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Increased Proinflammatory Cytokine Production by Chronic Hepatitis B Patients with Mutant Hepatitis B Virus: Plausible Mechanisms Underlying Severe Liver Diseases in These Patients

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Abstract

Hepatitis B virus (HBV) is a noncytopathic virus and billions of HBV-infected patients live uneventful lives and do not suffer from notable liver damage. However, HBV also causes progressive liver diseases characterized by hepatic inflammation, hepatic fibrosis, and liver cancer in millions of HBV-infected patients. The goal of this study was to evaluate the role of mutant HBV in HBV pathogenesis. In a cohort of 360 chronic HBV-infected patients, mutations at T1762/A1764 of HBV genome were detected in most of the patients with HBV-induced liver cirrhosis and hepatocellular carcinoma. To explore if mutations at T1762/A1764 of HBV genome has any role in progressive liver disease, peripheral blood mononuclear cells (PBMCs) and antigen-presenting dendritic cells (DCs) were isolated from five chronic hepatitis B (CHB) patients with mutations at T1762/A1764 and five comparable patients of CHB without mutations at T1762/A1764. DCs were pulsed with hepatitis B surface antigen (HBsAg). The levels of cytokines produced by PBMCs and DCs as well as nitrite production by DCs were evaluated.

Significantly higher levels of interleukin-12, tumor necrosis factor-alpha, interferon-gamma, and transforming growth factor-beta were detected in cultures of PBMCs, DCs, and HBsAg-pulsed DCs from CHB patients with mutations at T1762/A1764 compared with those without mutations ($p < 0.05$). DCs of all CHB patients with mutations produced significantly higher levels of nitrite compared with those without mutation at T1762/A1764 ($p < 0.001$). This study discusses the inflammatory potential of mutant HBV that may be responsible for diverse levels of pathogenicity of HBV. Further studies involving larger cohorts would provide more insight into these unresolved issues about HBV pathogenesis and these insights may aid in developing immune therapy for CHB patients.

Keywords: chronic hepatitis B, viral mutation, host immunity

Background

Hepatitis B virus (HBV) infection is a highly complex and complicated health problem. About two billion people in the world have been infected by HBV at some point in their lives. However, >90% of the two billion HBV-infected people do not exhibit visible features of liver injury (24). In contrast, about 300 million people are chronically infected by HBV and about 20% of them would develop progressive liver diseases such as chronic hepatitis B (CHB; HBV DNA and hepatitis B surface antigen [HBsAg] in the blood with elevated alanine aminotransferase [ALT]), liver

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cirrhosis (LC; distortion of normal architecture of hepatocytes due to progressive fibrosis), and hepatocellular carcinoma (HCC; primary cancer of the hepatocytes) (27). Worldwide, >800,000 people die annually due to HBV-related liver diseases (11).

Since HBV is a noncytopathic virus and does not produce toxins, the process through which HBV-infected people develop inflammation, fibrosis, and carcinogenesis is still elusive (28). The noncytopathic nature of HBV has been demonstrated in HBV transgenic mice (HBV TM), an animal model of HBV carrier state, and also in patients with chronic HBV infection (5). Almost all lines of HBV TM have exhibited very high levels of HBV DNA, HBsAg, and hepatitis B e antigen (HBeAg), but none of these HBV TM has shown hepatic inflammation, fibrosis of the liver or cancer of the hepatocytes (5,13). Also, several patients with very high levels of HBV DNA and HBsAg do not exhibit evidence of liver damage; however, considerable number of patients with low HBV DNA exhibit complications such as LC (14,16,22). As the mechanism of HBV-induced liver damage has not been fully understood, it is assumed that liver damage may be due to anomalous immune responses to HBV (1,9). However, convincible evidences are yet to emerge.

Although the levels of HBV DNA, HBsAg, and HBeAg in sera have failed to show direct association with extents of liver damage, HBV is prone to undergoing mutations, deletions, and insertions due to the inherent properties of HBV replication systems (21,25). With the advent of recent molecular techniques, studies have shown that due to frequent alteration of HBV genome, there may be at least 10 HBV genotypes. Interestingly, some HBV genotypes are more pathogenic than the others. For example, HBV genotype C has shown more carcinogenic potential than HBV genotype B (12). Also, similar data about divergent pathogenic potentials have been recorded by HBV genotypes A and D (15). But almost nothing has been explored regarding underlying mechanisms.

In the past one decade, a patient pool of about 1500 chronic HBV-infected patients have been developed in Bangladesh to accomplish phase I/II/III clinical trials with a new and innovative immune therapeutic drug, NASVAC (6,7). HBV genotype and mutations of HBV genome were checked among 360 of these HBV-infected patients. Full genome sequences indicated that most patients with HBV genotype C developed LC and HCC, and majority of them had mutations at T1762/A1764 (20). In contrast, all asymptomatic HBV carriers with almost no liver damage did not have mutations at T1762/A1764. This provided us with an excellent opportunity to assess the implication of mutation of HBV genome at T1762/A1764 on a host's immunity.

Materials and Methods

For HBV genotyping, a total of 360 patients with CHB were enrolled. Full genome and partial genome sequencing along with analyses of pathological conditions revealed that progression of LC and HCC was mostly associated with presence of mutation at T1762/A1762 of HBV genome (20). In contrast, patients without mutations at T1762/A1764 remained mostly asymptomatic or progressed without any complications. Based on these realities, 10 patients were carefully selected from these patients in such a way that these patients do not differ in age, gender, levels of HBV DNA, HBV genotype, HBeAg-positivity, levels of ALT, levels of inflammation, and extent of fibrosis by liver biopsy. These 10 patients were grouped into two groups. Each group comprised five patients with almost similar clinical profiles. Written consent was obtained from all patients after the nature and purpose of the study had been explained to them. Institutional approval was obtained from the institutional review board.

Study design

We opted to check immune modulatory capacity of two populations of immunocytes in patients with and without mutations at T1762/A1764. One of these were peripheral blood mononuclear cells (PBMCs). PBMC represents a lump population of immunocytes. We also checked the immune status of antigen-presenting dendritic cells (DCs).

Based on these observations, we checked inflammatory cytokine production by PBMC and DC. Also, nitrite production by DC was evaluated to get initial insights into the role of DC in mutant HBV-induced inflammation, if any.

Isolation of PBMCs and peripheral blood DCs

PBMCs and antigen-presenting DCs were isolated and enriched by methods that have been previously described (2–4). PBMCs were isolated from freshly drawn heparinized whole blood by Ficoll–Hypaque (Sigma, St. Louis, MO) density gradient centrifugation. The cells were retrieved from the interface and washed three times. Finally, PBMCs were resuspended in RPMI 1640 (Nipro, Osaka, Japan) plus 10% autologous serum. The use of fetal calf serum (FCS) was avoided to prevent the immune stimulatory effect of FCS. The viability of PBMCs was checked with the trypan blue exclusion test.

Isolation of DCs from peripheral blood

DCs were isolated from PBMCs by culturing PBMC in RPMI 1640 with 10% autologous sera with human-grade granulocyte macrophages colony-stimulating factor (800 U/mL) and interleukin (IL)-4 (400 U/mL (Pepro Tech EC Ltd., Margravine Road, London, UK) for 7 days, as described in previous studies (2–4). DCs were then retrieved from cultures and washed three times with phosphate-buffered saline (PBS).

Preparation of HBsAg-pulsed DCs

Preparation of HBsAg-pulsed DCs have been optimized in our laboratory in the past three decades. DCs were cultured with HBsAg (1 μg/mL; Center for Genetic Engineering and Biotechnology, CIGB, Havana, Cuba) for 48 h. HBsAg-pulsed-DCs were pelleted and washed five times in PBS (2–4).

Culture of PBMC, DC, and HBsAg-pulsed DC

PBMCs (1×10^6 cells/mL), DCs (2×10^5 cells/mL), and HBsAg-pulsed DCs (1×10^6 cells/mL) were suspended and their viabilities were assessed. PBMCs and DCs were cultured in the presence of HBsAg (1 μg/mL, Center for

Genetic Engineering and Biotechnology, CIGB, Havana, Cuba) for 72 h at an atmosphere of 95% air and 5% CO_2 in humid conditions at 37°C (4,23,29). The culture supernatant was collected for estimation of cytokines and nitrite.

Cytokines in culture supernatants

Cytokines in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) method using commercial kits, according to the manufacturer's instructions (RD Bioscience System, Minneapolis, MN) (2,3).

Nitrite production and measurement

This was done according to methods that have been accomplished in our laboratory (3,29). HBsAg-pulsed DCs and *Staphylococcus aureus* Cowan Strain 1 (0.0075%) were cultured at 37°C for 48 h.

A commercial kit was used to measure nitrite in the culture supernatants (Griess Assay Kit No kit-C; Wako, Osaka, Japan). Supernatants were incubated with Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min resulting in color development, which was measured by an ELISA reader at 540 nm. The data were expressed as micromole per milliliter after calibration with standard sera containing known amounts of nitrites.

Statistical analysis

The data have been shown as median and range. For the statistical analysis, paired *t*-test was used for normally distributed data. When the distribution was skewed, the Wilcoxon signed-rank test was used.

Results

In this study, the patients with CHB were selected in a way that ensured they had comparable ages, genders, levels of HBV DNA and ALT, HBeAg-positivity, HBV genotype, levels of inflammation, and fibrosis in liver biopsy (Table 1). All of them were presumed to have been infected with HBV during neonatal and perinatal stages since their mothers were HBsAg-positive, indicating a vertical transmission of HBV from mother to off-spring. The two groups differed in presence and absence of mutations at T1762/A1764. Thus, the role of mutation of HBV genome at T1762/A1764 could be reasonably assessed from the capacities of their immunocytes to produce various cytokines and other mediators.

Table 1. Clinical Profiles of the Patients

Parameters	*Patient without mutations at T1762/A1764 of HBV genome*	*Patient with mutations at T1762/A1764 of HBV genome*
Age	30.0 ± 3.4	29.6 ± 2.9
Gender (male:female)	3:2	3:2
HBV DNA (IU/mL)	14,600 ± 3049	14,400 ± 2509
HBeAg/anti-HBe	2:3	2:3
Serum ALT (IU/L)	56 ± 12.5	58.8 ± 3.77
Serum albumin (gm/dL)	3.08 ± 0.11	3.04 ± 0.11
Serum bilirubin (gm/dL)	0.62 ± 0.8	0.64 ± 0.09
Level of inflammation (liver biopsy)	Moderate inflammation	Moderate inflammation
Level of hepatic fibrosis (liver biopsy)	Moderate fibrosis	Moderate fibrosis

Data shown as mean and standard deviation. Statistical significance was not observed in any parameters between these two groups.

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

Increased cytokine production by PBMCs in CHB patients with mutations at T1762/A1764 than in patients without mutations

The levels of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta, and IL-12 produced in culture supernatants of PBMC of patients with CHB having mutations at T1762/A1764 were significantly higher than those detected in supernatants of CHB patients without mutations at T1762/A1764. The levels of IL-2 and IL-6 were higher in culture of patients of CHB with mutations, but the difference was not statistically significant (Table 2).

Increased cytokine production by DCs in CHB patients with mutations at T1762/A1764 compared with patients without mutations

The DCs used in this study were monocyte-derived DCs propagated by culturing monocytes with IL-4 and GM-CSF. Significantly higher levels of cytokines were detected in culture supernatant of DCs from CHB patients with mutations at T1762/A1764 compared with those without mutations ($p < 0.05$) (Table 3).

Increased nitrite production by HBsAg pulsed DCs from CHB patients with mutations at T1762/A1764

Nitrite was detected in the culture of HBsAg-pulsed DCs of all patients. However, the levels of nitrite were significantly higher in patients of CHB with mutations at T1762/A1764 compared with other patients without mutations at this region (Table 4).

Table 2. Increased Levels of IFN-γ, TNF-α, TGF-β, and IL-12 in Culture of PBMC from CHB Patients with Mutations at T1762/A1764 of HBV Genome

Cytokines in pg/mL	*Patient without mutations at T1762/A1764 of HBV genome*	*Patient with mutations at T1762/A1764 of HBV genome*
IFN-γ	360 (120–720)	3200 (1420–5400)[a]
TNF-α	130 (90–165)	620 (430–876)[a]
TGF-β	34 (24–55)	186 (98–298)[a]
IL-2	92 (56–134)	102 (43–156)
IL-6	32 (18–61)	49 (16–65)
IL-12	27 (16–43)	124 (68–190)[a]

Data of five patients shown.

The levels of cytokines are shown as range and median values.

[a]$p < 0.05$, compared with patients without mutations at T1762/A1764 of HBV genome.

CHB, chronic hepatitis B; IFN-γ, interferon-gamma; IL, interleukin; PBMC, peripheral blood mononuclear cell; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha.

Table 3. Increased Levels of Cytokine Production by DC with Mutations at T1762/A1764 Compared with Those Without Mutations

Cytokines in pg/mL	*Patient without mutations at T1762/A1764 of HBV genome*	*Patient with mutations at T1762/A1764 of HBV genome*
IFN-γ	90 (60–110)	240 (150–350)[a]
TNF-α	120 (60–180)	430 (150–600)[a]
TGF-β	32 (16–49)	142 (124–212)[a]
IL-2	22 (12–42)	24 (16–51)
IL-6	50 (32–72)	62.2 (34–112)
IL-12	66 (46–82)	168 (124–268)[a]

Data of five patients shown.
The levels of cytokines are shown as range and median values.
[a]$p < 0.05$, compared with patients without mutations at T1762/A1764 of HBV genome.
DC, dendritic cell.

Discussion

HBV was discovered >50 years ago. A potent vaccine against the HBV became available in the 1980s. Various direct-acting drugs that can block HBV replication or complete negate serum HBV DNA have widely been used in the past 40 years (11,24,27,28). However, there is still no curable therapy for different HBV-related pathologies. The underlying factors of these frustrating realities are mainly attributable to a major issue that has not been addressed till now: How can a noncytopathic, nontoxic, and noninvasive virus such as HBV cause inflammation and progressive liver damage?

Recent advances in molecular biology techniques have unmasked a fundamental but important development about HBV virology: HBV is not a homogeneous virus, rather it is highly heterogenous in terms of genotype, presence of deletion and insertion and, most importantly, site-specific mutations (13,16,22).

Since the 1990s, investigators have documented that mutations at T1762/A1764 are associated with the genesis of progressive diseases, and patients with HCC showed increased mutations at these regions (17,18,23,26,30). However, mechanisms underlying these observations were not elucidated. The main reasons for this were mainly related to not having a comparable population. These studies were mainly accomplished by molecular biologists and cancer specialists.

Table 4. Increased Nitrite Production by Dendritic Cells from Patients with Chronic Hepatitis B with Mutations at T1762/A1764

	Nitrite in culture of DCs (μMol/mL)
Patient without mutations at T1762/A1764 of HBV genome	5 (4–8)
Patient with mutations at T1762/A1764 of HBV genome	24 (12–32)[a]

The levels of nitrite are shown as median and range.
Data of five patients in each group have been cited.
[a]$p < 0.05$, compared with patients without mutations at T1762/A1764 of HBV genome.

In this study, the HBV genotype study included patients with different statuses of HBV infection. We had a group of patients with mutations at T1762/A1764 and another group of patients without these mutations. However, the basal characteristics, virological parameters, biochemical statuses, and liver biopsy findings were comparable between the two groups.

The study revealed that the immunocytes of CHB patients with mutations at T1762/A1764 induced significantly higher levels of proinflammatory cytokines and nitrite compared with similar patients without these mutations. These findings were found both in PBMC and DC. Proinflammatory cytokines induce an inflammatory mucosal milieu, which may potentiate progressive liver diseases such as LC and HCC. The link between mutations at T1762/A1764 and progressive liver diseases have been reported by several investigators; however, the present report seems to be one of the first analysis that clearly provided evidence of increased inflammatory potentials of mutant HBV (17,18,23,26,30). In fact, roles of proinflammatory cytokines in the course of liver damage in CHB patients have been shown by various investigators. Liver damage seems to be induced by the action of proinflammatory cytokines as described by Poovorawan *et al.* (19). Similarly, Falasca *et al.* also found increased production of IFN-γ, and TNF-α in patients with CHB with elevated ALT (10). Dunn *et al.* analyzed possible mechanisms related to cytokine-induced liver damage in CHB patients. They showed that natural killer cells may contribute to liver inflammation by the TNF-related apoptosis-inducing ligand-mediated death of hepatocytes and demonstrated that this nonantigen-specific mechanism can be switched on by cytokines produced during active HBV infection (8). Other factors may also be related to induction of severe liver diseases by mutant HBV and these remain to be elucidated in future.

This study is a pilot study that has shown how we may develop insights into the diverse nature of HBV-induced pathogenesis. There are notable limitations of this study. The sample size was small. However, accumulating two groups of patients with no significant difference in age, gender, HBV DNA, ALT, HBeAg positivity, genotype, levels of albumin and bilirubin is extremely tough in clinical situations. In addition, biopsy was taken in all patients. More importantly, all patients were treatment naive so the effect of therapy could not affect cytokine production or nitrite induction.

In addition to pathological significance of mutant HBV, this study has prognostic importance. HBV is a global problem with 300 million chronic HBV-infected patients; about 20% are likely to develop complications such as LC and HCC. Considering the impact of HBV on the health care system, WHO has recently formulated a HBV elimination program. The target is to increase diagnosis of patients, their follow-up and treatment from its present level of <10% to 65–90% by 2030. However, this goal is most likely unachievable for developing and resource-constrained countries. This study as well as our recently published articles about HBV genotype (21) indicate that certain specific genotypes and mutations are important predictors of LC and HCC. If these facts can be confirmed in a larger case–control study, these mutations would have more clinical and prognostic importance. This study opens a window into how we can select more vulnerable patients for therapy out of the millions of chronic HBV-infected patients.

Author Disclosure Statement

No competing financial interests exist.

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