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Suberoylanilide hydroxamic acid (SAHA) inhibits EGF-induced cell transformation via reduction of cyclin D1 mRNA stability

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ABSTRACT

Suberoylanilide hydroxamic acid (SAHA) inhibiting cancer cell growth has been associated with its down-regulation of cyclin D1 protein expression at transcription level or translation level. Here, we have demonstrated that SAHA inhibited EGF-induced Cl41 cell transformation via the decrease of cyclin D1 mRNA stability and induction of G0/G1 growth arrest. We found that SAHA treatment resulted in the dramatic inhibition of EGF-induced cell transformation, cyclin D1 protein expression and induction of G0/G1 growth arrest. Further studies showed that SAHA downregulation of cyclin D1 was only observed with endogenous cyclin D1, but not with reconstitutionally expressed cyclin D1 in the same cells, excluding the possibility of SAHA regulating cyclin D1 at level of protein degradation. Moreover, SAHA inhibited EGF-induced cyclin d1 mRNA level, whereas it did not show any inhibitory effect on cyclin D1 promoter-driven luciferase reporter activity under the same experimental conditions, suggesting that SAHA may decrease cyclin D1 mRNA stability. This notion was supported by the results that treatment of cells with SAHA decreased the half-life of cyclin D1 mRNA from 6.95 h to 2.57 h. Consistent with downregulation of cyclin D1 mRNA stability, SAHA treatment also attenuated HuR expression, which has been well-characterized as a positive regulator of cyclin D1 mRNA stability. Thus, our study identifies a novel mechanism responsible for SAHA inhibiting cell transformation via decreasing cyclin D1 mRNA stability and induction of G0/G1 growth arrest in Cl41 cells.

Introduction

Histone deacetylase (HDAC) inhibitor is one of the most important classes of anticancer agents. Suberoylanilide hydroxamic (SAHA), one of the well-known HDAC inhibitors, has been shown with highly effective in inducing cell growth arrest and apoptosis, particularly in cancer cell lines in comparison to relative normal cells (Ungerstedt et al., 2005). Accumulated evidence indicates that SAHA exerts its inhibition of cancer cell growth through both histone-dependent and histone-independent mechanisms (Bolden et al., 2006). SAHA treatment increases p21 transcription via the accumulation of acetylated histones H3 and H4, by which SAHA mediates the cancer cell growth arrest (Gui et al., 2004). It has also been reported that SAHA treatment promotes cell apoptosis by induction of p53 protein acetylation and the protein stability in cancer cell lines (Kai et al., 2010). However, the potential chemopreventive effect of SAHA on skin carcinogenesis has not yet been reported. Thus, we evaluated the potential inhibitory effect of SAHA on EGF-induced cell transformation in Cl41 cells.

Cyclin D1 overexpression has been observed in many human cancer tissues, including breast cancer, lung cancer, pancreatic cancer, and non-melanoma skin cancer, particularly in keratoacanthomas and squamous cell carcinomas (SCCs) (Musgrove et al., 2011). In addition, cyclin D1 in carcinogenesis has been well demonstrated in previous studies (Donnellan and Chetty, 1998). We have also found that knockdown of cyclin D1 impairs cell transformation induced by environmental carcinogens in mouse skin epidermal Cl41 JB6 cells and human immortalized keratinocyte HaCaT cells (Ding et al., 2009; Ouyang et al., 2008; Zhang et al., 2009). Cyclin D1 plays a critical role in cell growth and proliferation through regulation of cell cycle progression (Resnitzky and Reed, 1995). Cyclin D1 forms complexes with CDK4 and CDK6, which is an essential in G1/S progression. Cyclin D1 also activates cyclin E/CDK2 and indirectly inactivates the function of tumor suppresser gene retinoblastoma protein (Fu et al., 2004). Previous studies indicate that SAHA treatment downregulates cyclin D1 via repressing cyclin D1 transcription and translation (Kawamata et al., 2007; Yamaguchi et al., 2005). Our current studies demonstrate

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that SAHA inhibits EGF-induced Cl41 cell transformation via decreasing cyclin D1 mRNA stability and induction of G0/G1 growth arrest.

Materials and methods

Cell culture and reagents. Mouse epidermal Cl41 cells and their transfectants were cultured in 5% Fetal bovine Serum (FBS) MEM containing with 1% penicillin/streptomycin and 2 mM L-glutamine (Life Technologies), and were maintained at 37 °C in 5% CO_2 incubator. Human colon cancer cell lines HCT116 and HCT116 $p21^{-/-}$ were cultured in McCoy's 5A supplemented with 10% FBS. The cultures were dissociated with trypsin and transferred to new 25 cm^2 culture flasks twice a week. FBS was purchased from Life Technologies, Inc. (Gaithersburg, MD), MEM and McCoy's 5A were from Calbiochem (San Diego, CA), Luciferase assay substrate, CellTiter-Glo® Luminescent Cell Viability Assay kit and EGF were from Promega (Madison, WI).

Plasmids and cell transfection. AP-1-luciferase reporter, NF-κB-luciferase reporter, and cyclin D1 promoter luciferase reporter were described in our previous studies (Ouyang et al., 2006, 2007a; Zhang et al., 2009). JB6 Cl41 cells stably transfected with cyclin D1 promoter-driven luciferase reporter plasmid, AP-1-luciferase reporter plasmid, and NF-κB-luciferase reporter plasmid were described in our previous publications (Ouyang et al., 2006; Zhang et al., 2009). The expression vector pEGFP-C3-cyclin D1 was constructed as follows: pEGFP-C3 was digested with EcoRI and BamHI. The human cyclin D1 cDNA fragment was amplified using specific primers (forward: 5′-GCGAATTCCCATGGAACACCAGCTCCTG-3′; reverse: 5′-GCGGATC CTCAGATGTCCACGTCCCGCA-3′). The fragment containing human cyclin D1 open reading frame, EcoRI site, and BamHI site, was inserted to the clone site of pEGFP-C3. The construct was sequenced by Genewiz (South Plainfield, NJ). Cl41 cells were transfected with either p-EGFP-C3 (GFP) or pEGFP-C3-cyclin D1 (GFP-cyclin D1) expression plasmids using PolyJet™ DNA *in vitro* transfection reagent (SignaGen Laboratories, Rockville, MD).

Anchorage-independent growth. Soft agar colony formation assay was performed as described previously (Ouyang et al., 2008; Zhang et al., 2009). Briefly, 2.5 ml of 0.5% agar in basal modified Eagle's medium (BMEM) supplemented with 10% FBS and 20 ng/ml EGF was layered onto each well of 6-well tissue culture plates. 3×10^4 Cl41 cells or HCT116 cells were mixed with 1 ml of 0.5% agar BMEM supplemented with 10% FBS with or without 20 ng/ml EGF and layered on top of the 0.5% agar layer. The plates were incubated at 37 °C in 5% CO_2 for three weeks. The colonies were then counted under microscopy and those with 32 cells were scored. The results were presented as colonies/10^4 cells.

Cell proliferation assay. 2×10^3 Cl41 viable cells suspended in 100 μl medium containing 5% FBS were seeded into each well of 96-well plates and cultured till 70% confluent. The cells were treated with EGF (20 ng/ml) with or without SAHA at indicated doses for 24 h. The cell proliferation was determined using CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI) with a luminometer (Wallac 1420 Victor2 multilabel counter system). The results were expressed as proliferation index (relative luminescence signal to medium control).

Flow cytometry assay. Cl41 cells were cultured in 6-well plates until 70%–80% confluent. Cell culture medium was replaced with 0.1% FBS medium for 36 h. The cells were then treated with EGF (20 ng/ml) with or without SAHA at indicated concentrations in the medium containing 1% FBS. Cells were fixed in ice-cold 70% ethanol and stained with PI buffer (0.1% Triton X-100, 0.2 mg/ml RNase A, and 0.05 mg/ml PI) for 15 min. The samples were subjected to flow cytometry (Beckman) for cell cycle analysis.

Western blottings. Cl41 cells and their transfectants (24 h after transfection) were cultured in each well of 6-well plates with normal medium until 70%–80% confluence. Cell culture medium was replaced by medium with 0.1% FBS for 36 h. Following that the culture medium was changed to MEM with 1% FBS and cells were treated with SAHA for 0.5 h followed by treatment with SAHA and/or EGF for the indicated concentrations and time periods. After exposure to EGF and SAHA, cells were washed with ice-cold PBS, and then extracted with cell lysis buffer (10 mM Tris–HCl, pH 7.4, 1% SDS, 1 mM Na_3VO_4, and proteasome inhibitor). The cell extracts were separated on polyacrylamide–SDS gels, transferred and probed with each of the antibodies against GAPDH (Cell Signaling, Beverly, MA), GFP, cyclin D1, VHL, HuR (Santa Cruz Biotechnology, Santa Cruz, CA), Nucleolin and β-Actin (Sigma, St. Louis, MO). The protein bands specifically bound to the primary antibodies were detected using alkaline phosphatase-linked secondary antibody and ECF (enhanced chemifluorescence) western blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (Zhang et al., 2009).

Reverse transcription polymerase chain reaction (RT-PCR). Cl41 cells and their transfectants (24 h after transfection) were cultured in 6-well plates until 70%–80% confluence. Cell culture medium was changed to 0.1% FBS medium for 36 h and then changed to 1% FBS medium and cells were exposed to SAHA with or without EGF and Actinomycin D (Act D), in the same manner as the cells treated for western blotting assay. After treatment for indicated time periods, total RNAs were extracted from cells using Trizol reagent (Invitrogen, Carlsbad, California). Total cDNAs were synthesized using oligd$T_{(20)}$ primer by SuperScript™ First-Strand Synthesis system (Invitrogen, Carlsbad, California). cyclin D1, GFP-cyclin D1 and β-actin mRNA amounts presenting in the cells were determined by semiquantitative RT-PCR assay. Mouse cyclin D1 (forward 5′-TCCCTTGACTGCCGAGAAG-3′, reverse 5′-AGACCAGCCTCTTCCTCCAC-3′) and β-actin (forward: 5′-CCTGTGGCATCCATGAAACT-3′, reverse: 5′-GTGCTAGGAGCCAGAGCA GT-3′) primers (Invitrogen) were used to determine the mRNA amount of endogenous cyclin D1 and β-actin, respectively. Human cyclin D1 (forward: 5′-GAGGTCTGCGAGGAACAGAAGTG-3′, reverse: 5′-GAGGGCGGATTGGAAATGAACTTC-3′) primer (Invitrogen) was used to detect the mRNA amount of reconstitutive GFP-cyclin D1 in Cl41 cells. The PCR products were separated on 3% agarose gels, stained with EB, and scanned the images from a UV light, as described previously (Zhang et al., 2009).

Luciferase assay. Cl41 and its stable transfectants transfected with AP-1-luciferase reporter plasmid, NF-κB-luciferase reporter plasmid, and cyclin D1 promoter luciferase reporter plasmid, respectively, were seeded into 96-well plates and starved by replacing culturing medium with 0.1% FBS MEM prior to exposure to EGF and SAHA, at indicated time of periods and concentration. Following that, cells were lysed for luciferase assay using luciferase substrate as previously described (Ouyang et al., 2007b).

Statistical analysis. The student's *t*-test was used to determine the significance between treated and untreated group. The results are expressed as mean ± SD.

Results

SAHA inhibited anchorage-independent colony formation induced by EGF in Cl41 cells

SAHA possesses significant inhibitory activity on cell proliferation in various human cancer cell lines (Musgrove et al., 2011). However, the potential inhibitory effect of SAHA on tumor promoter EGF-induced cell transformation has not been explored yet. Cells are capable of yielding anchorage-independent growth when neoplastic transformation