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Inhibition of HBV replication by theophylline

Zirui Zheng[a,e,1], Junyan Li[c,1], Jing Sun[d,1], Ting Song[a], Congwen Wei[a], Yanhong Zhang[a], Guirong Rao[b], Guangming Chen[b], DeChao Li[c], Gongming Yang[d], Bao Han[d], Shi Wei[e,*], Cheng Cao[a,*], Hui Zhong[a,*]

[a] State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Biotechnology, Room 841, East District Building, No. 27 Taiping Road, Haidian District, Beijing 100850, China
[b] PLA Liver Disease Research Center, 458 Hospital of PLA, Guangzhou 510600, China
[c] Oral Clinical Professional, Jiamusi University, 154007, China
[d] Beijing Public Security Hospital, 100121, China
[e] Key Laboratory for Molecular Enzymology and Engineering, Ji Lin University, Changchun 130021, China

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ABSTRACT

We have suggested recently that ATM-Rad3-Related (ATR) DNA damage signaling pathway, which responds to single-strand breaks in DNA, was activated in response to HBV infection. ATR knockdown cells showed decreased HBV DNA yields, implying HBV infection and replication activate and exploit the activated DNA damage response. Host cell proteins may constitute an attractive target for anti-HBV-1 therapeutics, since development of drug resistance against compounds targeting these cellular cofactor proteins is unlikely. In this study, we show that one of the clinically used compounds of ATR and ataxia telangiectasia-mutated (ATM) kinases inhibitor, theophylline (Tp), significantly reduced the yield of HBV DNA, HBsAg and HBeAg in HepG2215 cell culture system, furthermore, Tp could also suppress serum HBV DNA and HBsAg levels in the HBV-transgenic mice. Consistent with this result, immunohistology also showed reduced intensity of HBsAg staining on livers from Tp-treatment group. Taken together, these data indicated the feasibility of therapeutic approaches that target host cell proteins by inhibiting a cellular gene that was required for HBV replication and provided a potential approach for the prevention and treatment of HBV infection.

1. Introduction

Hepatitis B virus (HBV) infection is a major public health problem, with approximately 350 million individuals chronically infected worldwide (Lee, 1997). HBV is highly endemic in sub-Saharan Africa, China and South-East Asia, and is the major risk factor for nearly all cases of primary hepatocellular carcinoma (Lavanchy, 2004; Wong and Lok, 2006). In spite of the availability of an effective and safe vaccine against HBV, infection by this virus is an important worldwide health problem (Beasley and Hwang, 1991; Kar et al., 2009). Although several pharmacological strategies are currently being implemented to treat affected patients, no effective antiviral therapy against HBV infection has yet been fully developed. The development of drug-resistance in HBV, including strains carrying resistance to multiple licensed agents is an emerging clinical problem. Thus, new drugs to be used alone or in combination with existing treatments are needed.

Eukaryotic cells employ multiple strategies of checkpoint signaling and DNA repair mechanisms to monitor and repair damaged DNA (Shiloh, 2003; Sancar et al., 2004; Bakkenist and Kastan, 2004). Virus replication presents the host cells with large amounts of exogenous genetic material, including DNA ends and unusual structures. Thus, infected cells recognize viral replication as a DNA damage stress and elicit DNA damage signal transduction, which ultimately induces apoptosis as part of host immune surveillance. However, recent reports have shown that viruses evolve a variety of mechanisms to manipulate DNA damage signaling for their replication and propagation. For example, Epstein-Barr virus abrogate the p53 checkpoint signaling pathway through the interaction of the BZLF1 protein and p53 to avoid apoptosis (Kudoh et al., 2005). Other viruses such as human immunodeficiency virus type 1 (Daniel et al., 2003; Zimmerman et al., 2006; Roshal et al., 2003), herpes simplex virus type 1 (Wilkinson and Weller, 2006) and human cytomegalovirus (Gaspar and Shenk, 2006) can activate and exploit a cellular DNA damage response, which aids viral replication. Adenovirus blocks ATM signaling and concatemer formation through targeting the DNA repair complex of MRE11-RAD50-NBS1 (MRN) for degradation and mislocalization (Liu et al., 2005; Stracker et al., 2002). The prominent role, which these DNA repair kinases play in virus replication and propagation, creates an opportunity to suppress virus replication by targeting host cell proteins.

* Corresponding authors. Tel.: +86 10 66931809; fax: +86 10 66931809.
E-mail addresses: shiw@jlu.edu.cn (S. Wei), cao_c@yahoo.com (C. Cao), towall@yahoo.com (H. Zhong).
[1] The first three authors contributed equally to this work.

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Previous reports show that the ATM-specific inhibitor ku55933 can inhibit HIV replication in primary T cells (Lau et al., 2005). Caffeine and caffeine-related methylxanthines, known inhibitors of DNA repair, could also suppress replication of infectious HIV-1 strains (Nunnari et al., 2005). These proof-of-the-principle experiments demonstrate that HIV-1 replication can be successfully suppressed by inhibition of host cell DNA repair proteins.

Based on this principle, we have previously established an in vivo infection system using the HL7702 cell line and showed that HBV infection elicited acute cellular DNA damage response dependent on ATR (Zhao et al., 2008b) and the inhibition of DNA damage response by caffeine and caffeine-related methylxanthines could lead to suppressed HBV replication (Zhao et al., 2008a). In this study, we extended our study by using the HepG2215 cell culture system and HBV genome transgenic mice as evaluation models. The HBV positive transgenic mouse, which allows HBV DNA replication, is an appropriate tool to evaluate anti-HBV drugs. We found that theophylline, one product of caffeine metabolism in vivo, was able to suppress HBV replication and HBV major antigen productions.

2. Materials and methods

2.1. Chemicals

Theophylline was obtained from Sigma (St. Louis, MO) and Lamivudine (LMV) Lamivudine was obtained from the Glaxo-SmithKline (GSK) Corporation (China). They were prepared in sterile physiological saline and stored at 4 °C until used.

2.2. Treatment of transgenic mice

HBV transgenic mice were provided by Infectious Disease Center of No. 458 Hospital (Guangzhou, China). The HBV-Tg mouse lineage was initially produced on a BALB/c background. The transgene in these mice consists of 1.3 copies of the HBV ayw complete genome. The HBV-Tg mice express high level of HBsAg in their serum and have detectable HBV DNA in their serum (Li et al., 2005; Gao et al., 2004). All the mice used for experiments were at the age of 8 weeks. The HBV-transgenic mice were intragastric administered with different doses of theophylline every day for 60 days. LMV was intragastrically administered at 100 mg/kg every day for 60 days. Sterile physiological saline was used as control. Serum samples were collected and assayed for HBV DNA and HBsAg in the appropriate mice every 2 weeks. Mice were then killed 30 days after drug treatment and the livers removed for histology analysis.

2.3. Serum HBV DNA quantification by real time PCR

Aliquots (50 μl) of serum were digested with proteinase K (1 mg/ml) in a total volume of 500 μl containing 50 mM Tris base (pH 8.0) and 1% sodium dodecyl sulfate at 37 °C overnight. Nucleic acids were extracted as described. Diagnostic kit from Da-An Gene Corp. using fluorescent hybridization probes was monitored to determine the HBV viral load and HBV cccDNA levels. The PCR reaction was carried out as follows: initially at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 15 s.

2.4. RNA isolation and RT-PCR assay

HepG2215 cells were treated with different doses of Tp for 10 days, total RNA were then extracted by Trizol Reagent (Invitrogen) according to the method described in the Manufacturer's manual. Reverse transcription was performed with total RNA as the template. The cDNAs were amplified for 25 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min with HBs gene-specific primers. Sequences for HBV primers: sense 5′-ATCCTGCTGCTATGCCTCATCTT-3′ antisense 5′-ACAGTGGGGGAAAGCCCTACGAA-3′, sequences for actin primers: sense 5′-CTCCATCCTGGCCTCGCTGT-3′ antisense 5′-GCTGTCACCTTCACCGTTCC-3′.

2.5. HBsAg and HBeAg assay

The levels of HBsAg and HBeAg proteins were determined by enzyme-linked immunosorbent assay using a HBV diagnostic kit (Shanghai Kehua Biotech Co. Ltd.). Assays were performed in triplicate independent experiments.

2.6. Southern blot analysis

Twenty micrograms of total DNA was digested with HindIII and separated on a 1.2% agarose gel. The gels were treated as described (Chou et al., 2005) and the DNA samples were transferred onto nylon membranes (Amersham, Freiburg, Germany). After ultraviolet crosslinking and prehybridization, the membranes were hybridized with [^{32}P] labeled DNA probe generated by a random-primed labeling kit (Amersham, Freiburg, Germany) using full-length HBV DNA.

2.7. Immunohistology

Tissues were fixed in 4% formaldehyde and paraffin-embedded. Five micrometres sections were H&E stained, using Mayers hematoxylin (Merck, Germany). Immunohistological detection of HBsAg was performed on paraffin sections using HBsAg antibody from LifeSpan Biosciences Inc.

2.8. Statistical analysis

Differences between mean values were analyzed using the Student's *t*-test. Standard deviations were analyzed by the χ^2 method. All statistical analyses were performed by statistical software.

3. Results

3.1. Tp treatment suppressed HBsAg and HBeAg protein production in HEPG2215 cells

To evaluate the antiviral effects of Tp on the cell culture system, a derivative of the human HepG2 hepatoma cell line, HepG2215, which has been stably transformed with two copies of the HBV genome, was used. The HepG2215 cells were chosen because of the production of viral replicative intermediates and their capacity to support cccDNA formation. Before identifying the effective dose that interfered with HBV gene expression and replication, it is critical to use concentrations that are not overtly cytotoxic, since any impairment to cell functions would affect virus replication. Thus various concentrations of Tp were tested in the culture medium of HepG2215 and percentage survival was determined by the ratio of the number of treated cells divided by the number of untreated cells. As shown in Fig. 1A, Growth was not inhibited after 10 days of treatment with 2.5 mM Tp, and there was no obvious apoptotic cell death detected by flow cytometry staining (data not shown) of both the Tp treated and the control groups. The effects of Tp on DNA damage signaling pathways in HepG2 215 cells were also analyzed: the dose 1.1 mM nearly totally abrogates the phosphorylation of target proteins with ATR or ATM kinase activity such as Chk1 at Ser-345 and p53 at Ser-15 in response to UV radiation (Fig. 1B). Based on these results, we then selected three different dose of Tp with the highest dose at 2.5 mM to evaluate its antiviral effect. HBsAg and HBeAg concentrations in the culture media of theophylline-treated and control cells were measure by ELISA using HBsAg and HBeAg

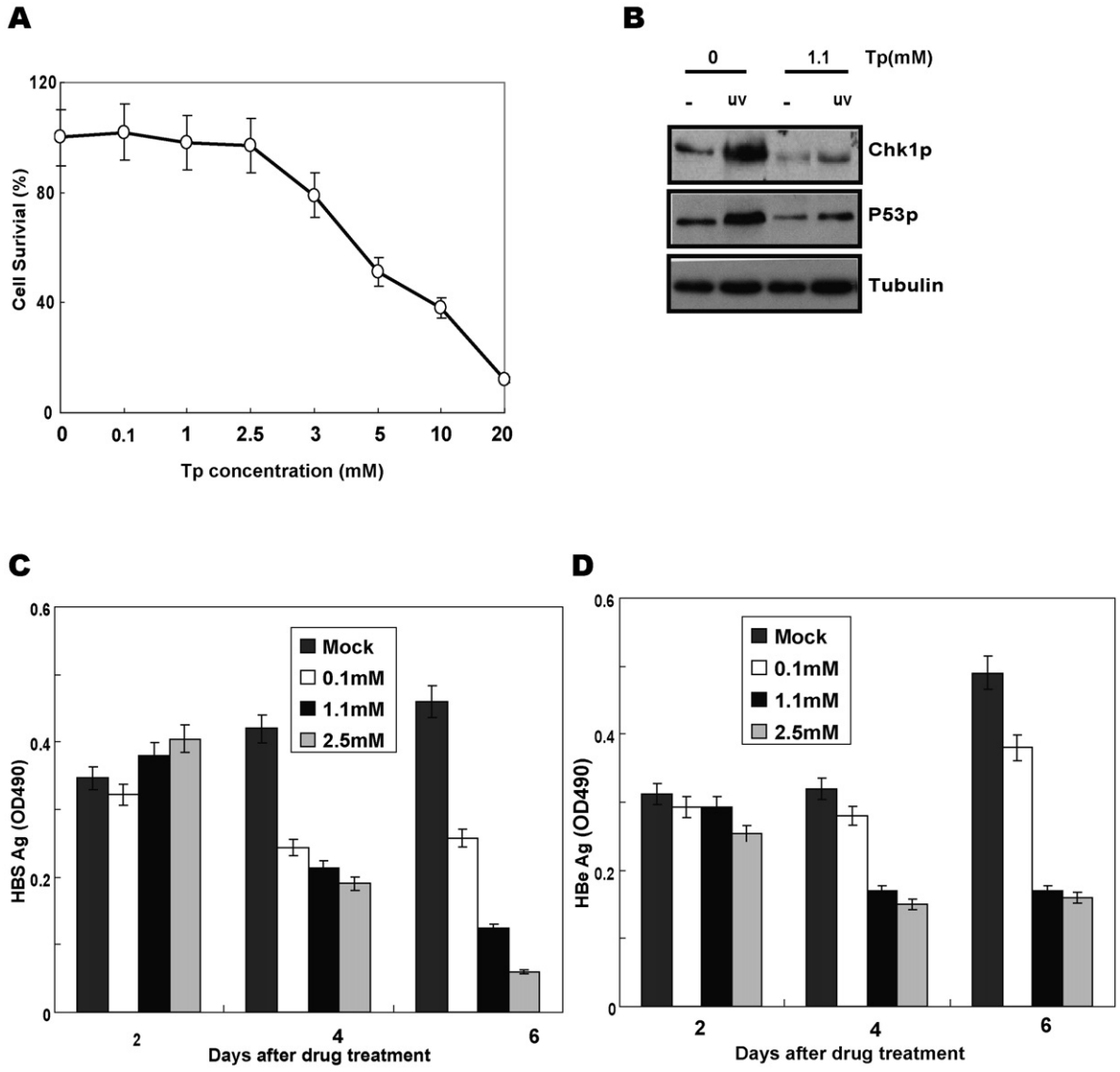

Fig. 1. Theophylline treatment suppressed HBsAg and HBeAg proteins productions. (A) HepG2215 cells were treated with Tp of different concentrations for 10 days and surviving cells were counted with trypan blue staining. (B) Cell lysates were prepared at 2 h after UV radiation at 50 MJ/cm^2 in the presence of 1.1 mM Tp and subjected to immunoblot assay by using antibodies to the indicated proteins. β-Tubulin was used as the equal loading control. (C) HepG2215 cells were treated with the indicated concentrations of Tp for different times and HBsAg levels were determined by ELISA. (D) HepG2215 cells were treated with the indicated concentrations of Tp for different times and HBeAg levels were determined by ELISA.

diagnostic kit. After prolonged treatment for 6 days, a reduction in the HBsAg and HBeAg production could be observed with Tp at a concentration as low as 0.1 mM, and 1.1 mM Tp reduced the levelS of HBsAg and HBeAg production by about 80% and 69%, respectively ($p < 0.01$; Fig. 1B and C). These results suggested that Tp had a similar inhibitory effect on the production of the viral protein HBsAg and HBeAg.

3.2. Tp treatment reduced levels of HBV-RNA and replicative intermediates in HepG2 cells

The antiviral activity of Tp was also tested using the human hepatoma cell line HepG2 transfected with a HBV head-to-tail dimmer plasmid that express all viral proteins, RNAs, produce replicative intermediates, and secrete mature virions (Sells et al., 1987).

In order to elucidate the antiviral effects of Tp on an ongoing infection, Tp was added to the medium of the cells 2 days after transfection. To get an idea of the level at which HBV propagation is inhibited by Tp, the time course of Tp-mediated reduction of the viral products was assayed. All experiments were repeated at least twice. When no Tp was added to the cells, the levels of viral RNAs, replicative intermediates, secreted HBeAg and HBsAg increased up to the end of the observation period, whereas intracellular HBeAg and HBsAg was highest at the earliest time point measured, and then slightly decreased (Fig. 2). Viral RNA levels were reduced to 63% and 65% of those in untreated control load at 36 h with 1 mM and 2 mM Tp treatment (Fig. 2A). In contrast to late reduction of viral RNA by Tp, the level of HBV RNA was not reduced 18 h and 24 h after addition of Tp. The levels of replicative intermediates were sharply reduced 24 h and 48 h after Tp treatment (Fig. 2B). The levels of secreted and intracellular HBeAg and HBsAg were also reduced by Tp treatment (Fig. 2C and D), whereas the level of intracellular β-actin protein was not affected (data not shown), indicating the specificity of the Tp-induced antiviral activity for HBV in our system. Taken together, treatment of HBV-transfected cells with 1 mM and 2 mM Tp specifically reduced expression of replicative DNA intermediates and viral RNAs (Fig. 2A and B). The inhibition was strongest for replicative intermediates and lower for the viral

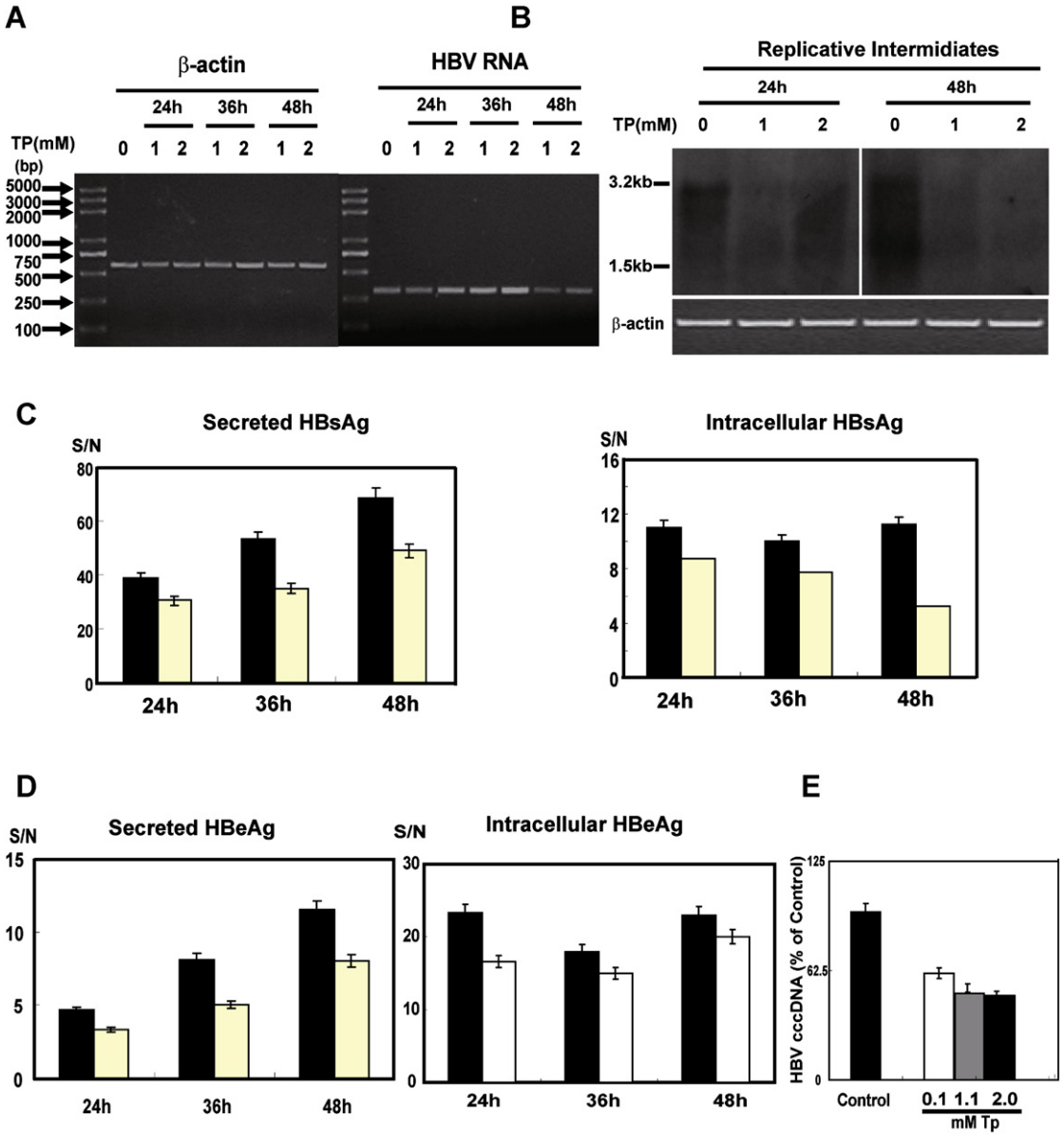

Fig. 2. Kinetics of Tp-induced reduction of the levels of all HBV products in HepG2 cells concomitantly with dimer DNA. (A) Levels of HBV RNA were determined by semi-quantitated RT-PCR analysis.Total RNA was used as template to synthesize cDNA. HBV-specific primers were then applied. β-Actin was used as equal loading control. (B) Levels of replicative DNA intermediates were determined by southern blot hybridized with ^{32}P-labeled HBV-DNA probe. β-Actin was used as loading control. (C and D) secreted products and intracellular HBeAg and HBsAg. Forty hours after transfection of HepG2 cells with HBV-DNA and the pGFP control gene construct the variation of the transfection efficiency was controlled in every individual sample by using an enzyme assay for GFP expressed (data not shown). Thereafter, the cells were treated with 1 mM Tp for 18 h, 24 h or 36 h. The amount of HBeAg and HBsAg was determined by enzyme immunoassays. S/N indicates the ratio between antigen concentration in media from transfected and mock-transfected cells. (E) HepG2 22'5 cells were treated with or without Tp for 6 days. HBV DNA was treated with Plasmid-Safe ATP-dependent DNase and then HBV cccDNA levels were measured by real time PCR.

RNAs. The amount of secreted viral HBeAg and HBsAg was also significantly reduced in Tp-treated cells (Fig. 2C and D). The kinetics of these effects suggest that Tp induces in vitro at least two distinct HBV-specific antiviral activities, one affecting the steady-state level of viral RNAs and one that of replicative DNA intermediates. These data demonstrate for the first time that Tp treatment can specifically reduce in a cell culture system the amount of all intracellular and extracellular HBV products tested.

The observation that Tp effectively reduced the level of viral replicative intermediates raised the possibility that Tp might also affect the formation of HBV cccDNA. To examine the effect of Tp on the amount of cccDNA produced, viral nucleic acid was extracted from HepG2 15 cells at day 6 after Tp treatment and then was treated with Plasmid-Safe ATP-dependent DNase (PSAD) to reduce the relaxed circular DNA (rcDNA). The products were further amplified by real time PCR with primers spanning the two direct repeat regions of HBV genome. Viral cccDNA levels were reduced to 62% and 57% of those in untreated control load with 0.1 mM and 2 mM Tp treatment, indicating that Tp can exert a potent inhibitory effect via its effects on cccDNA formation (Fig. 2E).

3.3. *The expression of HBsAg protein in HBV-transgenic mice was inhibited by Tp treatment*

Having established that Tp had antiviral effects in cultured HepG2215 cells, we then proceeded to evaluate its antiviral effect in the HBV-transgenic mice. Mice were randomly assigned to either treatment or control groups ($n = 5$). Treatment was for 60 days with either saline, LMV (100 mg/kg/day), or Tp (5, 20 or 40 mg/kg/day) by intragastric administration. Serum samples were collected and

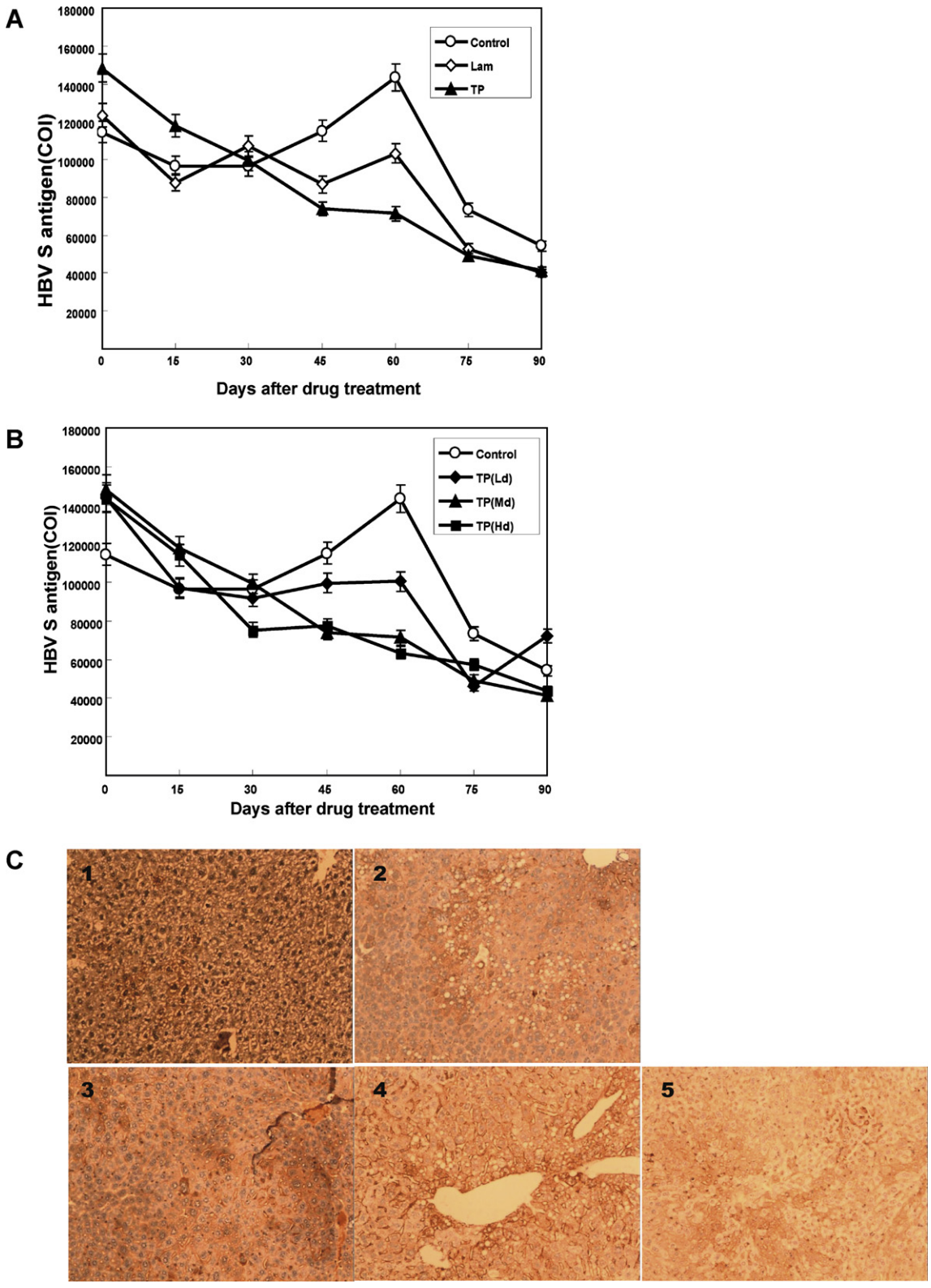

Fig. 3. The expression of HBsAg protein in HBV-transgenic mice was inhibited by Tp treatment. (A) Serum HBsAg levels were measured by ELISA assay in HBV-transgenic mice treated with LMV or Tp for different times. (B) Serum HBsAg levels were measured by ELISA assay in HBV-transgenic mice treated with Tp of different concentrations for different times. (C) Representative immunohistochemical staining of HBsAg on liver from HBV-transgenic mice treated with (1) saline, (2) LMV 100 mg/kg/day, (3) Tp 5 mg/kg/day, (4) Tp 20 mg/kg/day, (5) or Tp 40 mg/kg/day. (1–5) Original magnification ×200.

assayed for HBV HBsAg level in the appropriate mice every 2 weeks. As was shown in Fig. 3A, a 39% decrease in serum HBsAg levels was observed at 60 days following LMV treatment, while 20 mg/kg Tp treatment for 60 days led to 56% reduction in serum HBsAg levels; furthermore, this inhibitory effect of Tp on HBsAg levels was also shown in a dose-dependent manner (Fig. 3B).

Liver sections from mice were also stained for HBsAg by immunohistochemical staining at 30 days after Tp treatment.

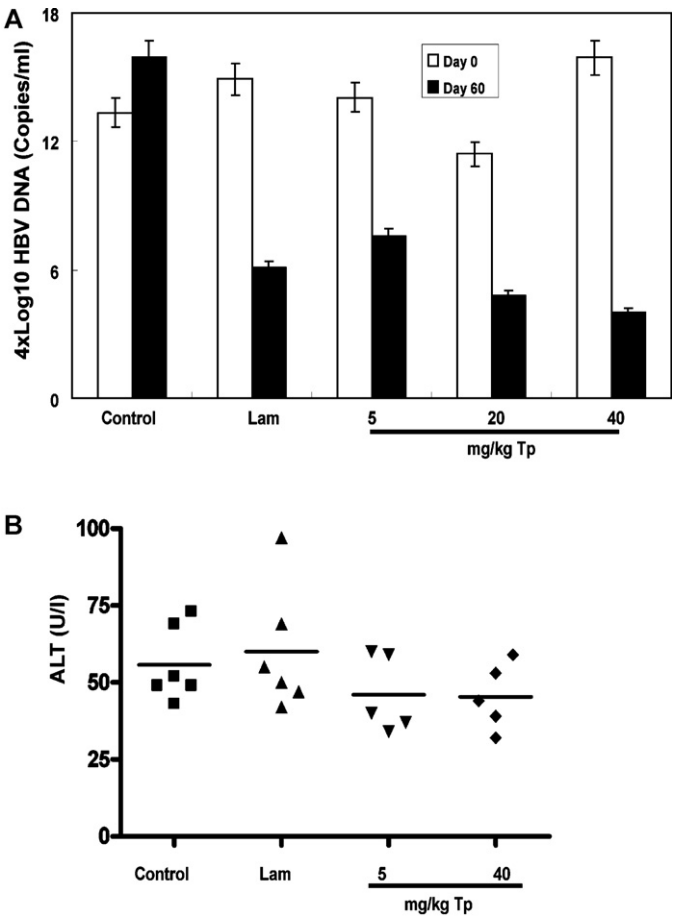

Fig. 4. HBV replication in HBV-transgenic mice was inhibited by Tp treatment. (A) Cells were treated with or without Tp at the indicated doses for 60 days. Serum DNA was extracted and HBV DNA levels were measured by FQ-PCR assay in HBV-transgenic mice. (B) Liver damage was assessed by measuring mouse serum ALT after 60 days of Tp treatment.

In concert with decreased serum HBsAg protein levels, the intensity of HBsAg (Fig. 3C) staining in LMV and Tp treatment mice showed a significant decrease compared with control group.

3.4. HBV replication in HBV-transgenic mice was inhibited by Tp treatment

Circulating HBV DNA was also monitored in the HBV-transgenic mice using real-time PCR. As expected, serum HBV DNA remained constant in all groups after saline injection, while serum HBV DNA was inhibited after Tp treatment in a dose-dependent manner. 20 mg/kg Tp treatment for 60 days reduced the level of serum HBV DNA by about 59% ($p < 0.01$; Fig. 4A).

In addition, liver damage was measured by serum ALT levels. HBV-transgenic control mice exhibited normal or slightly higher ALT levels compared with high dose Tp-treated mice at days 60, albeit without significance difference ($p = 0.08$; Fig. 4B).

4. Discussion

In this study, we found that Tp could suppress HBV replication in an HBV-producing cell line and in the HBV-transgenic mice. Our current results demonstrated first that Tp suppressed synthesis of viral replicative intermediates and HBV main protein production in cell culture medium. HBV Transgenic mice evaluation model results also illustrated that Tp could be effective at a dose as low as 5 mg/kg and in this study was most effective at doses around 20 mg/kg. These effective doses are comparable with the suggested Tp clinical doses for asthma therapy. The data presented suggest that some DNA damage response proteins are implicated in modulating HBV infections and therefore might be used for the treatment of drug-resistant viral strains that have developed as a result of current anti-HBV therapeutic approaches. In addition, targeting cellular proteins that have a low mutation rate may not lead to the rapid emergence of HBV strains that are resistant to inhibitors of these proteins.

The ATM and ATR kinases are targets of a known inhibitor of DNA repair, namely, caffeine (CF). CF was shown to inhibit the activity of these kinases in vitro and the response to cellular DNA damage controlled by ATM and ATR. Theophylline (Tp) is a product of CF metabolism in vivo and has been clinically used for the treatment of asthma. Both we and other groups have demonstrated that Tp exhibits CF-like effects on the DNA damage response. The effect of CF is mediated by the inhibition of its cellular target, the ATR kinase, that is involved in retroviral integration (Daniel et al., 2003; Zimmerman et al., 2006; Roshal et al., 2003; Lau et al., 2005; Nunnari et al., 2005). Our previous reports suggest that Tp exert much strong inhibitory effect on HBV replication compared to CF and its other metabolism products (Zhao et al., 2008a and unpublished data), the precise role of ATR in HBV replication is unclear. The integration of HBV DNA into chromosomes has been reported. Interestingly, treatment with Tp had an inhibitory effect on HBV replication even at a concentration as low as 0.1 mM, a concentration too low to impact ATM/ATR kinase activity. Therefore, the antioxidant function of Tp may have an inhibitory effect on the HBV infection process, and further experiments should be done in this direction.

Unlike most viruses, HBV RNA transcription and protein production are effectively separated from viral genome replication due to the presence of a long-lived population of covalently closed viral template genomes in the host cell nucleus (cccDNA) (Locarnini, 2004). Intracellular HBV replication takes place in viral nucleocapsids located in the cytoplasm. As a result, most compounds that inhibit HBV DNA replication (e.g. nucleoside analogues), do not typically alter HBV protein production, indicating a novel mechanism of action of Tp against HBV. Our studies found that Tp could inhibit HBV DNA replication as well as the production of major HBV proteins. The potency of Tp against HBsAg and HBeAg was similar to that observed against HBV replicative intermediates in the same experiment. Our unpublished data also show that Tp treatment could lead to reduced HBV cccDNA level in a cell culture system. Taken all data together, Tp may play a dual protective role against HBV infection.

Acknowledgements

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