

Effects of Hepatitis B Surface and Hepatitis B Core Antigens from Hepatitis B Virus Genotypes B & C on In Vitro Apoptosis

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Background and Aims: To initially explore the underlying pathogenesis of the relationship between genotypes of hepatitis B virus (HBV) and its clinical manifestations.

Methods: The S and C genes of HBV from 60 serum samples, infected by HBV of genotypes B or C were amplified by PCR. The products were recombined with vector pEGFP-C1, which is an internal reference for transfection, to construct the eukaryotic expression recombinant plasmids, followed by cloning and subcloning. Then they were transfected into hepatocarcinoma cell HepG2. The increment rates and apoptosis rates of these transfected cells were determined by MTT and flow cytometer, respectively.

Results: The 120 eukaryotic expression recombinant plasmids were all constructed successfully. As an internal reference for transfection, EGFP confirmed that large S protein and C protein of HBV had been expressed in all HepG2 cells. It was found by flow cytometer that the apoptosis rates of HepG2 cells transfected by pEGFP-C1/HBs or pEGFP-C1/HBc from HBV-genotype C samples were all significantly higher than that from HBV-genotype B samples ($P=0.009$ & $P=0.001$, respectively).

Conclusions: HBV of genotype C can induce more serious cell apoptosis than HBV of genotype B. Difference in apoptosis may be an important reason that HBV of genotype C can induce more severe liver injury than HBV of genotype B.

Keywords: HBV, Genotype, Apoptosis, In Vitro System

Introduction

Hepatitis B virus (HBV) is the most common virus which can induce chronic hepatitis, and more than 350 million people in the world are chronic carriers of this virus (^{1, 2}). HBV can be classified into eight genotypes from A to H, according to difference of its gene sequence. It has been found that HBV genotype is related to clinical manifestation of hepatitis B. For example, HBV of genotype C can induce more severe liver injury than HBV of genotype B. The ALT abnormality, HBeAg positive rate and severe patient proportion in patients infected with HBV of genotype C are all higher than that in patients infected with HBV of genotype B (³⁻⁹). HBV of genotype B is associated with better response to therapy than genotype C (¹⁰).

However, the reason for this relation is still not very clear. Few reports paid close attention to this pathogenicity. As all know, the pathogenesis of hepatitis B is not only autoimmune response, but also apoptosis induced by HBV (¹¹⁻¹⁵). Thus in this paper, apoptosis was used to initially study the pathogenesis of the relationship between HBV

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genotype and clinical manifestations of hepatitis B. For this purpose, apoptosis induced by HBV of genotype B and HBV of genotype C was compared. The large S protein and HBeAg/core protein of HBV were studied because they can well represent the pathogenicity of HBV and it is easy for them to mute.

Materials and Methods

Reagents

The pMD18-T vector, restriction endonuclease EcoRI and KpnI, and Agarose Gel DNA Purification Kit and MiniBEST Plasmid Purification Kit were all made by TaKaRa Ltd., Japan. The eukaryotic expression plasmid pEGFP-C1 was made by Clontech Ltd., USA.

Primers

Design of primers was based on PreS1/PreS2/S gene and PreC/C gene of HBV in GenBank. In front of the primers, cutting sites for endonuclease EcoRI and KpnI were made, respectively. Moreover, a base of A was added in upstream primer (PS1 and PC1) between encoding sequence and cutting site for EcoRI, in order that PreS1/PreS2/S gene and PreC/C gene would not shift while vector pEGFP-C1 expressed green fluorescence. Primers for PreS1/PreS2/S gene of HBV: PS1 (5'-GAA TTC A ATG GGA GGT TGG TCT TCC-3'), PS2 (5'-GGT ACC TTA AAT GTA TAC CCA AAG AC-3'). Primers for PreC/C gene of HBV: PC1 (5'-GAA TTC A ATG CAA CTT TTT CAC CTC TG-3'); PC2 (5'-GGT ACC CTA ACA TTG AGG CTC CCG-3'). The length of DNA fragments amplified by these primers was 1203bp (PreS1/PreS2/S gene) and 639bp (PreC/C gene) respectively.

Patients

Serum samples were randomly collected from 60 patients with chronic viral hepatitis B. Randomization was carried out through random digits table. Among these patients, 30 patients were infected with HBV of genotype B, while 30 patients were infected with HBV of genotype C. The HBV genotypes were determined by nested PCR with multiplex pairs of genotype-specific primers⁽¹⁶⁾. Informed written consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.

Amplification of HBV gene fragments by PCR

HBV-DNA was extracted from serum samples by extraction kit, and was amplified by PCR. Ingredients of PCR mixture were as follows: PS1/PC1 (50 pmol/ μ l) 1 μ l, PS2/PC2 (50 pmol/ μ l) 1 μ l, Taq enzyme (5U/ μ l) 0.25 μ l, 10 $\times$ PCR buffer (containing Mg²⁺) 5 μ l, dNTP (2.5 mM) 4 μ l, sterile DDW 37 μ l, DNA template 2 μ l and total 50 μ l. PCR condition: preoperatively denaturalized at 95°C for 5 min, then amplified for 35 cycles of 94°C 1 min, 58°C 1 min and 72°C 1 min, lastly extended for 7 min at 72°C. The 8 μ l PCR product was taken for gel electrophoresis at 1.5% agarose. Negative control was HBV-DNA-negative serum. PCR products were purified by Agarose Gel DNA Purification Kit.

Construction and identification of recombinant plasmids

PCR products were recombined with T vector and pEGFP-C1 in order. Recombinant T vectors were identified by enzyme incision. After being cut by EcoRI and KpnI for 3 hrs, they were taken into 1.5% agarose for gel electrophoresis. Eukaryotic expression recombinant plasmids were identified respectively by PCR, enzyme incision and sequencing. In sequencing, the gene sequences of these plasmids were bilaterally identified by common primers EGFP-C-F and EGFP-C-R, and then were compared with GenBank by Blastn program.

Transfection of eukaryotic expression recombinant plasmids into hepatoma carcinoma cell HepG2

Negative control was HepG2 cells which were not transfected.

Identification of transfection efficiency

The HepG2 cells were cultivated for 48h and digested by 0.25% trypsin. Then 2 $\times$ 10⁵ cells were played on the cover slips in bottom of 6-hole culture plate and were cultivated continuously. After 48 hrs, the little cover slips were taken to be observed and were photographed directly under fluorescence microscope with 488 nm excited light.

Detection of apoptosis by flow cytometer

The 2 $\times$ 10⁵ cells were taken out after being cultivated for 48 hrs in 6-hole-plate. All of these cells were digested by 0.25% trypsin, centrifugated in room temperature with 1500 rpm for 10 min and washed by 0.1M PBS once. Then they were fixed by 70% cold alcohol and were taken in flow cytometer to detect cell cycle and apoptosis. Operation of flow cytometer was submitted to China Academy of

T.C.M. The conditions were as follows. Cytometer: XL A27153; List gating: Disabled; Total count: 10,000; Parameters: PI; Gating parameters: Ratio gate, Diploid and PI×FS.

Statistical analysis

SPSS 11.0 was used to statistically analyze the data. Measurement data were expressed as $\bar{x} \pm SD$. Comparison of data was analyzed by independent samples *t*-test. Difference was defined as $P < 0.05$.

Results

Identification of recombinant T vector

The circular plasmids including S gene were cut into two clear straps with length of about 2600bp and 1200bp, while the circular plasmids including C gene were cut into two clear straps with length of about 2600bp and 640bp. All of the results agreed with expectation. It was initially believed those recombinant T vectors were successfully constructed.

Identification of eukaryotic expression recombinant plasmid

After gel electrophoresis for PCR products, a clear strap was at the site of about 1200bp or 640bp, which agreed with exception. There is no strap in negative control DDW. After enzyme incision, circular plasmids were all cut into two clear straps with length of about 4700bp and 1200bp/640bp, which agreed with expectation. After sequencing, the gene sequence of these plasmids, besides objective fragment, were completely the same as pEGFP-C1 in GenBank. Compared by Blastn program, the objective fragments sequence of plasmids were the same as S and C gene sequence of HBV of genotype B or C in GenBank, with the homology of 97%-98%. All of these methods proved that eukaryotic expression recombinant plasmids were successfully constructed.

Determination of transfection efficiency by observing green fluorescent under fluorescence microscope

Cover slips were taken out from culture plates to be played on slide by forceps, and were observed under fluorescence microscope. Green fluorescence could be intensively seen in HepG2 cells which were transfected by eukaryotic expression recombinant plasmid or pEGFP-C1 plasmids, but could not be seen in HepG2 cells without transfection. The results indicated that plasmid pEGFP-C1 had commendably played a role of internal reference for

transfection. It could determine the completion and efficiency of transfection. It just proved that the transfection had succeeded.

Detection of apoptosis by flow cytometer

The cell apoptosis of HepG2 cells were detected by flow cytometer (Table 1, Figure 1). The results indicated that apoptosis rates of HepG2 cells transfected by HBV were all clearly higher than that transfected by blank plasmid pEGFP-C1 and that without transfection ($P < 0.001$, $P < 0.001$). It marked further that S and C protein of HBV can promote cell apoptosis. Compared with independent sample *t*-test, apoptosis rates of HepG2 cells transfected by S or C protein of HBV of genotype C were clearly higher than that of HBV of genotype B ($P = 0.009$, $P = 0.001$).

Table 1. Cell apoptosis of HepG2 cells ($\bar{x} \pm SD$).

Cell kinds	N	Apoptosis rates (%)
1. Transfected by S protein of HBV of genotype B	30	5.88±1.54
2. Transfected by S protein of HBV of genotype C	30	7.04±1.80
3. Transfected by C protein of HBV of genotype B	30	8.21±1.47
4. Transfected by C protein of HBV of genotype C	30	10.84±3.90
5. Transfected by pEGFP-C1	30	3.35±1.45
6. Without transfection	30	3.02±1.22

There was notable difference between 1 and 2 ($P = 0.009$), 3 and 4 ($P = 0.001$). Apoptosis rates of the cells transfected by HBV were all clearly higher than those cells transfected by pEGFP-C1 and those cells without transfection ($P < 0.001$, $P < 0.001$).

Discussion

Most of scholars believe that genotypes of HBV are related with clinical manifestation of hepatitis B. Chu *et al.* ⁽⁷⁾ reported that rate and factors of HBsAg seroconversion and rate of reactivation of hepatitis B differed between genotype B and genotype C patients, and that genotype C and reactivation of hepatitis B were associated with increased risk of cirrhosis. Lin ⁽⁸⁾ thought that genotype C has a higher risk of hepatocellular carcinoma (HCC) development than genotype B. Wen ⁽⁹⁾ believed that genotype of HBV correlates with its clinical manifestation and genotype C of HBV can lead to more severe hepatitis than genotype B of HBV. Chien ⁽¹⁰⁾ reported that HBV of genotype B is

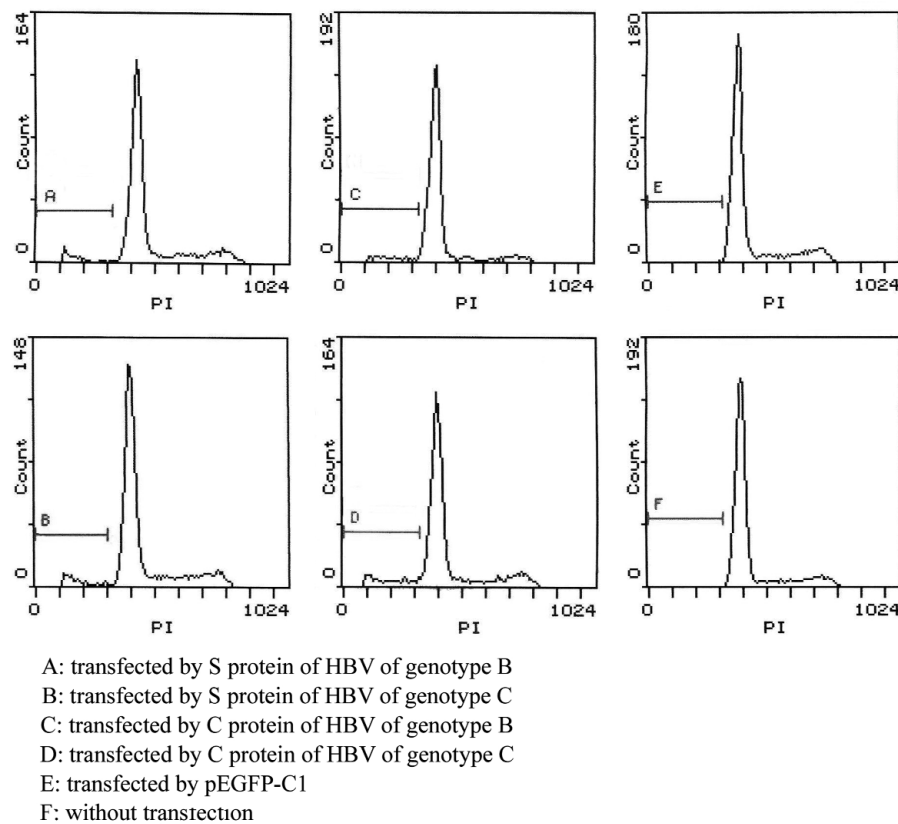


Figure 1. Apoptosis rates of different kinds of cells.

associated with better response to therapy than genotype C.

However, it is not very clear why it can do in this way. Only a few reports paid close attention to this pathogenesis. In 1999, Lindh⁽¹⁷⁾ found that HBV of genotype C has a higher mutation rate than HBV of genotype B on A-1896 and TGA-1762-1764. He thought the mutation may be useful as a marker for progressive liver damage, but seem to contradict that down-regulation of HBeAg production is the major effect of this mutation.

It was found many years ago that HBV can aggravate pathogenic condition of hepatitis through apoptosis. Ryo⁽¹⁸⁾ believed that fas-mediated apoptosis may be one of the triggers for the induction of fulminant hepatic failure. Xin⁽¹⁹⁾ found that apoptosis of hepatocytes correlated with liver fibrosis of CH and TGF- β 1 may play an important role between apoptosis and liver fibrosis. McPartland⁽²⁰⁾ believed that apoptosis is implicated as a major pathogenic mechanism in chronic hepatitis B and C. Because apoptosis is an important etiological factor for viral hepatitis, we tried to begin with apoptosis to explore the mechanisms by which

genotypes of HBV are related with clinical manifestation of hepatitis B. In other words, does HBV of genotype C induce more severe pathogenic condition through promoting more apoptosis than HBV of genotype B? Results showed that the apoptosis rates of HepG2 cells transfected by S or C protein of HBV of genotype C were clearly higher than that of HepG2 cells transfected by S or C protein of HBV of genotype B ($P=0.009$, $P=0.001$).

It indicated that HBV of genotype C can induce more severe apoptosis than HBV of genotype B indeed. The pathogenesis of relation between genotypes of HBV and clinical manifestation of hepatitis B may be due to it in some sense. At least, difference in apoptosis between two genotypes of HBV may be an important factor for the pathogenesis. However, more factors about the pathogenesis possibly exist. For example, does the injury induced by autoimmune response also play an important role in the pathogenesis? In the next step, we are prepared to compare the difference in autoimmune response for peripheral eukomonocyte in vitro system between HBV of genotype B and C.

References

1. Dunn C, Brunetto M, Reynolds G, Christophides T, Kennedy PT, Lampertico P, et al. Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. *J Exp Med* 2007; **204**: 667-80.
2. Cougot D, Wu Y, Cairo S, Caramel J, Renard CA, Levy L, et al. The hepatitis B virus X protein functionally interacts with CREB-binding protein/p300 in the regulation of CREB-mediated transcription. *J Biol Chem* 2007; **282**: 4277-87.
3. Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, et al. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; **37**: 19-26.
4. Ni YH, Chang MH, Wang KJ, Hsu HY, Chen HL, Kao JH, et al. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology* 2004; **127**: 1733-8.
5. Hagiwara S, Kudo M, Minami Y, Chung H, Nakatani T, Fukunaga T, et al. Clinical significance of the genotype and core promoter/pre-core mutations in hepatitis B virus carriers. *Intervirology* 2006; **49**: 200-6.
6. Chan HL, Wong ML, Hui AY, Hung LC, Chan FK, Sung JJ. Hepatitis B virus genotype C takes a more aggressive disease course than hepatitis B virus genotype B in hepatitis B e antigen-positive patients. *J Clin Microbiol* 2003; **41**: 1277-9.
7. Chu CM, Liaw YF. Genotype C hepatitis B virus infection is associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than genotype B: a longitudinal study of hepatitis B e antigen-positive patients with normal aminotransferase levels at baseline. *J Hepatol* 2005; **43**: 411-7.
8. Lin CL, Chen JD, Liu CJ, Lee PH, Chen PJ, Lai MY, et al. Clinicopathological differences between hepatitis B viral genotype B- and C-related resectable hepatocellular carcinoma. *J Viral Hepat* 2007; **14**: 64-9.
9. Wen ZL, Tan DM, Hou ZH. Relation between genotype of HBV and its clinical manifestation by PCR with genotype-specific primers (in Chinese). *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 2004; **29**: 50-3.
10. Chien RN, Lin CY, Yeh CT, Liaw YF. Hepatitis B virus genotype B is associated with better response to thymosin alpha1 therapy than genotype C. *J Viral Hepat* 2006; **13**: 845-50.
11. Kim KH, Seong BL. Pro-apoptotic function of HBV X protein is mediated by interaction with c-FLIP and enhancement of death-inducing signal. *EMBO J* 2003; **22**: 2104-16.
12. Baumert TF, Yang C, Schurmann P, Kock J, Ziegler C, Grulich C, et al. Hepatitis B virus mutations associated with fulminant hepatitis induce apoptosis in primary Tupaia hepatocytes. *Hepatology* 2005; **41**: 247-56.
13. Nakamoto Y, Kaneko S. Mechanisms of viral hepatitis induced liver injury. *Curr Mol Med* 2003; **3**: 537-44.
14. Liang X, Liu Y, Zhang Q, Gao L, Han L, Ma C, et al. Hepatitis B virus sensitizes hepatocytes to TRAIL-induced apoptosis through Bax. *J Immunol* 2007; **178**: 503-10.
15. Foo NC, Ahn BY, Ma X, Hyun W, Yen TS. Cellular vacuolization and apoptosis induced by hepatitis B virus large surface protein. *Hepatology* 2002; **36**: 1400-7.
16. Wen ZL, Tan DM. Detection for genotypes of hepatitis B virus in Hunan Province of China by nested PCR with multiplex pairs of primers. *World Chin J Digestol* 2004; **12**: 332-5.
17. Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. *J Infect Dis* 1999; **179**: 775-82.
18. Ryo K, Kamogawa Y, Ikeda I, Yamauchi K, Yonehara S, Nagata S, et al. Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. *Am J Gastroenterol* 2000; **95**: 2047-55.
19. Xin S, Zhao J, Wang L. Study on relationship between the apoptosis of hepatocytes and liver fibrosis of chronic viral hepatitis (in Chinese). *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 2000; **14**: 31-3.
20. McPartland JL, Guzail MA, Kendall CH, Pringle JH. Apoptosis in chronic viral hepatitis parallels histological activity: an immunohistochemical investigation using anti-activated caspase-3 and M30 cytodeath antibody. *Int J Exp Pathol* 2005; **86**: 19-24.