Gene silencing by *S*-adenosylmethionine in muscle differentiation

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Abstract A well-characterised experimental system, the *myogenin* gene in C2C12 muscle cell culture, was chosen to better understand the methylation mechanism underlying the regulation of gene expression. We already demonstrated that demethylation dynamics of a specific CpG site in the 5′-flanking region of *myogenin* well correlates with gene expression and terminal differentiation. Here we demonstrate that *S*-adenosylmethionine-sulphate-*p*-toluenesulphonate (SAM) inhibits *myogenin* expression and myoblast differentiation by delaying the demethylation of specific CpG in differentiating myoblasts. These results suggest new perspectives in methylation mechanisms and the use of SAM in the partial silencing of gene expression, as it could be required in disease treatment. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: DNA methylation; *S*-Adenosylmethionine cycle; Transcription regulation; Gene silencing; Muscle differentiation

1. Introduction

The differentiation of muscle cells is a widely used system to study the regulation of gene expression. Most of the myogenic transcription factors are known and in particular the interaction between the basic helix–loop–helix (bHLH) myogenic factors, like *myogenin* [1] or *MyoD* [2], and the HLH repression factors like the *Id* [3] family, are known.

Many studies have demonstrated that DNA methylation plays a central role in the regulation of gene expression [4–6] but the exact molecular mechanism acting in this modulation is still not well understood [7]; in particular, little is known about the methylation of myogenic factors. The studies on methyl binding proteins [8], demethylase activity [9], and interaction of the methylation machinery with histone acetylation, established a connection between gene expression, CpG methylation and chromatin structure [10]. Besides regulating the expression of housekeeping and tissue-specific genes [11–13], methylation has also been shown to be involved in tumourigenesis [14–16], and senescence [17].

The involvement of the DNA methylation process with the regulation of gene expression in muscle differentiation has been demonstrated using 3-deazaadenosine, which blocks the *S*-adenosylmethionine (SAM) metabolic pathway inducing hypomethylation and promotes muscle differentiation [18,19]. Recently we demonstrated the tight correlation between the demethylation dynamics of a specific CpG site in the 5′-flanking region of *myogenin*, its expression and myoblast differentiation [20].

The possibility of silencing the genes regulated by DNA methylation, through the administration of exogenous SAM, could open a new pathway in the treatment of pathologies characterised by gene overexpression. Administering SAM to C2C12 cells under differentiating conditions, we found that myoblast differentiation was repressed without affecting cell growth; the repression is coherent with the inhibition of *myogenin* expression, although *Id3* expression is not affected. Finally the methylation of the 5′-flanking region was also studied with a *Hpa*II/polymerase chain reaction (PCR) multiplex assay. We showed that the demethylation dynamics of *myogenin* was delayed in cells exposed to SAM. For the first time it was possible to observe the direct silencing of a gene by administrating the methyl donor to the cells. The SAM uptake into cells, which was indicated as controversial [21], has been verified through a high performance liquid chromatography (HPLC) analysis [22].

2. Materials and methods

2.1. Chemicals

Restriction enzymes and oligo-d$(T)_{16}$ were purchased from Boehringer Mannheim (Mannheim, Germany). M-MuLV (Moloney murine leukaemia virus) reverse transcriptase (cloned), Super Taq (*Thermus thermophilus* DNA polymerase, licensed Hoffmann-La Roche), human placental ribonuclease inhibitor, buffer for reverse transcription, buffer for PCR and ΦX/*Hae*III marker were obtained from HT Biotechnology (Cambridge, UK). Oligonucleotides used as primers were synthesised by M-Medical Genenco (Firenze, Italy). All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

The C2C12 mouse muscle cell line [23] was cultured in F14 medium supplemented with 50 μg/ml neomycin and 10% foetal calf serum (growth medium, GM), or with 1% foetal calf serum (differentiation medium, DM) which induces differentiation with the appearance of myotubes and of creatine kinase (CK) activity, as previously described [24]. Flasks were coated with 0.2% gelatin to favour differentiation. 24 h after plating, cells were either shifted to DM, or reefed with GM according to the experimental design. SAM was added to DM according to the experimental design. Cultures were reefed every second day

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Abbreviations: GM, growth medium (with 10% foetal calf serum); DM, differentiation medium (with 1% foetal calf serum); CK, creatine kinase; SAM, *S*-adenosylmethionine; MM, molecular weight marker

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with the appropriate medium. The times indicated refer to medium shift (or change) as day 0.

2.3. Differentiation assay

Cells to be used for enzymatic test, either in GM or DM, were rinsed twice with phosphate buffered saline (PBS) and frozen at −80°C until used. The frozen cells were scraped into 1 ml of 50 mM Tris–HCl (pH 7.2) and 1 mM dithiothreitol, sonified for 15 s in ice and centrifuged. Supernatant was used for CK (EC 2.7.3.2) and total protein content assay [25]. The results were expressed as the average of at least three experiments in mU CK/μg proteins ± S.E.M.

2.4. HPLC analysis

Cell cultures were rinsed twice with PBS and frozen at −80°C. After thawing, cells were scraped into 1 ml of deionised water and sonified for 15 s in ice. The macromolecules were precipitated using 1.5 M PCA solution at 4°C for 1 h adjusting the pH to 4–5 with KOH and then centrifuged for 15 min at 9000×*g*. The supernatant was freeze-dried and later subjected to HPLC analysis [26]. The HPLC measurements were carried out using a Varian HPLC system (Varian, Walnut Creek, CA, USA) equipped with a ProStar 210 pump and a UV detector (ProStar 320) set to 254 nm.

Remote control of the HPLC system, data acquisition and calculation of peak areas were performed via computer-based data system (Varian Star 5.3). The samples were injected through an injection valve with a 20 μl sample loop. The mobile phase was pumped at a flow-rate of 1.00 ml/min. The samples were dissolved in water and injected onto the reverse-phase column (Alltima, C18-LL 100A 5u, length 150 mm, i.d. 4.6 mm).

The mobile phases were (A) 10 mM ammonium formate and (B) acetonitrile (HPLC grade, Aldrich). A 20 min gradient from 0–10% B followed by 90% A and 10% B for 20 min was applied.

Standard samples of SAM, SAH dissolved in water were run before and after the experimental samples.

2.5. RNA extraction and expression assay

Total RNA extraction was performed by the acidified phenol procedure [27,28]. For the expression studies by reverse transcription (RT)-PCR, we performed a reverse transcription with 1 μg of total RNA, using 50 pmol of oligo-d(T)$_{16}$ per sample, with 50 U per sample of M-MuLV reverse transcriptase at 42°C for 1 h, followed by heat inactivation at 94°C for 5 min. The total reaction volume was 20 μl per sample in the assay buffer as indicated by the manufacturer. The subsequent amplification reactions were carried out as previously described [20] for mouse *myogenin* (GenBank, accession number M95800), and for mouse γ-actin (GenBank, accession number L21996) cDNAs. For the amplification of *Id3* (GenBank, accession number M60523) cDNA we used MM*ID3*P5 (5′-CTACTCTCCACATGAAGGCGCTGAGCC-3′, nucleotides (nt) 46–73) as forward primer and MM*ID3*M1 (5′-TTTCTCCAAGGAAACCAGAAGAACAGC-3′, nt 784–810) as backward primer, obtaining an amplified fragment of 765 bp. The use of γ-actin as internal standard acted as control for the processing of equal quantity samples.

2.6. DNA extraction and methylation assay by multiplex HpaII/PCR

Genomic DNA was extracted using a standard phenol/chloroform method followed by ethanol precipitation [28]. Genomic DNA was treated separately with the following restriction endonucleases:

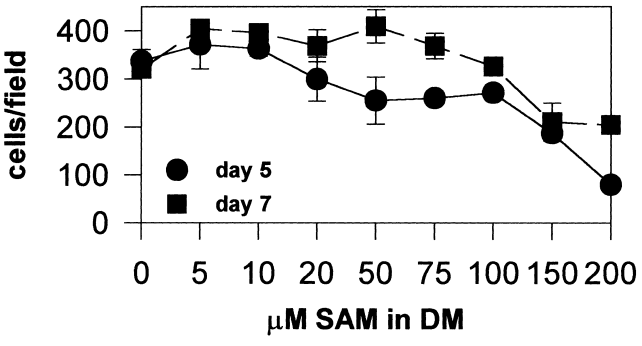

Fig. 1. Cell growth after 5 or 7 days in culture in differentiation medium.

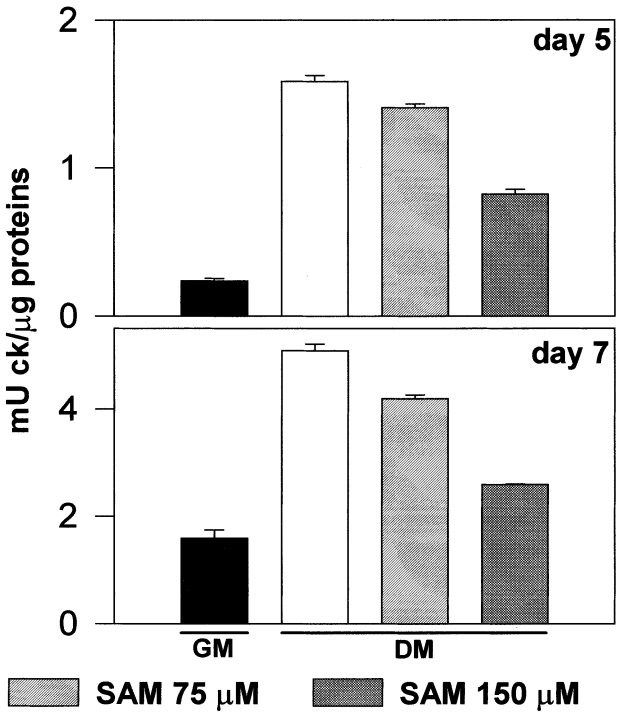

Fig. 2. Cell differentiation evaluated as CK activity after 5 (upper) or 7 (lower) days in culture in growth medium, in differentiation medium and in the presence of SAM.

(i) *Eco*RI, which has no recognition sites internal to the amplified fragments of *myogenin* gene; (ii) *Hpa*II, which has a recognition site internal to the 5′-flanking region and three recognition sites internal to exon 1, and is methylation-sensitive (i.e. it fails to cut if the CCGG recognition sequence is methylated at any C). Exon 3, which possesses no *Hpa*II or *Eco*RI recognition sites, was used as internal standard. In each case, 1.5 μg of genomic DNA were digested overnight at 37°C with 5 U of enzyme, and then with 3 U more for an additional 6 h, in a final volume of 40 μl of the buffer provided by the manufacturer. For the amplification of the mouse *myogenin* gene, we used the primers and the conditions previously described [20].

2.7. Gel electrophoresis and analysis of PCR product

Aliquots of the PCR products (15 μl) were examined by electrophoresis in 1.5% agarose gel. Each gel was scanned by a CCD camera and acquired on the BioImage computerised densitometer. The specificity of the fragments was assessed by restriction analysis.

3. Results and discussion

Cells were exposed to SAM at concentrations ranging from 5 to 200 μM in DM. The cultures were stopped at day 5 and day 7 after the shift to the SAM-containing medium. The growth resulted slightly higher than in controls at the lower concentrations and decreased in a dose-dependent manner with a three-fold reduction in SAM 200 μM at day 7 (Fig. 1). Up to the concentration of 150 μM SAM, the growth rate was almost unchanged.

Unlike the growth, myoblast differentiation, evaluated as percent of fused nuclei, was inhibited by SAM at the concentration of 20 μM (data not shown). The inhibition increased at higher concentrations. Therefore, SAM seemed to inhibit differentiation without affecting cell growth at concentrations below 150 μM.

Given this data, we chose the concentrations of 75 and 150 μM for the following experiments, judging it a good compro-

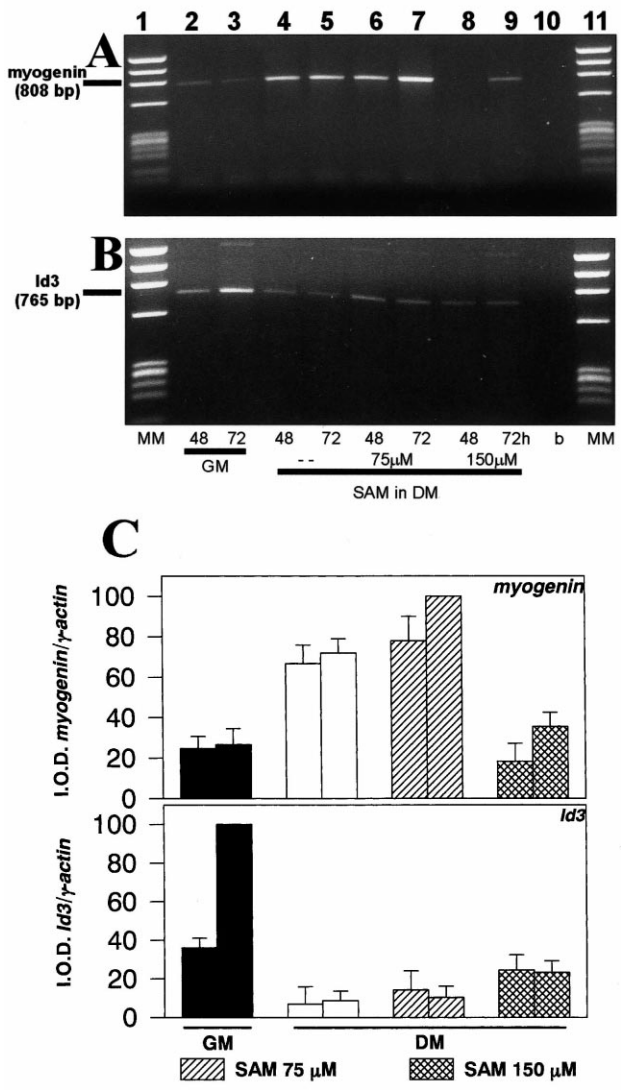

Fig. 3. Expression of *myogenin* (A) and *Id3* (B) after 48 and 72 h in culture in GM (lanes 2 and 3), in DM (lanes 4 and 5), and in DM in the presence of 75 μM SAM (lanes 6 and 7) and 150 μM SAM (lanes 8 and 9). In lane 10 there is a negative control of the amplification (b) and in lanes 1 and 11 a ϕX/*Hae*III molecular weight marker (MM) is shown. C: Graphic representation of *myogenin* (upper) and *Id3* (lower) expression, obtained plotting the optical density (OD) values of the PCR fragments normalised with the OD values of γ-actin (not shown).

mise and borderline conditions between the inhibition of differentiation and a normal cell growth. We performed further differentiation assays comparing the CK activity in cells exposed to 75 and 150 μM of SAM, with control cells cultivated in GM or DM (Fig. 2).

To assess the SAM effective cellular uptake, we performed HPLC assays on cell extracts from myoblasts cultivated 48 h with or without 100 μM SAM in DM, verifying a three-fold increase in SAM-exposed cells.

Myogenin expression in cells cultured in the presence of 75 and 150 μM SAM for 48 or 72 h, is shown in Fig. 3A. As already demonstrated [20] the expression at both times was lower in GM than in DM (lanes 2–5). 75 μM SAM did not affect the transcription (lane 6) although it affected differentiation, on the contrary transcription seemed slightly higher than in DM at a later time (lane 5–7). *Myogenin* expression in the cells treated with 75 μM SAM is apparently in contrast with differentiation data. Since it has already been established that myogenin levels reflect mRNA synthesis [29], such a contrast could either lie in the post-transcriptional regulation or could be explained by the involvement of other gene(s) which contribute to the regulation of terminal muscle differentiation. 150 μM SAM, however, sharply repressed the transcription of *myogenin* (lanes 8 and 9). For further confirmation of these results, we also investigated the expression of *Id3*, a negative regulator of differentiation (Fig. 3B). It was expressed at high levels in GM (lanes 2 and 3) and poorly expressed in DM (lanes 4 and 5). SAM did not have an effect on *Id3* as it did on *myogenin*. Nevertheless the transcription of *Id3* in DM was enhanced (lanes 6–9). These data confirmed the involvement of the modulation of gene expression in SAM-mediated differentiation inhibition. Fig. 3C, shows the plotted integrated optical density values of signals in previous electrophoresis, normalised to γ-actin.

We performed the *Hpa*II/PCR assay to investigate the effect of SAM on *myogenin* gene methylation (Fig. 4). The fragment of 499 bp, amplified from the 5′-flanking region of the gene, has a single CCGG site that was fully methylated in GM (lanes 11 and 12) and in DM after 24 h, but becomes completely demethylated in DM after 48 h (lanes 13 and 14). Samples extracted from cells grown in the presence of SAM, both 75 and 150 μM, show the amplification of this fragment, indicating that the investigated site did not undergo demethylation until 48 h (lanes 15–18). The fragment of about 990 bp visible in lanes relative to *Eco*RI-digested samples (lanes 2–9) and in some *Hpa*II-digested samples, was due to the amplification of a fragment recognised by MyoP1 and MyoM7 primers, but it did not affect the efficiency of the reaction. We already demonstrated that this CCGG site appears demethylated in C2C12 cells in DM at 48 h, and that culture conditions resulting in higher *myogenin* expression and myoblast differentiation, also result in earlier demethylation [20]. Here we demonstrate that the repression of differentiation and inhibition of *myogenin* expression, correlate in a delay of methylated conditions, and that SAM carries out its effects, inhibiting the demethylation in a specific manner. These results also stress the hypothesis that active demethylation acts in balance with AdoMet-dependent methylation; in fact the inhibitors of the AdoMet cycle can anticipate the demethylation whereas SAM can delay it. SAM [20] may be considered as a drug

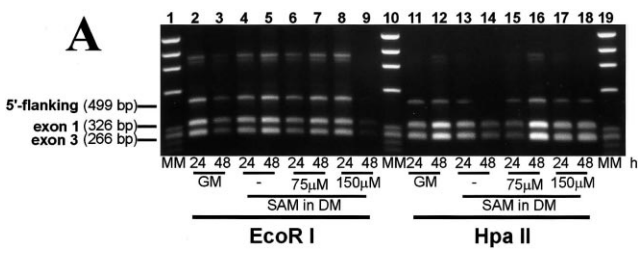

Fig. 4. *Myogenin* methylation patterns in C2C12 cells. On the left is the *Eco*RI panel, as positive controls, and on the right the *Hpa*II panel, as methylation-sensitive experiments in GM (lanes 2, 3, 11 and 12), DM (lanes 4, 5, 13 and 14), DM in the presence of 75 μM SAM (lanes 6, 7, 15 and 16) and DM in presence of 150 μM SAM (lanes 8, 9, 17 and 18). In lanes 1, 10 and 19 a ϕX/*Hae*III MM is shown.

with a well-characterised action, without relevant side-effects, so that it can be assumed that it does not carry out a widespread methylation, which could result in a toxic behaviour.

The observed effects, increase the hypothetical use of SAM in the treatment of diseases characterised by abnormal expression of genes independent by sequence mutation; in particular we consider the possibility that SAM can also act in modulating the expression of genes involved in some pathologies.

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