Simvastatin exerts anti-hepatitis B virus activity by inhibiting expression of minichromosome maintenance protein 7 in HepG2.2.15 cells

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Abstract. Simvastatin (SIM), a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, has been reported to inhibit the activity of hepatitis B virus (HBV), however, the mechanism underlying its antiviral function remains unknown. Minichromosome maintenance (MCM) 7, a component of the MCM complex, has been reported to act as an important host factor aiding virus genome replication in host cells. The present study demonstrated that downregulation of MCM7 inhibited the expression of proteins transferred by adenoviral vectors. This suggests an association between MCM7 and viral DNA expression. Thus, the current study aimed to investigate whether SIM affected MCM7 expression. Notably, the results of the present study indicated that following exposure to SIM the protein expression levels of MCM7 in HepG2.2.15, a human HBV-transfected liver cell line, was decreased. In addition, the HBV DNA replication in the cell line was suppressed. As quantitative polymerase chain reaction experiments demonstrated that SIM did not downregulate the mRNA expression level of MCM7, the current study further investigated whether SIM affects the translation of MCM7. Western blot experiments indicated that SIM improved the activation of eukaryotic initiation factor-2α (eIF2α), a protein synthesis initiation factor, and upregulated the upstream factors of eIF2α, protein kinase RNA-like endoplasmic reticulum kinase, which is regulated by the liver kinase B1 (LKB1)-AMP-activated protein kinase (AMPK) signaling pathway. These results indicated that SIM induced HBV downregulation via an MCM-dependent mechanism, and SIM may inhibit MCM7 expression by increasing the phosphorylation of eIF2α, which is mediated by the LKB1-AMPK signaling pathway.

Introduction

Hepatitis B virus (HBV) infection is a major global health issue, which has affected >2 billion people and results in 0.5-1.2 million mortalities per year. There are ~350 million HBV chronic carriers worldwide. Infection by HBV results in acute and chronic liver diseases and may lead to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1,2). Two types of therapeutic agents are available for treatment of HBV infection, interferon (IFN), and nucleoside and nucleotide analogues (NA) (3). IFN exerts its antiviral action by targeting the double-stranded RNA-activated protein kinase R (PKR) (4). PKR phosphorylates eukaryotic initiation factor-2α (eIF2α), a protein synthesis initiation factor, and reduces the level of viral protein synthesis (5). Theoretically, IFN may be an ideal agent for treatment of HBV infection, however, the response rate of interferon α is only 30-40% in HBV envelope antigen-positive patients after 4-6 months IFN treatment (1). Other disadvantages of IFN include its side effects and high costs (6). Patients who use IFN may require symptomatic treatment, dose modification or discontinuation of therapy (1). NAs, including lamivudine, tenofovir, telbivudine and adefovir, have strong antiviral effects, however, the development of drug resistance has limited their clinical applications (7,8).

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, also known as statins, are widely used in the treatment of hypercholesterolemia, however, they have also been reported to inhibit hepatitis C virus (9) and cytomegalovirus (10). HBV has also been observed to be inhibited by statins, including simvastatin (SIM) (11). However, the mechanism underlying the inhibition of HBV by SIM remains to be elucidated. Previously, it has been demonstrated that statins inhibit vascular smooth muscle cell growth by downregulating minichromosome maintenance (MCM) proteins (12). MCM proteins have ten conserved factors functioning in gene replication (13). Of the ten conserved factors, MCM2-7 are connected to each other to form a complex. The MCM2-7 complex acts

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as a replicative DNA helicase to regulate the initiation of DNA synthesis (14,15). The complex is important in restricting DNA replication to a single round per cell cycle (16). During the G1 phase cell division cycle (Cdc) 6 and DNA replication factor Cdt1 recruit the MCM2-7 complex to form an origin recognition complex at the replication origin. During S phase, the MCM2-7 complex is phosphorylated by the Cdc7-Dbf4 kinase (17) and then changed conformation, resulting in its association with Cdc45 at the replication origin. The formation of Cdc45-MCM complex initiates the duplex DNA unwinding and recruits various replication proteins to the unwound DNA, initiating DNA synthesis (18-20). MCM proteins interact with each other to form various complexes, including MCM2-7, MCM4/6/7, MCM2/4/6/7, or MCM3/5 (21). Biochemical investigations into these complexes have indicated that only the dimeric complex of the MCM4/6/7 heterotrimer has DNA helicase, single-stranded DNA binding, and DNA-dependent ATPase activities (22).

The MCM complex is an important host replication factor that participates in the genome replication of viruses in host cells, such as the influenza virus (23). It has been demonstrated that nuclear MCM7 is correlated with hepatitis B virus infection (P=0.020) (24). Our preliminary experiments also indicated that the expression of exogenous green fluorescent protein (GFP), which was transfected by adenoviral vectors, was decreased when MCM7 was silenced by small interfering RNA (siRNA) in murine normal fibroblast NIH3T3 cells. It indicated that MCM7 silencing may contribute to the inhibition of adenoviral vectors, a DNA virus. Thus, the present study hypothesized that SIM attenuated the expression of HBV DNA via an MCM-dependent mechanism.

The current study demonstrated for the first time, to the best of our knowledge, that SIM suppressed HBV expression levels by reducing the expression of MCM7 protein at the translational level. The results of the present study also demonstrated that the translational inhibition of MCM7 induced by SIM may be associated with the increasing phosphorylation of eIF2α. In addition, the LKB1-AMPK signaling pathway may be involved in the phosphorylation of eIF2α as a result of SIM. Overall, the findings of the current study demonstrated that decreasing MCM7 expression by SIM at the translational level may contribute to inhibition of HBV.

Materials and methods

Reagents. SIM was purchased from Merck Millipore (Darmstadt, Germany). SIM (4 mg) was dissolved in 100 μl ethanol and 150 μl 0.1 M NaOH, incubated at 50°C for 2 h, and adjusted to pH 7.0 with HCl. The final volume was corrected to 1 ml by adding absolute ethyl alcohol. Antibodies against MCM7 (sc-9966; 1:500), GFP (sc-8334; 1:1,000) phosphorylated (p)-retinoblastoma (Rb; Ser567) (sc-32824; 1:500), Rb (sc-50; 1:1,000), cyclin D1 (sc-4074; 1:1,000), tumor protein P53 (p53; sc-126; 1:1,000), LKB1 (sc-32245; 1:500), β-actin (sc-47778; 1:1,000) and horse-radish peroxidase (HRP)-conjugated anti-rabbit (sc-2370; 1:5,000) or anti-mouse (sc-2383; 1:5,000) IgG were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against cyclin-dependent kinase inhibitor 1B (p21; 10355-1-AP; 1:1,000), cyclin-dependent kinase inhibitor 1B (p27; 25614-1-AP; 1:1,000) and

PERK (20582-1-AP; 1:1,000) were purchased from Wuhan Sanying Biotechnology (Wuhan, China). p-eIF2α (Ser51; #9721; 1:1,000) and p-AMPKα (Thr172; #2531; 1:500) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for the siRNA transfection. The Cell Cycle Detection kit (KGA511-KGA512) was purchased from Nanjing KeyGen Biotech, Co., Ltd. (Nanjing, China). MG132 (M8699) and lamivudine (Y0000426) was purchased from Sigma-Aldrich (Merck Millipore).

Cell culture. The HepG2.2.15 human HBV-transfected liver cells line used in the present study was kindly provided by School of Basic Medical Sciences, Fourth Military Medical University (Xi'an, China). HepG2.2.15 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a water-saturated atmosphere of 5% CO_2. SIM was added into the medium at different concentrations and the medium was changed every two days. Cells were divided into three groups according to the concentrations of SIM added into the medium: i) Control group (not treated with SIM); ii) low concentration group (treated with 5 μM SIM); and iii) high concentration group (treated with 40 μM SIM).

Cell proliferation assay. Cell proliferation was evaluated using a cell growth curve and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Exponentially growing cells ($3.0x10^4$ cells/well) were seeded into 96-well plates (Corning Incorporated, Corning, NY, USA) and cultured in DMEM supplemented with 10% FBS. After 24 h, cells were added to the medium containing different concentrations of SIM (control, 5, or 40 μM) and cultured for 7 days at 37°C. To create cell growth curves, cell numbers were counted at indicated times. The MTT assay was performed by adding 20 μl MTT solution (5 mg/ml) into each well. Following incubation for an additional 4 h at 37°C, the medium was removed and 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck Millipore) was added to each well to dissolve the resultant formazan crystals. The absorbance was measured at 570 nm using a microplate reader (PerkinElmer, Inc., Waltham, MA, USA).

Cell cycle analysis. Cells were seeded in six-well plates and treated with 5 or 40 μM of SIM for 1 and 4 days at 37°C in a humidified atmosphere of 5% CO_2. Cells were subsequently collected by trypsinization. Samples of at least $1x10^6$ cells were stored in ice-cold 70% ethanol for at least 2 h at 4°C, washed twice with phosphate-buffered saline (PBS) and stained with a solution containing 50 mg/ml propidium iodide (PI) and 50 mg/ml RNase at room temperature for 30 min in the dark. Cell cycle analysis was performed by using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the CellQuest Pro software, version 6.0 (BD Biosciences).

Protein extraction and western blot analysis. Following treatment with 5 or 40 μM SIM in 100 mm diameter cell culture dishes for 1 and 4 days, cells were washed twice with ice-cold PBS, and lysed in radioimmunoprecipitation lysis buffer with

protease inhibitor and phosphatase inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany). Cell lysates were centrifuged at 12,000 x g for 20 min at 4°C and the supernatant was harvested. The protein concentration was determined using a Pierce BCA Protein assay kit (Thermo Fisher Scientific, Inc.). Protein samples (100 μg) were separated electrophoretic ally using a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% (w/v) non-fat dry milk in 1 M Tris buffer saline (pH 7.4), 5 M NaCl and 0.1% Tween-20 (TBST) for 1 h at 37°C. Subsequently, the membrane was incubated at 4°C overnight in 5% non-fat dry milk in TBST containing primary antibodies. Subsequent to washing with TBST three times for 10 min each time, the membrane was incubated with a HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies for 1 h at room temperature. Signals were detected using the Immobilon™ Western Chemiluminescent HRP Substrate reagent (EMD Millipore).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following exposure of the cells to 5 or 40 μM SIM for 1 and 4 days, total RNA was extracted from cells using E.Z.N.A®. Total RNA Kit I (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's protocols. Total RNA (2 μg) was reverse transcribed using the PrimeScript™ RT Master Mix kit (RR036a; Takara Biotechnology, Co., Ltd., Dalian, China) according to the manufacturer's protocols. qPCR was performed using an iQ5 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Reactions were performed using GoTaq qPCR Master mix (Promega Corporation, Madison, WI, USA). The thermocycling parameters were as follows: One cycle of 50°C for 2 min; one cycle of 95°C for 10 min; and 40 amplification cycles of 95°C for 15 sec and 60°C for 1 min. The following primers were used: MCF7, F 5'-GCT GATTGCCGTACAAGAG-3' and R 5'-AGCAGGGTACTG GTTCTG-3'; GAPDH, F 5'-CTCCTCCACCTTTGACGC TG-3' and R 5'-TCCTCTTGTGCTCTTGCTGG-3'. MCM7 gene expression were defined as MCM7 mRNA expression normalized to GAPDH mRNA expression. The $2^{-\Delta\Delta Cq}$ method was used to calculate relative expression levels (25).

Silencing of MCM7 by small interfering RNAs. siRNA (100 pmol) and 5 μl Lipofectamine™ 2000 were diluted in 245 μl of opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.), respectively. Diluted siRNA and Lipofectamine™ 2000 reagents were mixed and incubated at room temperature for 20 min. The siRNA-lipid complex was added to cells which had been grown to 70-80% confluence in six-well plates and the cells were incubated at 37°C in a 5% CO$_2$ incubator. The sequences of sense and antisense primers (obtained from Thermo Fisher Scientific, Inc.) were as follows: Sense, 5'-AUC GGAUUGUGAAGAUGAATT-3' and antisense, 5'-UUC AUCUUCACAAUCCGAUTT-3' for MCM7 siRNA; and sense, 5'-UAGCGACUAAACACAUCAATT-3' and antisense, 5'-UUGAUGUGUUUAGUCGCUATT-3' for the negative control siRNA.

Detection of HBV DNA in HepG2.2.15 cells and cell culture supernatants. Expression levels of HBV DNA in HepG2.2.15 cells and cell culture supernatants were collected by centrifugation at 3,220 x g for 5 min at room temperature (22-25°C) and were quantified by fluorescence qPCR (FQ-PCR) using the HBV PCR kit purchased from DAAN Gene Co., Ltd. (Guangzhou, China) according to the manufacturer's protocols. The thermocycling parameters were as follows: One cycle of 45°C for 10 min; one cycle of 95°C for 15 min; and 40 amplification cycles of 95°C for 15 sec and 58°C for 1 min.

Statistical analysis. Statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). At least three replicate experiments were conducted for each group and results were presented as the mean ± standard deviation. Statistical differences between groups were determined by using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

MCM7 silencing inhibited HBV DNA replication. Our preliminary experiments indicated that the expression of GFP was decreased when MCM7 was silenced by siRNA in NIH3T3 cells transfected with AdGFP (Fig. 1A). In the present study, MCM7 was silenced by transfecting siRNA into HepG2.2.15 cells to investigate the association between MCM7 and HBV DNA replication. The MCM7 protein expression level was downregulated following siMCM7 transfection (Fig. 1B). In addition, protein concentrations of HepG2.2.15 and siMCM7-transfected HepG2.2.15 cells were determined to normalize the expression level of HBV DNA. The results indicated that the HBV DNA expression level was significantly decreased 7 days after downregulating MCM7 expression (P<0.05; Fig. 1C). These results indicated that MCM7 was associated with increased HBV DNA replication and that silencing of MCM7 resulted in downregulation of HBV DNA expression.

SIM treatment inhibited MCM7 protein expression. HepG2.2.15 cells were treated with 5 or 40 μM SIM (C$_{25}$H$_{38}$O$_5$; molecular weight, 418.57 Da; Fig. 2A). Protein expression levels of MCM7 protein were determined by western blotting at 1 and 4 days after treatment. Notably, on day 1 of the treatment the expression levels of the MCM7 protein was slightly downregulated only in the high (40 μM) dose SIM treatment group, while, on day 4, the protein expression levels of MCM7 decreased significantly in low (5 μM) and high (40 μM) dose SIM treatment groups (Fig. 2B). Proteasome inhibitor MG132 was used to detect whether MCM7 protein was degraded or not by SIM and no difference was observed following the use of MG132 (data not shown).

SIM suppressed HepG2.2.15 cell proliferation. To investigate the effect of SIM on HepG2.2.15 cell proliferation, HepG2.2.15 cells were treated with 5 or 40 μM SIM for 1 and 4 days. Cell morphology changed after four days treatment with 40 μM SIM. Cells became small and rounded and ceased proliferation, compared with the cells in the control group (Fig. 3A). MTT assay and cell growth curve indicated that 40 μM SIM suppressed HepG2.2.15 cell proliferation, particularly 4 days after SIM treatment (Fig. 3B and C).
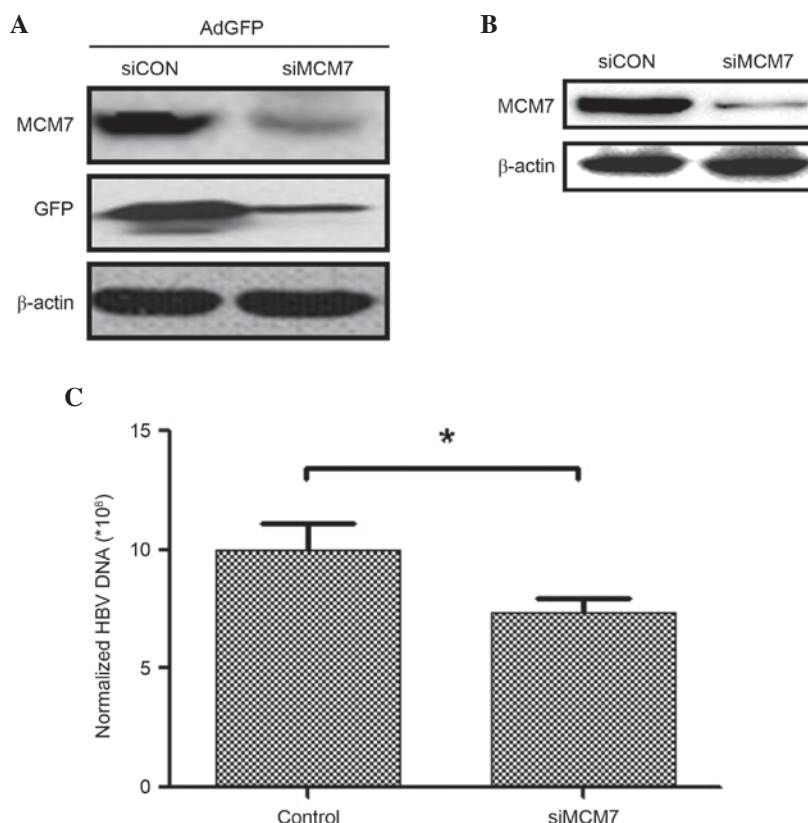
Figure 1. MCM7 silencing inhibited HBV DNA replication. (A) NIH3T3 cells transfected with the adenoviral vector AdGFP were transfected with MCM7-specific siRNA (siMCM7) or control siRNA (siCON). The levels of GFP in these cells were quantified by western blot analysis. (B) HepG2.2.15 cells were transfected with siMCM7 or siCON, and the efficiency of the siRNAs was evaluated by western blot analysis. (C) HBV DNA levels in HepG2.2.15 cells transfected with siMCM7 or siCON were determined by fluorescence quantitative polymerase chain reaction, and the results were normalized by the protein concentrations of each sample (quantity of HBV DNA in siCON or siMCM7 cell samples/protein concentrations of siCON or siMCM7 cell samples). Data wererepresented as the mean ± standard deviation from three independent experiments. Significance was analyzed using two-tailed Student's t-test. *P<0.05 vs. the siCON group. MCM7, minichromosome maintenance 7; HBV, hepatitis B virus; GFP, green fluorescent protein; siRNA, small interfering RNA; siMCM7, siRNA MCM7; siCON, siRNA control.
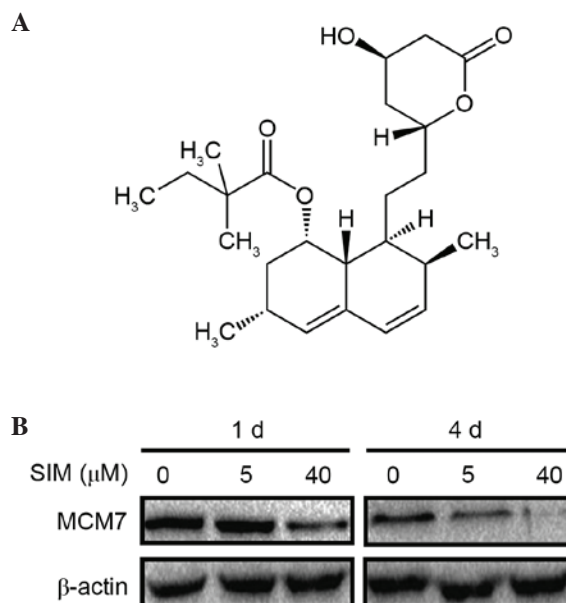

Figure 2. SIM inhibited MCM7 protein expression. (A) Chemical structure of SIM. (B) MCM7 expression levels were analyzed by western blot analysis. Cells were treated with SIM (0, 5 and 40 μM) for 1 and 4 days. Total proteins were extracted and β-actin served as an internal control. Representative data from three independent experiments is presented. SIM, simvastatin; MCM7, minichromosome maintenance 7.

To further investigate the effects of SIM on HepG2.2.15 cell proliferation, the cell cycle was analyzed by PI. Flow cytometry demonstrated that after 4 days SIM treatment, the G_1/S cell cycle arrest was more notable in the 40 μM group
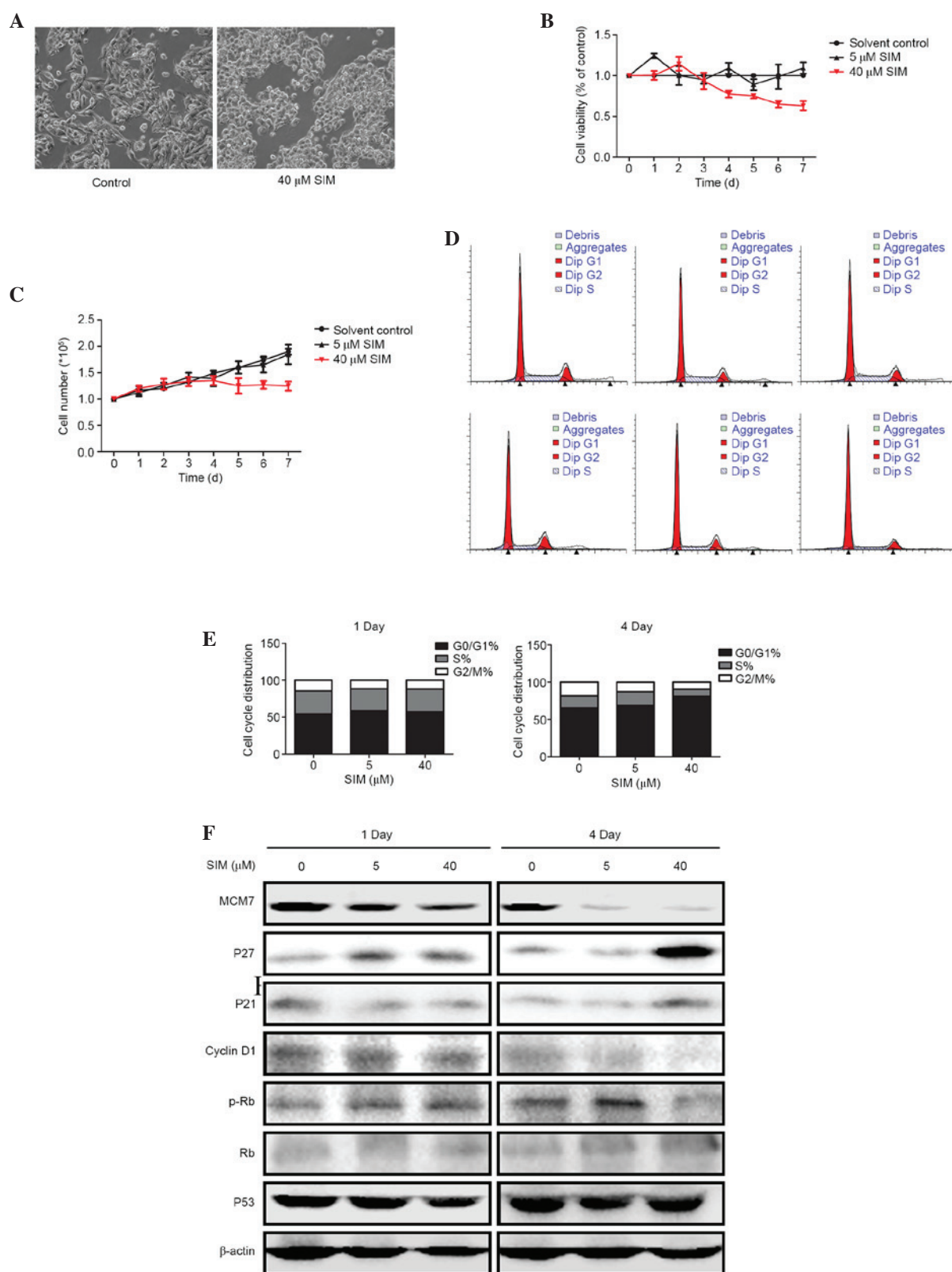
Figure 3. SIM suppressed HepG2.2.15 cell proliferation. (A) Morphological changes of HepG2.2.15 cells treated with SIM. Dose- and time-dependent inhibition of HepG2.2.15 cell proliferation by SIM was evaluated by (B) MTT assay and (C) cell growth curve. (D and E) HepG2.2.15 cells were treated with 0, 5 and 40 μM SIM for 1 and 4 days. Cell cycle distribution was detected by using a FACSCalibur flow cytometer. (F) Proteins involved in G_1/S transition were analyzed by western blot analysis. Cells were treated with SIM (0, 5 and 40 μM) for 1 and 4 days. Total proteins were extracted and equal protein loading was indicated by β-actin. Data are presented as the mean ± standard deviation of three independent experiments. SIM, simvastatin; MCM7, minichromosome maintenance 7; Rb, retinoblastoma; p, phosphorylated; p27, cyclin-dependent kinase inhibitor 1B; p21, cyclin-dependent kinase inhibitor 1; p53, tumor protein 53.

compared with those in the control and low (5 μM) dose groups (Fig. 3D and E), while on day 1 there was no significant G_1/S

cell cycle arrest observed. This indicated that SIM generated G_1/S cell cycle arrest in a dose- and time-dependent manner. To
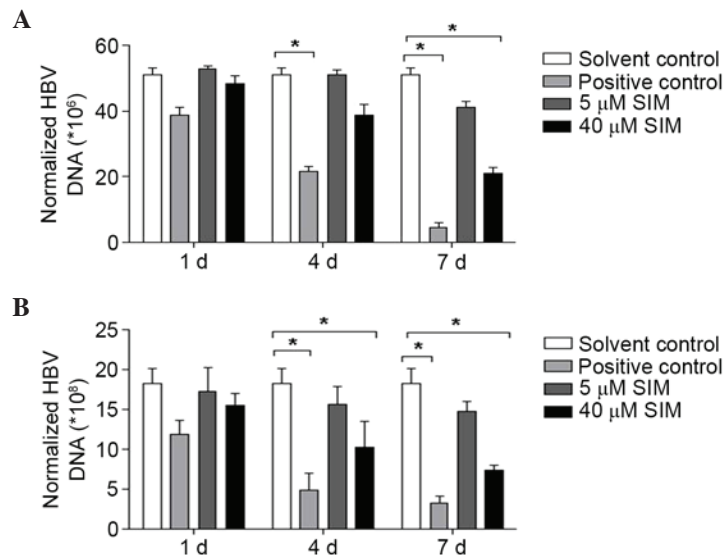
Figure 4. SIM repressed HBV DNA replication in HepG2.2.15 cells and their culture supernatants. (A) HepG2.2.15 cell culture supernatants and (B) HepG2.2.15 cells were harvested for quantification of HBV DNA levels by quantitative polymerase chain reaction following treatment with SIM (0, 5 and 40 μM) for 1, 4 and 7 days. The results were normalized by protein concentrations of each sample. Data were presented as the mean ± standard deviation from three independent experiments. Significance was analyzed using two-tailed Student's t-test. *P<0.05 vs. the untreated control group. SIM, simvastatin; HBV, hepatitis B virus.

further investigate how expression of cell cycle proteins were changed in SIM-treated HepG2.2.15 cells, western blotting was used to examine the expression levels of proteins involved in G_1/S transition. The results demonstrated that MCM7 was notably downregulated by SIM in the cells treated with 5 or 40 μM, while cyclin D1 and p-Rb were downregulated, and p27 and p21 were upregulated, only in cells treated with high dose (40 μM) SIM after 4 days. However, expression levels of Rb and p53 were not notably different in either group (Fig. 3F).

SIM repressed HBV DNA replication in HepG2.2.15 cells and their culture supernatants. HepG2.2.15 cells were divided into four groups: i) Solvent control group (treated with solvent); ii) positive control group (treated with 5 μM lamivudine); iii) low dose (5 μM) SIM group; and iv) high dose (40 μM) SIM group. FQ-PCR was conducted to assess the level of HBV DNA replication in culture supernatants and cells at 1, 4 and 7 days after treatment with SIM. After 4 days SIM treatment, the expression of HBV DNA in HepG2.2.15 cell culture supernatant were downregulated (without statistical significance), while after 7 days SIM treatments, the HBV DNA expression was significantly downregulated in the 40 μM SIM group (P<0.05; Fig. 4A). Similar results were observed for the cells, at 4 and 7 days after SIM treatment, HBV DNA expression was downregulated, with a statistically significant difference identified in the 40 μM SIM group (P<0.05; Fig. 4B).

LKB1-AMPK signaling pathway may be involved in the anti-HBV effect of SIM. To investigate the underlying mechanism of the effect of SIM on MCM7, MCM7 mRNA expression levels were assessed by RT-qPCR at 1 and 4 days after HepG2.2.15 cells were treated with SIM. Notably, the results were contrary to results obtained for MCM7 protein expression levels. The expression level of MCM7 mRNA was increased by SIM in a dose- and time-dependent manner (Fig. 5A). Thus, the present study investigated whether SIM

affects the translation of MCM7. As initiation factor eIF2α is commonly involved in the translation, eIF2α expression following SIM treatment was investigated. Western blots indicated that after 4 days of treatment with high dose (40 μM) SIM, HepG2.2.15 cells indicated increased expression of protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) upstream of p-eIF2α and eIF2α, which can phosphorylate eIF2α directly. As PERK is downstream of the LKB1-AMPK signaling pathway, further investigations were conducted into the activation of LKB1 and AMPK by western blotting. The results demonstrated that the expression levels of LKB1 and p-AMPK were increased after 4 days treatment with SIM (40 μM) in HepG2.2.15 cells, however, expression of these factors exhibited no notable changes after 1 day or in low dose (5 μM) treatment groups (Fig. 5B).

Discussion

HBV infection remains a major health problem, particularly in developing countries. Due to varying response rates, drug resistance and side effects, to investigate novel effective therapeutic agents with fewer side effects is required. SIM is a widely used therapeutic agent in the treatment of hypercholesterolemia, and has been reported to inhibit the level of HBV DNA (11), however, the underlying mechanism remains unknown. Elucidation of a mechanism underlying the effect of SIM on inhibiting HBV DNA replication may enable development of novel therapies to treat HBV infections. In the present study, it was determined that SIM induced HBV downregulation involved a MCM-dependent mechanism, and SIM may inhibit MCM7 expression via increasing eIF2α phosphorylation, which is mediated by the LKB1-AMPK signaling pathway.

Numerous studies have reported that statins have activities that prevent the replication of viruses, including influenza virus and HBV (9,10,23). SIM has been demonstrated to
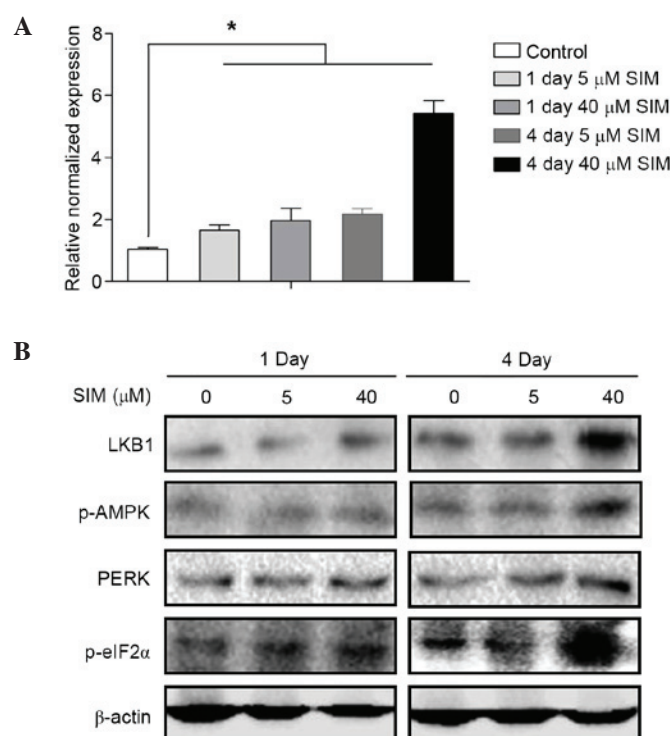
Figure 5. LKB1-AMPK pathway may be involved in the anti-HBV effect of SIM. (A) HepG2.2.15 cells were treated with SIM (0, 5 and 40 μM) for 1 and 4 days and MCM7 mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction. (B) HepG2.2.15 cells were treated with SIM (0, 5 and 40 μM) for 1 and 4 days. Total proteins were extracted and the expression levels of LKB1, p-AMPK, PERK and p-eIF2α were examined by western blot analysis. Equal protein loading was evaluated by β-actin. Data were presented as the mean $\pm$ standard deviation from three independent experiments. Significance was analyzed using two-tailed Student's t-test. *P<0.05 vs. the untreated control group. LKB1, liver kinase B1; AMPK, AMP-activated protein kinase; HBV, hepatitis B virus; SIM, simvastatin; PERK, protein kinase RNA-like endoplasmic reticulum kinase; eIF2α, eukaryotic initiation factor-2α; p, phosphorylated.

have a function to inhibit HBV (11). Consistently, the present study detected that the expression levels of HBV were downregulated in HepG2.2.15 cells and their supernatants following treatment with SIM. As SIM itself has an effect on inhibiting cell growth, the total protein concentration of cells in each group was examined as normalization. HBV copy number per unit protein concentration was calculated to infer the treatment effects on HBV DNA replication.

As HBV genomes are considerably small, they cannot encode large numbers of genes alone. Thus, once they infect host cells, viruses use a number of proteins from host cells termed 'host factors' to aid their replication. The MCM complex, which controls DNA helicase in host cells, has previously been reported as an important 'host factor' (23). Kawaguchi and Nagata (23) demonstrated that the MCM complex is important in the replication of HBV in host cells. These effects suggest that MCM complex may be an important antiviral target. Notably, a previous study demonstrated that at orvastatin, a type of statin, inhibits the expression of MCM proteins in vascular smooth muscle cells (12). Thus, it was hypothesized that SIM may inhibit the replication of HBV DNA via downregulation of MCM7 protein. The results of the present study demonstrated that SIM downregulated MCM7 protein expression in HepG2.2.15 cells, and HBV DNA expression was decreased in cells following MCM7 silencing.

Furthermore, the present study aimed to investigate how SIM regulates MCM7. It was demonstrated that the mRNA expression levels were increased while MCM7 protein expression was decreased following SIM treatment. This indicates that certain variations may have occurred during the process of translation or protein degradation. Proteasome inhibitor MG132 was used to detect whether MCM7 protein was degraded or not by SIM and no difference was observed following the use of MG132 (data not shown). In addition, the results demonstrated that SIM promoted phosphorylation of eIF-2α and expression of PERK. Phosphorylation of eIF-2α was one of the most well-studied mechanisms that regulate translation (26,27). eIF2 contains three subunits, α, β and γ (28) and it moves Met-tRNAi to the ribosome to form the ternary complex eIF2-GTP-Met-tRNAi (29). Due to GTP hydrolysis, eIF2-GDP complex is released from the ternary complex. The eIF2-GDP complex remains in an inactive state until the GTP exchange factor, eIF-2B, catalyzes GDP to GTP; then the eIF2-GTP complex is regenerated and a new round of transport is started. Phosphorylation of eIF-2α at residue Ser51 inhibits eIF-2β activity, resulting in the suppression of translation initiation (27). When cells suffer from various stress conditions, including anoxia and medication, phosphorylation of eIF2α is initiated and translation initiation is repressed. eIF2α is also associated with IFN, which has been identified to protect cells from viral infection (5,30). IFN can activate certain key biological functions of PKR, a double-stranded RNA-dependent protein kinase. PKR phosphorylates eIF-2α and the phosphorylated eIF-2α is key in the antiviral mechanism of the host (31). PKR is

one of the four mammalian kinases that can phosphorylate eIF-2α, the other three kinases are general control nonde-repressible 2, PERK (27), and heme-regulated inhibitor kinase (32,33). It was assumed that SIM may inhibit HBV DNA in a similar manner as IFN exerts its antiviral functions. PERK is commonly activated via ER stress (34). In eukaryotic cells, ER is understood to be important in folding and maturing most secreted and transmembrane proteins (35). Whether unfolded proteins can enter the ER or not depends on cell differentiation programs, environmental conditions and the physiological state of the cell. Unfolded proteins accumulate in the ER and induce a coordinated adaptive program termed the unfolded protein response. When cells are under stress conditions, the ER cannot lead to efficient folding of proteins, thus, unfolded proteins accumulate in the lumen of ER, ER stress is initiated, then PERK consequently is activated, and eIF2α is phosphorylated, attenuating protein synthesis (35).

A previous study has reported that the LKB1-AMPK pathway is able to regulate the activation of PERK (36). The results of the present study demonstrated increased PERK and p-eIF2α expression levels, coupled with activation of LKB1-AMPK signaling pathway. LKB1, also known as serine/threonine-protein kinase 11, phosphorylates and activates AMPK proteins and is implicated as a central regulator of cell polarity and energy metabolism in a variety of systems (37,38). Perhaps certain associations exist between ER stress and the LKB1-AMPK signaling pathway. Further research is required to elucidate whether these exist or not.

In conclusion, this is the first study, to the best of our knowledge, to indicate that the anti-HBV activity of SIM may be, at least in part, mediated by inhibition of MCM7 expression in HepG2.2.15 cells. Phosphorylation of eIF2α and activation of PERK and the LKB1-AMPK pathway may be important in SIM-mediated inhibition of MCM7 expression. Future research may determine whether or not SIM can inhibit other viruses via the MCM-dependent mechanism. MCMs may be a novel target for antiviral therapy in the future.

Acknowledgements

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