Relationship between Molecular State of Serum HBV-DNA and Clinical Features of Hepatitis B Virus Carriers

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SATO, S., YAMADA, M., MIYAZAKI, Y., MATSUDA, K., KANNO, A., ISHII, M., OHORI, H. and TOYOTA, T. *Relationship between Molecular State of Serum HBV-DNA and Clinical Features of Hepatitis B Virus Carriers*. Tohoku J. Exp. Med., 1993, 171 (4), 309-317 ——— Molecular species of serum hepatitis B virus (HBV)-DNA in HBV carriers were classified by Southern blot hybridization into three types; type I with two bands of 4.0 kb and 3.2 kb, type II with the two bands of type I plus the smear between 4.0 kb and 3.2 kb, and type III with a broad band below 4.0 kb. A total of 51 HBV carriers were classified into three groups (group I, $n=19$ with type I; group II, $n=12$ with type II; and group III, $n=20$ with type III). Serum aminotransferase levels of group I were significantly lower than those of groups II and III. Liver pathology revealed that 18 of the 19 (94.7%) group I patients showed nonspecific reactive hepatitis (NSRH), while 11 of the 12 (91.7%) group II patients and 19 of the 20 (95.0%) group III patients showed chronic persistent hepatitis (CPH) or chronic active hepatitis (CAH). Immunohistochemical study showed that hepatitis B core antigen (HBcAg) was localized in the nucleus of hepatocytes in most of patients with type I while it was localized in both the nucleus and cytoplasm in those with types II and III. Since the smear between 4.0 kb and 3.2 kb is specifically found in groups II and III, HBV-DNA with this smear may be related to active hepatitis. ——— Southern blot hybridization; HBV-DNA; HBV carriers; HBcAg

Chronic infection of hepatitis B virus (HBV) to the liver causes chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. On the other hand, it is accompanied by healthy HBV carrier state with no or minimal active hepatitis. Because HBV replication is high in healthy carrier state, absence of hepatitis is considered due to the lack of immune response to HBV antigens (Mondelli et al. 1982). However, the possibility that molecular species of HBV may alter the activity of hepatitis still exists.

HBV has a double-stranded DNA (a plus and a minus strands) in the viral particle. Southern blot analysis of HBV-DNA in the sera of HBV carriers

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demonstrated that several molecular species were detected in serum HBV-DNA (Imazeki et al. 1984; Lok et al. 1985; Scotto et al. 1985). It was stated previously that serum HBV-DNA of HBV carriers could be categorized into three distinct molecular states. These molecular states represented the length of the plus strand of HBV-DNA. The patients who had HBV with incomplete, short plus stranded DNA appeared to have chronic liver disease with active inflammation (Yamada et al. 1993), although the number of patients examined was insufficient to draw a positive conclusion.

This paper addresses the issue of the relationship between the molecular variation of serum HBV-DNA and clinical features of type B hepatitis. The results imply that HBV-DNA in the form of a smear between 4.0 kb and 3.2 kb by Southern blot analysis is related to active hepatitis.

MATERIALS AND METHODS

Patients and serological tests

Serum samples were obtained from 51 HBV carriers positive for hepatitis B e antigen (40 males and 11 females; 30.2 ± 13.5 years of age, mean $\pm$ s.d.) at the time of liver biopsy. Hepatitis B surface antigen (HBsAg) and antibody against HBsAg were examined by radioimmunoassay (Abbott Laboratories, North Chicago, IL, USA). Hepatitis B e antigen (HBeAg) and antibody against HBeAg (HBeAb) were examined by enzyme-linked immunoassay (Abbott Laboratories). The carriers were histologically categorized as follows: asymptomatic carriers with nonspecific reactive hepatitis (NSRH; $n=20$), those with chronic persistent hepatitis (CPH; $n=9$), and those with chronic active hepatitis (CAH; $n=22$). Sera and liver specimens from ten patients with liver diseases negative for all HBV markers were used to determine the specificity of HBV-DNA analysis and immunohistochemistry for HBeAg.

HBV-DNA spot hybridization

Spot hybridization of serum HBV-DNA was performed using a cloned HBV-DNA labeled with biotin as described previously (Yamada et al. 1993).

Southern blot analysis of HBV-DNA

Extraction of HBV-DNA from serum and Southern blot analysis were performed as described previously (Yamada et al. 1993). Briefly, the DNAs obtained from sera were electrophoresed through a 1% agarose gel and transferred to nitrocellulose filter (Advantec Toyo Corp., Tokyo). The filter was dried for 3 hr at 80°C and then was prehybridized for 4 hr at 42°C in 50% formamide, 5X SSC (1X SSC is 150 mM NaCl, 15 mM sodium citrate), 5X Denhardt's solution (1X Denhardt's solution is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone and 0.02% Ficoll 400), 25 mM sodium phosphate (pH 6.5), and 500 μ g/ml sonicated salmon sperm DNA (Pharmacia, Uppsala, Sweden). The prehybridization solution was removed and then the filter was hybridized for 18 hr at 42°C in 45% formamide, 5X SSC, 1X Denhardt's solution, 20 mM sodium phosphate (pH 6.5), 5% dextran sulfate, 200 μ g/ml sonicated salmon sperm DNA, and a cloned HBV-DNA labeled with biotin (Yamada et al. 1993). The filter was then washed twice in 2X SSC with 0.1% sodium dodecyl sulphate (SDS), twice in 0.2X SSC with 0.1% SDS for 3 min at room temperature, twice in 0.16X SSC with 0.1% SDS for 15 min at 50°C, and once in 2X SSC. After incubation in a blocking buffer (100 mM Tris-HCl, 150 mM NaCl, 3% bovine serum albumin, pH 7.6) for 1 hr at 65°C, the filter was incubated for 10 min in streptavidinalkaline phosphatase conjugate which was subsequently visualized with nitroblue tetrazolium and

5-bromo-4-chloro-3-indolylphosphate.

Immunohistochemistry

Immunohistochemical study was performed as described previously (Kanno et al. 1987). Formalin-fixed and paraffin-embedded sections of the liver were stained for HBcAg by the avidin-biotin-peroxidase complex (ABC) method (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA). Each section was initially incubated with 1:100 diluted rabbit anti-HBc IgG (DAKO Corp., Santa Barbara, CA, USA) at room temperature for 60 min. After rinsing, the sections were then incubated with 1:100 diluted biotinylated goat anti-rabbit IgG (DAKO Corp.) at room temperature for 30 min, followed by incubation in a solution of 1:500 diluted ABC. The complex was made visible with diaminobenzidine reaction. Specificity of the reaction was confirmed by absorbing the anti-HBc rabbit serum with purified HBcAg particles (Onodera et al. 1982), blocking the HBcAg-positive liver specimens with anti-HBc human serum, and staining several liver specimens obtained from HBsAg-negative patients. The percentage of HBcAg positivity was determined by counting 1,000 hepatocytes under a light microscope.

Statistical analysis

One-way analysis of variance (ANOVA) test or χ^2 test was employed for statistical analysis. A probability of $p < 0.05$ was regarded as statistically significant.

RESULTS

Quantitation of serum HBV-DNA by spot hybridization

Known concentrations of HBV-DNA were hybridized with a biotin-labeled HBV-DNA probe. The concentrations were roughly correlated with the spot density in the range from 1 to 100 pg/50 μ l (Fig. 1). For Southern blot analysis, the DNA concentration of every serum sample was adjusted to 20 pg/50 μ l.

Southern blot hybridization patterns of serum HBV-DNA

The same three hybridization patterns as those reported previously (Yamada et al. 1993) were reproducibly identified in this study. Type I consisted of two distinct bands of 4.0 kb and 3.2 kb. Type II consisted of a smear between 4.0 kb and 3.2 kb in addition to the two bands of type I. Type III consisted of a broad band below 4.0 kb (Fig. 2). HBV carriers were classified into three groups (group

Fig. 1. Spot hybridization of serum HBV-DNA with a biotin-labeled HBV-DNA probe. Known concentrations of HBV-DNA were hybridized with a cloned HBV-DNA labeled with biotin. The grade of $\pm$, 1+, 2+, 3+, 4+ and 5+ corresponded to 1, 2.5~5, 10, 20, 50 and 100 pg/50 μ l of HBV-DNA, respectively.

I, n=19 with type I; group II, n=12 with type II; and group III, n=20 with type III). The sera from 10 control subjects negative for all HBV markers did not show any bands or stains.

Clinical data in groups I, II and III

There were no statistical difference on age and sex among these three groups. In the biochemical tests of the liver, the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly lower in group I than those in groups II and III. The other biochemical data did not show a statistically significant difference among the three groups (Table 1). The relationship between Southern blot hybridization patterns of serum HBV-DNA and pathologic changes in the liver was also examined. Eighteen of the 19 (94.7%) group I patients had NSRH, while 11 of the 12 (91.7%) group II patients and 19 of 20 (95.0%) group III patients had either CPH or CAH. In 22 patients with CAH, 15 patients showed type III blotting pattern in their serum HBV-DNAs (Table 2).

Localization of HBcAg in hepatocytes

A total of 32 liver specimens from 51 HBV carriers were stained for HBcAg in hepatocytes. HBcAg was localized either in the nucleus only (Fig. 3-A) or in both the cytoplasm and nucleus (Fig. 3-B). In 5 of the 6 (83.3%) type I patients, HBcAg was localized in the nucleus, while in 23 of the 26 (88.5%) patients with types II and III, HBcAg was localized in both the cytoplasm and nucleus. Furthermore, HBcAg was dominated in the cytoplasm in type III (Table 3).

Fig. 2. Three typical Southern blot hybridization patterns of serum HBV-DNA. The DNAs were electrophoresed through a 1% agarose gel, blotted onto nitrocellulose filter, and hybridized with a cloned HBV-DNA labeled with biotin. I, II and III indicate type I, type II and type III, respectively.

TABLE 1. *Clinical data of patients in each group*

	Patient					ANOVA test
	Group I (n=19)	Group II (n=12)	Group III (n=20)			
Age (years)	27.0±18.1	36.3± 8.8	29.7± 9.3			n.s
Sex (M/F)	14/5	9/3	17/3			n.s*
T.B. (mg/100 ml)	0.8± 0.2	0.9± 0.4	0.9± 0.3			n.s
AST (IU/liter)	27.2±11.8	191.7±211.6	131.4±121.8			$p<0.01$
ALT (IU/liter)	26.5±14.3	26.0±169.5	200.4±159.5			$p<0.01$
LDH (IU/liter)	390.5±88.9	44.8±115.8	411.4± 86.5			n.s
ALP (IU/liter)	147.6±80.1	112.6± 33.7	114.7± 52.6			n.s
γ -GTP (IU/liter)	34.0±20.8	60.9± 54.9	58.7± 37.7			n.s
ChE (IU/liter)	314.1±58.2	277.3± 58.1	264.6± 46.6			n.s
T.P. (g/100 ml)	7.3± 0.4	7.6± 0.4	7.5± 0.5			n.s
HBV-DNA (grade)	2.6± 0.5	2.6± 0.9	2.3± 1.3			n.s

Results are expressed as mean±s.d. n.s., not significant.

* χ^2 test

TABLE 2. *Pathological diagnosis and Southern blot hybridization patterns of serum HBV-DNA*

Group	No. tested	Pathological diagnosis		
		NSRH	CPH	CAH
I	19	18 (94.7%)	1 (5.3%)	0 (0%)
II	12	1 (8.3%)	4 (33.4%)	7 (58.3%)
III	20	1 (5.0%)	4 (20.0%)	15 (75.0%)

NSRH, nonspecific reactive hepatitis; CPH, chronic persistent hepatitis; CAH, chronic active hepatitis.

$\chi^2=40.5$ ($p<0.001$)

Changes of serum ALT levels and Southern blot pattern of HBV-DNA in the sera from a HBV carrier

In one HBV carrier whose sera were sequentially analysed for biochemical, serological, and virological features, his HBV-DNA at first showed type I when serum ALT levels were slightly elevated. With flare-up of hepatitis, HBV-DNA turned to type II and liver biopsy revealed chronic active hepatitis (Fig. 4).

DISCUSSION

This paper presented the relationship between the molecular state of serum HBV-DNA and clinical features of HBV carriers. The serum HBV-DNAs were

Fig. 3. (A) Nuclear HBcAg expression in hepatocytes of HBV carrier with nonspecific reactive hepatitis. Immunoperoxidase staining, $\times 200$.
 (B) Nuclear and cytoplasmic HBcAg expression in hepatocytes of HBV carrier with chronic active hepatitis. Immunoperoxidase staining, $\times 200$.

TABLE 3. *HBcAg in hepatocytes and Southern blot hybridization patterns of serum HBV-DNA*

Group	No. tested	HBcAg localization		
		N only	N > C	N < C
I	6	5 (83.3%)	1 (16.7%)	0 (0%)
II	9	1 (11.1%)	3 (33.4%)	5 (55.5%)
III	17	2 (11.8%)	2 (11.8%)	13 (76.4%)

N, nucleus; C, cytoplasm. N > C means HBcAg is more abundant in the nucleus than the cytoplasm.

$\chi^2 = 16.1$ ($p < 0.005$)

Fig. 4. Changes of serum ALT levels and Southern blot pattern of HBV-DNA in the sera from a HBV carrier who experienced exacerbation of hepatitis.

categorized previously into three types by Southern blot hybridization. These three types did not represent the different amount of DNA but a different molecular state of HBV-DNA; 4.0 kb form was a relaxed circular, fully double-stranded DNA, 3.2 kb form was a linear, fully double-stranded DNA, and the smear between 4.0 kb and 3.2 kb was the sum of relaxed circular, double-stranded DNAs with various length of plus strands (Yamada et al. 1993). Viral particles with the DNA molecules are eventually secreted into blood at any phase of intracellular assembly of HBV-DNA. Therefore, these three types may represent the different replication state of HBV in hepatocytes.

Serum aminotransaminase levels of groups II and III were significantly higher than those of group I. Histologically, group I patients had mainly minimal hepatitis, while groups II and III patients had active hepatocyte injury. HBcAg was present in the cytoplasm in patients with types II and III, while it was mainly in the nucleus in those with type I. These results indicate that types II and III relate to active hepatitis. Since the smear between 4.0 kb and 3.2 kb is specific in types II and III, this smear may be related to active hepatitis.

The exact mechanism of hepatocyte injury remains to be elucidated. HBcAg is present either in the nucleus or in the cytoplasm (Yamada and Nakane 1977; Akiba et al. 1987). In addition, HBcAg is localized mainly in the cytoplasm and/or on the plasma membrane when hepatocytes are damaged by lymphocytes (Chu and Liaw 1987; Hsu et al. 1987; Kojima et al. 1987). Therefore, replication of HBV in hepatocytes may result in expression of HBcAg on hepatocyte surface together with HLA-class I antigen. These two antigens will be recognized by cytotoxic T lymphocytes and subsequent injury of hepatocytes is induced (Montano et al. 1982; Chu and Liaw 1987; Ferrari et al. 1987). Our result that HBcAg in hepatocytes from patients who had types II and III HBV-DNA was localized in the cytoplasm also supports the contention that the smear between 4.0

kb and 3.2 kb is related to immune-mediated lysis of hepatocytes infected with HBV.

In this study, full length of relaxed circular and linear DNAs were found in viral particles from NSRH patient. On the other hand, viral particles contained heterogeneous DNA molecules with complete minus strands and partial plus strands in most cases with active hepatitis (Table 2). Imazeki et al. (1984) and Fowler et al. (1984) demonstrated that there was a full length of HBV molecules in serum. On the other hand, viral particles with partially double-stranded DNA molecules (Kam et al. 1982) or those with complete minus strands without associated plus strands (Scotto et al. 1985) were also found in serum. Clinical significance of these species of virus has not been evaluated, and the reason why packaging of such heterogeneous HBV-DNA into viral particles is not known. It is well known that increased HBV replication occurs immediately before hepatocyte injury (Tong et al. 1987). In this highly replicative phase, the incomplete plus strands of HBV may be packaged into viral particles before complete synthesis of the plus strands occurs.

Our results clearly demonstrated the close association of the molecular state of HBV-DNA in serum and the activity of hepatitis. Therefore, analysis of serum HBV-DNA by Southern blot hybridization is a useful marker for viral replication and the degree of hepatitis.

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