Hepatitis B virus inhibits the expression of complement C3 and C4, *in vitro* and *in vivo*

CHENGLIANG ZHU¹, HUI SONG², FENGXIA XU², WEI YI¹, FANG LIU³ and XINGHUI LIU²

¹Department of Clinical Laboratory, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060;

²Department of Clinical Laboratory, Gongli Hospital, The Second Military Medicine University, Shanghai 200135; ³The State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, P.R. China

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Abstract. The immune system serves an important function in Hepatitis B virus (HBV) infection, and the complement system is a major component of innate immunity. However, the regulatory effect of HBV on complement proteins has not yet been fully elucidated. The present study focused on investigating the impact of HBV on the expression of complement proteins C3 and C4. A total of 226 patients with a clinical diagnosis of HBV infection were enrolled in the study, including 153 with chronic hepatitis B (CHB) and 73 with hepatocellular carcinoma (HCC), whereas 116 healthy individuals were included as a control group. Immunoturbidimetric detection was performed to determine the levels of complement C3 and C4 in the serum of the patients with HBV and the control group. The results revealed that the mean $\pm$ standard deviation C3 and C4 content was 1.223 ± 0.237 and 0.226 ± 0.052 g/l for the control group, 0.687 ± 0.150 and 0.145 ± 0.070 g/l for the patients with CHB, and 0.829 ± 0.332 and 0.174 ± 0.088 g/l for the patients with HCC, respectively. The levels of complement C3 and C4 in the patients with CHB or HCC were significantly lower than the control group ($P < 0.05$). The HBV infectious clone pHBV1.3 was used to transfect Huh7 cells; Huh7 cells transfected with the pBlue-ks empty vector were used as the blank control. The changes in mRNA and protein expression of complements C3 and C4 were detected by RT-PCR and western blotting. When compared with the control cells, the Huh7 cells transfected with pHBV1.3 exhibited reduced C3 and C4 mRNA and protein expression levels. It was concluded that HBV can inhibit the expression of complement C3 and C4 *in vitro* and *in vivo*, which may lay the foundation for revealing the pathogenesis of HBV.

Introduction

Hepatitis B virus (HBV) is a DNA virus belonging to the Hepadnaviridae family with an enveloped nucleocapsid containing a partially double-stranded relaxed circular DNA of ~ 3.2 kb in length with four partially overlapping open-reading frames (S/PreS, C/PreC, P and X) that encode for the viral proteins (1). HBV infection can cause acute and chronic infection, which may ultimately lead to cirrhosis and hepatocellular carcinoma (HCC) (2-4). Worldwide, more than two billion people are infected with HBV, of which 350 million are chronic HBV carriers. Chronic HBV infection is a major risk factor for liver disease, including liver cancer. The total number of people dying from liver fibrosis and HCC caused by HBV each year has reached one million (5,6).

The mechanisms involved in the progression and development of HBV-related HCC are not fully understood. Currently, the pathogenicity of HBV is not considered to be attributable to the direct killing of liver cells; instead, it is attributed to the immune dysfunction that occurs subsequent to HBV infection (7). The complement system consists of a group of globulins with enzymatic activity and no heat tolerance; the most important components of the complement system are C3 and C4. As part of the body's innate immune system, these components are involved in regulating the body's immunity against invasion from foreign pathogens (8). However, viruses have developed a number of strategies to evade attack by the complement components (9); for example, certain types of viruses can incorporate the regulatory proteins for host complement into their viral envelope and regulate the expression of these proteins in infected cells (10-12).

In the present study, the serological levels of C3 and C4 were compared between healthy controls and patients with chronic hepatitis B (CHB), and the molecular mechanisms at the cellular level were explored, which is expected to lay the foundation for understanding the pathogenesis of HBV.

Materials and methods

Subjects. A total of 226 patients with a clinical diagnosis of HBV hepatitis from Renmin Hospital of Wuhan University (Wuhan, China) from March 2010 to January 2016 were

Correspondence to: Dr Xinghui Liu, Department of Clinical Laboratory, Gongli Hospital, Second Military Medicine University, Pudong New Area, Shanghai 200135, P.R. China
E-mail: syliuxh@163.com

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included in the present study, including 136 male and 90 female patients, with a mean age of 53.7 ± 14.2 years; this included 153 cases of CHB and 73 cases of HCC. A total of 116 healthy individuals were selected to form the control group, including 73 male and 43 female patients, with a mean age of 49.8 ± 13.6 years. The included patients did not exhibit disease of the heart, brain, kidneys and other vital organs, or infection with any other hepatotropic virus. The present study was approved by the Ethical Committee of Renmin Hospital of Wuhan University and written informed consent was obtained from all patients.

Cell culture and transfection. Huh7 cells (American Type Culture Collection, Manassas, VA, USA) were cultured with RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/l streptomycin in a 37°C incubator with 5% CO_2 . Prior to transfection, the Huh7 cells were seeded in 6-well plates. For transfection, 2.4 μg pHBV1.3 or pBlue-ks plasmid (Agilent Technologies, Inc., Santa Clara, USA) DNA and 5 μl Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) were diluted in 100 μl serum- and antibiotic-free RPMI-1640 medium. Subsequent to incubation at room temperature for 20 min, the obtained transfection solution was added to the cell culture medium, and the cells were incubated for 48 h.

Reverse transcription-semiquantitative polymerase chain reaction (RT-sqPCR). The transfected Huh7 cells were collected for total RNA extraction, which was performed using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol; the total RNA was subsequently subjected to sqPCR with primers for C3 and C4. The sequences of the primers were as follows: C3 forwards, 5'-ACGGCATCCTCTGTCATCT-3', reverse 5'-ACG GCATCCTCTGTCATCT-3'; C4 forwards 5'-CGAGGACAG GTAGTGAAAGG-3', reverse 5'-GGCCAGGGTTGTAAA TGG-3'; β -actin forwards 5'-ATGATATCGCCGCGCTCG-3', reverse 5'-CGCTCGGTGAGGATCTTCA-3'. β -actin served as an internal reference for densitometry ImageQuant[™] TL 7.0 software (GE Healthcare, Chicago, IL, USA). The PCR products were detected by 1% agarose gel electrophoresis.

Western blotting. Huh7 cells were harvested and sonicated in radioimmunoprecipitation assay lysis buffer (BioTeke Corporation, Beijing, China) and the protein concentration was determined using the Coomassie brilliant blue G250 method. SDS-PAGE (12%) was performed using 30 mg protein mixed with equal volume of sample loading buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, and protease inhibitor cocktail, pH 7.4). Subsequent to electrophoresis, the separated proteins were transferred onto a nitrocellulose (NC) membrane, which was blocked with 5% skimmed milk for 2 h, followed by incubation with C3 (cat. no. C6025) and C4 monoclonal antibodies (cat. no. SAB1403623) for 2 h at room temperature (dilution, 1:2,000; Sigma Aldrich; Merck KGaA, Darmstadt, Germany). The NC membrane was washed three times in PBS with Tween (PBST), followed by incubation with a horseradish

peroxidase-labeled goat anti-rabbit secondary antibody (cat. no. 7074; dilution, 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature; then the membrane was washed four times with PBST. Development was performed using an electrogenerated chemiluminescence system (Pierce; Thermo Fisher Scientific, Inc.).

Measurement of complement C3 and C4. Assays for Complement C3 and C4 in serum of patients with HBV infection and healthy controls were conducted using a Human Complement C3 and Complement C4 Multiplex EFSIA kit (cat. no ABIN1774745; Beijing 4A Biotech Co., Ltd, Beijing, China) according to the manufacturer's protocol.

Statistical analysis. Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The measurement data were presented as the mean $\pm$ standard deviation. A one-way analysis of variance was used for comparisons of C3 and C4 levels between the 3 disease groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The serum levels of complement C3 and C4 are decreased in patients with HBV. The clinical parameters of the controls and case subjects enrolled in the study are included in Table I. The serum levels of complement C3 and C4 for the healthy subjects and the patients with HBV were detected by an immunoturbidimetric assay. As included in Table I, the serum levels of C3 and C4 were 1.223 ± 0.237 and 0.226 ± 0.052 g/l in the healthy controls, 0.687 ± 0.150 and 0.145 ± 0.070 g/l in the patients with CHB, and 0.829 ± 0.332 and 0.174 ± 0.088 g/l in the patients with HCC, respectively. Compared with the healthy control group, the levels of complement C3 and C4 in the patients with CHB and HCC were significantly lower ($P < 0.05$), as demonstrated in Figs. 1 and 2.

HBV inhibits the mRNA and protein expression of complement C3 and C4. To investigate the molecular mechanism of HBV in regulating the expression of complement C3 and C4, the HBV infectious clone pHBV1.3 was transfected into the Huh7 cells. The empty vector pBlue-ks was used as a negative control. Huh7 cells transfected with pHBV1.3, an HBV infectious clone, synthesize and secrete HBV viral particles, as previously described (13).

The expression levels of C3 and C4 mRNA were detected using RT-sqPCR. The results showed that the expression levels of C3 and C4 mRNA were reduced in Huh7 cells transfected with pHBV1.3 (C3/ β -actin ratio of 0.96 and C4/ β -actin ratio of 0.90; data not shown) compared to Huh7 cells transfected with pBlue-ks (C3/ β -actin ratio of 0.33 and C4/ β -actin ratio of 0.29; data not shown, representative images of the PCR products are included in Figs. 3 and 4). In order to test the effect of HBV on the protein expression of C3 and C4, the protein expression levels of C3 and C4 were detected by western blotting. The results demonstrated that the expression levels of C3 and C4 proteins were reduced in Huh7 cells transfected with pHBV1.3 (C3/ β -actin ratio of 0.78 and C4/ β -actin ratio of 1.09; data not shown) compared to Huh7 cells transfected with pBlue-ks (C3/ β -actin ratio of 0.36 and C4/ β -actin ratio of 0.31; data not

Table I. Clinical parameters of the subjects enrolled in the study.

Clinical parameters	Healthy controls (n=116)	Chronic HBV patients (n=153)	HCC patients (n=73)
Age (years)	49.8±13.6	46.2±14.7	58.3±14.2
Gender (male/female)	73/43	97/56	49/24
BMI	25.2±1.6	24.9±1.4	24.4 ± 1.5
ALT (IU/l)	<30	168.3±114.6	63.3±44.7
AST (IU/l)	<30	216.2±116.7	72.5±58.1

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma.

Figure 1. Comparison of the serum levels of complement C3 for patients with HBV infection and healthy controls. *P<0.05. HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

Figure 2. Comparison of the serum levels of complement C4 for patients with HBV infection and healthy controls. *P<0.05. HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

Figure 3. Effect of pHBV1.3 on the mRNA expression of complement C3 as determined by reverse transcription-polymerase chain reaction.

shown, representative images of the western blots are included in Figs. 5 and 6), indicating that HBV could inhibit the mRNA and protein expression of C3 and C4.

Discussion

The complement system is the first line of immune defense against foreign pathogens in the host, including the defense against viruses (14). The complement constituents adhere to the surface of the pathogen, promoting the phagocytosis of the

Figure 4. Effect of pHBV1.3 on the mRNA expression of complement C4 as determined by reverse transcription-polymerase chain reaction.

Figure 5. Effect of pHBV1.3 on the protein expression of complement C3 as determined by western blotting.

Figure 6. Effect of pHBV1.3 on the protein expression of complement C4 as determined by western blotting.

host cell, the formation of the membrane attack complex, the dissolution of pathogens and the release of anaphylatoxin to cause inflammation and promote the elimination of pathogens (15,16). However, in the process of evolving with the host, certain viruses have established strategies to escape the complement system, including encoding the membrane complement regulatory proteins from the host, and entering the host cells using the membrane complement receptor of the host (17-20).

In the present study, serum was collected from clinically diagnosed HBV-infected patients and healthy controls, and the levels of C3 and C4 were determined using immunoturbidimetric assays. Statistical analysis indicated that the expression levels of C3 and C4 in patients with HBV were significantly lower than in the healthy controls, and the

serum levels of C3 and C4 in the patients with HCC were higher than in the patients with CHB. To investigate the molecular mechanism of HBV in regulating the expression of C3 and C4, pHBV1.3 was transfected into the Huh7 cells. As an HBV infectious clone, cells transfected with pHBV1.3 can synthesize and secrete HBV viral particles, and HBV DNA can be detected in the cell culture supernatant (13). RT-PCR and western blot analyses were applied to detect the changes in the mRNA and protein expression levels of C3 and C4; the results demonstrated that HBV suppressed the expression of C3 and C4 *in vivo*.

The serum levels of complement components C3 and C4 in the patients with CHB were decreased. We hypothesize that there are two possible explanations: Firstly, the liver synthesizes the majority of blood proteins with the exception of γ -globin, and damage to the liver would reduce the synthesis of C3 and C4 (21); secondly, the infection with HBV may induce the formation of various antigen-antibody complexes (22), leading to the activation of the complement system and resulting in the excessive consumption of the complement components, including C3 and C4.

The complement system is widely involved in the body's defense against the invasion of foreign pathogens, the maintenance of the internal environment and the regulation of immunity (23); however, HBV can incorporate the complement regulatory protein of the host into its outer membrane to evade the host's complement attack (11,12). HBV may also inhibit the expression of complement C3 and C4 through the aforementioned hypotheses. Additionally, the decline in the synthesis by liver cells and excessive complement component consumption would lead to reduced levels of complement C3 and C4 in the serum of the patients with CHB. However, the serum levels of C3 and C4 in the patients with HCC were higher than in the patients with CHB, which may be associated with the alteration in the expression level following the malignant transformation of the liver cells; the mechanism for this change requires further investigation.

In summary, the present study demonstrated that HBV can downregulate the synthesis and secretion of C3 and C4 both *in vitro* and *in vivo*. The detection of C3 and C4 in the serum of patients with HPV infection may provide a basis for the diagnosis of HBV-associated diseases.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author's contributions

CZ participated in the cell culture, transfection and reverse transcription-semiquantitative polymerase chain reaction. HS and FX participated in the sample collection and measurement of complement C3 and C4, WY and FL performed the western blotting and statistical analysis. XL participated in the design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This work was approved by the Ethical Committee of Renmin Hospital of Wuhan University (Wuhan, China), and all patients provided written informed consent.

Consent for publication

Written informed consent for publication was obtained from these patients.

Competing interests

The authors declare that they have no competing interests.

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