

VIRAL HEPATITIS

N-terminal and C-terminal cytosine deaminase domain of APOBEC3G inhibit hepatitis B virus replication

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Supported by the National Natural Science Foundation of China, No. 30271170 and 30571646, and the National Key Basic Research Program of China, No. 20014CB510008 and 2005CB522900

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Received: 2006-07-27 Accepted: 2006-11-03

The expression of hepatitis B virus core antigen (HBcAg) in the transfected cells was determined by Western blot analysis. Core-associated HBV DNA was examined by Southern blot analysis. Levels of HBV DNA in the sera of mice as well as HBV core-associated RNA in the liver of mice were determined by quantitative PCR and quantitative RT-PCR analysis, respectively.

RESULTS: Human APOBEC3G exerted an anti-HBV activity in a dose-dependent manner in HepG2 cells, and comparable suppressive effects were observed on genotype B and C as that of genotype A. Interestingly, the N-terminal or C-terminal cytosine deaminase domain alone could also inhibit HBV replication in HepG2 cells as well as Huh7 cells. Consistent with *in vitro* results, the levels of HBsAg in the sera of mice were dramatically decreased, with more than 50 times decrease in the levels of serum HBV DNA and core-associated RNA in the liver of mice treated with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain as compared to the controls.

CONCLUSION: Our findings provide probably the first evidence showing that APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain could suppress HBV replication *in vitro* and *in vivo*.

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Key words: Cytosine deaminase domain; Apolipoprotein B mRNA-editing enzyme catalytic-polypeptide 3G; Hepatitis B virus; Antiviral therapy

Lei YC, Tian YJ, Ding HH, Wang BJ, Yang Y, Hao YH, Zhao XP, Lu MJ, Gong FL, Yang DL. N-terminal and C-terminal cytosine deaminase domain of APOBEC3G inhibit hepatitis B virus replication. *World J Gastroenterol* 2006; 12(46): 7488-7496

<http://www.wjgnet.com/1007-9327/12/7488.asp>

Abstract

AIM: To investigate the effect of human apolipoprotein B mRNA-editing enzyme catalytic-polypeptide 3G (APOBEC3G) and its N-terminal or C-terminal cytosine deaminase domain-mediated antiviral activity against hepatitis B virus (HBV) *in vitro* and *in vivo*.

METHODS: The mammalian hepatoma cells HepG2 and Huh7 were cotransfected with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression vector and 1.3-fold-overlength HBV DNA as well as the linear monomeric HBV of genotype B and C. For *in vivo* study, an HBV vector-based mouse model was used in which APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression vectors were co-delivered with 1.3-fold-overlength HBV DNA *via* high-volume tail vein injection. Levels of hepatitis B virus surface antigen (HBsAg) and hepatitis B virus e antigen (HBeAg) in the media of the transfected cells and in the sera of mice were determined by ELISA.

INTRODUCTION

Hepatitis B virus (HBV) infects more than 350 million people worldwide and is a leading cause of end-stage liver disease and of hepatocellular carcinoma^[1]. HBV is non-cytopathic for hepatocytes; however, most newly HBV-

infected adult patients develop acute hepatitis because of a strong immune response that clears HBV from the liver, whereas approximately 5% of newly HBV-infected adult patients generate insufficient immunity and become chronically infected^[1,2]. HBV and other hepadnaviruses replicate their partially double-stranded DNA genome within cytoplasmic core particles by reverse transcription of encapsidated pregenomic RNA and thus are related to retroviruses^[3].

The cytidine deaminase APOBEC3G (A3G), which is encoded within a cluster of seven related editing enzymes (APOBEC3A-G) on chromosome 22, provides a broad innate immunity against exogenous and endogenous retroelements^[4-10]. It has been shown that the propagation of HIV-1 strains lacking the accessory protein virion infectivity factor (VIF) is suppressed in non-permissive cells due to expression of the cytidine deaminase APOBEC3G^[5,11]. Encapsidated into the retroviral particle, APOBEC3G induces massive C→U deamination of single-stranded retroviral DNA, resulting in DNA degradation or lethal G→A hypermutation^[6-8]. HIV overcomes this innate defense barrier in T-cells with the help of the HIV-encoded VIF protein that specifically targets A3G to proteosomal degradation^[12-14]. In addition, APOBEC3F (another cytidine deaminase of APOBEC family) is a potent retroviral restrictor, but its activity, unlike that of APOBEC3G, is partially resistant to HIV-1 VIF and results in retroviral hypermutation. Moreover, APOBEC3F and APOBEC3G appear to be coordinately expressed in a wide range of human tissues and are independently able to inhibit retroviral infection. Thus, APOBEC3F and APOBEC3G are likely to function alongside one another in the provision of an innate immune defense, with APOBEC3F functioning as the major contributor to HIV-1 hypermutation *in vivo*^[15]. Recent evidence suggests that some of the HIV restriction exerted by A3G may be independent of its cytidine deaminase activity^[16-18].

Interestingly, APOBEC3G can also interfere with the HBV life-cycle in co-transfected cells^[19,20]. However, reduced levels of encapsidated viral pgRNA rather than extensive editing was found to be the major contributing factor^[19], APOBEC3G-mediated editing did occur but was only detected in a minority of clones produced in the transfected HepG2 hepatoma cells^[20]. In addition, the C-terminal cytosine deaminase domain catalytically inactive APOBEC3G derivatives no longer inhibited VIF-defective HIV-1 but did conserve wild-type levels of activity on HBV^[19]. As a result, the mechanisms of APOBEC3G blocking HBV production are unclear.

A previous study shows that APOBEC family members contain a domain structure characteristic of cytidine deaminases^[21]. A short α -helical domain is followed by a catalytic domain (CD), a short linker peptide and a pseudocatalytic domain (PCD). In APOBEC3G, the entire unit is duplicated to form the domain structure helix1-CD1-linker1-PCD1-helix2-CD2-linker2-PCD2. Each catalytic domain contains the conserved motif H-X-E-(X)27-28-P-C-X-X-C in which the His and Cys residues coordinate Zn²⁺ and the Glu serves as a proton shuttle in the deamination reaction^[22]. Based on the molecular

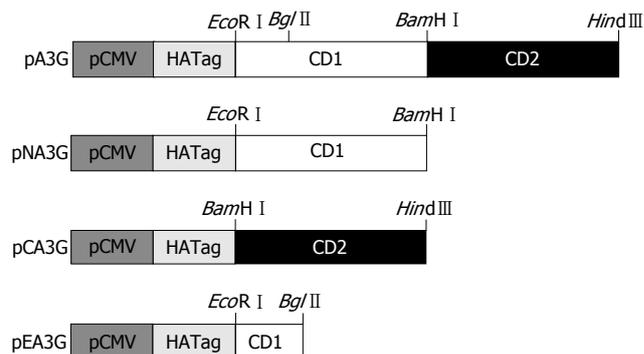


Figure 1 Construction of APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain expression plasmids. Full-length APOBEC3G sequence was cloned into *EcoR* I/*Hind* III restriction sites of the CMV-driven expression vector fused with a hemagglutinin fusion epitope tag at its N-terminal end (pXF3H) to construct APOBEC3G expression plasmid (pA3G). pA3G was digested with *EcoR* I/*Bam* H I and *Bam* H I/*Hind* III, these fragments were inserted into pXF3H to construct the N-terminal and C-terminal cytosine deaminase domain expression plasmids (pXFNA3G and pXFCA3G), respectively. pA3G was digested with *EcoR* I/*Bgl* II, and used for the construction of the N-terminal region of APOBEC3G which did not contain any cytosine deaminase domain plasmid (pXFA3G). pCMV: CMV promoter; CD1: N-terminal cytosine deaminase domain; CD2: C-terminal cytosine deaminase domain.

structure of APOBEC3G and recent research results, we speculated that the N-terminal or C-terminal cytosine deaminase domain of APOBEC3G could also display HBV inhibition activity.

In the present study, we performed a detailed analysis of the inhibitory effect of APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain on hepatitis B virus replication in cell culture and in an HBV vector-based mouse model.

MATERIALS AND METHODS

Plasmid constructs

For construct expression vectors coding for human APOBEC3G (pXFA3G), total RNA was extracted from the peripheral blood mononuclear cells (PBMCs). RT-PCR amplification of human APOBEC3G sequence was carried out using forward primer 5'-CGGAATTC AAGCCTCACTTCAGAAACAC-3' and reverse primer 5'-CGAAGCTTTCTGCCTTCCTTAGAGACTG-3'. The PCR product was cloned into *EcoR* I/*Hind* III restriction sites of the CMV-driven expression vector fused with a hemagglutinin fusion epitope tag at its carboxy terminal end (pXF3H). For construction of the N-terminal and C-terminal cytosine deaminase domain expression plasmids, the unique restriction enzyme site *Bam* H I in the linker area of APOBEC3G, two cytosine deaminase domains were used. pXFA3G was digested with *EcoR* I/*Bam* H I and *Bam* H I/*Hind* III, these fragments were inserted into pXF3H to construct the N-terminal and C-terminal cytosine deaminase domain expression plasmids (pXFNA3G and pXFCA3G), respectively. Next, pXFA3G was digested with *EcoR* I/*Bgl* II, and used for construction of the N-terminal region of APOBEC3G which did not contain any cytosine deaminase domain plasmid (pEA3G) (Figure 1). Replication competent of wild-type HBV 1.3-fold-overlength plasmid (subtype, ayw) and linear monomeric HBV genomes of

genotype B and genotype C with *Sap* I ends have been constructed previously in our laboratory.

Cell culture, transfection and harvesting

HepG2 and Huh7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 mL/L fetal calf serum (Invitrogen, CA, USA) at 37°C in a humidified atmosphere containing 50 mL/L CO₂. The cells were plated at a density of 4.5×10^5 cells per well in 6-well plates 18 h prior to transfection. Transfection of cells was performed with lipofectamine 2000 (Invitrogen, USA) following the manufacturer's guidelines. On d 3 after transfection, the cells were removed from the culture dish *via* treatment with trypsin-EDTA, resuspended in culture medium, washed with phosphate-buffered saline (PBS), pelleted, and resuspended in 1 mL chilled lysis buffer (140 mmol/L NaCl, 1.5 mmol/L MgCl₂, 50 mmol/L Tris-HCl [pH 8.0]) containing 5 mL/L NP-40. Nuclei were removed *via* centrifugation for 5 min at 2000 r/min in an eppendorf centrifuge, and the supernatant was cleared of cell debris by centrifugation for another 5 min at 14 000 g.

HBV vector-based mouse model

For the *in vivo* experiments, 6 to 8-wk-old female BALB/c mice were used. A total of 30 mice were randomly divided into 5 groups (6 mice/group). Replication-competent pHBV1.3 (10 µg) and APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression vectors (10 µg) or pXF3H (10 µg) control plasmid DNA were co-injected into the tail vein of mice in a volume of Ringer's injection equivalent to 9% of the mouse body weight^[23,24], the total volume was delivered within 5 s. The mice sera were collected at an indicated time after hydrodynamic injection, and secreted hepatitis B virus surface antigen (HBsAg) levels and HBV DNA content were measured. All mouse experiments were carried out according to the guidelines established by the Institutional Animal Care and Use Committee at the Tongji Medical College, Huazhong University of Science and Technology.

HBsAg and HBeAg assays and Western blot analysis

Levels of HBsAg and HBeAg in the media of the transfected cells, and in the sera (1:100 dilution) of the treated mice were determined using an ELISA kit (Shanghai Shiye Kehua Company, China). For Western blot analysis, cytoplasmic lysates were incubated with 1 vol 2 × loading buffer containing 100 mL/L beta-mercaptoethanol for 10 min at 95°C before loading on a 125 g/L SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane *via* electroblotting. The membranes were incubated with HBV core-specific rabbit antiserum (Santa Cluz, USA) or with anti-hemagglutinin fusion epitope monoclonal antibody (Santa Cluz, USA), followed by horseradish peroxidase-conjugated mouse anti-rabbit antibody. Proteins were visualized by Enhanced Chemiluminescence (Roche, Germany).

HBV DNA purification and analysis

The method for purification of cytoplasmic core-associated HBV DNA was adapted from Pugh *et al*^[25]. Briefly, HepG2 cells were disrupted in lysis buffer (100 mmol/L Tris-HCl, pH 8.0, 2 mL/L NP-40). The cell lysate was clarified by

centrifugation at 13 000 g for 1 min to pellet nuclei and insoluble material. The supernatant was adjusted to 6 mmol/L MgOAc₂ and incubated for 2 h at 37°C with 200 µg/mL DNase I and 100 µg/mL RNase A. Following digestion, the lysate was centrifuged for 1 min at 13 000 g. The supernatant was incubated for 1 h at 55°C after addition of 10 mmol/L EDTA, 10 g/L SDS, 100 mmol/L NaCl and 200 µg/mL proteinase K. Finally, the sample was extracted with phenol:chloroform. The DNA was ethanol precipitated, resuspended in TE at pH 8 (10 mmol/L Tris-HCl, pH 7.5; 1 mmol/L EDTA) and digested with 100 µg/mL RNase A for 30 min at 37°C. The purified DNA was subjected to Southern blot analysis. DNA samples were loaded onto 13 g/L agarose gels, blotted onto nylon membranes, and probed with a Dig-labeled full-length HBV genome in EasyHyb hybridization solution (Roche, Germany).

For quantitative PCR, 100 µL of mouse serum was adjusted to 6 mmol/L MgOAc₂ and incubated for 2 h at 37°C with 200 µg/mL DNase I and 100 µg/mL RNase A. Following proteinase K digestion, the sample was extracted with phenol:chloroform. HBV DNA levels were analyzed with the Light Cycle real-time PCR system (Roche, Germany), using the primer sequences as follows: 5'-TCA-CAATACCGCAGAGTC-3' (nt231-248, forward) and 5'-AGCAACAGGAGGGATACA-3' (nt569-552, reverse). The pHBV1.0 vector containing the full-length HBV genome was used as a standard curve to calculate HBV copies per milliliter of serum.

RNA isolation and analysis

For quantitative RT-PCR, approximately 20 mg of liver tissue was obtained from each mouse for total RNA extraction with RNeasy Total RNA kit (QIAGEN, Germany), according to the manufacturer's protocol. cDNA was synthesized from 2 µg of total RNA with oligo(dT)₁₈ primer in a total volume of 20 µL. Quantitative RT-PCR was performed with the Light Cycler real-time PCR system (Roche, Germany). The PCR primers were as follows: GAPDH, 5'-GTTGTCTCCTGCGACTTCA-3' (forward) and 5'-GGTGGTCCAGGGTTTCTTA-3' (reverse); HBV, 5'-TCACAATACCGCAGAGTC-3' (nt231-248, forward) and 5'-AGCAACAGGAGGGATACA-3' (nt569-552, reverse). A standard curve was constructed by the simultaneous amplification of serial dilutions of the expression plasmid encoding HBV used as templates. Target cDNAs were normalized to the endogenous RNA levels of the GAPDH. Quantitative amplification was carried out using the SYBR Green kit (Invitrogen, USA). Gene expression was determined using the relative quantification: $\Delta\Delta C_T = (C_{\text{HBV}} - C_{\text{T GAPDH}})_{\text{Test}} - (C_{\text{HBV}} - C_{\text{T GAPDH}})_{\text{Control}}$. C_T is the fractional cycle number that reaches a fixed threshold, C_{Test} is the test of interest, and C_{Control} is the reference control (RNA from control group). ΔC_T is the difference between gene expression in the treated cells and the reference control cells. The fold increase was calculated using $2^{\Delta\Delta C_T}$ ^[26].

RESULTS

Inhibitory effect of APOBEC3G on HBV DNA replication in cell culture

Various amounts of a CMV-driven expression vector

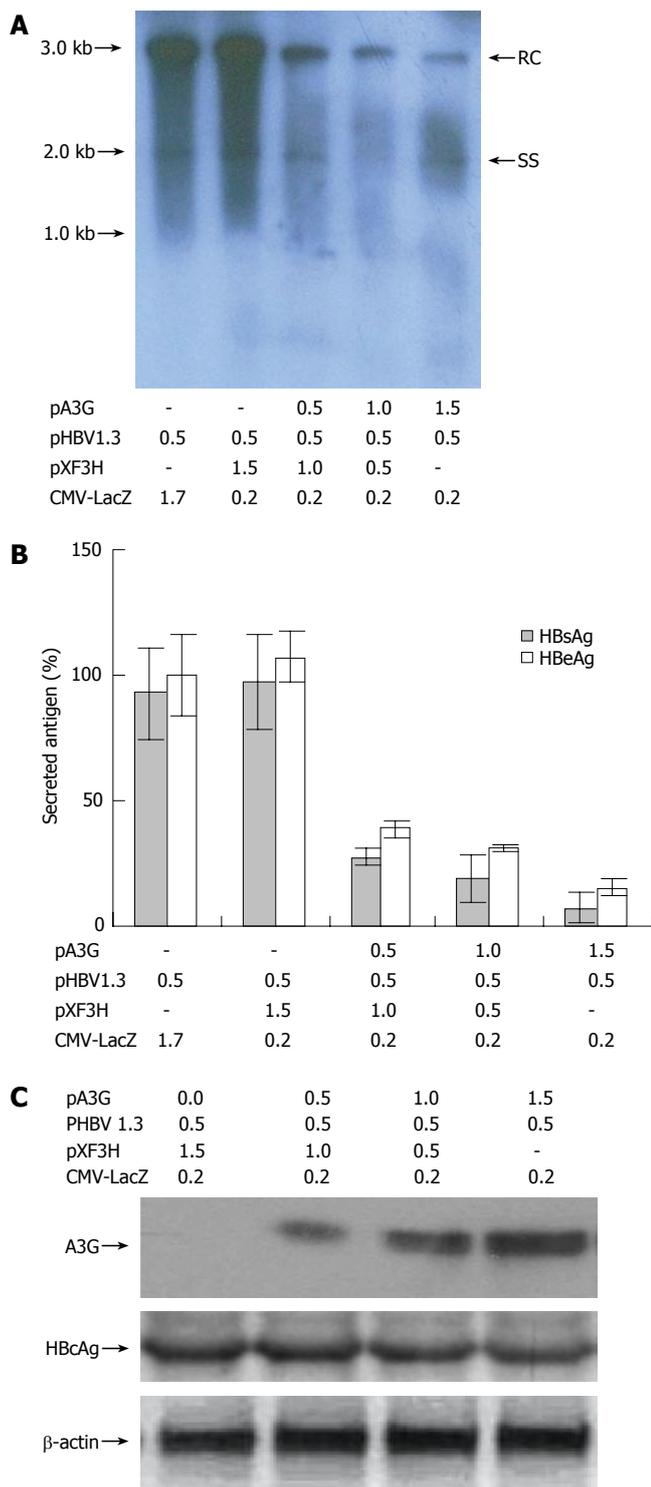


Figure 2 Effect of APOBEC3G on HBV replication in co-transfected HepG2 cells. (A) Human hepatoma HepG2 cells were transiently co-transfected with pHBV1.3 and various amounts of a CMV-driven expression vector encoding A3G or with empty vector pXF3H and pCMV-LacZ using Lipofectamine 2000 reagents. The cells were harvested 3 d after transfection. HBV core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates. Viral replicative DNA intermediates were analyzed by Southern blotting using a Dig-labeled full-length HBV DNA probe. (B) HBsAg and HBeAg levels were determined in the media of co-transfected HepG2 cells by ELISA, normalized to the activity of co-transfected β -galactosidase in the cell lysates. The mean \pm SE of six independent experiments is shown (error bar indicates standard error). (C) Western blot analysis of cytoplasmic extracts from HepG2 cells co-transfected with the indicated plasmids. Numbers at the end or top of the lines indicate the amount of transfected plasmid DNA in micrograms. HBV: hepatitis B virus; pA3G: APOBEC3G expression plasmids; RC: relaxed circular DNA; SS: single-stranded DNA; HBcAg: hepatitis B virus core antigen; HBsAg: hepatitis B virus surface antigen; HBeAg: hepatitis B virus e antigen.

encoding A3G (pA3G) and empty vector (pXF3H) were co-transfected into the human hepatoma cells HepG2 together with replication-competent 1.3-fold-overlength HBV and with pCMV-LacZ to normalize for the transfection efficiency. Three days after transfection, core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates and analyzed with Southern blotting. HBsAg and HBeAg levels in the media of co-transfected cells were determined by ELISA. As shown in our previous study^[27], A3G reduced the level of HBV-replicative intermediates in a dose-dependent manner (Figure 2A). In a series of six independent experiments, the expression of A3G resulted in a dose-dependent decrease in the levels of extracellular production of HBsAg and HBeAg even after correction for transfection efficiency by determination of the co-transfected β -galactosidase (Figure 2B). However, the expression of core protein did not change (Figure 2C).

Suppression of the replication of HBV clinical isolates of different genotypes by APOBEC3G

Based on sequence divergence, HBV can be classified into at least 8 genotypes^[28]. These results, in agreement with our and other's results^[19,27], showed that APOBEC3G inhibited the replication of a laboratory clone of genotype A (subtype ayw). Further testing concerned whether or not APOBEC3G could also inhibit the replication of HBV with different genotypes from clinical isolates. As genotypes B and C are the most prevalent genotypes in China, this study mainly focused on these 2 genotypes. A previous study has demonstrated that linear monomeric HBV genomes with *Sap* I ends can initiate a full replication cycle, leading to viral replication and antigen expression^[29].

In order to examine the effect of APOBEC3G on different genotypes of clinical isolates, a full-length HBV genome of genotype B or C was amplified from the patient's serum by PCR and cloned into pUC19 vector. After digestion with *Sap* I, the linear monomeric HBV genomes were transfected into HepG2 cells with APOBEC3G expression plasmid (pA3G) or the control plasmid (pXF3H). Three days after transfection, HBV core-associated DNA was analyzed by Southern blot. As shown in Figure 3, pA3G could also inhibit the replication of HBV genotypes B and C from clinical isolates to a similar extent as on genotype A (pHBV1.3).

Inhibitory effect of N-terminal and C-terminal cytosine deaminase domain of APOBEC3G on HBV DNA replication in cell culture

APOBEC family members contain a domain structure characteristic of cytosine deaminases^[21]. A short α -helical domain is followed by a catalytic domain (CD), a short linker peptide and a pseudocatalytic domain (PCD). In APOBEC3G, the entire unit is duplicated to form the domain structure helix1-CD1-linker1-PCD1-helix 2-CD2-linker2-PCD2. Each catalytic domain contains the conserved motif H-X-E-(X)27-28-P-C-X-X-C in which the His and Cys residues coordinate Zn^{2+} and the Glu serves as a proton shuttle in the deamination reaction^[22]. A recent study has shown that APOBEC3G can interfere with the HBV life-cycle, but APOBEC3G-mediated

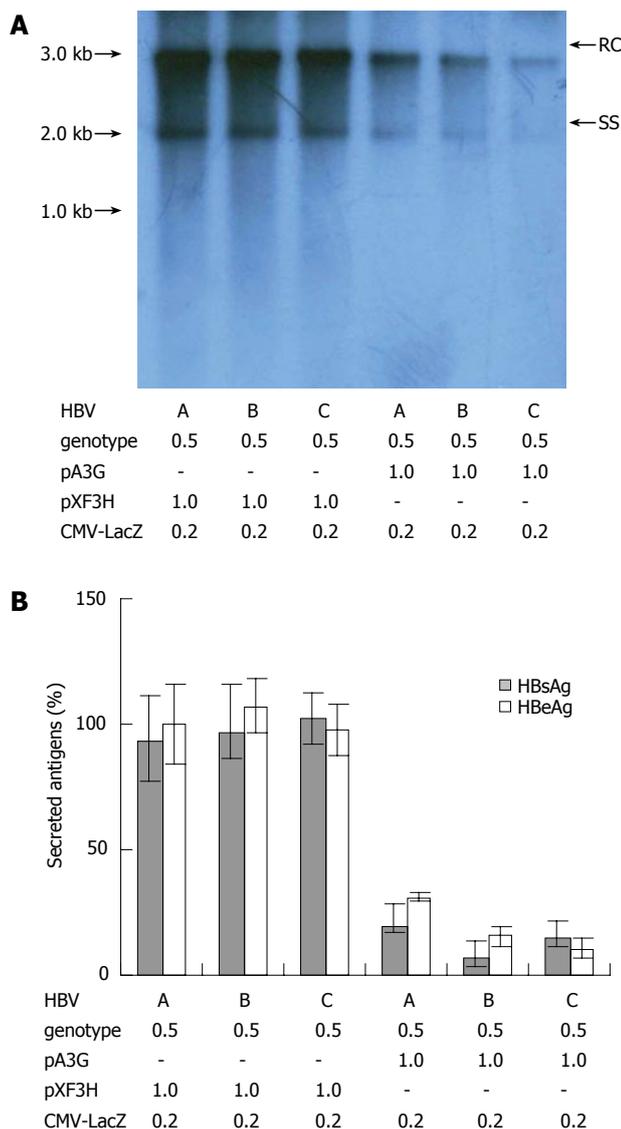


Figure 3 Suppression of replication of HBV clinical isolates of different genotypes by APOBEC3G. (A) pHBV1.3 or the linear monomeric HBV genomes of genotype B and C were transiently co-transfected into HepG2 cells with the CMV-driven expression vector encoding A3G or empty vector pXF3H and pCMV-LacZ using Lipofectamine 2000 reagents. The cells were harvested 3 d after transfection. HBV core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates. Viral replicative DNA intermediates were analyzed by Southern blotting using a Dig-labeled full-length HBV DNA probe. (B) HBsAg and HBeAg levels were determined in the media of co-transfected HepG2 cells by ELISA, normalized to the activity of co-transfected β -galactosidase in the cell lysates. The mean \pm SEM of six independent experiments is shown (error bar indicates standard error). pA3G: APOBEC3G expression plasmids; RC: relaxed circular DNA; SS: single-stranded DNA.

editing of nucleic acids does not seem to represent the major contributing factor^[10]. We speculated that the N-terminal or C-terminal cytosine deaminase domain of APOBEC3G could also display HBV inhibitory activity.

To verify this hypothesis, APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression plasmids were co-transfected with replication-competent 1.3-fold-overlength HBV DNA into HepG2 and HuH7 cells, and with pCMV-LacZ to normalize for the transfection efficiency. HBV core-associated viral DNA was prepared 3 d after transfection and analyzed

by Southern blotting. HBsAg and HBeAg levels were determined by ELISA. As expected, N-terminal or C-terminal cytosine deaminase domain of APOBEC3G expression plasmid (pNA3G and pCA3G) could also reduce the level of replicative HBV intermediates and HBsAg and HBeAg levels in the media of co-transfected HepG2 cells, while the N-terminal region of APOBEC3G, which did not contain any cytosine deaminase domain (pEA3G), had no inhibitory effect on HBV production, and the same results were observed in another human hepatoma cell Huh7 (Figures 4A and 4B). In a series of six independent experiments, the expression of A3G and its N-terminal or C-terminal cytosine deaminase domain, but not of pEA3G, resulted in a consistent decrease of secreted HBsAg and HBeAg even after correction for transfection efficiency by determination of the co-transfected galactosidase (Figure 4C). Compared with the empty vector control and normalized to the amount of secreted HBsAg, the N-terminal or C-terminal cytosine deaminase domain decreased the formation of intracellular HBV-replicative intermediates in this series to approximately 60% and thus achieved a similar level of inhibition as A3G (Figure 4D). In all of these experiments, pEA3G showed no apparent inhibition of HBV replication (Figure 4D). However, Western blot analysis, using the polyclonal anti-HBV core antibody, indicated that the levels of core protein were unaffected by APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain (Figure 4E).

Inhibition of HBV replication in mice by N-terminal or C-terminal cytosine deaminase domain of APOBEC3G

To examine whether the N-terminal or the C-terminal cytosine deaminase domain of APOBEC3G could also display HBV inhibition *in vivo*, replication-competent HBV plasmid (pHBV1.3) was co-transfected with the expression plasmids of N-terminal or C-terminal cytosine deaminase domain of APOBEC3G or the empty control plasmid (pXF3H) to mouse liver by hydrodynamic injection. As previously shown^[30], hydrodynamic injection of pHBV1.3 into mouse resulted in HBV replication in the liver and secretion of viral antigens to the serum. Consistent with *in vitro* experiments, serum HBsAg and viral DNA in mice that received pHBV1.3 were suppressed dramatically at different time points by the N-terminal or the C-terminal cytosine deaminase domain of APOBEC3G compared to the mice treated with control plasmid (Figures 5A and 5B). Real-time PCR quantification of core-associated HBV RNA in the liver of mice treated with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain of APOBEC3G expression plasmid showed approximately 50 times decrease compared to the controls (Figure 5C).

DISCUSSION

Our study describes the inhibitory effects of human APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain on HBV DNA production in mammalian hepatoma cells as well as in HBV vector-based mouse model. Our data confirm and extend the recent results by Turelli *et al*^[19] who reported A3G-mediated inhibition of HBV DNA production in human

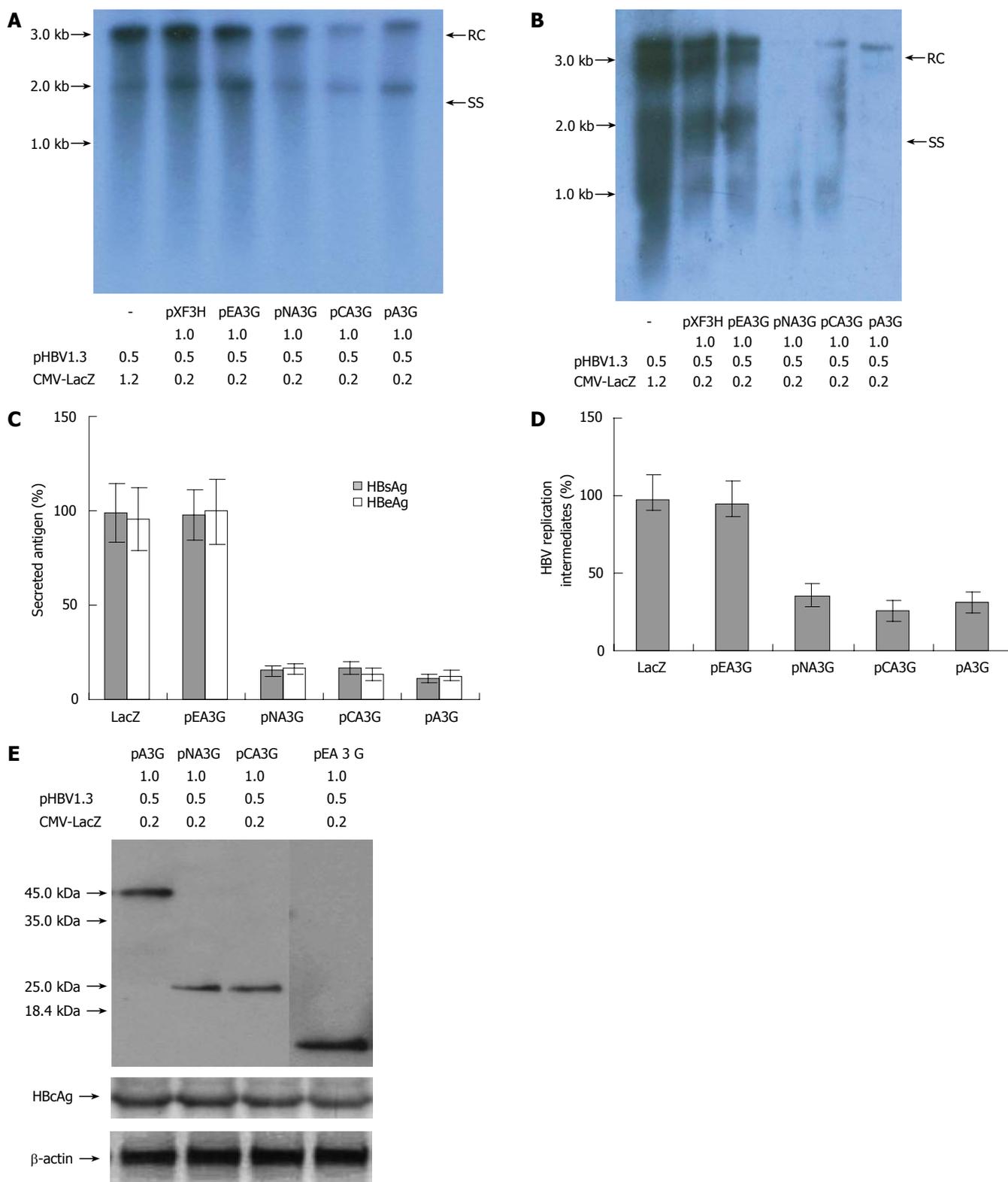


Figure 4 Inhibitory effect of the N-terminal and C-terminal cytosine deaminase domain on HBV production in the co-transfected cells. (A and B) Human hepatoma HepG2 and Huh7 cells were transiently co-transfected with the pHBV1.3 and CMV-driven expression vector encoding A3G and its N-terminal or C-terminal cytosine deaminase domain or with the empty vector pXF3H and pCMV-LacZ using Lipofectamine 2000 reagents. The cells were harvested 3 d after transfection. HBV core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates. Viral replicative DNA intermediates were analyzed by Southern blotting using a Dig-labeled full-length HBV DNA probe. (C) HBsAg and HBeAg levels were determined in the media of co-transfected HepG2 cells by ELISA, normalized to the activity of co-transfected β -galactosidase in the cell lysates. The mean \pm SE of six independent experiments is shown (error bar indicates standard error). (D) The hybridized HBV DNA from the replicative intermediates was quantified by radiophosphorimaging, normalized to the amount of secreted HBsAg (normalized for co-transfected β -galactosidase activity) and calculated relative to the empty vector control (pXF3H). The mean \pm SE of six independent experiments is shown. (E) Anti-HA monoclonal antibody for Western blot analysis of cytoplasmic extracts from HepG2 cells co-transfected with the indicated plasmids. Numbers at the end or top of the lines indicate the amount of transfected plasmid DNA in micrograms. pXF3H: empty vector; pA3G: APOBEC3G expression plasmids; pNA3G: N-terminal cytosine deaminase domain of APOBEC3G expression plasmids; pCA3G: C-terminal cytosine deaminase domain of APOBEC3G expression plasmids; pEA3G: N-terminal region of APOBEC3G which does not contain any cytosine deaminase domain plasmid; HBV: hepatitis B virus; RC: relaxed circular DNA; SS: single-stranded DNA; HBcAg: hepatitis B virus core antigen; HBsAg: hepatitis B virus surface antigen; HBeAg: hepatitis B virus e antigen.

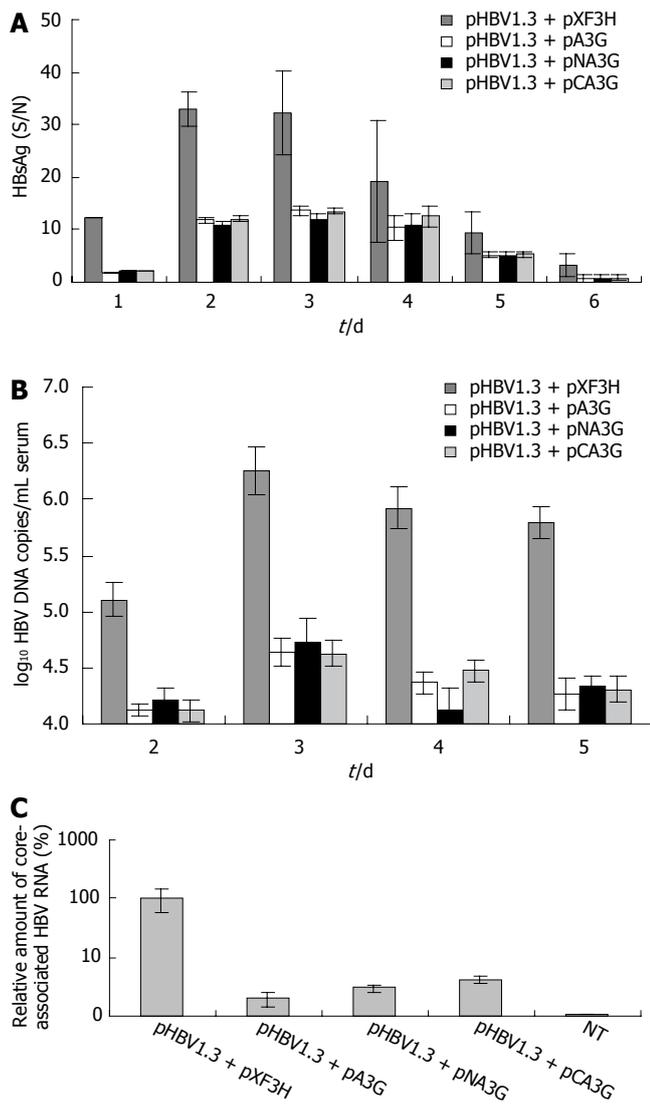


Figure 5 The N-terminal or C-terminal cytosine deaminase domain of APOBEC3G inhibits HBV replication and gene expression in mice. (A) Serum HBsAg levels in the mice treated with APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain expression vector. HBsAg levels (S/N, S: absorbance of the sample, N: absorbance of the negative control) in the sera of BALB/c mice were significantly reduced after treatment with APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain expression plasmids (error bar indicates standard error) in the indicated time points. (B) Real-time PCR quantification of HBV DNA in the sera of mice treated with APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain expression plasmid. BALB/c mice were co-injected with pHBV1.3 vector (10 μ g) and the indicated expression plasmids (10 μ g). HBV DNA levels of sera were determined by quantitative PCR ($n = 6$ per treatment group). (C) Real-time PCR quantification of core-associated HBV RNA in the liver of mice treated with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression plasmids. BALB/c mice were co-injected with pHBV1.3 vector (10 μ g) and the N-terminal or C-terminal cytosine deaminase domain expression vector of APOBEC3G (10 μ g) 3 d after injection, and the levels of the liver HBV RNA were determined by quantitative RT-PCR, normalized to GAPDH mRNA and reported as a ratio of HBV mRNA/GAPDH mRNA (\pm SD) ($n = 6$ per treatment group). NT: non-treated group; pXF3H: empty vector; pA3G: APOBEC3G expression plasmids; pNA3G: N-terminal cytosine deaminase domain of APOBEC3G expression plasmids; pCA3G: C-terminal cytosine deaminase domain of APOBEC3G expression plasmids; HBsAg: hepatitis B virus surface antigen.

HuH-7 hepatoma cells. Previous studies suggested that G-to-A hypermutation could influence HBV pathogenesis. Specific G-to-A changes yielded HBeAg-negative HBV variants, often isolated from patients with acute fulminant

hepatitis, as well as HBV vaccine escape mutants^[31,32]. However, there is no evidence so far that APOBEC3G-induced lethal hypermutation represents an important innate defense mechanism down-regulating hepadnavirus production.

APOBEC3G site-directed mutation experiments showed that both the N- and the C-terminal cytosine deaminase domains were required for inhibiting the infectivity of HIV-based retroviruses^[6,7], and the C-terminal cytosine deaminase domain of APOBEC3G governed the catalytic activity and the retroviral hypermutation specificity, while the N-terminal deaminase domain was to perform other aspects of retroviral restriction^[33]. Another study suggested that the N-terminal deaminase domain of APOBEC3G mediates encapsidation and RNA binding, while the C-terminal cytosine deaminase domain mediates cytidine deaminase activity^[17].

Although APOBEC3G could inhibit HBV replication, extensive editing was not found to be the major contributing factor^[19,20]. Each catalytic domain contains the conserved motif in which the His and Cys residues coordinate Zn²⁺ and the Glu serves as a proton shuttle in the deamination reaction^[22]. In this study, we found the N-terminal and C-terminal cytosine deaminase domain of APOBEC3G could inhibit HBV DNA replication and gene expression in cell culture and the HBV vector-based mouse model. Together with the fact that catalytically inactive APOBEC3G derivatives no longer inhibited VIF-defective HIV-1 but did conserve wild-type levels of activity on HBV, confirming the hypothesis that APOBEC3G might act on HBV and retroviruses through different mechanisms. As a result, we speculated that the RNA binding activity might play a role in these processes, for example, APOBEC3G might bind to the pregenome RNA or interfere with the binding of the ϵ subunit and RT domain of HBV DNA polymerase, but this needs further investigation.

Given the high heterogeneity of HBV sequences, HBV can be classified into 8 genotypes according to the sequence divergence^[28]. Our results and other group showed that APOBEC3G inhibited the replication of genotype A. Whether APOBEC3G could also inhibit the replication of HBV with different genotypes (genotypes B and C) from clinical isolates is a great concern. As we expected, pXFA3G could also inhibit the replication of the most prevalent genotypes B and C from clinical isolates to a similar extent as on pHBV1.3, thereby indicating the inhibitory effect of APOBEC3G is different than that of the siRNAs which is sequence-specific.

The hydrodynamic delivery of nucleic acids in the mouse was described by Liu *et al*^[23], who showed that the vast majority of the injected nucleic acid was delivered to the liver by this technique. Yang *et al*^[30] first demonstrated that hydrodynamic injection of a replication-competent HBV vector resulted in high levels of HBV replication in the livers of the injected mice. In the vector-based model, HBV replicates in the liver of immunocompetent mice for 7-10 d, resulting in detectable levels of HBV RNA and antigens in the liver and of HBV DNA and antigens in the serum. Several reports have documented the use of the HBV vector model to examine the *in vivo* activity

of co-HDI-administered HBV-targeted unmodified siRNAs^[34,35] or vector-expressed short hairpin RNAs^[36] in silencing HBV gene expression. Most notably, we have demonstrated *in vivo* activity of APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression vector via standard intravenous administration. This is probably the first demonstration of APOBEC3G *in vivo* activity in a hepatitis animal model with a clinically viable route of administration. Although we observed a more than 50 times reduction in serum HBV levels after treatment with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain in an HBV mouse model, the potential contribution of toxicity from the APOBEC3G has not been ruled out and will need to be investigated further.

During the course of an acute infection, HBV DNA clearance apparently occurs through noncytopathic mechanisms in which interferon γ (IFN- γ) and tumor necrosis factor α (TNF- α) play an important role^[37,38]. APOBEC3G might participate in this type of antiviral response. Although APOBEC3G is not normally expressed in the liver, but a recent study has shown that APOBEC3G is induced by IFN- α stimulation in human hepatocytes^[39]. It is possible that it is induced by IFN in the course of HBV infection. Several studies have shown that IFNs inhibit HBV replication *in vitro* and *in vivo* in human hepatoma cells and HBV transgenic mice^[40-42]. Currently, IFN- α is an approved treatment for chronic hepatitis B. We speculate that APOBEC3G might be responsible for the anti-HBV action of IFNs under hepatic inflammation. Further analyses will be necessary to determine whether APOBEC3G plays roles in the human innate defense against hepatitis viruses *in vivo*.

Our study demonstrated that APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain effectively inhibited HBV replication in the culture cells and mammalian liver, showing that such an approach could be useful in the treatment of HBV infection. However, whether suppression of viral replication by APOBEC3G plays a role during natural HBV infection seems speculative at present, because expression of APOBEC3G in human liver tissue has not yet been shown. Nevertheless, a better understanding of the mechanisms of A3G action may help identify new therapeutic strategies against chronic hepatitis B.

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S- Editor Wang GP L- Editor Kumar M E- Editor Bai SH