Role of histone deacetylase expression levels and activity in the inflammatory responses of patients with chronic hepatitis B

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Abstract. Histone acetylation has been demonstrated to serve a pivotal role in numerous inflammatory diseases. The present study examined histone acetylation in patients with chronic hepatitis B (CHB) and CHB with liver failure by detecting histone deacetylase (HDAC) activity. Mice with acute liver failure (ALF) were treated with the HDAC inhibitor entinostat (MS275) and alterations in HDAC activity and pro-inflammatory cytokine expression levels were detected. The effect of HDAC1 silencing on LPS-treated RAW264.7 murine macrophages was examined using specific small interfering RNA sequences, and the acetylation level of the non-histone nuclear factor-κB (NF-κB) p65 subunit was additionally examined. The results demonstrated that serum levels of alanine aminotransferase, aspartate aminotransferase and total bilirubin, and the expression levels of pro-inflammatory cytokines, were significantly increased in patients with CHB. Aberrant histone acetylation and HDAC activity were identified in patients with CHB, with their levels associating with disease severity. MS275 treatment may decrease HDAC activity and inhibit the production of cytokines; however, acetylation levels of H3 and H4 were enhanced. Acetylation levels of NF-κB p65 were decreased in lipopolysaccharide-treated cells and ALF mice, and were promoted by MS275 treatment and HDAC1 silencing. In conclusion, alterations in HDAC activity and expression levels demonstrated a greater effect on inflammation compared with histone acetylation; therefore, the underlying mechanisms may be associated with the acetylation of non-histones. These results provide a potential novel therapeutic strategy for the treatment of CHB.

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Abbreviations: CHB, chronic hepatitis B; HDACs, histone deacetylases; HATs, histone acetyltransferases; HDACi, histone deacetylase inhibitor; NF-κB, nuclear factor-κB; PBMCs, peripheral blood mononuclear cells; SPF, specific pathogen-free

Key words: chronic hepatitis B, liver failure, histone deacetylases, histone acetylation, inflammation, entinostat

Introduction

Epigenetics refers to heritable alterations in gene expression that do not involve coding sequence modifications (1). Epigenetic modifications may be grouped into three primary categories: DNA methylation, histone modifications and nucleosome positioning (2). Histone acetylation has been the most thoroughly investigated modification. It is regulated by the opposing activities of two enzymes, histone deacetylases (HDACs) and histone acetyltransferases (HATs). HAT-induced histone acetylation is associated with activation of transcription via relaxation of the chromatin structure, whereas deacetylation by HDACs induces a more condensed or inactive chromatin state, leading to gene repression (3,4).

Hepatitis B virus (HBV) infection is a global public health problem affecting >350 million individuals worldwide (5). In China, there are ~93 million individuals who have been infected with HBV, of which 20 million are chronic hepatitis B (CHB) patients (6). CHB infection is a primary cause of hepatic dysfunction. It is hypothesized that HBV is not directly cytopathic and that the host immune response is responsible for the disease. A human leucocyte antigen class I-restricted T cell response against HBV peptides expressed on the surface of liver cells serves an important role in the pathogenesis of liver damage (7). In addition to the primary damage caused by immunity, inflammatory cytokines are involved, particularly in severe liver damage.

Epigenetic regulation of gene expression is now regarded as a novel approach for disease treatment (8). Histone acetylation modification was demonstrated to serve pivotal roles in numerous inflammatory diseases, including rheumatoid arthritis (9), COPD (10) and allergic skin inflammation (11). However, the role of histone acetylation modification in CHB, particularly in liver failure, remains unclear. In the present study, the association between HDAC activity and disease severity in CHB patients was investigated. In addition, an acute liver failure (ALF) model was induced in mice and the RAW264.7 murine macrophage cell line was used to evaluate the effect of acetylation regulation under inflammatory conditions.

Materials and methods

Patients. A total of 60 patients with CHB were recruited from the Department of Infectious Diseases, Renmin Hospital of Wuhan University (Wuhan, China) between January and December 2013. Informed consent was obtained from all participants in the study. Additionally, healthy blood samples (~30) were obtained from the Blood Bank of the Renmin Hospital of Wuhan University. The patients were divided into two groups: CHB and CHB with liver failure (n=30/group). CHB and CHB with liver failure were diagnosed using guidelines of CHB and acute-on-chronic liver failure (12,13). The present study received ethical approval from the Clinical Research Ethics Committee of Renmin Hospital of Wuhan University Patients with hepatitis A, C and E, autoimmune liver disease, drug-induced hepatitis, alcoholic liver disease, and fatty liver disease infections were excluded from the present study.

Processing of blood samples. Peripheral blood mononuclear cells (PBMCs) were extracted from the blood samples of patients using a peripheral blood mononuclear cell separation fluid kit (Lengton Biological Technology, Shanghai, China) according to the manufacturer's protocol. Blood serum and PBMCs were stored at -80˚C until required.

Processing of liver tissue samples. Liver tissue samples were obtained from patients with CHB and CHB with liver failure. Samples were fixed in 10% buffered formalin for 24 h, embedded in paraffin and sliced into 5-μm thick sections. Tissue sections were dewaxed in xylene and rehydrated in a series of dilutions of alcohol. Following this, sections were placed in 3% H_2O_2 for 20 min to block endogenous peroxidase activity and boiled in sodium citrate buffer (pH=6.0) for 15 min. The sections were subsequently blocked with 10% normal goat serum (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) in a humidified chamber at 37˚C for 20 min to reduce non-specific binding, following which they were incubated with a rabbit anti-histone deacetylase 1 (HDAC1) primary antibody (cat. no. 5356S; Cell Signaling Technology, Inc., Danvers, MA, USA) at a 1:500 dilution at 4˚C overnight. The sections were subsequently incubated with a biotin-labelled goat anti-rabbit IgG secondary antibody (dilution, 1:100; cat. no. BA1003; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 1 h at 37˚C. Following this, sections were stained with 3,3-diaminobenzidine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), counterstained with Mayer's hematoxylin, dehydrated and mounted.

Animals. 30 specific pathogen-free (SPF) male C57BL/6 mice (weight, 16 to 30 g), were purchased from the Animal Experimental Center, Hubei Medical University (Wuhan, China). All animals were housed in a light-controlled room (12-h light/dark cycle) at a temperature of 25˚C and humidity of 45 to 50% with free access to food and water. All animal experiments procedures were performed in accordance with the institutional guidelines of the Animal Care and Use Committee of Renmin Hospital of Wuhan University (Hubei, China). The experimenter possessed an experimental animal application certificate.

Model production and sample collection. Animals were randomly divided into three groups: Healthy (control), model and entinostat (MS275)-treated. A mouse ALF model was induced by administration of D-Galactosamine (D-Gal; (Sigma-Aldrich; Merck KGaA) and lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA). Mice in the model group (n=10 received 400 mg/kg D-Gal and 100 μg/kg LPS by intraperitoneal injection. In addition to D-Gal and LPS, mice in the MS275-treated group (HDAC inhibitor; HDACi; n=10) received 1 mg/kg MS275 by intraperitoneal injection 2 h prior to ALF induction. Mice in the control group (n=10) were injected intraperitoneally with normal saline as a control. The time of administration of D-Gal and LPS was referred to as the baseline (time point 0). All animals were sacrificed following general anesthesia by intraperitoneal injection of 30 mg/kg pentobarbital sodium (Sigma-Aldrich; Merck KGaA) for orbital blood and hepatic tissue collection at the 24 h time point.

Cell culture. The RAW264.7 murine macrophage cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 20 U/ml penicillin and 20 μg/ml streptomycin in an incubator at 37˚C with 5% CO_2 under a humidified atmosphere.

Transient transfection of small interfering (si)RNA into RAW264.7 cells. HDAC1 (forward, 5'-GUUCUAUUCGCC CAGAUAATT-3' and reverse, 3'-UUAUCUGGGCGAAUA GAACTT-3') and non-specific control (forward, 5'-UUC UCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGA CACGUUCGGAGAATT-3') siRNA were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were seeded at a density of $5x10^5$ cells per well into 6-well cell culture clusters for 4 h prior to siRNA transfection. siRNA was diluted with Opti-Minimum Essential Medium® (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated at room temperature for 15 min with Oligofectamine™ reagent (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The final concentration in the culture was 100 nM. Following 1 h, siRNA-transfected and control cells were treated with 1 μg/ml LPS and cultured for an additional 48 h.

Analysis of blood samples and cell supernatants. Blood samples were collected in medical anticoagulant tube from patients with CHB and with CHB and liver failure. The samples were mixed and centrifuged at 600 x *g* for 30 min, at room temperature, then the supernatant were collected and preserved in -20˚C. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) levels were measured using a Hitachi Automatic Analyzer (Hitachi, Ltd., Tokyo, Japan). Tumor necrosis factor-α (TNF-α; cat. no. BMS223HS) and interferon-γ (IFN-γ; cat. no. BMS228/BMS228TEN) serum levels were determined using ELISA kits (eBioscience, Inc., San Diego, CA, USA) following the manufacturer's protocol. Plasma thromboplastin antecedent (PTA) data were taken from medical records.

Detection of HDAC activity. The activity of HDAC was measured using an HDAC assay kit (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from RAW264.7 cells and mouse hepatic tissue, and reverse-transcribed using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). According to the manufacturer's protocol, qPCR was performed with cDNA using gene-specific primers, the SYBR®-Green Master Mix kit (Takara Bio, Inc.) and a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers (Table I) were developed using Primer Express software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Quantification cycles (Cq) were determined from the amplification plots, and target gene expression was normalized against the Cq of the GAPDH housekeeping gene using the $2^{-\Delta\Delta Cq}$ method (14).

Western blot analysis. Cells were washed twice using phosphate-buffered solution (Beijing Solarbio Science and Technology Co., Ltd.). The appropriate amount (200 μl) of Mammalian Protein Extraction Reagent (M-PER; cat. no. 78503; Thermo Fisher Scientific, Inc.), mixed with cOmplete™ EDTA-free Protease Inhibitor Cocktail Tablets (cat. no. 5892791001; Roche Diagnostics, Basel, Switzerland), was added to each well (6-well plate) and the plates were shaken gently using a constant temperature shaker (IS-RDD3; Suzhou Jiemei Electronic Co., Ltd., Suzhou, China) for 15 min at 4˚C. The lysates were collected and transferred to a microcentrifuge tubes for centrifugation at 12,000 x *g* for 30 min at 4˚C to pellet the cell debris. The supernatant was collected and stored at -20˚C. Total protein (50 μg) was subjected to 10% SDS-PAGE and subsequently transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was incubated at 4˚C overnight with the following monoclonal primary antibodies: Mouse anti-HDAC1 (dilution, 1:1,000; cat. no. 5356S), mouse anti-HDAC2 (dilution 1:1,000; cat. no. 5113P), rabbit anti-histone H3 (dilution, 1:1,000; cat. no. 9715S), mouse anti-histone H4 (cat. no. 2935), rabbit anti-nuclear factor-κB (NF-κB) p65 (dilution, 1:1,000; cat. no. 8242S), rabbit anti-acetyl-histone H3 (dilution, 1:1,000; cat. no. 9649S), rabbit anti-acetyl-H4 (dilution, 1:1,000: cat. no. 2594S), rabbit anti-acetyl-NF-κB p65 (dilution, 1:1,000; cat. no. 3045S) and rabbit anti-β-actin (dilution, 1:1,000; cat. no. 4970S), (all from Cell Signaling Technology, Inc.). This was followed by incubation with the following secondary antibodies for 1 h at room temperature in the dark: IRDye 800CW goat anti-mouse 926-32210 (cat. no. C50316-03, dilution, 1:10,000; LI-COR Biosciences, Lincoln, NE, USA), IRDye 800CW goat anti-rabbit 926-32211 (cat. no. C50602-08, dilution 1:10,000; LI-COR Biosciences). Proteins were visualized using an ODYSSEY® infrared imaging system (LI-COR Biosciences, USA). β-actin served as an internal control. Densitometry analysis was performed using the Odyssey software application (version, 3.0.29; LI-COR Biosciences).

Statistical analysis. Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of the variance was performed, with multiple comparisons between groups compared using the Student-Newman-Kuels method for post hoc tests. Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Increased inflammatory cytokine levels in patients with CHB and CHB with liver failure. Serum levels of ALT (Fig. 1A), AST (Fig. 1B) and TBil (Fig. 1C) increased significantly in the CHB and CHB with liver failure groups compared with the control group (P<0.05). In addition, serum levels of ALT, AST and TBil were markedly increased in the CHB with liver failure group compared with the CHB group (P<0.05). PTA levels decreased in the CHB and CHB with liver failure groups compared with the control group (P<0.05), and levels in the CHB and liver failure group were markedly reduced compared with the CHB group (P<0.05; Fig. 1D). As measured by ELISA, serum levels of TNF-α (Fig. 1E) and IFN-γ (Fig. 1F) were markedly increased in CHB patients compared with the control group (P<0.05). Compared with the CHB group, TNF-α and IFN-γ levels in the CHB with liver failure group increased significantly (P<0.05).

Increased HDAC activity and HDAC expression levels in patients. HDAC activity was detected in the sera of the patients. As presented in Fig. 2A, HDAC activity increased markedly in the CHB and CHB with liver failure groups compared with the control group (P<0.05). HDAC activity in the CHB with liver failure group was markedly increased compared with the CHB group (P<0.05). The expression of HDAC1 in the liver tissue was detected by immunohistochemistry. HDAC1 was primarily located in the nucleus, and increased positive staining was observed in the CHB with liver failure group compared with the CHB group (Fig. 2B). To observe alterations in HDAC expression, the protein expression levels of HDAC1 and HDAC2 in PBMCs were measured by western blot analysis. As presented in Fig. 2C, the expression levels of HDAC1 and HDAC2 increased significantly in the CHB and CHB with liver failure groups compared with the control group (P<0.05), and were increased in the CHB with liver failure group compared with the CHB group (P<0.05).

Acetylation levels of H3/H4 are increased in PBMCs in patients. To investigate histone acetylation alterations in patients with CHB, the acetylation levels of H3 and H4 in PBMCs were assessed. As presented in Fig. 2D, the acetylation levels of H3 and H4 were markedly increased in the CHB and CHB with liver failure groups compared with the control group (P<0.05). In addition, the acetylation levels of H3 and H4 were markedly increased in the CHB with liver failure group compared with the CHB group (P<0.05).

MS275 alleviates liver injury and the production of inflammatory cytokines in ALF mice. Serum levels of ALT increased significantly in the ALF group compared with the control group (P<0.05) and decreased markedly in the MS275-treated group compared with the ALF group (P<0.05;

Table I. List of primer sequences used for quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
HDAC-1	TGTTGCTCGCTGCTGGACTTA	ATCTTCATCCCCACTCTCTTCG
HDAC-2	GGTCGTAGGAATGTTGCTGAT	AAGCCAATGTCCTCAAACAGG
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
CSF	TTACTTTTCCTGGGCATTGTGG	CAGGAGGTTCAGGGCTTCTTTG
IL-1β	TGCCACCTTTTGACAGTGATG	ATGTGCTGCTGCGAGATTTG
Clc-2	ACCTGAATCGGAACCAAAT	TGAAAGGGAATACCATAACATC
GAPDH	AGGAGCGAGACCCCACTAACA	AGGGGGGCTAAGCAGTTGGT

HDAC, histone deacetylase; TNF-α, tumor necrosis factor-α; CSF, cerebrospinal fluid protein; IL-1β, interleukin-1β; Clc-2, chloride channel 2.

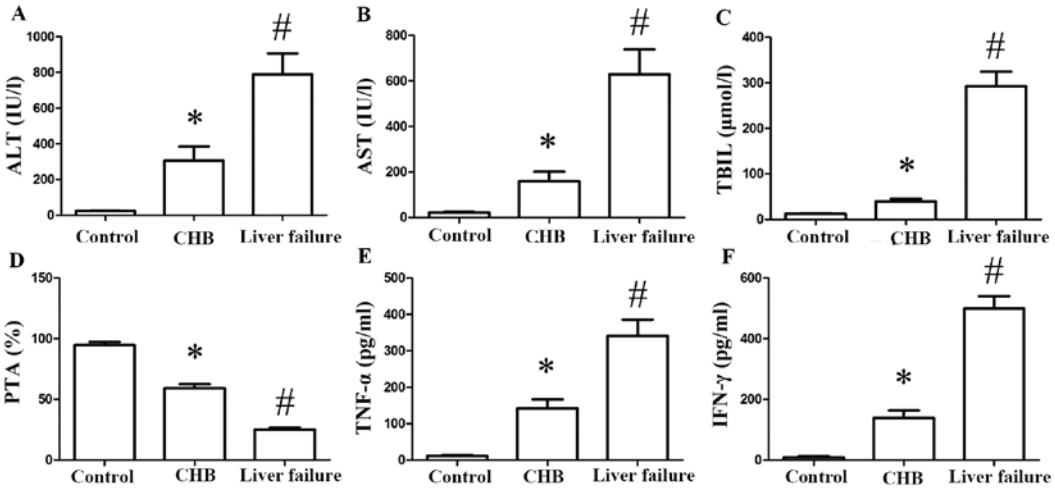

Figure 1. Liver function and cytokine production in CHB patients and CHB patients with liver failure. Serum levels of (A) ALT, (B) AST and (C) TBil. (D) PTA levels. As measured by ELISA, serum levels of (E) TNF-α and (F) IFN-γ. Data are presented as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. CHB group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBil, total bilirubin; PTA, plasma thromboplastin antecedent; TNF-α, tumor necrosis-α; IFN-γ, interferon-γ; CHB, chronic hepatitis B.

Fig. 3A). Serum levels of TNF-α (Fig. 3B) and IFN-γ (Fig. 3C) were markedly increased in the ALF group compared with control group (P<0.05), and were significantly decreased in the MS275-treated group compared with the ALF group (P<0.05).

MS275 inhibits HDAC activity and HDAC expression levels in ALF mice. The present study assessed HDAC activity in the sera of ALF mice. HDAC activity increased significantly in the ALF group compared with the control group (P<0.05), and HDAC activity in the MS275-treated group was markedly reduced compared with the ALF group (P<0.05). To observe the alterations in HDAC expression, mRNA expression levels of HDAC1 and HDAC2 in liver tissue were measured by RT-qPCR. The expression levels of HDAC1 and HDAC2 increased significantly in the ALF group compared with the control group (P<0.05), and were decreased in the MS275-treated group compared with the ALF group (P<0.05; Fig. 4A).

MS275 promotes the acetylation of H3/H4 in the ALF mice. To investigate alterations in histone acetylation, the acetylation levels of H3 and H4 in liver tissue were detected in ALF mice. As presented in Fig. 4B, the acetylation levels of H3 and H4 were markedly increased in the ALF group compared with the control group (P<0.05), and were enhanced in the MS275-treated group compared with the ALF group (P<0.05).

MS275 promotes the acetylation of NF-κB p65 in the ALF mice. To investigate alterations in non-histone acetylation, the acetylation levels of NF-κB p65 in liver tissue were detected in ALF mice. As presented in Fig. 4C, the acetylation levels of p65 were decreased in the ALF group compared with the control group (P<0.05), and were enhanced in the MS275-treated group compared with the ALF group (P<0.05).

siRNA inhibits HDAC1 expression and HDAC activity in RAW264.7 cells. To investigate the function of HDAC

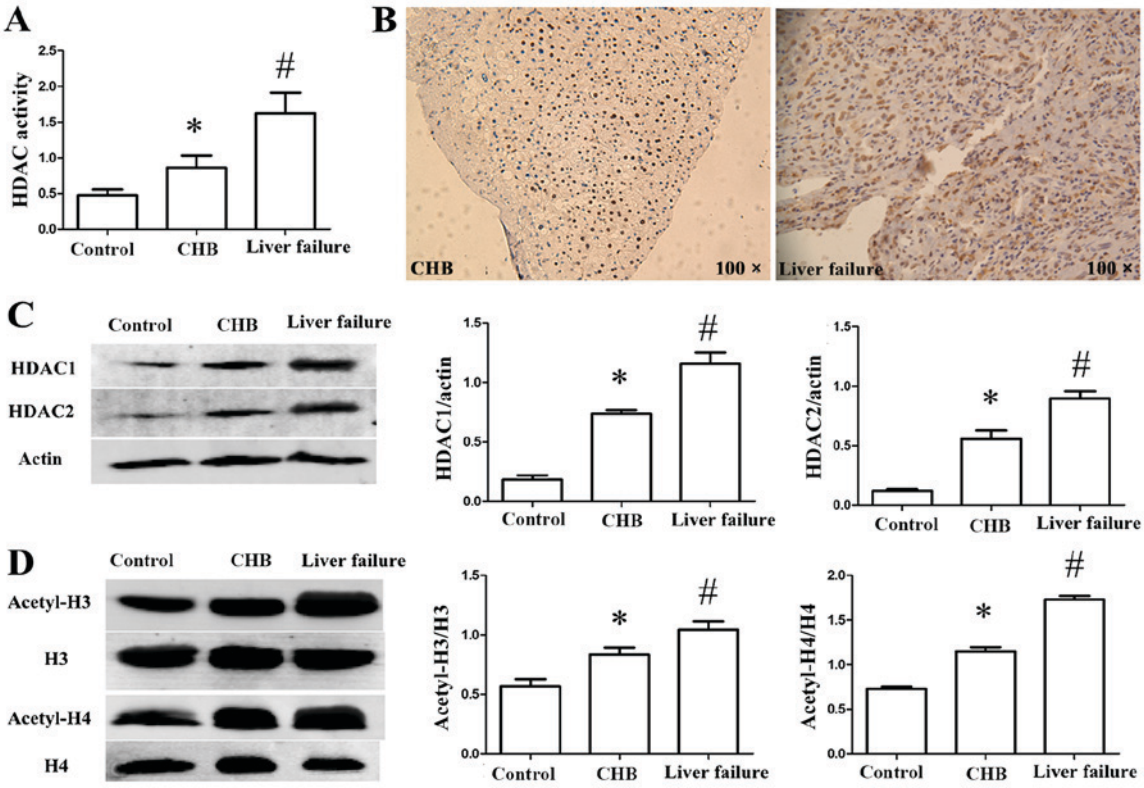

Figure 2. Acetylation modification in CHB patients and CHB patients with liver failure. (A) Quantification of HDAC activity in patients. (B) Immunohistochemistry of HDAC1 expression levels in liver tissue of patients; expression levels were decreased in CHB patients compared with those with CHB with liver failure. Magnification, x100. (C) Western blot analysis of HDAC1 and HDAC2 protein expression levels in PBMCs of patients. β-actin served as an internal control. (D) Histone acetylation levels of PBMCs in patients, as detected by western blot analysis. The relative protein expression levels from three independent experiments are represented as the density of histone bands normalized to that of acetyl-histone bands. Data are presented as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. CHB group. CHB, chronic hepatitis B; HDAC, histone deacetylase; PBMCs, peripheral blood mononuclear cells.

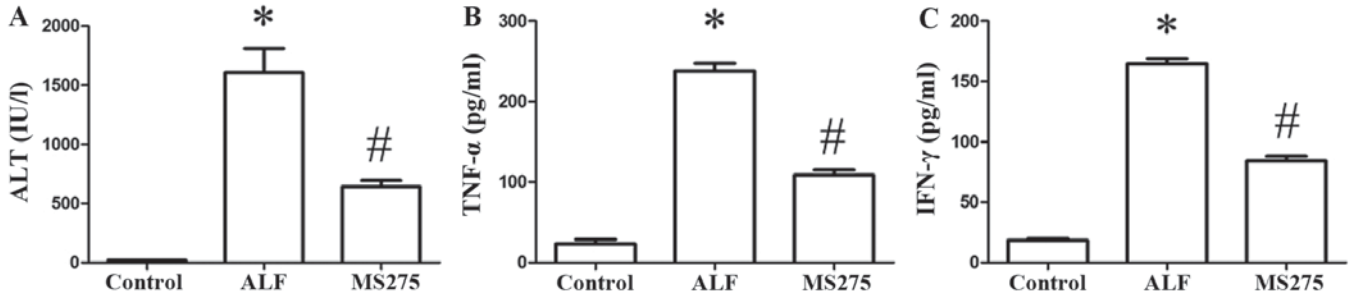

Figure 3. Effect of MS275 treatment on cytokine production. Serum levels of (A) ALT, (B) TNF-α and (C) IFN-γ in ALF and MS275-treated mice, compared with control mice. Data are presented as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. ALF group. ALT, alanine aminotransferase; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MS275, entinostat; ALF, acute liver failure.

in inflammatory responses, siRNA was transfected into RAW264.7 cells to silence HDAC1. siRNA transfection significantly inhibited HDAC1 expression and decreased HDAC activity in LPS-treated cells compared with control cells (Fig. 5A).

siRNA inhibits the production of cytokines in RAW264.7 cells. To evaluate the effect of HDAC1-silencing on cytokine production in RAW264.7 cells, the mRNA expression levels of TNF-α, cerebral spinal fluid (CSF) protein, chloride channel (Clc)-2 and interleukin (IL)-1β were measured by RT-qPCR. Expression levels of TNF-α, CSF, Clc-2 and IL-1β decreased significantly in siRNA-transfected cells compared with LPS-treated cells (P<0.05; Fig. 5B).

siRNA promotes acetylation of H3/H4 in RAW264.7 cells. Acetylation of H3/H4 in RAW26.7 cells was assessed. Compared with the control group, the acetylation levels of H3/H4 in LPS-treated cells were increased significantly (P<0.05), and enhanced in siRNA-transfected cells compared to LPS-treated cells (P<0.05; Fig. 6A).

siRNA promotes acetylation of NF-κB p65 in RAW264.7 cells. In addition to histone acetylation, acetylation of NF-κB p65

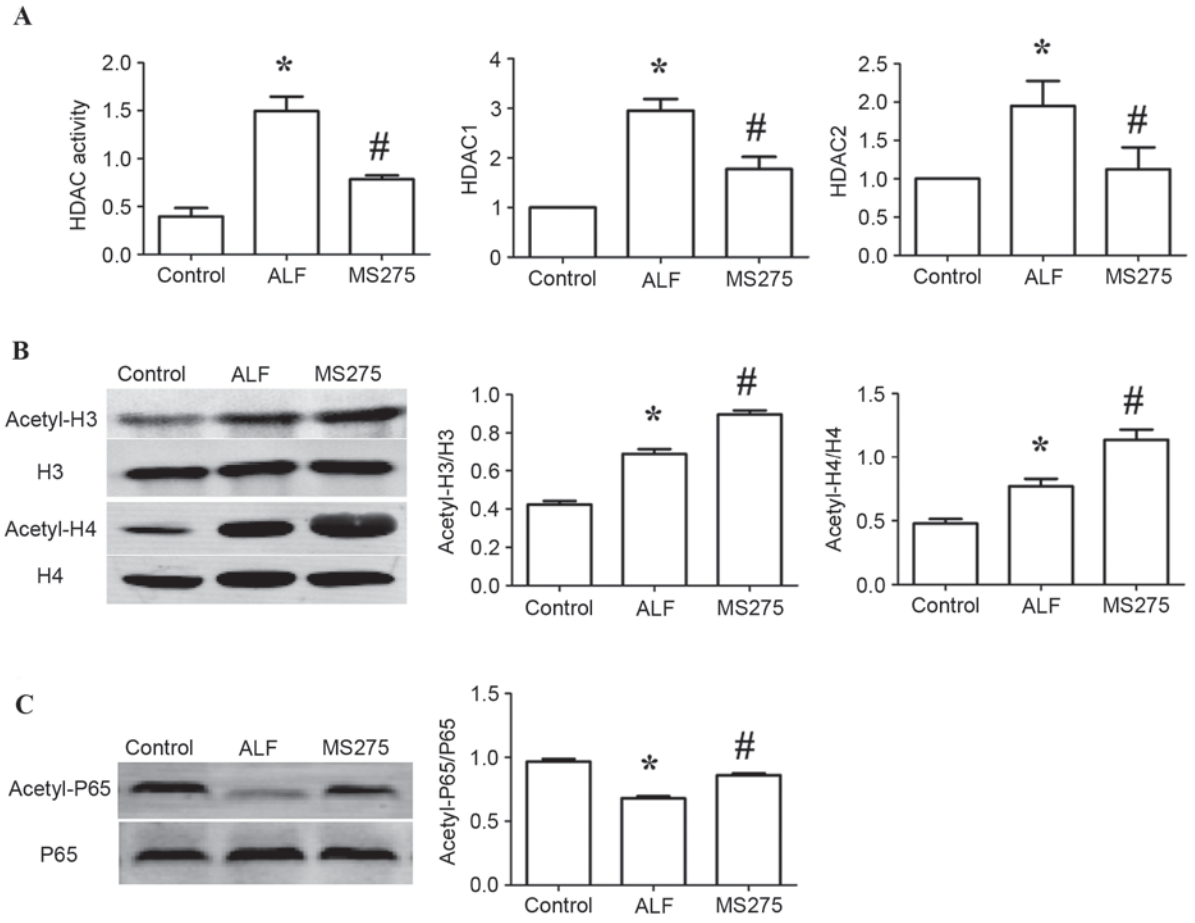

Figure 4. Effect of MS275 on histone/non-histone acetylation in ALF mice. (A) HDAC activity in mice and mRNA expression levels of HDAC1 and HDAC2 in mouse liver tissue, as detected by reverse transcription-quantitative polymerase chain reaction. (B) Histone acetylation levels in the liver tissue of mice. Histone and acetyl-histone protein expression levels were detected by western blot analysis. The relative protein expression levels from three independent experiments are represented as the density of histone bands normalized to that of acetyl-histone bands. (C) Nuclear factor-κB p65 protein acetylation level as detected by western blot analysis. Data are presented as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. ALF group. ALF, acute liver failure; HDAC, histone deacetylase; MS275, entinostat.

in liver tissue was examined. The acetylation levels of NF-κB p65 decreased in LPS-treated cells compared with the control group (P<0.05), and were enhanced in the MS275-treated group compared with the LPS-treated group (P<0.05; Fig. 6B).

Discussion

Epigenetic mechanisms have been identified as a primary determination of gene expression and regulate complex physiological and pathological processes. In addition to methylation, histone acetylation is considered a key component of epigenetic regulation. The nucleosome is composed of an octamer of four core histones, an H3/H4 tetramer and two H2A/H2B dimers, surrounded by 146 bp of DNA (15). This architecture of chromatin is strongly influenced by histone acetylation. Histone acetylation is controlled by HATs and HDACs. To date, 18 members of the HDAC family have been identified (16). The class I (HDAC1, 2, 3 and 8) and class II (HDAC4, 5, 6, 7, 9, 10 and 11) isoforms are zinc-dependent, whereas class III HDACs (Sirtuins1, 2 and 7) are nicotinamide adenine dinucleotide-dependent.

The present study demonstrated that HDAC activity, and HDAC1 and HDAC2 expression levels, increased significantly in patients with CHB, particularly in those with liver failure. These results indicated that the aberrant status of HDAC activity and expression levels may be associated with the pathogenesis of CHB.

Additionally, the acetylation levels of H3 and H4 were assessed; acetylation levels were markedly increased in CHB patients, particularly in those with liver failure. These results indicated that the acetylation of histone is associated with the disease progression of CHB.

Histone acetylation has previously been demonstrated to be associated with activation of transcription, whereas deacetylation is associated with gene repression (3,4). Previous studies have additionally revealed that the acetylation of histones is closely associated with activation of gene transcription (17-20), which may promote inflammatory responses by enhancing the expression levels of proinflammatory genes (21). The present study demonstrated increased acetylation levels of H3 and H4, and serum HDAC expression levels and activity, in PBMCs of patients with CHB and liver failure. This indicated that increased acetylation levels of histones and HDAC activity are associated with the progression of CHB. HDACis, including valproic acid, suberolyanilide hydroxamic acid and peroxiredoxin, may suppress the expression levels

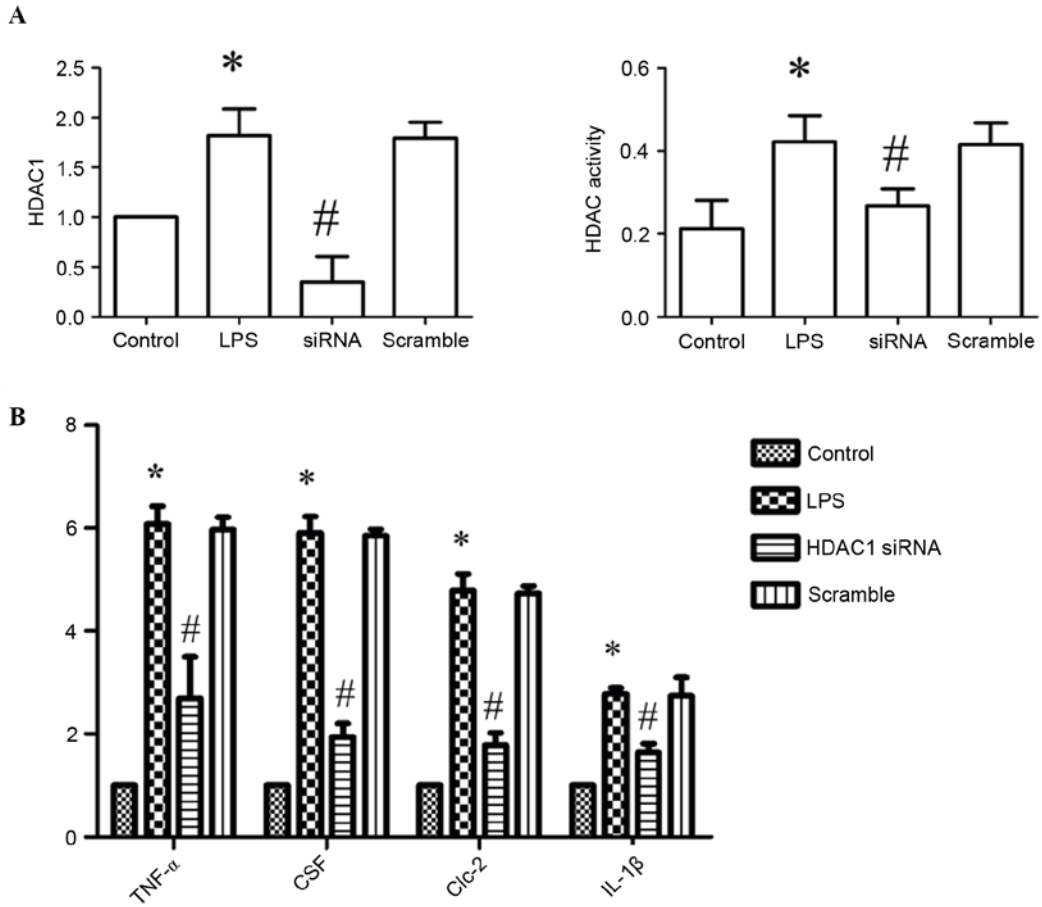

Figure 5. Effect of silencing HDAC1 on HDAC activity and cytokine production. (A) HDAC1 expression levels and HDAC activity in RAW264.7 cells. (B) Expression levels of TNF-α, CSF, Clc-2 and IL-1β, as assessed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. LPS-treated group. LPS, lipopolysaccharide; HDAC, histone deacetylase; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; CSF, cerebrospinal fluid protein; Clc-2, chloride channel-2; IL-1β, interleukin-1β.

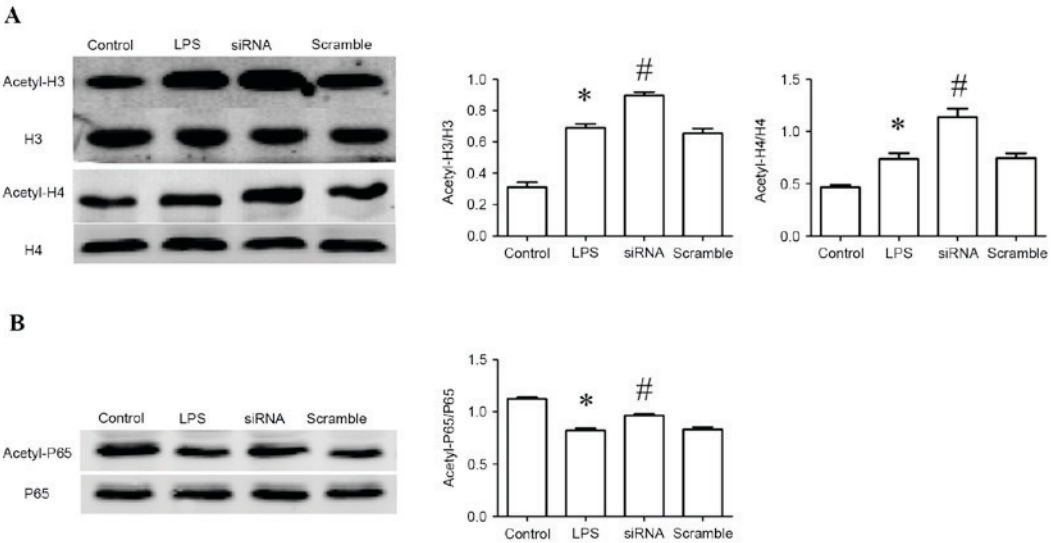

Figure 6. Effect of silencing HDAC1 on histone/non-histone acetylation in RAW264.7 cells. (A) Histone acetylation levels, as detected by western blot analysis. The relative protein expression levels from three independent experiments are represented as the density of histone bands normalized to that of acetyl-histone bands. (B) Nuclear factor-κB p65 acetylation levels, as detected by western blot analysis. Data are presented as the mean ± standard deviation. *P<0.05 vs. control group; #P<0.05 vs. LPS-treated group. HDAC, histone deacetylase; LPS, lipopolysaccharide; siRNA, small interfering RNA.

of proinflammatory cytokines and increase the survival rate of mice in a septic shock model (22-24). The results of the present study suggested that inhibiting the activity of HDACs may be beneficial for the treatment of inflammation.

However, histone acetylation levels and HDACis require further investigation. Acetylation of histones is associated with activation of transcription via relaxation of the chromatin structure, whereas deacetylation induces a condensed or inactive chromatin state, leading to gene repression. This may explain the increased histone acetylation levels observed in CHB patients. HDAC expression levels and activity were expected to increase due to the deacetylation of histones and the subsequent activation of transcription. However, the effect of transcription regulation was interfered by the acetylation of non-histones and/or other target genes of MS275 (only HDAC1 and HDAC2 were detected in this study). Histone acetylation levels increased following MS275 treatment, indicating that endogenous HDAC expression levels and activity affects deacetylation of histones.

To investigate the roles of acetylation in inflammatory responses, the present study examined the effect of MS275 treatment on ALF mice, and silencing of HDAC1 in LPS-treated RAW264.7 cells. MS275 is a class I-specific HDAC inhibitor, which has previously been used as an antitumor drug (25). In the present study, MS275 was demonstrated to protect liver tissue and inhibit the production of pro-inflammatory cytokines in ALF mice. In addition, the expression levels of HDAC1 and HDAC2, and HDAC activity, were decreased by MS275 treatment. However, the acetylation levels of H3 and H4 were enhanced by MS275 administration. These effects were additionally observed in LPS-treated RAW264.7 cells.

These results indicated that HDAC1 serves a role in the inflammatory response and that MS275 may represent a potential therapeutic agent for the treatment of inflammation. Acetylation of histones may not be involved in this anti-inflammatory effect, as acetylation of H3 and H4 were promoted by MS275 and HDAC1 siRNA, potentially activating gene transcription. Therefore, alterations in HDAC activity and expression levels may contribute more to inflammation, compared with histone acetylation.

In addition to histones, non-histones are hypothesized to be modified by HDACs and HATs. A previous study reported that >1,750 proteins are acetylated at their lysine residuals (26). Furthermore, >60 transcription factors were identified to be acetylated, including signal transducer and activator of transcription proteins, NF-κB, p53 and forkhead box O. The acetylation of these proteins regulates multiple processes, including gene expression and protein activity (27). To evaluate whether the acetylation of non-histones is involved in the anti-inflammatory effect of MS275 treatment and HDAC1 silencing, the acetylation levels of NF-κB p65 were detected in ALF mice and LPS-treated RAW264.7 cells. The results demonstrated that the acetylation levels of NF-κB p65 decreased in LPS-treated cells and ALF mice, and were promoted by MS275 treatment and HDAC1 silencing. This indicated that the acetylation of non-histones may be associated with the anti-inflammatory effects of MS275 treatment and HDAC1 silencing.

It has previously been reported that acetylation of histones affects HBV replication (28,29). In patients with CHB, the HBV infection is the original cause of liver injury. The immune system eliminates the virus by initiating an inflammatory response. In the present study, the association between acetylation of histones and HBV DNA load was not evaluated; acetylation modification of histones/non-histones in the inflammatory process was examined *in vitro*, *in vivo* and in CHB patients. Therefore, inhibition of histone/non-histone acetylation may have an anti-inflammatory effect.

In conclusion, the present study demonstrated aberrant histone acetylation, and HDAC activity and expression levels, in patients with CHB; these were associated with the severity of the disease. Additionally, MS275 treatment and HDAC1 silencing had an anti-inflammatory effect by decreasing the expression levels of pro-inflammatory cytokines. Alterations in HDAC activity and expression levels demonstrated a greater effect on inflammation compared with histone acetylation; therefore, the underlying mechanisms may be associated with the acetylation of non-histones. These results provide a potential novel therapeutic strategy for the treatment of CHB.

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