INTRAHEPATIC HBV DNA AND COVALENTLY CLOSED CIRCULAR DNA (cccDNA) LEVELS IN PATIENTS POSITIVE FOR ANTI-HBc AND NEGATIVE FOR HBsAg

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Abstract. Covalently closed circular DNA (cccDNA) is a unique episomal replicative intermediate molecule of hepatitis B virus (HBV) which plays a key role in viral persistence. The aim of this study was to prove cccDNA persistence in the liver tissue of patients negative for hepatitis B surface antigen (HBsAg) and positive for antibody to hepatitis core antigen (anti-HBc). Intrahepatic HBV DNA and cccDNA were determined using real-time and semi-nested PCR assays on the liver tissues of 35 patients who were negative for HBsAg and positive for anti-HBc with or without anti-HBs. HBV DNA was detected in the liver tissue of 4 out of 35 patients who were positive for anti-HBc. None of the samples harbored cccDNA. In this study population, which is of Asian origin, very low levels of HBV DNA were detected in a small percentage of patients with anti-HBc. Even using the highly sensitive semi-nested PCR assay, HBV cccDNA was not detectable in any anti-HBc positive patients either with or without anti-HBs.

Key words: hepatitis B virus, HBV DNA, cccDNA, real-time PCR

INTRODUCTION

Hepatitis B virus (HBV) remains a major health problem worldwide. It is a leading cause of death and more than 350 million people world-wide are chronically infected (Lee, 1997). The diagnosis of HBV infection is based on the detection of hepatitis B surface antigen (HBsAg) and antibody to hepatitis core (anti-HBc) in sera. Once a patient's serum has become HBsAg negative and displays anti-HBc and anti-HBs, it indicates previous exposure without ongoing infection. Based on serology, some individuals appear to produce only anti-HBc (isolated anti-HBc). They might have occult HBV infection characterized by either low levels of anti-HBs or low concentrations of HBV genome which can only be detected in serum or liver tissue using a highly sensitive molecular biology

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technique (Raimondo *et al*, 2007). Occult HBV infection may have an impact on clinical outcome (Dickson *et al*, 1997; Mindikoglu *et al*, 2006). De novo HBV infection in liver transplantation cases has been reported in over three quarters of recipients who received organs from donors negative for HBsAg and positive for anti-HBc (Dickson *et al*, 1997). Reactivation of HBV after cytotoxic chemotherapy has been documented in this group of patients (Mindikoglu *et al*, 2006). These findings suggest persistent infection of liver tissue with this dormant virus.

The molecular basis of persistent HBV infection is related to its unique life cycle in hepatocytes. Covalently closed circular DNA (cccDNA), a replicative form of HBV DNA, is an important intermediate molecule in the HBV life cycle. Upon infection, the virus transports its partially double-stranded DNA into the nucleus of hepatocytes and converts itself into a cccDNA molecule which is a stable episome and serves as a template for viral genomic transcription. Newly synthesized viral DNAs are translated into capsid and polymerase proteins and subsequently encapsidated and reverse-transcribed into new partially double-stranded viral genomes. DNA-containing nucleocapsids eventually are enveloped and secreted from the cell as mature virus, or recycled in the nucleus (Zoulim, 2005). This process helps maintain the cccDNA pool in the nucleus of infected cells. The presence of cccDNA in hepatocytes plays a key role in viral persistence (Caruntu and Molagic, 2005).

To date, several studies have been conducted focusing on cccDNA detection in patients with chronic hepatitis B infection with (Werle-Lapostolle *et al*, 2004; Yuen *et al*, 2005) or without treatment (Laras *et al*, 2006), patients with acute self-limited hepatitis B infection (Yuki *et al*, 2003) and patients with a hepatoma (Wong *et al*, 2006). Results of cccDNA analysis in the liver tissues of patients positive for anti-HBc with or without anti-HBs, were mostly obtained among western populations who usually acquired infection during adulthood (Mason *et al*, 1998; Caruntu and Molagic, 2005; Yuen *et al*, 2005). In contrast, most HBV carriers in Asian populations have been infected during the neonatal period (Ahn *et al*, 2005; Yuen *et al*, 2004, 2008). The aim of this study has been to investigate cccDNA persistence in the liver tissue of Asian patients positive for anti-HBc antibody and negative for HBsAg who supposedly have been infected since childhood.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The patients were informed as to the study's objective and written consent was obtained prior to enrollment.

Patients and samples

Patients with a clinical indication for undergoing needle liver biopsy at King Chulalongkorn Memorial Hospital between January 2005 and December 2007 were enrolled in this study. Indications for liver biopsy were as follows: baseline histological evaluation for chronic hepatitis C treatment, suspected autoimmune hepatitis, primary biliary cirrhosis, nonalcoholic steatohepatitis and undetermined cases of hepatitis. Liver tissue from patients known to be negative for HBsAg and positive for anti-HBc antibody were included in the study. Exclusion criteria were patients with liver tissue known to be positive for HBsAg and a history of malignancy. Percutaneous liver biopsies were stored in liquid nitrogen. Serum samples

were kept at -70ºC until analysis. Control liver biopsies were obtained from two patients with chronic HBV infection, one hepatitis B e antigen (HBeAg) positive patient and one HBeAg negative patient. Liver tissue from two patients with no serological evidence of hepatitis B infection were used as negative controls.

Serology test for markers of HBV infection

Thirty-five serum samples were determined by enzyme-linked immunosorbent assay (ELISA) for HBsAg, anti-HBs and anti-HBc detection using the Murex HBsAg Version 3, Murex anti-HBs and Murex anti-HBc, respectively (Murex, Biotech Limited, Dartford, Kent, England).

HBV DNA extraction from serum and liver tissue

HBV DNA was extracted from serum and liver biopsy samples as previously described (Theamboonlers *et al*, 1999). Briefly, 100 µl of serum samples were incubated with 400 µl lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0 and 0.5% SDS) and 20 mg/ml proteinase K at 37ºC for 1 hour. After incubation, DNA was extracted by adding a mixture of phenol -chloroform -isoamyl alcohol and subsequent centrifugation. This step was repeated once. HBV DNA was precipitated with 2.0 M sodium acetate and absolute ethanol. The DNA pellets were dried and resuspended in 30 µl sterile distilled water. For liver biopsies, a piece of liver tissue was homogenized in 100 µl 1x phosphate buffered saline (PBS). Lysis buffer was added to the homogenate and DNA extraction performed as described above (Theamboonlers *et al*, 1999).

HBV DNA and cccDNA level detection

Real-time PCR assay. Real-time PCR was performed to quantitatively verify HBV DNA levels in the serum and liver biopsies. The quantitative method was adapted from a procedure described previously (Payungporn *et al*, 2004). In brief, HBV DNA standard plasmid was constructed by inserting the fragment nt 2814-475 of the *preS* region into the pGem®-T Easy Vector. After determining the concentration of the plasmid using a spectrophotometer at OD_{260}, 10^2 to 10^{10} copies/µl of plasmid DNA were prepared to determine the standard curve for quantitative HBV DNA levels. The 12.7 µl reaction mixture contained 1.0 µl of DNA, 5.0 µl of 2.5x MasterMix and 0.5 µl of 25 µM magnesium chloride solution (5 PRIME Mastermix, 5 PRIME GmbH, Hamburg, Germany), 0.5 µl of 25 µM forward primer (*PreS1*F+: 5′-GGG TCA CCA TAT TCT TGG GAA C-3′), 0.5 µl of 25 µM reverse primer (*PreS1*R2: 5′-CCT GAG CCT GAG GGC TCC AC-3′), 0.2 µl of 10x SYBR Green (QIAGEN, Hilden, Germany) and distilled water. Amplification was performed using LightCyCler™ (Roach, Basel, Switzerland) for 40 cycles comprised of denaturation at 94ºC for 25 seconds, primer annealing at 60ºC for 20 seconds and extension at 72ºC for 25 seconds. A single fluorescent signal was obtained once per cycle at 78ºC after the extension step.

For quantitative determination of cccDNA concentrations in liver biopsies, real-time PCR was performed in an adaptation of a previous report (He *et al*, 2002). The chosen primer set was suitable for cccDNA but not genomic DNA amplification. The 15 microliter reaction mixture was comprised of 1.0 µl of DNA sample extracted from liver biopsies, 6.0 µl of 2.5x MasterMix, 0.3 µl of 25 mM magnesium chloride solution (5 PRIME Mastermix, 5 PRIME GmbH, Hamburg, Germany), 0.75 µl of 25 µM forward primer (cccHBV F: 5′-ACT CTT GGA CTC TCA GCA ATG-3′) 0.75 µl of 25 µM reverse primer (cccHBV

R: 5′-CTT TAT ACG GGT CAA TGT CCA-3′), 0.24 µl of 10x SYBR Green (QIAGEN, Hilden, Germany) and distilled water. Upon activation of the Taq DNA polymerase at 95ºC for 10 minutes, amplification was performed for 40 cycles, comprised of denaturation (94ºC, 15 seconds), annealing (59ºC, 30 seconds) and extension (72ºC, 60 seconds). The fluorescent signal was obtained once per cycle at 80ºC after the extension step.

The β - globin gene was used as an internal standard. The primer sequence for the β - globin gene has been previously described (Shadrina *et al*, 2007). Real-time PCR was performed in 10 µl reaction volumes containing 1 µl of DNA, 5 µl of 2.5x MasterMix solution (5 PRIME Mastermix, 5 PRIME GmbH, Hamburg, Germany), 0.1 µl of 25 µM forward primer (Beta-globin F: 5′-GTGCACCTGACTCC TGAGGAGA-3′), 0.1 µl of 25 µM reverse primer (Beta-globin R: 5′-CCTTGATACCAACCTG CCCAG-3′), 0.25 µl of 10x SYBR Green (QIAGEN, Hilden, Germany) and distilled water. Upon activation of the Taq polymerase at 95ºC for 3 minutes, the amplification reaction was performed for 40 cycles of denaturation at 95ºC for 10 seconds, primer annealing at 60ºC for 15 seconds and extension at 72ºC for 20 seconds. The fluorescent signal was obtained at 78ºC.

The standard curve for cccDNA and β - globin was created in a manner identical to the HBV DNA detection method using pGem-T® Easy Vector inserted with the amplicon obtained with the appropriate primer set.

Conventional PCR assay. Samples with HBV DNA concentrations below the detection level of real-time PCR (less than 100 copies/µl) were subjected to further analysis by semi-nested PCR. For HBV DNA detection, the first round PCR was performed using primers *PreS1*F+ and *PreS1*R2 and the second round using primers *PreS1*F+ and R2 (5′- CCA GGA CAA GTT GGA GGA C - 3′).

For cccDNA detection, the first round PCR was performed using forward primer (x101: 5′-TCT GTG CCT TCT CAT CTG - 3′) and reverse primer (CO2: 5′-GTG AGG TGA ACA ATG TTC CG - 3′). The second round PCR was performed using forward primer x101 and reverse primer (Xi3: 5′-GGC ACA GCT TGG AGG CTT G-3′).

First and second round PCR amplifications were performed under the following conditions: initial denaturation at 94ºC for 3 minutes followed by 35 cycles of denaturation at 94ºC for 30 seconds, primer annealing at 55ºC for 30 seconds for HBV DNA and at 60ºC for 30 seconds for cccDNA, extension at 72ºC for 1.30 minutes and a final extension step at 72ºC for 7 minutes.

The total 25-µl reaction mixture was comprised of 2 µl of a DNA solution, 10 µl of 2.5x (5 PRIME Mastermix, 5 PRIME GmbH, Hamburg, Germany), 0.5 µl of each 25 µM primer and sterile water.

The PCR-amplified products were subjected to electrophoresis in 2% agarose gel stained with ethidium bromide and visualized under UV light.

RESULTS

HBV and cccDNA analysis were performed on liver tissue obtained from 16 of 18 patients with isolated anti-HBc and 19 of 20 patients positive for anti-HBc and anti-HBs. There were 11 males (69%) with isolated anti-HBc antibody and 14 males (74%) with both anti-HBc and anti-HBs. The mean age ± SD of the patients in the first group was 49.0 ± 11.5 years (range

22 - 66) and in the second group was 50.8 ± 13.4 years (range 27 - 77). Indications for liver biopsy were isolated elevation of transaminase enzymes (n = 6; 17%), suspected autoimmune hepatitis (n = 6; 17%), suspected non-alcoholic steatohepatitis (n = 4; 11%) and pretreatment evaluation for hepatitis C infection (n = 19; 54%).

Liver tissues serving as positive and negative controls were randomly selected from 1 HBeAg positive and 1 HBeAg negative chronic hepatitis B patient each and from 2 patients negative for serological markers for HBV infection. Serum HBV DNA concentrations were determined at $1.6x10^7$ and $2.0x10^7$ copies/µl, tissue HBV DNA levels were found at $1.3x10^7$ and $2.6x10^4$ copies/µl and cccDNA were detected at levels of $1.7x10^6$ and $3.2 x10^4$ copies/µl in the liver samples of HBeAg positive and negative chronic HBV infected patients, respectively. When compared with β - globin concentrations detected in liver samples, total intrahepatic HBV DNA and cccDNA levels were 164.6 and 21.5 copies/cell in the HBeAg positive patient and 0.6 and 0.7 copies/cell in the HBeAg negative patient, respectively.

Amplification of β - globin in liver specimens was performed. The median concentration of β - globin was $9.9x10^4$ (range $3.6x10^3$-$5.8x10^5$) copies/assay. HBV DNA detection in sera of anti-HBc positive individuals was attempted by real-time PCR. HBV sequences could not be amplified in the serum samples of any of the patients. The DNA extracted from liver tissue was analyzed using the same PCR procedure. HBV sequences were not detected in any of the samples using real-time PCR. However, 4 of 35 patients (11.6%) were positive for HBV DNA in their liver tissue upon amplification by semi-nested PCR. Three HBV DNA positive tissue samples were derived from patients expressing only anti-HBc and one sample was obtained from a patient positive for anti-HBc and anti-HBs.

Quantitative PCR analyses for detection of cccDNA in liver tissue were performed on all patient samples. Neither real-time nor semi-nested PCR could amplify cccDNA in any of the specimens. The results are summarized in Table 1.

DISCUSSION

Although the crucial role of cccDNA in persistent HBV infection is well known, the current understanding of this replicative form of HBV has been mostly obtained from animal studies conducted over the past two decades. Only little data has been obtained from actual patients. A difficulty in studying cccDNA in humans stems from the necessity of a liver biopsy, particularly in patients during the quiescent phase of infection.

The intrahepatic HBV DNA concentrations in control specimens were 164.6 copies/cell in the HBeAg positive HBV infected patient and only 0.6 copies/cell in the HBeAg negative HBV infected patient, consistent with findings previously reported for similar patients (Werle-Lapostolle *et al*, 2004). HBV DNA was detected in 4 of 35 liver samples from anti-HBc positive patients by highly sensitive semi-nested PCR assay. This finding suggests a very small amount of HBV DNA in the liver of anti-HBc positive individuals. This could either indicate lack of persistent HBV infection in the hepatocytes, or more likely, lower levels of HBV genome in patients with protective immunity to HBV virus. The percentage of positive samples was quite low when compared to that reported in other studies (Table 2) (Mason *et al*, 1998; Yuki *et al*, 2003; Murakami *et al*, 2004; Werle-Lapostolle *et al*, 2004; Yuen *et al*,

Table 1
Demographic data and the results of liver cccDNA, HBV DNA in the control and study group patients.

		Control group		Study group		
	Technique	HBeAg+ CHB	HBeAg- CHB	Isolated anti-HBc	Positive anti-HBc and anti-HBs	Total
Number of patients		1	1	16	19	35
Male, *n* (%)		1	0	11 (69)	14 (74)	25 (71)
Mean age ± SD (yrs)		28	36	49.0 ± 11.5	50.8 ± 13.4	50.0 ± 12.4
Undetermined cause of hepatitis (ALT > 40 IU/l), *n* (%)		-	-	1 (16)	5 (26)	6 (17)
Suspected autoimmune hepatitis, *n* (%)		0	0	3 (19)	3 (16)	6 (17)
Suspected non-alcoholic steatohepatitis, *n* (%)		0	0	3 (19)	1 (5)	4 (11)
Pretreatment evalutation for HCV infection, *n* (%)		0	0	9 (56)	10 (53)	19 (54)
Serum HBV DNA (copies/µl)	Real-time	$1.6x10^7$	$2.0x10^7$	Undetectable	Undetectable	Undetectable
Tissue HBV DNA (copies/cell)	Real-time	$1.3x10^7$	$2.6x10^4$	Undetectable	Undetectable	Undetectable
Tissue cccDNA (copies/µl)	Real-time	$1.7x10^6$	$3.2x10^4$	Undetectable	Undetectable	Undetectable
Tissue HBV DNA (copies/cell)	Real-time	164.6	0.6	Undetectable	Undetectable	Undetectable
	Semi-nested PCR	Positive	Positive	3 of 16	1 of 19	4 of 35
Tissue cccDNA (copies/cell)	Real-time	21.5	0.7	Undetectable	Undetectable	Undetectable
	Semi-nested	Positive	Positive	Negative	Negative	Negative

Table 2
Comparative results of the patients positive for anti-HBc antibody and negative for HBsAg.

Country		Total N	Total biopsy	Setting of patients	Median time from HBsAg loss to biopsy (range)	Serum HBV DNA	Tissue HBV DNA	Tissue cccDNA	Refrence
Thailand		35	35		NA[c]				
	Group I[a]		16			Undetectable	3/16	Undetectable	This study
	Group II[b]		19			Undetectable	1/19	Undetectable	
Japan		14	9	Acute self limited hepatitis B	7.2 yrs (1.8 - 9.5)				Yuki *et al*, 2003
	Group I		1			0/1	1/1	1/1	
	Group II		8			2/8	8/8	8/8	
Multicenter (France, Australia, Germany, Hong Kong, USA)		7	7	HBsAg seroconverter	NA				Werle-Lapostolle *et al*, 2004
	Group I		0			NA			
	Group II		7			NA	5/7	5/7	
USA		7	7	Chronic hepatitis B with HBsAg clearance	Mean 28 months (3 - 67)	1/7	6/7	3/7	Mason *et al*, 1998
Japan		10	10	HCV infection	NA				Murakami *et al*, 2004
	Group I		3			3/3	3/3	0/3	
	Group II		7			3/7	7/7	1/7	
Hong Kong		96	16	Chronic hepatitis B with HBsAg clearance	48 months (26-128)	1/92			Yuen *et al*, 2004
	Group I						2[e]	2[e]	
	Group II						4[e]	4[e]	
Hong Kong[d]		298	29	Chronic hepatitis B with HBsAg clearance	48 months (14-128)		29/29	27/29	Yuen *et al*, 2008
	Group I		16			NA			
	Group II		13			NA			

[a]Isolated antiHBc; [b]anti-HBC and anti-HBs positive; [c]Data not available; [d]Some samples overlap with Yuen *at el*, 2004; [e]Total number of patients was not shown

2004, 2008). This may be explained by differences in the study population. Most studies reported detection of HBV in the liver of patients with a documented history of acute or chronic HBV infection whose serum subsequently became clear of HBsAg and HBV DNA over 1-9 years (Mason *et al*, 1998; Yuki *et al*, 2003; Yuen *et al*, 2004; Ahn *et al*, 2005; Yuen *et al*, 2008). However, none of the patients in this study had previously been diagnosed with either acute or chronic HBV infection. The mode of HBV transmission may be different. Most patients in western countries and Japan usually acquire infection during adulthood whereas in the Far East, vertical transmission or infection during early childhood is more common. This is believed to have been the mode of transmission with most patients participating in this study as well (Mason *et al*, 1998; Yuki *et al*, 2003; Werle-Lapostolle *et al*, 2004). These patients may have a longer quiescent infection, a longer period of host immune manipulation, and less HBV in liver tissue than those investigated in the other studies.

Four of the liver tissue samples from anti-HBc positive patients were positive for HBV genome. However, we were unable to detect cccDNA molecules in any specimen. The presence of an extremely small amount of this replicative form of virus cannot be ruled out. An undetectable level of cccDNA may result from the host's immune response, which can suppress viral replication to an extremely low level. In contrast, with immuno-suppressed individuals, HBV DNA replication may increase, causing a flare up of hepatitis, as seen in patients receiving cytotoxic chemotherapy (Mindikoglu *et al*, 2006). Therefore, this PCR assay may not be sensitive enough to detect cccDNA in this population. It may be more applicable to immuno-suppressed patients which require further investigation. In conclusion, there were extremely low levels of HBV DNA detectable in liver tissue of patients only expressing anti-HBc; cccDNA could not be amplified from either PCR assay performed in this study.

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