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Histone deacetylase inhibitor (HDACi) upregulates activin A and activates the Smad signaling pathway in melanomas

Jeeyong Lee, Jiwon Ko, Jae Youn Yi*

Division of Basic Radiation Bioscience, Korea Institute of Radiological and Medical Sciences, Seoul, 01812, Republic of Korea

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ABSTRACT

Background: Histone deacetylase (HDAC) is an enzyme that regulates gene expression, cell cycle arrest, apoptosis and modulation of various pathways. HDAC inhibitors (HDACis) can modulate these pathways by hyper-acetylating target proteins, thereby acting as cancer chemotherapeutic agents.
Objective: One of HDACis, suberoylanilide hydroxamic acid (SAHA), has been found to regulate the Smad signaling pathway, by an as yet unclear mechanism. This study therefore investigated the mechanism by which SAHA regulates Smad signaling in the melanoma cell line SK-Mel-5.
Methods: Cell proliferation was assessed by MTT assays and fluorescence activated cell sorter (FACS) analysis. The activation of Smad signaling pathway was assessed by western blots analysis. The transcriptions of target genes were checked by RT-PCR and dual luciferase assay.
Results: Treatment with SAHA inhibited the proliferation of SK-Mel-5 cells, enhanced the phosphorylation of R-Smad, and up-regulated p21 protein. Surprisingly, R-Smad was also activated by conditioned medium from SAHA-treated SK-Mel-5 cells. An analysis of the conditioned medium showed that activin A was responsible for the activation of R-Smad. SAHA treatment enhanced the level of activin A mRNA, increasing the level of activin A in the secretome. The activity of the SAHA-treated secretome could be eliminated by pre-incubation with antibody to activin A. In addition, activin A supplemented medium could mimic the effect of the SAHA-treated secretome.
Conclusion: These findings indicate that the anti-cancer function of SAHA is mediated, at least in part, by the upregulation of activin A.

1. Introduction

Protein function is affected by post-translational modifications, including phosphorylation, methylation and acetylation [1]. Levels of protein acetylation are controlled by two enzymes: histone acetyltransferase (HAT) and histone deacetylase (HDAC) [2]. Although HAT is involved primarily in histone acetylation, it also acetylates non-histone proteins, including p53, STAT3 and microtubules [3–5]. Acetylation status, in turn, affects gene transcription.

Various types of cancer show abnormal acetylation profiles [6]. For example, HDAC is overexpressed in cutaneous T-cell lymphomas (CTCL) and acute myeloid leukemia (AML), resulting in a decrease in acetylation state (hypo-acetylation) [7]. Because hypo-acetylation may reduce the expression of both pro-apoptotic and tumor suppressor genes, histone deacetylase inhibitors (HDACis) may act as novel anticancer agents. Drugs that affect the activity of HDAC include MS-275, TSA, TPX, valproic acid, depsipeptide, and suberoylanilide hydroxamic acid (SAHA) [8]. Because HDACis inhibit HDAC activity, they induce various cellular activities, including apoptosis, cell cycle arrest, generation of reactive oxygen species (ROS), inhibition of angiogenesis, and autophagy [8]. Although some HDACis are moderately toxic to normal cells, they show selectivity against several tumor types. For example, SAHA and other HDACis have shown selective toxicity toward malignant cells while sparing surrounding normal cells [9].

Melanoma is one of the most aggressive types of skin cancer, highly resistant to chemotherapeutic agents and with a low patient survival rate [10]. Targeted therapies, such as vemurafenib, dabrafenib, and trametinib, have greater initial impact on overall survival, but patients usually relapse within 6 months [11]. HDACi,

* Corresponding author at: Division of Basic Radiation Bioscience, Korea Institute of Radiological and Medical Sciences, 75 Nowon-ro, Nowon-gu, Seoul, 01812, Republic of Korea.
E-mail address: yjy_71@kcch.re.kr (J.Y. Yi).

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SAHA, was recently reported to synergistically augment apoptotic events when used simultaneously with other anticancer treatments, including conventional chemotherapies agents and radiation [12], suggesting that HDACis may be useful in treating melanoma patients. The mechanism by which HDACis induce cell cycle arrest has been shown to involve the acetylation of p53 [13]. Melanoma acquisition of drug resistance has been found to be accompanied by cross-resistance to apoptosis [14]. Pretreatment of drug-resistant melanomas with an HDACi restored their sensitivity to apoptosis by inducing apoptotic gene programs. Melanoma cross-resistance to apoptosis may be mediated by secreted molecules, suggesting that HDACi-induced apoptosis may be due to the ability of HDACis to alter the melanoma secretome. However, the precise mechanism underlying the ability of HDACis to induce cell cycle arrest remains to be clarified.

This study was therefore designed to analyze the effects of the HDACi SAHA on the secretome of a melanoma cell line. SAHA-treated medium induced R-Smad phosphorylation and downstream p21 activation, suggesting that alterations in the secretome may play a significant role in SAHA-mediated cellular effects. These findings may help reveal the detailed mechanism by which SAHA induces cell cycle arrest, and may contribute to the use of HDACis in the treatment of patients with refractory melanoma.

2. Materials and methods

2.1. Reagents and antibodies

TGF-beta1 was purchased from R&D Systems (USA), SB431542 from Tocris Bioscience (UK), and activin A from PeproTech (USA). SAHA (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) and used at the indicated concentrations. Antibodies against p21, phospho-Smad2, Smad2, and Smad3 were purchased from Cell Signaling Technologies (USA); antibodies against phospho-Smad3 and activin A from Abcam (UK); and antibody against β-actin from Santa Cruz Biotechnology (USA).

2.2. Cell culture

SK-Mel-5, A375, Mv1Lu, R1B, DR26 and 293T cell lines were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and ZellShield™ (Minerva Biolabs GmbH, USA) in a humidified incubator containing 5% CO_2. Where indicated, cells were treated with 0.5 ng/ml TGF-beta1 or 50 ng/ml activin A for 1 h.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays

Six well plates were prepared with the indicated cell lines. After the appropriate treatment, the samples were incubated for 3 days. Subsequently, 20 ml MTT solution (Amresco, USA, 5 mg/ml) was added and the incubation was continued for another 4 h. Finally, the medium was carefully removed, 200 μl dimethylsulfoxide (DMSO) was added and the optical density of each well was measured at 570 nm with a microplate reader. The results reported are the average of triplicate samples.

2.4. Western blot analysis

Cells were extracted with lysis solution (Cell Signaling Technology), and the protein concentrations of the lysates were determined using a BCA Protein Assay kit (Pierce, USA) according to the manufacturer's instructions. Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8–12% gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.2% Tween-20 (PBST) for 1 h, and incubated with primary antibodies overnight at 4 °C. After three washes with PBST for 1 h, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Protein bands were detected with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotechnology, UK) and X-ray film (AGFA, Belgium).

2.5. Fluorescence activated cell sorter (FACS) analysis

For analyzing the cell cycle distribution, SK-Mel-5 cells were treated with SAHA (0.4 nM) and incubated for 24 h. Subsequently, the cells were trypsinized and washed with ice-cold PBS. Seventy percent chilled ethanol was used to fix the cells for 30 min at 4 °C. Cells were rewashed with chilled PBS twice and resuspended in 500 μl of PBS containing 100 μg/ml RNase and 40 μg/ml of propidium iodide. Each sample was incubated at 4 °C for overnight. Becton–Dickinson fluorescence-activated cell sorter (FACS) was used to perform flow cytometric analysis using CellQuest software (Becton Dickinson, USA).

2.6. Treatment with conditioned medium

The SK-Mel-5 and 293T (control) cell lines were cultured in serum-free medium and treated with 2 nM SAHA for 0, 4 and 24 h. The media were collected and debris removed by brief centrifugation. These conditioned media were incubated with 293T, Mv1Lu, and Mv1Lu derivative cell lines.

2.7. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultures of SK-Mel-5 cell lines using TRI Reagent (Molecular Research Center, USA) according to the manufacturer's instructions. Briefly, melanoma cell samples were extracted in 1 ml of TRI Reagent, mixed with 0.2 ml of chloroform, and centrifuged at 12,000 X g for 15 min at 4 °C. The aqueous phase containing RNA was transferred to a new tube and mixed with 0.5 ml isopropyl alcohol to precipitate RNA, with the latter recovered by centrifugation at 12,000 X g for 10 min at 4 °C. The RNA pellet was washed briefly in 1 ml of 75% ethanol and centrifuged at 7500 × *g* for 5 min at 4 °C. Each resulting total RNA pellet was dissolved in 0.1% diethyl pyrocarbonate (DEPC)-treated water, and its quality and quantity were assessed. cDNA was prepared from total RNA by reverse transcription using Superscript II Reverse Transcriptase (Invitrogen, USA) and diluted to 20 ng/μl. All PCR reactions were performed with the primers GDF-1 (sc-39764), GDF-3 (sc-39766), activin-A (sc-39783), activin-B (sc-43861), GDF-11 (sc-44724) and Nodal (sc-45478) (Santa Cruz Biotechnology, USA) and primers for GAPDH, 5′-ACCACAGTC-CATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). Thermal cycling conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 10 s and extension at 72 °C for 30 s. The final reaction mixtures were analyzed with 2% agarose gel.

BioRad CFX96 (Bio-Rad Laboratories, USA) was used for qRT-PCR analysis. Each reaction was done in a 20 μl reaction buffer containing AMPIGENE® qPCR Green Mixes (Enzo Life Sciences, USA) with 400 nmol/l activin A or GAPDH primers. Triplicate reactions were performed using a program of 95 °C for 5 s and 60 °C for 30 s (40 cycles). The quantification of the samples was calculated using the comparative cycle threshold (ΔΔCt) method.

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