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Changes of serum sPD-1 levels in HBeAg-positive chronic hepatitis B patients with entecavir treatment and correlation with curative effect

Chunhua BI[1], Deyu HUANG[1], Jing JIANG[1], Yueping JIANG[2], Hui WANG[1], Cheng BIAN[1], Zibin TIAN[2,*]

[1]Department of Infectious Diseases, Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, P.R. China
[2]Department of Gastroenterology, Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, P.R. China

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Background/aim: This study aimed to assess expression changes of serum sPD-1 levels in HBeAg-positive chronic hepatitis B patients with entecavir treatment and explore the correlation with the curative effect.

Materials and methods: A total of 220 HBeAg-positive CHB patients and 207 healthy individuals were included. sPD-1 levels and virological and biochemical responses were assessed at baseline and at 12, 24, and 48 weeks in CHB patients after antiviral therapy.

Results: sPD-1 levels in HBeAg-positive CHB patients before treatment were significantly lower than those of healthy controls. After entecavir treatment, sPD-1 levels increased gradually over time. At 48 weeks after entecavir treatment, sPD-1 amounts in the HBeAg seroconversion group were significantly higher than the values of the HBeAg-negative and HBeAg-positive groups. sPD-1 levels in patients with alanine aminotransferase (ALT) returning to normal were significantly higher than in those with ALT not returning to normal. sPD-1 levels in treated patients with no HBV-DNA were significantly higher than in those with HBV-DNA remaining positive. sPD-1 was negatively correlated with ALT and HBV-DNA at 12, 24, and 48 weeks after entecavir treatment.

Conclusion: sPD-1 may play a certain role in chronic hepatitis B and has a close relationship with the curative effect of entecavir.

Key words: sPD-1, chronic hepatitis B, entecavir, curative effect

1. Introduction

Hepatitis B virus (HBV) belongs to Hepadnaviridae. Persistent HBV infection remains a challenging global health problem. After HBV intrusion into the adult body, about 10% of infected individuals develop a chronic infection called chronic hepatitis B (CHB) (1). Currently, there are more than 370 million CHB patients, a number projected to increase by 4 million per year. CHB is the main factor responsible for cirrhosis and hepatocellular carcinoma, and it accounts for approximately 1 million deaths annually (2). Currently, immune tolerance is considered the most important factor leading to CHB, but its mechanism in the process of HBV infection remains incompletely understood (3,4).

In CHB, persistent exposure to high concentrations of viral antigens leads to various degrees of T-cell function impairment, even T-cell exhaustion (5). Recent animal models of chronic viral infection have indicated that the interaction between programmed death-1 (PD-1), a negative regulator of activated T-cells, and its ligand, PD-L, plays a critical role in T-cell exhaustion (6,7). In addition to membrane-bound PD-1 on T cells, circulating soluble PD-1 (sPD-1) has been described (8). Little is known about the origin and physiological functions of sPD-1; however, it has already been used as an antagonist of PD-1 signaling in experimental studies (9). It was shown that sPD-1 blocks the PD-1/PD-L pathway and is related to T-cell function regulation in aplastic anemia (10). However, the biological role of sPD-1 in CHB infection remains unknown.

In this study, changes of serum sPD-1 levels and their correlation with the curative effect were assessed in hepatitis B e antigen (HBeAg)-positive CHB patients before and after treatment with entecavir; in addition, the biological significance of sPD-1 in CHB was assessed.

2. Materials and methods

2.1. Study population

A total of 220 HBeAg-positive CHB patients and 207 healthy blood donors were included in this study. All patients were treated at the Affiliated Hospital of Qingdao University from February 2012 to May 2016. Diagnosis was carried out according to criteria for viral hepatitis

* Correspondence: lucia6666@163.com

described by "The Guideline of Prevention and Treatment for Chronic Hepatitis B" (2015 version) (11). Diagnostic criteria for HBeAg-positive chronic hepatitis B were: serum HBsAg- and HBeAg-positive, HBV-DNA-positive, and ALT persistently or repeatedly increased or hepatitis lesions in liver histological examination. The patients had no obvious heart, brain, nerve, mental, or thyroid disease and no diabetes or loss of compensated liver disease. Patients with concomitant hepatitis virus and HIV-infection liver diseases were excluded. None of the patients received antiviral or immunosuppressive therapy, or were exposed to hepatotoxin. Subjects with other liver diseases or liver cirrhosis were also excluded. There were 119 male and 101 female patients aged 21–58 years, averaging 41 ± 15 years old.

Meanwhile, 207 healthy individuals were identified from the physical examination center as the healthy control group. They had no previous history or current evidence of liver disease and had normal serum ALT levels. They were also negative for HBsAg, anti-hepatitis A virus, anti-HCV, and anti-HIV IgM antibodies. The controls included 107 male and 100 female healthy individuals aged 23–60 years, averaging 40 ± 14 years old. Age and sex distribution were not significantly different between the two groups ($P > 0.05$).

2.2. Antiviral therapy and follow-up

The patients received entecavir (0.5 mg/day). Virological and biochemical response and immunological parameters were assessed at baseline and at 12, 24, and 48 weeks during the antiviral therapy. Each assessment included alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), HBV-DNA, HBeAg, and sPD-1 detection. An adverse event inquiry was completed.

2.3. Ethics statement

The study protocol was approved by the ethics committee of the Affiliated Hospital of Qingdao University. Each patient provided a signed written informed consent form.

2.4. Reagents

The following reagents were used: sPD-1 ELISA Kit (Shanghai Enzyme-Linked Biotechnology Co. Ltd.); HBV Kit (Abbott Company); Hepatitis B Virus Nucleic Acid Extraction and Purification Kit (Shanghai Renaissance Long March Medical Science Co. Ltd.); ALT, AST, and TBIL determination kits (North Controlled Biotech Corp).

2.5. Main outcome measures and methods

2.5.1. Detection of HBV markers

HBV markers included HBsAg, HBsAb, HBcAb, HBeAg, and HBeAb. HBV markers were measured by the chemiluminescence method (Anthos 2010, Austria), according to the manufacturer's instructions (Sino-American Biotech Co. Ltd, Shanghai, China). The samples meeting the experimental requirements were screened after test results were analyzed; the medical history of each patient was then taken.

2.5.2. Serum sPD-1 content measurement

The levels of serum sPD-1 were assessed with the ELISA kit described above, strictly in accordance with the manufacturer's instructions.

2.5.3. Liver function test

The detection of liver enzymes, including serum ALT, AST, and TBIL, was carried out using routine automated techniques (upper limit of normal levels: 50 U/L, 40 U/L, and 17.1 μmol/mL, respectively) (HITACHI 7600.210, Japan).

2.5.4. Detection of HBV-DNA levels

Serum HBV viral load was quantitated by real-time quantitative PCR, strictly in accordance with the manufacturer's instructions. The primers were: HBV forward 5'-GAC CAC CAA ATG CCC CTA T-3'; HBV 508 reverse, 5'-AAG CGC TGC GTG TAG TTT CT-3'. The probes were: HBV FL, 5'-GAV GCA GGW CCC CTA GAA AAA AAA-fluorescein-3'; HBV LC, 5'-LVRde-TCC CTC GCC TCG CAG ACG AAG TRC TS-phosphate-3'. The sensitivity of this test was 10^2 IU/mL. Values below the detection limit were assumed to be 10^2 IU/mL.

2.6. Statistical analysis

All statistical analyses were carried out with SPSS 20.0 software. Data are mean ± standard deviation (SD). Analysis of variance and t-test were performed for comparison between groups. Correlation analyses between sPD-1, ALT, and HBV-DNA were assessed by Pearson correlation analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical outcomes

The serological characteristics of the 220 HBeAg-positive CHB patients before entecavir treatment and at 12, 24, and 48 weeks of entecavir treatment, as well as those of healthy controls, are shown in Table 1.

3.2. Changes of sPD-1, ALT, and HBV-DNA

The expression levels of sPD-1 in HBeAg-positive CHB patients before treatment were significantly lower than those of healthy controls ($t = 22.792$, $P = 0.000$). With time after entecavir treatment, sPD-1 increased gradually; values before treatment and at 12, 24, and 48 weeks after treatment were significantly different ($F = 199.849$, $P = 0.000$). Indeed, sPD-1 levels at 12, 24, and 48 weeks were significantly higher than baseline values ($t = -2.951$, -11.739, -22.238; $P = 0.003$, 0.000, 0.000, respectively). However, sPD-1 levels at 48 weeks after treatment remained lower than control values, with a statistically significant difference ($t = 3.867$, $P = 0.000$). With increasing treatment time, ALT and HBV-DNA levels decreased gradually and

were statistically significant (F = 309.495, 4260.24; P = 0.000, 0.000, respectively). Specifically, ALT and HBV-DNA amounts in patients at 48 weeks were significantly lower than baseline levels (t = 18.671, 113.41; P = 0.000, 0.000, respectively); however, ALT at 48 weeks remained significantly higher compared with control values (t = –12.182, P = 0.000).

3.3. Correlation between sPD-1 levels and HBeAg, ALT, and HBV-DNA

3.3.1. Correlation between sPD-1 levels and HBeAg

At 48 weeks after entecavir treatment, the 220 CHB patients were divided into 3 groups according to HBeAg levels: HBeAg seroconversion, HBeAg-negative (HBeAg-negative and no seroconversion), and HBeAg-positive. As shown in Table 2, sPD-1 expression levels in the 3 groups at 48 weeks were significantly higher than baseline amounts (t = 16.883, 7.948, 266.784; P = 0.000, 0.000, 0.000, respectively). There were no statistically significant differences in sPD-1 amounts among the 3 groups before treatment (F = 0.055, P = 0.9470). However, the 3 groups showed significantly different values at 48 weeks after treatment (F = 22.034, P = 0.000). Precisely, sPD-1 levels in the HBeAg seroconversion group were significantly higher than those of HBeAg-negative and HBeAg-positive groups (t = 2.378, 7.005; P = 0.020, 0.000, respectively). Meanwhile, sPD-1 amounts in the HBeAg-negative group were significantly higher than the values obtained for patients with continuously positive HBeAg (t = 2.013, P = 0.046).

3.3.2. Correlation between sPD-1 levels and ALT

At 48 weeks after entecavir treatment, ALT returned to normal in 180 cases and remained higher in 40 patients. The expression levels of sPD-1 in the two patient groups at 48 weeks were significantly higher than baseline amounts (t = 21.223, 7.711; P = 0.000, 0.000, respectively). There was no difference in sPD-1 levels between the two groups before treatment (t = 0.671, P = 0.503). Interestingly, sPD-1 levels in patients with ALT that had returned to normal levels were significantly higher than those obtained from patients with continuously higher ALT (t = 2.866, P = 0.004) (Table 3).

3.3.3. Correlation between sPD-1 levels and HBV-DNA

At 48 weeks after antiviral therapy, HBV-DNA was not detected in 204 cases and remained positive in 16 cases. The expression levels of sPD-1 in the two groups at 48 weeks were significantly higher than baseline values (t = 21.892, 4.673; P = 0.000, 0.000, respectively). There was no difference in sPD-1 amounts between the two groups before treatment (t = 0.441, P = 0.660). However, sPD-1 levels in patients with no HBV-DNA detection were significantly higher than those obtained for patients retaining positive HBV-DNA (t = 2.159, P = 0.032) (Table 4).

Table 1. Serological characteristics of HBeAg-positive chronic hepatitis B patients (before and after entecavir treatment) and healthy controls (mean ± SD).

Groups	ALT (U/L)	HBV-DNA (Ig IU/mL)	sPD-1 (ng/L)
Healthy controls	21.6 ± 4.6	Negative	51.18 ± 10.58
Baseline	268.4 ± 184.7	7.70 ± 0.62	32.01 ± 6.05
12 weeks	55.5 ± 32.8*	3.02 ± 0.65*	33.86 ± 7.12*
24 weeks	41.9 ± 22.8*	2.46 ± 0.55*	39.69 ± 7.60*
48 weeks	35.1 ± 15.7*	2.25 ± 0.41*	47.58 ± 8.44*

*Significant change compared with baseline, P < 0.05.

Table 2. Expression levels of sPD-1 in CHB patients with different HBeAg outcomes (mean ± SD).

Groups	Cases	sPD-1 before treatment (ng/L)	sPD-1 at 48 weeks (ng/L)	t	P
HBeAg seroconversion	54	32.07 ± 6.10	53.35 ± 6.97	16.883	0.000
HBeAg-negative	21	31.59 ± 6.53	48.98 ± 7.60	7.948	0.000
HBeAg-positive	145	32.04 ± 6.00	45.23 ± 8.01	266.784	0.000
F		0.055	22.034		
P		0.947	0.000		

Table 3. Expression levels of sPD-1 in CHB patients with different ALT outcomes (mean ± SD).

Groups	Cases	sPD-1 before treatment (ng/L)	sPD-1 at 48 weeks (ng/L)	t	P
ALT returned to normal	180	32.13 ± 6.113	48.34 ± 8.21	21.223	0.000
ALT not returned to normal	40	31.42 ± 5.72	44.15 ± 8.72	7.711	0.000
t		0.671	2.866		
P		0.503	0.004		

Table 4. Expression levels of sPD-1 in CHB patients with different HBV-DNA outcomes (mean ± SD).

Groups	Cases	sPD-1 before treatment (ng/L)	sPD-1 at 48 weeks (ng/L)	t	P
HBV-DNA-negative after treatment	204	32.06 ± 6.12	47.92 ± 8.35	21.892	0.000
HBV-DNA-positive after treatment	16	31.36 ± 5.23	43.27 ± 8.71	4.673	0.000
T		0.441	2.159		
P		0.660	0.032		

3.4. Correlation analysis of sPD-1, ALT, and HBV-DNA in patients with HBV

Before entecavir treatment in CHB patients, sPD-1 and the clinical biochemical indicators ALT and HBV-DNA were not related. sPD-1 showed negative correlations with ALT ($r = -0.366$, $P = 0.000$; Figure 1) and HBV-DNA ($r = -0.393$, $P = 0.000$; Figure 2) at 12, 24, and 48 weeks after entecavir treatment in CHB patients. Their regression equations are $Y = -0.134X + 46.288$ and $Y = -5.926X + 55.661$, respectively.

3.5. Adverse reactions

Creatine kinase amounts were increased slightly in 4 cases during entecavir treatment and relieved after rest, without discontinuation of entecavir treatment. Other individuals showed no overt adverse reactions.

4. Discussion

The pathogenesis of CHB is complex, and the host immune response cannot effectively eliminate the hepatitis B virus. Recent evidence has suggested that T cells play an important role in CHB patients, with decreased cellular immune function (12). There are various degrees of T-cell function impairment, even T-cell exhaustion, in chronic HBV infection (5). PD-1 is an inhibitory receptor on the surface of activated T cells (13). The combination of PD-1 with PD-L1 is an immune suppression signal. This inhibitory signal can negatively regulate activation, proliferation, and cytokine production in T cells when delivered to them. This signal transduction pathway is considered to be related to the chronic infection of CHB (14). Studies have shown that PD-1 and PD-L1 counts in peripheral blood mononuclear cells of CHB patients are increased (15,16).

An increasing number of studies demonstrate that many costimulatory molecules exist in both cell membrane and soluble forms, e.g., CD40, 4-1BBL, OX40L, CD86, CD80, and CTLA-4. sPD-1 is the soluble form of PD-1, with extracellular domains like IgV structures on the cell surface portion of PD-1 (8). Molecules on the cell membrane mediate costimulatory signals through direct receptor–ligand interactions; on the other hand, soluble protein factors play important regulatory roles in the immune response, like the function of cytokines. Not only do they affect near-end cells, but they also bind receptors on the far-end cell surface. These features make them participate in occurrence and development of diseases, with a far more important role than body membrane surface molecules.

Studies have demonstrated that sPD-1 has a role in blocking the PD-1/PD-L pathway in immune-related diseases. Recently, it was found that sPD-1 has a strong ability to bind PD-ligand, since it has a PD-1 extracellular domain. This can compete with PD-1 and restore the killing and secretion functions of T-cells (17,18). Wu et al. assessed the abnormal activation of T cells in patients with aplastic anemia and demonstrated that sPD-1 can block membrane PD-1 in T cells, which results in abnormal proliferation and activation of T cells (10). Wan et al. found abnormally high expression of sPD-1 in the peripheral blood and synovial fluid of rheumatoid arthritis patients, with sPD-1 expression level closely related to the body concentration of rheumatoid factor (19). Other

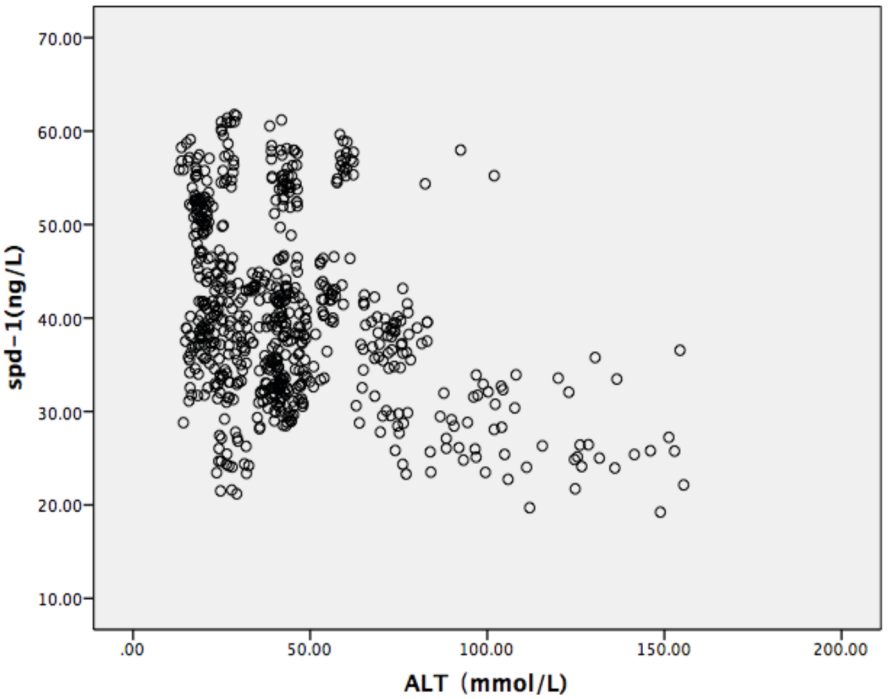

Figure 1. Correlation between sPD-1 and ALT at 12, 24, and 48 weeks after entecavir treatment in CHB patients.

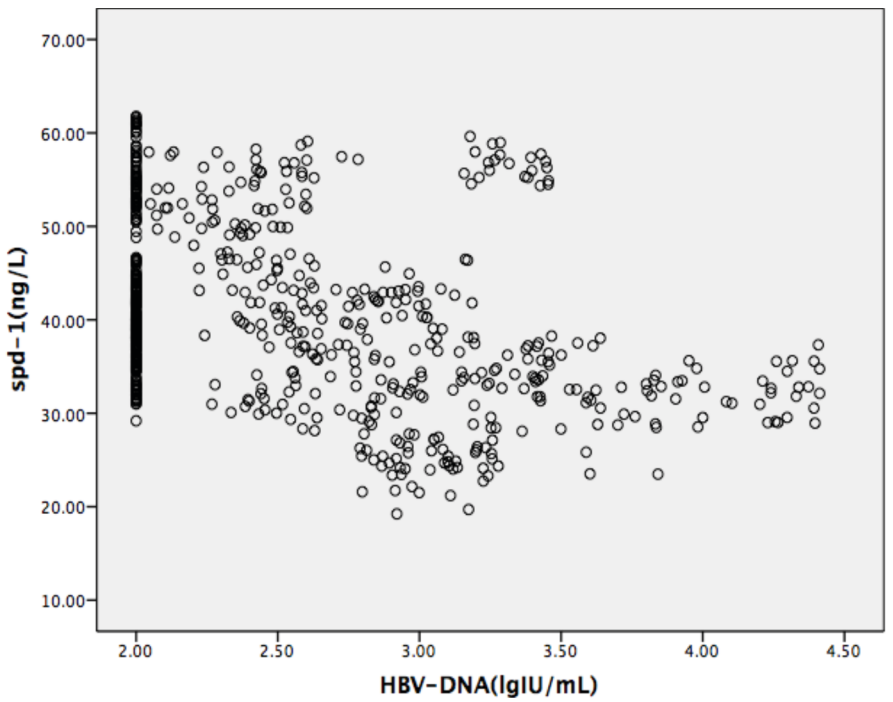

Figure 2. Correlation between sPD-1 and HBV-DNA at 12, 24, and 48 weeks after entecavir treatment in CHB patients.

studies also indicated that sPD-1 has a role in blocking the PD-1/PD-L pathway in hepatocellular carcinoma. He et al. deemed that sPD-1 could block the PD-1/PD-L1 (B7. H1) pathway to improve tumor escape in a mouse model of liver cancer (20). Wang et al. (21) showed that sPD-1 is associated with pathological injury in chronic HCV infection.

However, the biological role of sPD-1 in hepatitis B virus infection remains unknown. Immune factors also play an important role, and the PD-1/PD-L1 pathway increases in CHB. Since sPD-1 could block the PD-1/PD-L1 pathway, it might play an important role in the development of CHB. As shown above, serum sPD-1 levels in HBeAg-positive chronic hepatitis B patients were reduced, indicating that sPD-1 may play a certain role in the development and progression of chronic hepatitis B. In CHB patients, sPD-1 levels were decreased, thus increasing PD-1 activity and potentially decreasing T-cell function.

Entecavir is one of the most effective anti-HBV drugs used extensively worldwide. It inhibits viral replication by substituting a similar nucleoside, which is required in the HBV replication process, therefore terminating chain elongation. Meanwhile, studies have shown that entecavir plays a role in regulating the immune system. You et al. demonstrated the restoration of the T-lymphocyte subpopulation in CHB patients treated with entecavir (22). However, changes of serum sPD-1 levels in CHB before and after treatment with entecavir are poorly understood, as well as the relationships of sPD-1 with ALT, HBeAg, and HBV-DNA.

We evaluated HBeAg-positive CHB patients before and after treatment with entecavir for 48 weeks. As shown above, sPD-1 levels increased gradually with treatment time. This indicated that entecavir antiviral might increase sPD-1 expression and restore T-cell function. Meanwhile, the expression of sPD-1 remained lower than baseline at 48 weeks after treatment, which may be related to the incomplete recovery of the CHB patients at 48 weeks after entecavir treatment. Interestingly, sPD-1 levels in the HBeAg seroconversion group were significantly higher than those of the HBeAg-negative and HBeAg-positive groups at 48 weeks, suggesting that sPD-1 is basically associated with HBeAg prognosis. These findings indicated that sPD-1 could be used as a valid predictive factor for HBeAg in the late stage of seroconversion. The expression levels of sPD-1 in patients with ALT returned to normal were significantly higher than those in patients with ALT remaining abnormal; meanwhile, sPD-1 amounts in patients with no HBV-DNA were significantly elevated compared with values obtained for patients still HBV-DNA-positive. These findings indicate that sPD-1 has a close relationship with the curative effect of entecavir. In this study, sPD-1 was negatively correlated with ALT and HBV-DNA at 12, 24, and 48 weeks of entecavir treatment in CHB patients. This suggests that sPD-1 levels can be used for disease assessment; furthermore, a given drug regimen can be stopped quickly when a bad therapeutic effect appears, which would save money and time.

In summary, this study suggests that sPD-1, as a marker of T-cell function, may play a certain role in CHB and has a close relationship with the curative effect of entecavir treatment in CHB patients. These findings provide new insights for understanding the pathogenesis and treatment of HBV.

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