Archives of Biochemistry and Biophysics 590 (2016) 1–9

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Histone deacetylase inhibitors reduce WB-F344 oval cell viability and migration capability by suppressing AKT/mTOR signaling *in vitro*

Peng Zhang [a, 1], Xiaofeng Zhu [a, 1], Ying Wu [b], Ronglin Hu [a], Dongming Li [c], Jun Du [d], Xingyuan Jiao [a, *], Xiaoshun He [a, **]

[a] Organ Transplant Center, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China
[b] Department of Biostatistics, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China
[c] Department of Hepatobiliary Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China
[d] Department of Microbial and Biochemical Pharmacy, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, Guangdong, China

ARTICLE INFO

Article history:
Received 25 March 2015
Received in revised form
3 November 2015
Accepted 4 November 2015
Available online 10 November 2015

Keywords:
Oval cells
AKT/mTOR signaling pathway
Histone deacetylase inhibitor
Rapamycin
Cell proliferation
Apoptosis

ABSTRACT

Histone deacetylase (HDAC) can blockDNA replication and transcription and altered HDAC expression was associated with tumorigenesis. This study investigated the effects of HDAC inhibitors on hepatic oval cells and aimed to delineate the underlying molecular events. Hepatic oval cells were treated with two different HDAC inhibitors, suberoylanilidehydroxamic acid (SAHA) and trichostatin-A (TSA). Cells were subjected to cell morphology, cell viability, cell cycle, and wound healing assays. The expression of proteins related to both apoptosis and the cell cycle, and proteins of the AKT/mammalian target of rapamycin (mTOR) signaling pathway were analyzed by Western blot. The data showed that HDAC inhibitors reduced oval cell viability and migration capability, and arrested oval cells at the G0/G1 and S phases of the cell cycle, in a dose- and time-dependent manner. HDAC inhibitors altered cell morphology and reduced oval cell viability, and downregulated the expression of PCNA, cyclinD1, c-Myc and Bmi1 proteins, while also suppressing AKT/mTOR and its downstream target activity. In conclusion, this study demonstrates that HDAC inhibitors affect oval cells by suppressing AKT/mTOR signaling.

1. Introduction

Oval cells are progenies of the hepatic stem cells that are thought to reside in the terminal branches of the biliary tree, termed the Canals of Hering. Oval cells are named according to their characteristic morphology, which features an ovoid nucleus. These cells are small (relative to hepatocytes), and have a high ratio of nuclei to cytoplasm [1,2]. After severe liver injury resulting in hepatocyte and cholangiocyte necrosis/apoptosis, these dual-potential oval cells will proliferate and differentiate into hepatocytes or cholangiocytes to replace those lost cells [3–6]. Oval cell activity has been observed in various conditions, such as acute liver necrosis, hemochromatosis, chronic cholestatic diseases, alcoholic liver disease, and chronic viral hepatitis [7–11]. Recently, the concept that hepatocellular carcinoma (HCC) originates from oval cells (acting as tumor-initiating cells) has gained considerable attention in the field [12,13]. Oval cells participate in liver regeneration, fibrogenesis and carcinogenesis. Thus, targeting of oval cells and their activity could provide a novel strategy for control of HCC recurrence or metastasis, and could have utility as a therapeutic tool [14–16]. However, it remains to be determined how oval cell activity is regulated and controlled.

WB-F344 cells express a phenotypic repertoire of both hepatocytes and bile duct epithelial cells compared with those of normal hepatocytes and “oval” cells isolated from liver treated with chemical carcinogens. The phenotypic properties of a cultured liver epithelial cell line bear a close resemblance to those of “oval” cells; thus, it is considered to be an ideal *in vitro* model for oval cells.

Histone deacetylases (HDAC) are a group of enzymes that

Abbreviations: HDAC, Histone deacetylase; SAHA, suberoylanilidehydroxamic acid; TSA, trichostatin-A; HCC, hepatocellular carcinoma; AKT, Protein Kinase B; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO, dissolved in dimethyl sulfoxide; FBS, fetal bovine serum; IC_{50}, 50% inhibitory concentration.

* Corresponding author. Organ Transplant Center, The First Affiliated Hospital, Sun Yat-sen University, No. 58 Zhongshan Er Road, Guangzhou 510080, China.
** Corresponding author.
E-mail addresses: jiaoxingyuan@hotmail.com (X. Jiao), gdtrc@163.com (X. He).
[1] These two authors contributed equally to this work.

http://dx.doi.org/10.1016/j.abb.2015.11.004
0003-9861/

remove acetyl groups from histones and in turn block DNA replication and transcription. They therefore play an important role in the regulation of gene expression and cell proliferation. As such, HDAC inhibitors have been used to treat a range of different human diseases, including cancer. Indeed, vorinostat (suberoylanilidehydroxamic acid, SAHA) and trichostatin-A (TSA) have shown strong anti-proliferative effects as well as protective ability against intracellular events in various cells and cancers [17–20]. In particular, SAHA is currently one of the most advanced agents undergoing clinical development in the field of cancer treatment largely due to its low toxicity. Furthermore, it was approved by the US FDA for the treatment of cutaneous T-cell lymphoma [21]. Therefore we investigated the effects of HDAC inhibitors on oval cells to help understand the underlying molecular processes. The data could help the development of HDAC inhibitors as a potential tool in the treatment of hepatocellular carcinoma or other chronic liver diseases.

2. Materials and methods

2.1. Cell lines,culture, and drug treatment

The oval cell line WB-F344 derived from an adult male Fischer-344 rat, was obtained from the cell bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The morphological and biochemical properties of this cell line have stem cell features and have been previously characterized [22,23].

WB-F344 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12; Invitrogen, Carlsbad, CA, USA), which was supplemented with 10% endotoxin-free, heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 μg/ml streptomycin and 100 units/ml penicillin in a humidified incubator containing 5% CO_2 at 37 °C. The culture medium was changed every second day and cells were passaged using 0.05% trypsin plus 0.02% EDTA treatment. For drug treatment, WB-F344 cells were seeded in 6-well culture plates at 3×10^5density per well and cultured overnight. The following day, the cells were treated either with or without various concentrations of rapamycin (30 and 700 nM), TSA (0.4and 1.6 μM) or SAHA (1 and10 μM) for 24 hand 48 h before imaging with a digital camera that was mounted on a light microscope (Olympus DP-70, Japan). Rapamycin, LY294002, TSA and SAHA were purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma). 1,2-dioctanoyl-sn-glycero-3-phosphate (C8-PA), was purchased from Avanti Lipids (Alabaster, AL, USA) and dissolved in DMSO. Control cells were treated with an equal amount of DMSO used for drug treatment [not exceeding 0.1% (v/v)].

2.2. Cell viability assay

To assess changes in cell viability after drug administration, we performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, exponentially growing cells were plated in triplicate at 4×10^3 cells per well into 96-well plates and grown overnight. The cells were subjected to the following drug treatments: rapamycin (5, 10, 20, 40, 80, 160, 320, and 640 nM) for 24 h and; TSA (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μM) or SAHA (0.312, 0.625, 1.25, 2.5, 5, 10, 20, and 40 μM) for 48 h, or 0.4 μM TSA or 10 μM SAHA for up to 72 h. In order to establish that AKT is the agent responsible for the proliferative activity of these cells and also the cross-talk between AKT and mTOR, the cells were treated with either rapamycin (RAP, 30 μM), LY294002 (LY, 20 μM)or a mixture of rapamycin (RAP, 30 μM) and LY294002 (LY, 20 μM) for 48 h. In order to assess whether the observed apoptotic effect of HDACinhibition is related to the mTOR pathway signaling, WB-F344 cells were treated with rapamycin or C8-PA to either deactivate or activate p-mTORWB-F344 cells. Cells were treated with TSA (0.4 μM); SAHA (10 μM); TSA (0.4 μM) +rapamycin (50 μM); SAHA (10 μM) +rapamycin (50 μM); TSA (0.4 μM) + C8-PA (300 μM); or SAHA (10 μM) + C8-PA (300 μM) for 24 h. At the end of experiments, 20 μl of 5 mg/ml MTT (Sigma Aldrich) was added to each well and the cells were cultured at 37 °C for an additional 4 h. The supernatant was then discarded and the resulting formazan crystals were dissolved by adding 150 μl DMSO to each well and incubated for 10 min at 37 °C. The optical density (absorbance rate) was measured using a Vmax Microplate Reader (Molecular Devices, Silicon Valley, CA, USA) at 490 nm. The percentage of cell viability was calculated using the following formula: percentage of cell viability = [(absorbance of the experimental well)−(absorbance of the blank)]/[(absorbance of untreated control well)−(absorbance of blank)] × 100%.IC_{50} (50% inhibitory concentration) was also calculated by a Logit model based on these cell viability data. The experiments were performed in triplicate and repeated at least twice.

2.3. Flow cytometry assay

WB-F344 cells were plated in 6-well plates at a density of 3×10^5 cells per well and incubated for 24 h at 37 °C. For cell cycle analysis, sub-confluent cells were treated with or without various concentrations of TSA (0.1 and 0.4 μM) or SAHA (1, 2.5 and 5 μM) for 48 h. The cells were then harvested, washed twice with ice-cold phosphate-buffered saline (PBS), fixed with 70% ethanol, treated with 1% RNase, and stained with propidium iodide (100 μg/ml final concentration; Sigma). The samples were then analyzed using a FACS scanner (Becton Dickinson) and the software, FlowJo (Tree Star, Ashland, OR, USA). Sub-G1 cells identified in flow cytometric histograms were considered apoptotic cells.

Annexin V Apoptosis Detection Kit I (BD Biosciences) was also used to confirm cell apoptosis according to the manufacturer's instructions. Cells at a density of 1×10^6 cell/ml were re-suspended with 1 × binding buffer and then 100 μl of the resulting cell suspension was mixed with 5 μl Annexin V and 5 μl 7-AAD. The samples were then analyzed for the proportion of apoptotic cells using a FACS scanner (Becton Dickinson) and the software FlowJo (Tree Star, Ashland, OR, USA).

2.4. Protein extraction and Western blotting

Protein extraction and Western blottingwereperformed as described previously [24]. Briefly, cells were lysed in a lysis buffer containing 1% NP-40, 20 mM Tris–HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 20 mg/ml aprotinin, and 5 mg/ml leupeptin. The lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer. Equal amounts (30 μg) of protein samples were separated on 10% sodium dodecylsulfate (SDS)–polyacrylamide gels by electrophoresis and subsequently transferred on to nitrocellulose membranes. Then, the membranes were blocked with 5% non-fat milk at room temperature for 2 h, and then incubated overnight with different primary antibodies at 4 °C. The blots were then subsequently incubated with a horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5000 for 1 h at room temperature. Specific immune complexes were detected with the Western Blotting PlusChemiluminescence Reagent (Life Science, Inc., Boston, MA).

Primary antibodies against AKT (pan), Phospho-AKT (Ser473), mammalian target of rapamycin (mTOR), Phospho-mTOR (Ser2448), p70 S6 Kinase, Phospho-p70 S6 Kinase (Thr389), S6 Ribosomal Protein, Phospho-S6 Ribosomal Protein (Ser235/236), 4E-