

Article

Hepatitis B Virus Down-Regulates Expressions of MHC Class I Molecules on Hepatoplastoma Cell Line

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Chronic HBV infection is associated with a 100-fold high risk of developing hepatocellular carcinoma. Tumor recognition is of the most importance during the immune surveillance process that prevents cancer development in humans. In the present study, the expressions of MHC class I molecules on hepatoplastoma cell line HepG2.2.15 were investigated to indicate the possible effects of HBV on the immune recognition during HBV-associated hepatocellular carcinoma. It was found that the expressions of MHC class I molecules HLA-ABC, HLA-E and MICA were much lower in HepG2.2.15 cells compared with HepG2 cells. The expressing HBV in human hepatoplastoma cell line significantly down-regulated the expressions of MHC class I molecules. Additionally, it was observed that in murine chronic HBsAg carriers the expression of classical MHC-I molecule on hepatocytes was down-regulated. These results demonstrated that HBV might affect the immune recognition during HBV-associated hepatocellular carcinoma such as the recognition of CD8⁺ T, NK-CTL and NK cells and prevent the immune surveillance against tumors. However, the effects of HBV down-regulation of MHC class I molecules on the target cells *in vivo* should be further studied. *Cellular & Molecular Immunology*. 2006;3(5):373-378.

Key Words: hepatitis B virus, MHC, hepatoplastoma cell line, immune surveillance

Introduction

Hepatitis B virus (HBV) is regarded as one of the most fatal human pathogens. Infection with HBV can cause transient and chronic liver inflammation. Many chronically infected people will gradually acquire severe liver cirrhosis and may eventually progress to hepatocellular carcinoma (1). With an estimated population of 350 million individuals chronically infected and approximately one million deaths annually in the world, it is of great importance to demonstrate the key points in the mechanism of HBV infection.

The analysis of human HBV infection was rather difficult because HBV can only infect human beings and chimpanzees. HBV cannot infect common immortalized cell lines and the infection of primates with HBV is expensive and ethically controversial (2). To study the molecular biology of HBV infection, a variety of replication and infection systems were

established for closely related viruses like avian (3, 4) and woodchuck HBV (5). Recent reports describe promising new systems for the study of HBV infection *in vivo* and *in vitro* (6-8). By microinjecting the intact or selected parts of the HBV genome into the fertilized eggs of inbred mice, different lines of HBV-transgenic mice have been developed. Most of the researched regarding immune responses to HBV infection are conducted on transgenic mouse models (11).

It has been well known that the host immune response to the virus has a critical role in the pathogenesis (9, 10). The immune response initiated by the T-cell response to viral antigens is fundamental for both viral clearance and disease pathogenesis in HBV infection. Recently, innate immune systems including NKT cells, antigen-presenting cells (APCs) and NK cells have also been demonstrated to be involved during HBV infection (11). It was reported that chronic HBV infection is associated with a 100-fold high risk of developing hepatocellular carcinoma (12). There is an effective immune surveillance process that prevents cancer development in humans, during which tumor recognition is of the most importance. Whether HBV does effects on the immune recognition during HBV-associated hepatocellular carcinoma is deserved to be investigated.

HepG2.2.15 cell line, a HepG2 human hepatoma cell line derivative, is a well-established, steadily HBV expressing hepatoplastoma cell line, which permanently transfected with a plasmid containing two head-to-tail dimers of the HBV genome. HepG2.2.15 can not only release high level of HBsAg and HBeAg into supernatants, but also support the

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assembly and secretion of replicative intermediates of HBV DNA and Dane particles during culture (13). The stably transfected cell line HepG2.2.15 serves as an effective system for the study of HBV *in vitro*. In addition, HBV transgenic mice which contain the gene encoding S, pre-S and X domains of HBV, and express HBsAg in serum, liver and kidney tissues were used for the study of HBV infection *in vivo*. In the present study, the expressions of MHC-I molecules on hepatoplastoma cell line HepG2.2.15 and primary hepatocytes in HBV transgenic mice for the immune recognition were investigated to indicate the possible effects of HBV.

Materials and Methods

Cell lines and culture conditions

The human hepatoplastoma cell line HepG2 and its constitutively HBV expressing derivative HepG2.2.15 (serotype away, genotype D) were cultivated as described (14). HepG2.2.15 cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 200 mg/L G418 (Sigma, USA) in 5% CO₂ at 37°C. Subconfluent monolayer cells of HepG2.2.15 were detached from the culture dishes by trypsin (Invitrogen) treatment, then centrifuged at 800 rpm for 5 min and resuspended in the fresh media.

Animals

Eight-twelve weeks old male HBV transgenic mice C57BL/6J-TgN (AlbHBV) 44Bri were purchased from VITALRIVER Experiment Animal Company (Beijing) who purchased the mice from Jackson Lab and bred them for us. The littermates C57BL/6J mice were also obtained as the control mice. All mice were maintained under specific-pathogen-free and controlled conditions (22°C, 55% humidity, and 12-h day/night rhythm) in compliance with the regulations of animal care of University of Science and Technology of China.

Isolation of mouse hepatocytes

Primary mouse hepatocytes were isolated as described previously (35). Mice were anesthetized with sodium pentobarbital (30 mg/kg, *i.p.*), and the vena cava was cannulated. The liver was subsequently perfused with EGTA solution (5.4 mM KCl, 0.44 mM KH₂PO₄, 140 mM NaCl, 0.34 mM Na₂HPO₄, 0.5 mM EGTA, and 25 mM tricine, pH 7.2) and digested with 0.075% collagenase solution.

Flow cytometry

Hepatoplastoma cells detached from the dishes with Trypsin were filtered through a 40 µm cell strainer to obtain single-suspension cells. Mouse hepatocytes were isolated as described above. The surface phenotypes of cells were analyzed using fluorescence-conjugated mAbs including PE-conjugated mouse anti-human HLA-ABC, mouse anti-human MICA mAb (IgG), FITC-labeled goat anti-mouse IgG (Pharmingen, BD Biosciences), biotin-conjugated anti-H-2D^b

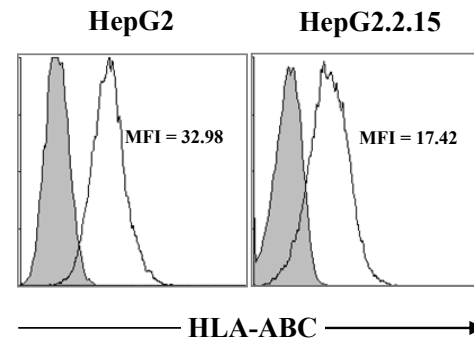


Figure 1. Expression of HLA-ABC on HepG2 and HepG2.2.15. By flow cytometry, the fluorescence intensity of HLA-ABC expression on HepG2.2.15 was compared with that of HepG2. The gray histogram was for the anti-isotype control, and the open histogram was for the detected HLA-ABC. MFI was shown as the mean fluorescence intensity of HLA-ABC subtracted the mean fluorescence intensity of the control. The result was representative of four independent experiments.

and FITC-labeled streptavidin (eBioscience). Firstly, cells were blocked with normal mouse immunoglobulin to saturate mouse Fc receptor for 30 min at 4°C. Then, the cells were stained with indicated labeled mAb or the control Ab at 4°C for 30 min, and then washed three times and acquired by FACSCalibur (Becton Dickinson) and analyzed with WinMDI 2.8 software.

RT-PCR

All reagents were from Invitrogen. All of the steps were performed using sterile technique in designated areas for RNA extraction and RT-PCR. RNA was extracted from hepatoplastoma cells (5×10^5) by Trizol Reagent according to the protocol provided by the manufacturer. The quantity and purity of mRNA were measured by reading absorbance at 260 and 280 nm with spectrophotometry. Total mRNA was reverse transcribed to cDNA in a reaction volume of 20 µl, containing 2 µg total mRNA, 5 µmol/L oligdT, 0.5 µmol/L dNTP, 4 µl 5× Buffer, 10 mmol/L DTT, 200 units of reverse transcriptase and distilled water (ultrapure, DNase and RNase free). Samples were then incubated at 37°C for 50 min and the reaction inactivated by heating at 70°C for 15 min. The standard reaction in a total volume of 50 µl contained 5 µl 10× PCR buffer, 2 µl complementary DNA template, 0.3 µmol/L forward and reverse primers, 0.2 µmol/L dNTP, 0.5 µl Taq enzyme. The step of PCR was 3 min at 94°C, and then 94°C 30 s, 58°C 50 s, 72°C 75 s for 32 cycles, and 7 min at 72°C. PCR primers for detecting mRNA of HLA-E, MICA and β-actin were as followed: HLA-E, 599 bp, P1 5'-GGC TCC CAC TCC TTG AAG TAT-3', P2 5'-GTG GCC TCA TGG TCA GAG AT-3'; MICA, 456 bp, P1 5'-CTG TCC TGG GAT GGA TCT GT-3', P2 5'-GTC TGC ATG CAT AGC GTG AT-3'; β-actin, 308 bp, P1 5'-CAA CTG GGA CGA CAT GGA GAA AAT-3', P2 5'-ATT GCC AAT GGT GAT GAC CT-3'.

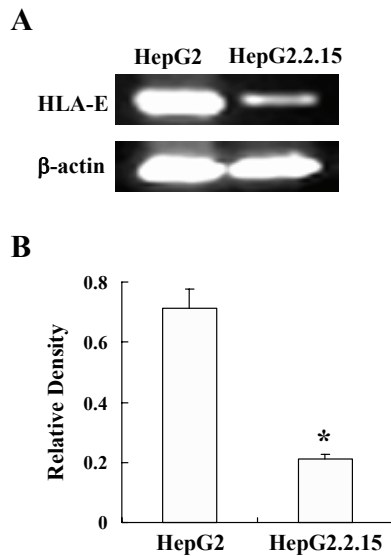


Figure 2. Expression of HLA-E mRNA in HepG2 and HepG2.2.15. RT-PCR was used to compare the level of HLA-E mRNA in HepG2.2.15 with that in HepG2. (A) The result of PCR production by gel electrophoresis. (B) Relative density was obtained by comparing HLA-E with its relevant β -actin. Data were shown as mean $\pm$ SEM from four independent experiments.

Results

Expression of HLA-ABC was down-regulated on HepG2.2.15
By flow cytometry, the expression of classical MHC-I molecules HLA-ABC on HepG2 and HepG2.2.15 cells was analyzed and the mean fluorescence intensity was obtained. As shown in Figure 1, the mean fluorescence intensity of HLA-ABC on HepG2 cells was much stronger than that of HepG2.2.15 cells (32.98 of HepG2 cells compared with 17.42 of HepG2.2.15 cells). It was demonstrated that the expressing HBV markedly inhibited the expression of HLA-ABC in hepatoplastoma cell line HepG2.2.15.

Expression of HLA-E mRNA was inhibited in HepG2.2.15
RT-PCR was used to compare the expression of HLA-E in HepG2 and HepG2.2.15 cells. From Figure 2, the expression of HLA-E mRNA was significantly reduced in HepG2.2.15 cells compared with HepG2 cells. The expression in HepG2 cells was about three times more than that of HepG2.2.15 shown by the relative density, which indicated that the expressing HBV also markedly inhibited the expression of HLA-E mRNA in HepG2.2.15.

Expressions of MICA in the level of protein and mRNA were down-regulated in HepG2.2.15

Firstly, the expression of MICA on cell surface was observed by flow cytometry. As shown in Figure 3A, the mean fluorescence intensity of MICA on HepG2 cells was much stronger than that of HepG2.2.15 cells (189.45 of HepG2 cells compared with 114.38 of HepG2.2.15 cells). The down-

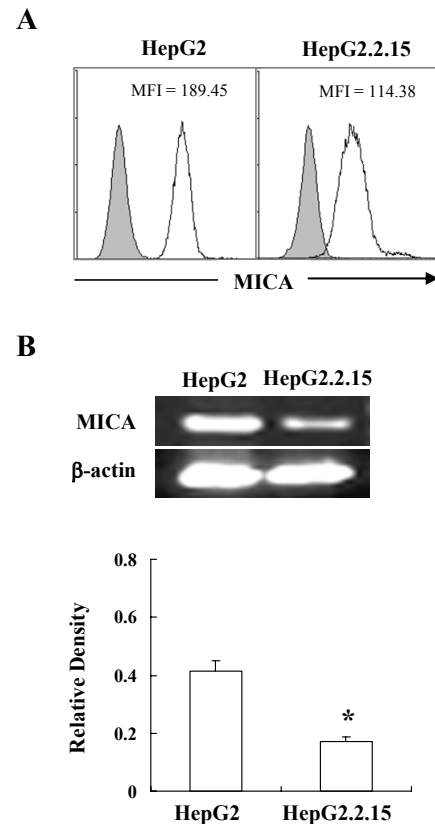


Figure 3. Expressions of MICA in the levels of protein and mRNA in HepG2 and HepG2.2.15. (A) The fluorescence intensity of MICA on HepG2 and HepG2.2.15 by flow cytometry. The gray histogram was for the control, and the open histogram was for MICA. MFI was shown as the mean fluorescence intensity of MICA subtracted the mean fluorescence intensity of the control. The result was representative of four independent experiments. (B) The level of MICA mRNA in HepG2 and HepG2.2.15. Relative density obtained by comparing MICA with its relevant β -actin showed the result of gel electrophoresis. Data were shown as mean $\pm$ SEM from four independent experiments.

regulation of membrane MICA by expressing HBV in HepG2.2.15 was confirmed.

It has been known that MICA is not only expressed on cell surface but also expressed as soluble. In addition to the protein of MICA on cell surface, expression of MICA mRNA was further investigated by RT-PCR. From Figure 3B, it was observed that the relative density of MICA mRNA in HepG2 cells was much higher than that of HepG2.2.15 cells, which demonstrated that the expressing HBV inhibited the expression of MICA in mRNA level in HepG2.2.15.

Expression of classical MHC-I molecule on hepatocytes was down-regulated from HBV transgenic mice

To investigate the effect of HBV on the expression of MHC-I molecules of hepatocytes *in vivo*, HBV transgenic mice as a model of murine chronic HBsAg carriers were used in the study. The expression of classical MHC-I molecule H-2D on

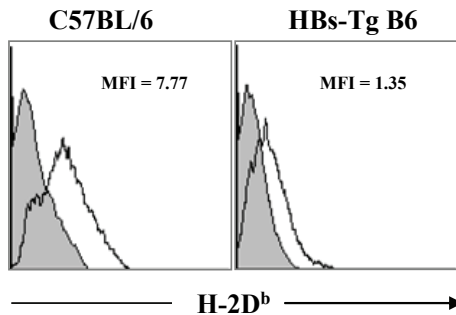


Figure 4. Expression of classical MHC-I molecule on hepatocytes in HBV transgenic mice. By flow cytometry, the fluorescence intensity of H-2D expression on primary hepatocytes from HBV transgenic mice was compared with that of wild mice. The gray histogram was for the negative control, and the open histogram was for the detected H-2D. MFI was shown as the mean fluorescence intensity of H-2D subtracted the mean fluorescence intensity of the control. The result was representative of three independent experiments.

primary hepatocytes was analyzed by flow cytometry. From the mean fluorescence intensity shown in Figure 4, the expression of H-2D on the hepatocytes from C57BL/6 mouse was stronger than that of HBV transgenic mouse. It was indicated that *in vivo* HBV also down-regulated the expression of classical MHC-I molecule on hepatocytes. In addition, the MHC-I chain-related molecule retinoic acid early transcript 1 (Rae-1) was also observed in our study. Rae-1 molecule was not expressed on primary hepatocyte from the HBV transgenic mice, which was as reported in wild mice (data not shown).

Discussion

In the present study, we found that the expressions of MHC class I molecules HLA-ABC, HLA-E and MICA were much lower in HepG2.2.15 cells compared with HepG2 cells. The expressing HBV in human hepatoplastoma cell line significantly down-regulated the expressions of MHC class I molecules. Further, it was observed that in murine chronic HBsAg carriers the expression of classical MHC-I molecule on hepatocytes was down-regulated.

MHC class I molecules are cell surface recognition elements expressed on virtually all somatic cells. It has been well known that the molecules present peptides generated within the cell and signal the cell's physiological state to effector cells of the immune system, both T lymphocytes and natural killer cells (15).

The classical HLA I molecules include the gene products of HLA-A, -B and -C loci (16). It is well known that the expression of classical HLA-I molecules are down-regulated by specific proteins produced by human cytomegalovirus (HCMV) and human immunodeficiency virus (HIV) during infection (17, 18), which will do negative effects on the activity of CD8⁺ T cells. As demonstrated in our study, HBV

also down-regulates the expression of classical HLA-I molecules to escape the recognition of CD8⁺ T cell. However, the negative signal for NK cells is reduced by the down-regulated classical HLA-I molecules, by which NK cells function. Such down-regulation of classical HLA I molecules might be compensated by viral-induced up-regulation of HLA-E in order to protect from NK cell activity.

HLA-E is referred to as non-classical or class Ib gene, which distinguished from its close relatives (the classical HLA class I genes) by expression patterns and low allelic polymorphism (19). The HLA-E complex, with bound peptides derived from other HLA class I signal sequences, was shown to interact with CD94/NKG2 heterodimers on NK cells and inhibits the killing activity of NK cells (20-22). Recent reports have suggested that HLA-E can also bind and present peptides from virus, mycobacterium or heat shock protein (Hsp) 60. Tomasec et al. found that HLA-E surface expression was upregulated by infection with HCMV (23). HCMV glycoprotein UL40 (gpUL40) could upregulate expression of HLA-E and inhibit NK cell lysis through CD94/NKG2 (23-25). Additionally, HLA-E could bind and present peptides with sequences completely distinct from HLA class I leader peptides. HLA-E could bind two viral peptides from Epstein-Barr virus (EBV) and influenza virus (26). The peptides from hepatitis C virus (HCV) (17) and HIV (18) could be presented by HLA-E on the cell surface. HLA-E-bound peptides derived from HCV inhibited cytotoxicity of NK cell through CD94/NKG2, and concluded that enhanced HLA-E expression was likely to be functionally relevant concerning down-regulation of NK cell activity during chronic hepatitis C infection. However, it was confirmed that the HLA-E expression was not enhanced but inhibited by HBV so that the inhibition of NK cell activity by HLA-E was weaker.

Recent reports have suggested that specific HLA-E/peptide complexes can induce not only the inhibition of NK cells through CD94/NKG2 receptors, but also the activation of cytotoxicity of CD8⁺ T cells through an interaction with the T cell receptor. A fraction of human CD8⁺ CTL, referred to as NK-CTL, are frequently composed of cells expressing one single TCR V β expansion, display a memory phenotype, and express HLA class I-specific inhibitory NK receptors (27). The $\alpha\beta$ TCR on NK-CTL could recognize HLA-E molecules, which revealed a novel type of TCR-mediated recognition, and HLA-E, bound to peptides derived from any of EBV, HCMV or classical HLA-I signal sequences, induced cytotoxicity to NK-CTL (28). It has been reported that upregulated HLA-E expression can induce HLA-E-restricted CTLs and may play a relevant defensive role during CMV infection, competing with down-regulation of classical HLA I molecules (28). From this point, the down-regulated HLA-E expression might affect the function of HLA-E-restricted CTLs during HBV infection. As the findings in this study, a viral protein US6 was reported to down-regulate not only expression of classical HLA I molecules but also HLA-E (25).

MICA, MHC class I chain-related molecule A, as a

ligand for the receptor NKG2D, is structurally analogous to a class I molecule but lacks the β_2m subunit. MICA is expressed in many carcinoma cells such as in lung, breast, ovary, prostate, colon cancer, but is usually absent from normal tissue (29, 30). Evidence clearly implicates that NKG2D recognition plays an important role in innate immune surveillance against tumor cells. Activation of NK cell through NKG2D triggers cell-mediated cytotoxicity and in some cases induces production of cytokines. A tumor-specific expression pattern of MICA has also been observed in human HCC, and NK cells recognize hepatoma cells *via* MICA-NKG2D interaction. MICA-NKG2D may serve as an efficient innate pathway of immune surveillance against HCC (31). In accord with that, the expression of MICA on HepG2 cells was observed. The down-regulated expression of MICA by expressing HBV would weaken the immune surveillance of NK cells *in vivo*. MICA can be released as a soluble form from the cell surface of tumor cells and the soluble form of MICA (sMICA) was found to sequester NKG2D in the cytoplasm and to inhibit cell-surface NKG2D expression and NKG2D mediated effector functions of immune cells in progressive malignant tumors (30, 32, 33). Significant levels of sMICA were detected in a subset of HCCs (34). Whether expressing HBV affects the expression of sMICA deserved further investigation.

The down-regulation of expressions of MHC class I molecules on hepatoplastoma cell line by HBV demonstrated that HBV might affect the immune recognition during HBV-associated hepatocellular carcinoma such as the recognition of CD8⁺ T, NK-CTL and NK cells, which might be related to the inhibited immune surveillance against tumors during HBV infection. The down-regulated classical MHC class I molecule on primary hepatocytes in murine chronic HBsAg carriers further confirmed the effect of HBV on the immune recognition *in vivo*. However, immunocyte functions are regulated by a balance of negative and positive signals and complex, so the effects of HBV down-regulation of MHC class I molecules on the target cells *in vivo* should be studied further.

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