Effect of Trichostatin A on Anti HepG2 Liver Carcinoma Cells: Inhibition of HDAC Activity and Activation of Wnt/β-Catenin Signaling

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Abstract

**Purpose:** To investigate the effect of deacetylase inhibitory trichostatin A (TSA) on anti HepG2 liver carcinoma cells and explore the underlying mechanisms. **Materials and Methods:** HepG2 cells exposed to different concentrations of TSA for 24, 48, or 72h were examined for cell growth inhibition using CCK8, changes in cell cycle distribution with flow cytometry, cell apoptosis with annexin V-FTIC/PI double staining, and cell morphology changes under an inverted microscope. Expression of β-catenin, HDAC1, HDAC3, H3K9, CyclinD1 and Bax proteins was tested by Western blotting. Gene expression for β-catenin, HDAC1 and HDAC3 was tested by q-PCR. β-Catenin and H3K9 proteins were also tested by immunofluorescence. Activity of Renilla luciferase (pTCF/LEF-luc) was assessed using the Luciferase Reporter Assay system reagent. The activity of total HDACs was detected with a HDACs colorimetric kit. **Results:** Exposure to TSA caused significant dose-and time-dependent inhibition of HepG2 cell proliferation (p<0.05) and resulted in increased cell percentages in G0/G1 and G2/M phases and decrease in the S phase. The apoptotic index in the control group was 6.22±0.25%, which increased to 7.17±0.20% and 18.1±0.42% in the treatment group. Exposure to 250 and 500nmol/L TSA also caused cell morphology changes with numerous floating cells. Expression of β-catenin, H3K9 and Bax proteins was significantly increased, expression levels of CyclinD1, HDAC1, HDAC3 were decreased. Expression of β-catenin at the genetic level was significantly increased, with no significant difference in HDAC1 and HDAC3 genes. In the cytoplasm, expression of β-catenin fluorescence protein was not obvious changed and in the nucleus, small amounts of green fluorescence were observed. H3K9 fluorescence protein were increased. Expression levels of the transcription factor TCF were also increased in HepG2 cells following induction by TSA, while the activity of total HDACs was decreased. **Conclusions:** TSA inhibits HDAC activity, promotes histone acetylation, and activates Wnt/β-catenin signaling to inhibit proliferation of HepG2 cell, arrest cell cycling and induce apoptosis.

Keywords: HepG2 cells - TSA - apoptosis - HDAC - β-catenin - histone acetylation

Introduction

Hepatic carcinoma, one of the most common malignant tumor in China, was found later (Quint et al., 2011; Chen et al., 2014; Page et al., 2014; Scaggiante et al., 2014). The incidence of HCC is increasing, particularly in China, due to the high prevalence of hepatitis virus infection, which confers a high risk of HCC (Ji et al., 2014; Li et al., 2014). Despite advances in diagnosis and treatment, HCC remains the third leading cause of cancer-associated mortality worldwide (Haghshenas et al., 2014; Scaggiante et al., 2014). Surgical resection is the most effective treatment for the majority of HCC patients, but the overall five year survival rate remains <12%. Surgical resection and VII section of Hepatic carcinoma is very difficult, so vast of patients lost the opportunity to surgery (Xu, 2010; Ker et al., 2011; Tanase et al., 2014). With the development of interventional treatment, chemotherapy drugs can directionally target hepatic carcinoma cells, which can prolong the life of patients with liver cancer (Osaki et al., 2013; Lee et al., 2014; Miyahara et al., 2014; Scaggiante et al., 2014; Tsai et al., 2014). However, certain percentage of hepatic carcinoma patients is not sensitive to chemotherapy and prone to resistance (Pathil et al., 2006). For most patients with liver cancer, it is lack of effective treatment. Therefore, seeking effective drugs to improve the effect of cancer chemotherapy is a focus of research.

Cancer epigenetics research shows that histone acetylation or deacetylation involved in tumorigenesis and affected gene expression and so on (Marks et al., 2001;
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Cell Signaling Technology (Danvers, MA, USA). The primary antibodies: HDAC1 (1:1000), HDAC3 (1:1000) antibody purchased from Cell Signaling Technology (Danvers, MA, USA); β-catenin (1:1000), Bax (1:1000), CyclinD (1:1000) antibody purchased from Sigma company. Luciferase Reporter Assay system reagent purchased from TaKaRa. HDACs Colorimetric kit purchased from Cell Signaling Technology (Danvers, MA, USA). The secondary antibodies: horseradish peroxidase (HRP-conjugated goat anti-rabbit IgG antibody, HRP-conjugated goat anti-mouse IgG antibody (Beyotime Institute of Biotechnology, Shanghai, China).

**CCK-8 assay**

For cell proliferation, a CCK-8 assay was done. Briefly, 1×10⁴ cells per well were plated in 96-well plates and cultured for different times. At the end of time, 20 µl CCK-8 was added to each well and then incubated at 37°C for 3h. Then plates were detected by 450nm on a spectrophotometric plate reader (Shanghai Precision and Scientific Instrument Co., Ltd., Shanghai, China).

**Cell morphology and imaging**

Logarithmic growth of HepG2 cells with 1×10⁴ cells/ml were seeded, the drug group were added TSA indifferent concentrations (250, 500 nmol/L), and control group added DMSO, incubated 24h, 48h, 72h, respectively. Cell morphology were observed by inverted microscope and photographed (Shanghai Optical Instrument Factory, Shanghai, China).

**Cell cycle assay**

The cells were seeded at a concentration of 2×10⁴ cells/ml and incubated for 24-72h with TSA at various concentrations. TSA dissolved in dimethyl sulfoxide was added to the medium in serial dilution. The cells were collected by centrifugation at 2500 r.p.m. for 5min, fixed in 70% ethanol then washed once with PBS and resuspended in 1ml of PBS containing 2.5µg/ml ribonuclease and 50µg/ml propidium iodide (Beyotime Institute of Biotechnology, Shanghai, China), incubated in the dark for 30min at room temperature and analyzed using flow cytometry (FCM).

**Apoptosis**

Quantitation of apoptotic cells by measurement of sub-G1DNA content using the propidium iodide method was carried out as described.

**Transient transfection and luciferase activity assays**

HepG2 cells were transiently transfected in triplicate using effect transfection reagent (TaKaRa). Plasmid encoding Renilla luciferase (pTCH/LEF-luc) was transfected for the normalization of transfection efficiency, unless otherwise indicated. Cells are trypsinized and evenly distributed into the wells of a six-well plate prior to designation of treatment condition. 24h after transfection the cells were treated with TSA indifferent concentrations (250, 500nmol/L), Supernatant were assayed for Renilla luciferase activities using the Luciferase Reporter Assay system reagent (TaKaRa), and assayed according to manufacturer’s directions.

**HDACs activity**

The activity of total HDACs was detected by HDACs Colorimetric kit. Dilute test samples (150µg of cell lysate) to 85µl (final volume) of ddH2O in each well (For background reading, add 85µl ddH2O only). Add 10µl of the 10X HDAC Assay Buffer to each well. Then add...
5µl of the HDAC colorimetric substrate to each well. Mix thoroughly. Incubate plates at 37℃ for 1h. Stop the reaction by adding 10µl of Lysis Developer and mix well. Incubate the plate at 37℃ for 30min. Read sample in an ELISA plate reader at 400nm.

Western blot analysis

The protein content of cell extracts was determined by the Bradford assay (Bio-Rad). A total of 20-30µg of protein was electrophoresed on 10-15% SDS-PAGE gels and transferred to PDVF membranes. Membranes were blocked, incubated with primary Abs at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:2000 dilutions). Labeled bands were detected by Immun-StarTM HRP Chemiluminescent Kit, and images were captured and the intensity of the Bands was quantified by the Bio-Rad VersaDocTM image system (Bio-Rad, Re-gents Park, NSW, Australia).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using TRIzol Reagent (Invitrogen). Reverse transcription PCR was carried out using MMLV transcriptase and Oligod (T) and the resulting cDNA products were used as templates for real-time PCR assays. Real-time RT-PCR was performed using the ABI Prism 7700 sequence detection system (PE Applied Biosystems). Twenty-five µl mixture was used for reaction. Fold change in gene expression was determined using the 2-ΔΔCT method with β-actin as endogenous control. Primer sequences were as follows: β-catenin: Forward: CCTCCACCTTGAC. Revers: TGCTCTAGATTAAATCTCCACATCGCTTTC. HDAC3: Forward: CGCGGATCCACCACCATGGCGCAGACGCAGG. Revers: TAATACGACTCACTAGAGGG. HDAC1: Forward: CGCGGATCCACCACCATGGCGCAGACGCAGG. Revers: TAATACGACTCACTAGAGGG. HDAC1: Forward: CGCGGATCCACCACCATGGCGCAGACGCAGG. Revers: TAATACGACTCACTAGAGGG.

Immunofluorescence

In a word, sterile glass slide placed in 6-well plate, then 1×10^7 cells were seeded. After 24h, treatment group were added TSA (250, 500 nmol/L), untreated group were added DMSO, incubate 24h, fixed with 4% paraformaldehyde, cell membrane was ruptured by 0.3% Triton X-100, closed with mountain goat serum (HyClone, Waltham, MA, USA), added antibody β-catenin (1:100), H3K9 (1:100), respectively. Overnight, anti-rabbit secondary fluorescent antibody was added and incubated for 1h, stained with PI (Beyotime Institute of Biotechnology, Shanghai, China), covered with 50% glycerol, imaging by fluorescent microscope.

Statistics analysis

The intensity of the immunoreactive bands was determined by a densitometer (Bio-Rad, Hercules, CA). Statistical significance of differences between control and treated samples was calculated by Student’s t-test (SSPS 17.0). p<0.05 were considered significant. All the experiments were repeated at least three times, each time with three or more independent observations.

Results

Growth studies

TSA inhibited the survival of HepG2 cells in various concentration ranged from 50 to 500nmol/L. Compared with the untreated group, cell proliferation was restrained significantly by TSA (50-500nmol/L) in treated group (p<0.01). In addition, HepG2 liver cancer cell growth inhibition was dose and time dependent (Figure1A). Shortly after addition of TSA (6-72h) HepG2 cells underwent a remarkable dose-dependent change of cell shape from polygonal to elongated, with cytoplasmatic extensions, differing from microscopic apoptosis. It caused cell morphology changes with numerous floating cells (Figure 1B).

Cell cycle perturbation and apoptosis were induced by TSA

To elucidate the pharmacological mechanism of TSA, HepG2 cells were treated with TSA in different concentrations. Flow cytometric analysis of the cell cycle revealed that the tested drugs, incubated with HepG2 cells for 24h had strong effects on the cell cycle progression in comparison with untreated cells (Figure 2A). Compared with those in control group, the HepG2 cells after treated with 250 nmol/L and 500 nmol/L TSA showed that the percentage in G0/G1 phase increased from (51.6±1.54) to (58.35±1.22)% and (61.4±0.94)% respectively; the percentage in S phase decreased from (35.3±0.72)% to (25.3±0.96)% and (17.5±1.1)% respectively; the percentage in G2/M phase increased from (16.3±0.7)% and (21.0±0.49)% respectively; the difference statistically significant (F=19.85, p<0.05). TSA could arrest the cell cycle of HepG2 cells in G0/G1 and G2/M phase. To further explore the underlying mechanisms, we studied the expression of cell cycle associated proteins. We found that TSA decreased the
HepG2 cells were incubated for 24h with TSA (250, 500nmol/L), expression of β-catenin, HDAC1, HDAC3 proteins was down-regulated, and the down-regulated was associated with the concentrations of TSA (Figure 4A). As shown in Figure 4B, after being treated for 24h with TSA (250, 500nmol/L), the activity of total HDACs was down-regulated, and the down-regulated was associated with the concentrations of TSA. In order to further validate up-regulated β-catenin protein by increasing genes expression, we used quantitative fluorescent PCR to determine β-catenin genes. The results showed the expression of β-catenin genes in treated group (250, 500 nmol/L) was up-regulated (Figure 3C). Since nuclear β-catenin was a hallmark of activated WNT/β-catenin signaling, we performed Immunofluorescence to determine the localization of β-catenin to further validate the activation of WNT/β-catenin signaling. As shown in Figure 3E, treated cells exhibited strong green fluorescence in the cytoplasm, and small amount of green fluorescence could be visible in the nucleus. Compared with the control group β-catenin no obvious changed localized in the cytoplasm. When it migrates to the nucleus, acts as a co-stimulatory protein for the TCF/LEF family of transcription factors. To further determine the changes due to treatment with TSA in β-catenin, we next examined activity of TCF transcription factor which dependent on β-catenin by luciferase reporter assay. Stimulation of TCF reporter activity was found when the cells were treated with TSA, suggesting that TCF-dependent transcriptional activity could be activated by TSA (Figure 3D).

**Inhibiting HDAC1 and HDAC3 enzymes and increasing histone acetylation**

We examined the activity of total HDACs being treated for 24h with TSA (250, 500 nmol/L), the activity of total HDACs was down-regulated, and the down-regulated was associated with the concentrations of TSA (Figure 4A). As shown in Figure 4B, after being treated for 24h with TSA (250, 500nmol/L), HDAC1 and HDAC3 protein was down-regulated, and the down-regulated was associated with the concentrations of TSA. We also determined genes of HDAC1 and HDAC3, compared with control group, the expression of HDAC1, HDAC3 gene had no significant changes (Figure 3C). The histone deacetylases (HDACs) opposed the action of histone acetyltransferases, removing acetyl groups from histone lysine tails, which resulted in chromatin compaction. The expression of H3K9 was proved to be up-regulated in manner of concentrations. We performed Immunofluorescence to determine the localization of H3K9 and further validate the histone acetylation was modified by histone deacetylases. As shown in Figure 4D, treated cells exhibited strong green fluorescence in the nucleus, in contrast, the control cells showed weak green fluorescence.
Discussion

In this experiment, we also found that the proliferation of HepG2 cells within different concentrations of TSA have been varying degrees inhibited, compared with the control, it has a statistically significant difference. TSA acted on HepG2 liver cancer cell growth inhibition was dose and time dependent (Figure 1A). The microscopic observation showed HepG2 liver cancer cells were induced by TSA in nmol/L levels. The changes as followed, cell population decreased significantly, scattered growth, no connection between each other, the refraction was strengthen, cell shape was circular, triangle, polygon and appearing a large number of floating cells (Figure 1B). Flow cytometry test results showed that 250nmol/L TSA group of apoptosis rate (7.17±0.20) %; 500nmol/L TSA of group of apoptosis rate (18.14±0.42) %, significantly higher than the control group (6.22±0.25)%. The experimental results showed that TSA can inhibit liver cancer cell proliferation and induce apoptosis markedly (Figure 2A and Figure 2B).

The abnormal proliferation of tumor is mainly caused by cell proliferation out of control, and thus inhibiting malignant cell proliferation is the key to regulate tumor development (Iguchi et al., 2007). TSA through the cell cycle to inhibit cell proliferation and development of tumor in leukemia cell lines. Cyclin D1 closely relate to the cell cycle, plays a crucial role in cell cycle G1 phase to S phase transition. The experimental results showed that down-regulation of the Cyclin D1 was consistent with cell cycle arrest, flow cytometry results and cell proliferation experiment results (Figure 2A and Figure 2C). It indicated that TSA reduced Cyclin D1 to adjust period, thus inhibited cell proliferation.

Histone acetylation and deacetylated modification is one of the key mechanisms of gene transcription regulation (Venturelli et al., 2007; Barbetti et al., 2013). The balance of the two kinds of enzyme activity regulates nucleosome core protein acetylation level and the expression of relating genes in eukaryotic cells. When the HAT and HDAC activity disorder will result in the expression of relating genes out of control (Bai and Merchant, 2007), eventually lead to the occurrence of tumor. Trichostatin A (TSA), derived from the streptomyacin metabolites, was first used as antifungal drugs (Herold et al., 2002). In recent years, research has shown that it is histone acetylation enzyme inhibitors (HDACi), specifically to inhibit I, II class histone acetylation enzyme (HDACs) activity. TSA can accumulate the acetylation of histone, change the chromosome structure, inhibit cell proliferation, induce tumor cell differentiation or apoptosis, and is a kind of new type anti-tumor drugs that has a broad prospect and targeted, but its mechanism remains to be further clarified. Studies found that the TSA obviously inhibited proliferation and promoted apoptosis in a wide variety of tumor. In this experiment, the activity of total HDACs was down-regulated, and the down-regulated was associated with the concentrations of TSA, and western blotting results demonstrated that histone acetylation enzyme HADC1, HADC3 significantly decreased in dosing group with concentration increasing, compared with the control group (Figure 4B). Then we checked histone H3K9, the acetylation level was on the rise (Figure 4B). Histone acetylation neutralize positive charge of histone, making mutually exclusive between the histone and the negatively charged DNA phosphate skeleton, the result is chromatin structure loosing chromatin, which is beneficial to β-catenin touching with TCF, and transcription factors, transcription complex, RNA polymerase is more close to the DNA promoter regions, stimulating downstream gene translation, including Bax, Bel-2, P21, P53, inducing cycle arrest and apoptosis (Feng et al., 2013).

According to previous reports, the constitutive activation of WNT/β-catenin signaling is believed to promote cell proliferation and tumorigenesis in tissues including the colon and the pancreas. However, the role of WNT/β-catenin signaling is multifaceted in human cancer cells. Interestingly, augmentation of WNT/β-catenin was found to be associated with the anti-tumor effects of HDACIs in both colon cancer and pancreatic cancer (Shao et al., 2012). It is generally believed that WNT/β-catenin signaling is constitutively activated in HepG2, and the aberrant activation of WNT/β-catenin signaling is associated with Hepatic carcinoma (Cui et al., 2001). However, to date, there has been no study of the effects of HDACIs on WNT/β-catenin signaling in HepG2. Thus, we treated human HepG2 cells with HDACis, TSA, and analyzed the subsequent effects on WNT/β-catenin signaling as well as anti-tumor effects against HepG2 cells. We have proved that TSA induce growth inhibition, apoptosis and cell cycle arrest in HepG2 cell lines. More importantly, we have demonstrated, to the best of our knowledge for the first time, that WNT/β-catenin signaling is strengthened by HDACis, and mediates the anti-tumor effects of HDACis against HepG2.

Recent studies have shown that transcriptional activity of Wnt/β-catenin signaling pathway was able to adjust by histone acetylation enzyme inhibitors, indicating Wnt/β-catenin signaling pathway may be potential of nonhistones.
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targets (Dahmani et al., 2011). In experiment, we found that the TSA played a role of anti-tumor accompanied by β-catenin protein expression. When chromatin is in a state of relaxation conditions, β-catenin can combine with TCF in the nucleus. After combination, N- and C- the end of β-catenin exposure, HAT protein CBP and p300 interact with β-catenin R10-C area, promoting the cofactor recruit, activating some transcription of Wnt target genes, such as Bax, Bcl-2, P21, P53, thus inhibiting hepatocellular carcinoma cell proliferation and promoting apoptosis of HepG2 (Mosimann et al., 2009). We detected Bax protein, increased in TSA treated group. At the same time TSA promoted the apoptosis of HepG2 cells (Figure 2C).

TSA can inhibit the activity of HDAC, promote the histone acetylation and, activate Wnt/β-catenin signaling pathway to inhibit the proliferation of HepG2 cell, inducing cycle arrest and apoptosis.

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