Biochemical and Biophysical Research Communications 377 (2008) 935–940

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Methyltransferase-inhibition interferes with neuronal differentiation of P19 embryonal carcinoma cells

Sukchul Hong [a], Jieun Heo [a], Sangjin Lee [a], Seok Heo [a], Sung-Soo Kim [b], Young-don Lee [b], Moosik Kwon [a], Sungyoul Hong [a,*]

[a] *Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea*
[b] *Departments of Anatomy, Ajou University School of Medicine, Suwon 443-749, Republic of Korea*

ARTICLE INFO

Article history:
Received 6 October 2008
Available online 26 October 2008

Keywords:
Methylation inhibition
Neuronal differentiation
Cell–cell interaction
P19 embryonal carcinoma
Retinoic acid

ABSTRACT

We have analyzed the importance of substrate methylation by *S*-adenosylmethionine-dependent methyltransferases for neuronal differentiation of P19 embryonal carcinoma cells. We show that treatment of cells with methyltransferase inhibitor adenosine dialdehyde (AdOx) interferes with neuronal differentiation. Retinoic acid (RA) and AdOx co-treated cells had a decreased number of neurites and a flattened morphology compared with cells differentiated by RA. Also, the amount of neuronal class III tubulin (Tuj1) decreased from 76% to 9.6% with AdOx-treatment. Gene expression levels of wnt-1, brn-2, neuroD, and mash-1 were also down-regulated by AdOx-treatment. But AdOx-treatment did not up-regulate BMP-4 and GFAP genes. Treatment of RA decreased E-cadherin expression during neuronal differentiation. However, in AdOx/RA co-treated cells, E-cadherin expression was restored to the control level. Also, mRNA expression of N-cadherin decreased with AdOx-treatment. Taken together, these data show that methylation reactions might influence the cell-fate decision and neuronal differentiation of P19 cells.

© 2008 Elsevier Inc. All rights reserved.

Methylation has many biological roles [1]. *S*-Adenosylmethionine (AdoMet) is a universal methyl donor in a wide variety of biological methylation reactions. *S*-Adenosylhomocysteine (AdoHcy) is formed after donation of the methyl group of AdoMet to a methyl acceptor, and is subsequently hydrolyzed to adenosine (Ado) and L-homocysteine (Hcy) by AdoHcy hydrolase [2]. One of the most important functions of AdoMet is as the methyl donor for nucleic acids. Genes can be inactivated by methylation of CpG loci, and this is sometimes associated with cellular differentiation [3]. Protein methylation is ascribed to repair functions of cell membranes, to stress adaptations or to cell motility [4]. The methylation reaction performed by AdoMet-dependent methyltransferases leads to the generation of two products: the methylated substrates and the by-product AdoHcy [5]. It is important to note that AdoHcy by itself functions as a potent inhibitor of AdoMet-dependent methyltransferases and thus must to be further broken down into adenosine and homocysteine by the enzyme *S*-adenosylhomocysteine hydrolase (SAHH). Inhibition of SAHH in turn leads to inhibition of all AdoMet-dependent methyltransferase reactions due to an increase in the intracellular concentration of AdoHcy [6]. Since attenuation of SAHH can affect methylation of phospholipids, proteins, DNA, RNA, and small molecules such as norepinephrine, it is an attractive pharmacological target [7,8]. One of the potent inhibitors of SAHH and therefore of AdoMet-dependent methyltransferases is adenosine dialdehyde (AdOx) [9]. Application of AdOx leads to inactivation of SAHH and therefore functions as a general inhibitor of AdoMet-dependent methyltransferases.

Neural development includes a cascade of genetic programs that precisely control stage-specific gene activities required for neural patterning, cell migration, and neuronal connectivity [10]. Proper regulation of stage-specific gene expression in the nervous system is not only controlled by the transcriptional machinery, but is also subject to modulation by epigenetic mechanisms such as DNA and protein methylation, histone modification, nucleosome, and chromatin remodeling.

P19 cells are embryonal carcinoma (EC) cells that serve as a model for studying differentiation processes, including commitment to cell lineage [11]. Following cell aggregation in the presence of retinoic acid (RA), P19 cells differentiate into a neuroectodermal lineage of neurons and glial cells. In a procedure that eliminates all dividing glial cells, a homogenous post-mitotic neuronal culture is maintained [12]. In the present study, we analyzed the effects of methylation inhibition by AdOx during P19

Abbreviations: AdOx, adenosine dialdehyde; bHLH, basic helix-loop-helix; BMP-4, bone morphogenetic protein 4; GFAP, glial fibrillary acidic protein; SAHH, *S*-adenosylmethionine hydrolase; AdoMet, *S*-adenosylmethionine; RA, retinoic acid; Tuj1, neuronal class III tubulin.

* Corresponding author. Fax: +82 31 290 7895.
E-mail addresses: syhong@skku.edu, syhong@skku.ac.kr (S. Hong).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.10.089

neuronal differentiation. We demonstrate that AdOx interferes with neuronal differentiation by causing morphological changes and by reducing gene expressions related to neuronal differentiation. AdOx also affected aggregate-formation and cadherin mRNA expression during neuronal differentiation.

Materials and methods

Cells and reagents. Mouse P19 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Chemicals including *all-trans* RA and AdOx, were purchased from Sigma Chemical Co. (St. Louis, MO), and all cell culture reagents including Dulbecco's Modified Eagle's Medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY).

Cell culture and differentiation. P19 embryonal carcinoma (EC) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen, San Diego, CA), in humidified 5% CO_2. Differentiation of P19 cells into neural cells were induced to form aggregates in bacterial-grade Petri dishes (SPL, Korea) with 1 μM of *all-trans*-retinoic acid (Sigma, St. Louis, MO) at a density of 1×10^5/ml in DMEM containing 5% FBS. After 4 days of RA-treatment, aggregates were dissociated by 0.25% trypsin-0.53 mM EDTA (Invitrogen, San Diego, CA), and re-plated in poly-D-lysine-coated (0.1 mg/ml) tissue culture dishes and then allowed to differentiate for 4–8 days. For AdOx-treated cells, 1 μM of AdOx was added to P19 cells with RA on the first day of induction and removed after 48 h. After 2 days of induction with reagents, the media was changed to fresh medium containing 1 μM RA.

Immunocytochemistry. After forming aggregates, dissociated P19 cells were plated on poly-D-lysine coated coverslip and allowed to differentiate into neurons for 3 days. Then, cells were fixed with 4% paraformaldehyde in PBS, dehydrated with 5% acetic acid in methanol, and permeabilized with Triton X-100/Tris-buffered saline. P19 cells were then stained with monoclonal antibody against a neural class III β-Tubulin (Tuj1) (1:500) (Covance, Princeton, NJ). The fluorescent-labeled antibody rhodamine-anti-mouse IgG (Chemicon, Billerica, MA, 1:200) was used as a secondary antibody. Hoechst 33258 was used to stain the nuclei in order to count the cell numbers. Quantification of percentage of cells immunoreactive for Tuj1 antigens was determined by capturing images random fields. Hoechst-staining nuclei and cells positive for the Tuj1 protein were counted.

Immunoblotting. P19 cells were homogenized in lysis buffer, containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride. After protein concentration was determined by the Bradford's method [13], the lysates were boiled in Laemmli sample buffer for 3 min, and 30 μg of protein were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) [14] on 15% slab gels. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, American Life Sciences, IL), and the membranes were blocked for 2 h in TBS, containing 0.1% Tween 20, and 5% (w/v) dry skim milk powder. The membranes were then incubated overnight with primary antibodies against unique β-Tubulin (βIII) or β-catenin. The membranes were then washed with TBS-T and incubated for 1 h with anti-mouse secondary antibody. (Zymed Laboratories, San Francisco, CA). Bound antibodies were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience, Pittsburgh, PA).

Reverse transcription polymerase chain reaction (RT-PCR). After harvesting 1×10^6 cells, total RNAs were obtained using TRIZOL reagent (Gibco-BRL). After the amounts of total RNAs were determined and RNAs were electrophoresed on formaldehyde-agarose gels, cDNA synthesis was performed using MMLV RTase (Rexgene, Korea). The polymerase chain reaction was performed with Super Taq polymerase (Rexgene, Korea) in a 20-μl reaction mixture containing 2 μl of reaction buffer, 1 μl of 5 mM dNTPs, 1 μl each of forward and reverse primers, and 1 μl of cDNA for the template. The sequences of primers and PCR reaction conditions are shown in Table 1.

Results

Inhibition of methylation by adenosine dialdehyde interferes with neuronal differentiation of P19 cells

To assess the effects of methylation inhibition on P19 cell neuronal differentiation, the morphology of P19 cells was observed during RA stimulation (Fig. 1). P19 cells formed many large aggregates in bacterial-grade dishes with RA stimulation (Fig. 1A), whereas P19 cells treated with AdOx for 48 h with RA formed few small aggregates, which had rough surfaces due to the sparse density of aggregates (Fig. 1B). At day 8, RA-stimulated P19 cells showed characteristic neuron morphology, *i.e.*, rounded cell bodies with long processes (Fig. 1C). However, AdOx-treated cells that were taken through the neuronal differentiation procedure showed an abnormal pattern of differentiation. At day 8 of differentiation, AdOx-treated cells showed decreased numbers of neurites and flattened and expanded morphology compared with RA-stimulated cells (Fig. 1D). The flattened morphology was similar to epithelial cells so inhibition of methylation during early neurogenesis of P19 cells seems to be important in the cell-fate determination. These findings indicate that methylation inhibition by AdOx interferes with neuronal differentiation of P19 cells. In order to confirm the importance of methylation in the neuronal differentiation process of P19 cells, immunocytochemistry was performed using antibody against neuronal class III tubulin (Tuj1). Neuronal differentiation induction conditions were the same as those described previously. After undergoing neuronal differentiation for 7 days, cells were fixed for immuno-staining 76% of RA-stimulated P19 cells expressed Tuj1 protein (Fig. 1K) and had extended neurites (Fig. 1E and G), whereas only 9.7% of Tuj1-positive cells (Fig. 1K) were detected and no neurites were observed in cultures of AdOx/RA co-treated P19 cells (Fig. 1H and J) in spite of the similar cell-density to RA-only treated cultures (Fig. 1F,I). These results correlate with the morphology observed in Fig. 1A–D. The levels of Tuj1 expression on day 4 were also determined by probing an immunoblot with antibody that recognizes Tuj1 (Fig. 1L). In RA-treated cultures, Tuj1 expression increased in a time-dependent manner. However, at 4d, AdOx/RA co-treated cultures showed dramatically decreased expression of Tuj1 compared with RA-treated cultures (Fig. 1L, Box, lane 3 and 5). These results indicate that methylation is importantly involved in the neuronal differentiation processes.

Inhibition of methylation attenuates transcription of developmental-regulatory genes during neuronal differentiation of P19 cells

The reduced expression of Tuj1 protein in AdOx-treated cells showed that blocking methylation for the first 2 days of the neuronal differentiation procedure inhibited neuronal differentiation of P19 cells. To further explore the molecular mechanisms, RT-PCR was used to examine the expression pattern of those developmental-regulatory genes, such as RARβ2, Oct-3, Wnt-1, Brn-2, NeuroD, and Mash-1 in P19 cells. Co-treatment of AdOx and RA for the first 2 days resulted in the down-regulated mRNA expression of neuron-positive markers such as Wnt-1, Brn-2, NeuroD, and Mash-1 (Fig. 2A). AdOx/RA co-treated cells also showed up-regulation of

Download English Version:

https://daneshyari.com/en/article/1935254

Download Persian Version:

https://daneshyari.com/article/1935254

Daneshyari.com