt 451–454) was unmethylated or methylated according to the expression level of the gene, as evident in Fig. 3. Electrophoresis of the *Hpa*II/PCR products clearly shows that SAM greatly reduced demethylation normally occurring in DM after 72 h (lanes 12 and 14). The graph (b) shows the optical densities of 5′-flanking versus internal standard PCR products (*Hpa*II-digested samples); histograms bars are expressed as percentage ± S.E.M., with the highest value taken as 100%. This experiment reinforces the observation that SAM could inhibit RNA expression of PS1 by the methylation of its gene promoter.

3.4. Protein synthesis

In Fig. 4 are shown APP (a), PS1 (b) and PS2 (c) Western Blots of SK-N-SH cell extracts; the right-hand panels (d–f) show the optical density values normalised versus the optical densities of 14-3-3β signals (a–c, lower panels). Histogram bars are expressed as percentage ± S.E.M., with the highest value taken as 100%. The 110 kDa APP isoform increased in DM and was only slightly reduced by SAM (panels a

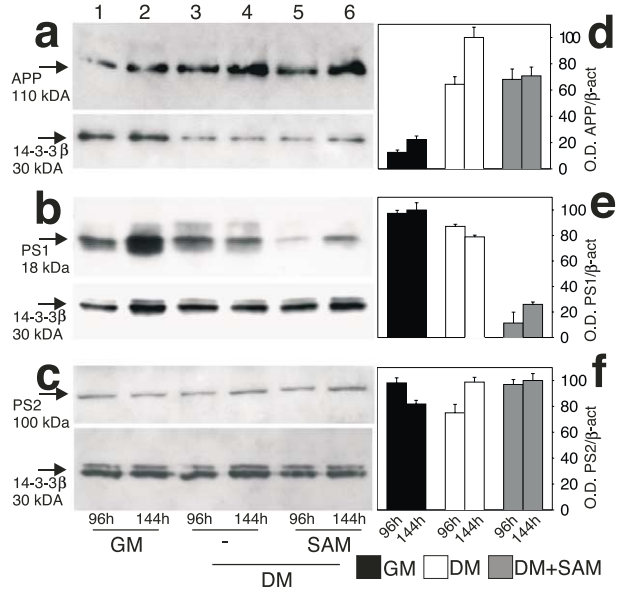

Fig. 4. SAM modulates protein synthesis in SK-N-SH. Protein levels of APP (a), PS1 (b) and PS2 (c) after 96 and 144 h in GM (lanes 1,2), DM (lanes 3,4) and in DM in the presence of 100 μM SAM (lanes 5,6). d–f: Densitometric quantification of experiments as in panels a–c (O.D. of protein bands normalised with O.D. of 14-3-3β bands).

and d). PS1 protein, in contrast, was synthesised at similar levels in GM and DM but was markedly reduced by SAM (panels b, lanes 5 and 6), in agreement with the results displayed in gene expression. PS2 protein synthesis seemed to be unmodified (panels c and f).

To confirm the efficacy of SAM treatment on β-amyloid processing, we wanted to demonstrate that PS1 promoter methylation, RNA downregulation and protein synthesis inhibition observed in the presence of SAM also resulted in a lowered secretion of Ab peptide. The final result of reducing β-amyloid production by six-fold is shown in Fig. 5.

3.5. Conclusions

The data presented in this paper suggest the possibility of therapeutically reducing Ab production. It is unclear whether Ab accumulation is due to its overproduction or to a clearance defect. However, the reduction of Ab formation has a good chance of preventing AD. We are convinced that the balance of presenilin activity or of their expression could be primarily responsible for Ab accumulation. The progressive SAM reduction observed in the elderly and the consequent methylation decrease, on the one hand, and our data showing that the PS1 gene is regulated by methylation, on the other, seem to substantiate our theory.

In *Hpa*II/PCR experiments on PS1 promoters, we showed that SAM downregulates PS1 expression, remethylating at least one CpG site. Under these conditions, Ab production was reduced about six-fold. PS1 is involved in Notch-1 cleavage [25], in neuronal differentiation [26] and probably in other physiological functions. We verified that SAM did not interfere with Notch-1 expression (data not shown). We cannot control correct Notch-1 processing, since protein levels in non-transfected cells are too low to be measured.

SAM acts in a physiological manner, partially silencing PS1, being a natural product and a basic body metabolite. We would like to stress that Ab accumulation in the central nervous system is a physiological change in ageing, excessive in AD, that should be lowered to levels not interfering with cognitive functions. The downregulation of PS1 expression by SAM is a mild intervention since a basal level of gene expression is maintained; this is in agreement with the slow Ab accumulation process in AD, due to minor changes in PS1 activity. This is a major point in favour of our method compared to the hypothesis of completely blocking PS1 activity.

The modulation of gene methylation processes could be the target for preventing AD or controlling the progression of the disease. The simplest explanation for HCY accumulation and SAM reduction in AD patients could be the reduction of B12, B6 and folic acid levels [27,28], since these vitamins are essential for the transformation of HCY to methionine. SAM reduction could be one of the main factors producing the typical metabolism modifications in ageing, indeed it is an emerging idea that other pathologies are associated with hyperhomocysteinaemia (e.g. vascular diseases).

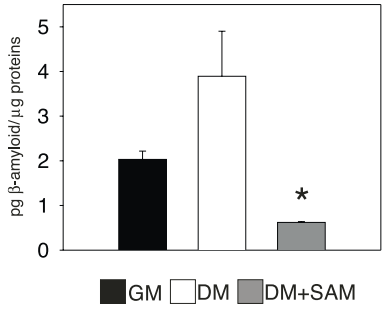

Fig. 5. Inhibition of β-amyloid(1–40) production. ELISA test performed on media collected from SK-N-SH cell cultures; $*P < 0.05$ compared to DM, $n = 4$ (ANOVA test).

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