The Expression of Programmed Death-1 in Circulating CD4+ and CD8+ T Cells during Hepatitis B Virus Infection Progression and Its Correlation with Clinical Baseline Characteristics

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Background/Aims: Programmed death-1 (PD-1) expression was investigated in CD4+ and CD8+ T cells from hepatitis B virus (HBV)-infected patients at the chronic hepatitis B (CHB) infection, liver cirrhosis (LC), and hepatocellular carcinoma (HCC) stages. Methods: PD-1 expression in circulating CD4+ and CD8+ T cells was detected by flow cytometry. The correlations between PD-1 expression and HBV viral load, alanine aminotransaminase (ALT) levels and aspartate aminotransferase (AST) levels were analyzed using GraphPad Prism 5.0. Results: PD-1 expression in CD4+ and CD8+ T cells was significantly increased in both the CHB group and advanced-stage group (LC plus HCC). In the CHB group, PD-1 expression in both CD4+ and CD8+ T cells was positively correlated with the HBV viral load, ALT, and AST levels. However, in the LC plus HCC group, significant correlations between PD-1 expression and the clinical parameters were nearly absent. Conclusions: PD-1 expression in peripheral CD4+ and CD8+ T cells is dynamic, changes with HBV infection progression, and is related to HBV viral load and liver function, especially in CHB. PD-1 expression could be utilized as a potential clinical indicator to determine the extent of virus replication and liver injury. (Gut Liver 2014;8:186-195)

Key Words: Programmed death-1; Hepatitis B virus; Hepatitis B, chronic; Liver cirrhosis; Carcinoma, hepatocellular

INTRODUCTION

Mounting data indicated that persistent antigen stimulation during chronic viral infections and tumor development led to T cell exhaustion, a state of dysfunction in which T cells get a progressive loss of effector function. It was suggested that reversing T cell exhaustion could restore effective immune response, and subsequently control tumor progress and virus amplification. Exhausted T cells are characterized by expressing multiple inhibitory receptors which dedicated complex layers of negative regulation. Programmed death-1 (PD-1), a lymphocyte antigen 4 (CTLA-4), and B and T lymphocyte attenuator (BTLA), currently constituting the immunoglobulin superfamily of coinhibitory molecules, play a prominent role in effectively and moderately regulating immune response in collaboration with costimulatory molecules as well as T cell receptor-antigen signals. However, overexpression of these co-inhibitory receptors may drive excessive inhibitory signals and contribute to T cell exhaustion. It has demonstrated that these coinhibitory receptors were up-regulated in patients along with chronic viral infections and associated with clinical characteristics. Some reports further revealed that blocking these coinhibitory molecules could temper the inhibitory signals, partially restore T cell function, and correspondingly release virus load and other clinical indicators. Thereby, blockade of inhibitory molecules could be adopted as a potential therapeutic approach in chronic viral infections.

PD-1 (CD279), of which the intracellular domain contains two tyrosine-based signaling motifs, immunoreceptor tyrosine-based inhibitory motif and immunoreceptor tyrosine-based switch motif, can recruit src homology domain 2-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2 and subsequently deliver negative signals to modulate activation and proliferation of T cells and production of cytokines dependent on interacting with its ligand PD-L (PD-L1 or PD-L2). It was identified that PD-1 overexpression not only accounted for exhaustion of T cells of patients infected with hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV), but...
but also played an important role in inhibiting the function of antigen-specific CD8+ T cells in tumor pathogenesis. Although a few of reports respectively described that expression levels of PD-1 were elevated on CD4+ or CD8+ T cells of CHB patients or CD8+ T cells of hepatocellular carcinomas (HCC) patients and to some extent related with HBV amplification or liver function, a systematic investigation of PD-1 expression pattern on T cells remains to be further elucidated during HBV infection progress from CHB to liver cirrhosis (LC), and then to HCC. Moreover, to comprehend such variation of PD-1 expression on T cells in the course of HBV infection is to benefit for not only developing valuable clinical indicator, but also estimating the appropriate phases for PD-1 blockade in the prospective therapy. For these reasons, we examined the PD-1 expression on peripheral CD4+ and CD8+ T cells from patients at the stage of CHB, LC, and HCC and analyzed its association with clinical baseline characteristics. Our results suggested that PD-1 expression on T cells presented dynamic change along with HBV infection progressing and its variation was correlated with HBV virus load as well as liver function.

MATERIALS AND METHODS

1. Study participants

The recruited samples include healthy donors (n=20) and patients with CHB (n=34), LC (n=18), and HCC (n=16). All patients were non-infected with HCV, hepatitis D virus (HDV), HIV, acute hepatitis B infection and excluded from other causes of liver disease. Healthy donors were also negative for HBV, HCV, HDV, and HIV-1 infection and other diseases. CHB was defined by persistent or elevation of alanine aminotransaminase (ALT) levels for 6 months. The diagnosis of LC was dependent on computed tomography (CT) acquiring images and laboratory findings. HCC was diagnosed by histology, α-fetoprotein levels, and CT imaging findings. The study was approved by the ethical committee of the Affiliated Infectious Hospital of Soochow University and informed consent forms were signed by all participants.

2. Serological assays for HBV markers, viral load, and basic clinical features

HBV serological markers, including hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B e antigen (HBeAg), and antihepatitis B e antibody, were detected using the assay kits (Abbott Ireland, Diagnostics Division, Sligo, Ireland). The HBV viral load was determined with HBV LC PCR Kit (Shanghai Shenyou Technology Co., Ltd., Shanghai, China). The routine biochemical assays, including liver function, albumin (ALB), total bilirubin, and so on were also detected.

3. Flow cytometric analysis

Monoclonal antibodies labeled with fluorochrome, CD3-ACP, CD4-PerCP, CD8-PerCP, and PD-1-PE were purchased from BD Pharmingen (San Diego, CA, USA). For each test, 50 μL fresh heparinized whole blood of patients or healthy donors was incubated with indicated antibodies (10 μL) for 15 minutes, then lysed with FACSTM lysing solution (BD Biosciences, San Jose, CA, USA), subsequently washed with phosphate buffered saline, fixed and eventually detected by BD FACSAria with DB FACS-Diva software supporting. Data were analyzed using FlowJo (Tree Star Inc., San Carlos, CA, USA).

4. Statistical analysis

The data were analyzed with GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA), and summarized and presented as mean±standard error of the mean (SEM). For comparisons Table 1. Clinical Characteristics of the Study Groups

| Characteristic | HC group | CHB group | LC group | HCC group |
|---------------|----------|-----------|----------|-----------|
| Cases         | 20       | 34        | 18       | 16        |
| Child-Pugh, A/B/C | IA       | IA        | 9/3/6    | 10/6/0    |
| BCLC, A/B/C   | IA       | IA        | IA       | IA        |
| Age, yr       | 42.2±5.8 | 38.5±3.65 | 51.6±3.78| 53.2±3.8  |
| Male/Female   | 14/6     | 25/9      | 12/5     | 14/2      |
| HBeAg, +/-    | IA       | IA        | IA       | IA        |
| HBV-DNA, log10 copies/mL | 6.6±0.22 | 5.9±0.34  | 5.4±0.28 | 5.4±0.28  |
| ALT, IU/L     | 21.7±6.5 | 155.6±39.7| 225.8±41.2|
| AST, IU/L     | 176.9±35.2| 121.2±9.87| 288.5±42.2|
| ALB, g/L      | 42.1±7.92| 32.9±1.60 | 32.9±1.60| 32.9±1.60 |
| TBIL, μmol/L  | 37.4±7.39| 39.3±2.12 | 57.6±18.12|

Data are presented as number or mean±SEM. The significance of differences (p-value) in the indicated group is shown. HC, healthy control; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; NA, not applicable; BCLC, Barcelona Clinic Liver Cancer; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransaminase; AST, aspartate aminotransferase; ALB, albumin; TBIL, total bilirubin.
of the collected data, nonparametric test (Mann-Whitney U test) and the chi-square test were performed and two-tailed p<0.05 was considered statistically significant. Nonparametric correlation test (Spearman) was applied for analyzing the correlation between PD-1 expression and virus load, ALT, or aspartate aminotransferase (AST) levels. The bar in every group in figures represents the mean±SEM.

Fig. 1. Gating strategy and expression profiles of programmed death-1 (PD-1) in peripheral CD4+ T cells of chronic hepatitis B (CHB) virus, liver cirrhosis (LC), and hepatocellular carcinoma (HCC) patients. (A) The gating strategies and representative results of PD-1 expression in CD4+ T cells are shown. (B) Statistical results for PD-1 expression in circulating CD4+ T cells from healthy control (HC), CHB, and LC plus HCC are shown. (C, D, E) The percentages of CD4+ PD-1+ T cells in CHB and LC plus HCC patients are organized according to hepatitis B virus (HBV) DNA load and alanine aminotransaminase (ALT) and aspartate aminotransferase (AST) levels, respectively, as indicated in the figures. SSC, side scatter; FSC, forward scatter; LC, liver cirrhosis.
RESULTS

1. Clinical baseline characteristics of study populations

The recruited participants in this study were classified into three groups: healthy control (HC; n=20), CHB (n=34), and LC plus HCC (n=34). Table 1 demonstrates age, gender, HBeAg serostatus, serum HBV DNA concentrations, liver function, ALB, and total bilirubin. On the basis of Child-Pugh classification, eighty LC patients were made up of nine cases at class A, three cases at class B, and six cases at class C. According to the Barcelona Clinic Liver Cancer staging classification, six patients at stage A, three patients at stage B, and seven patients at stage C consisted of HCC group.

2. Investigation of PD-1 expression on peripheral CD4⁺ and CD8⁺ T cells from HBV-infected patients

In order to elucidate PD-1 expression pattern on both CD4⁺ and CD8⁺ T cells with the disease progressing, CHB, LC, and HCC patients who represented different stages of HBV infection were enrolled and PD-1 expression was investigated by using flow cytometric analysis.

Gating strategies for CD4⁺ T cell subsets were shown in Fig. 1A. When the study patients were classified into two groups, chronic infection (CHB) and serious progress of disease (LC plus HCC), the results illustrated that compared with HC, the percentage of circulating CD4⁺ PD-1⁺ T cells was significantly increased in both of CHB and LC plus HCC, while there was no significant difference between the two patient groups (Fig. 1B). Surprisingly, it was further revealed that when patients were divided into subgroups according to the levels of clinical parameters such as serum HBV DNA level, ALT and AST, PD-1 expression on CD4⁺ T cells varied among these subgroups. The percentages of CD4⁺ PD-1⁺ T subsets in CHB patients with low serum HBV load, low ALT level or low AST level were correspondingly lower than those CHB with high serum HBV load, high ALT level or high AST level respectively (Fig. 1C-E). However, there were no significant differences of CD4⁺ PD-1⁺ T subsets between LC plus HCC groups with low serum HBV load, low ALT level or low AST level, and LC plus HCC groups with high serum HBV load, high ALT level or high AST level respectively (Fig. 1C-E). When compared with CHB and LC plus HCC, the significant differences of CD4⁺ PD-1⁺ T subsets were observed in subgroups with low serum HBV DNA, low ALT or low AST respectively.

The similar results were showed in CD8⁺ PD-1⁺ T subsets. The similar gating strategies were also adopted for detecting CD8⁺ T cell subsets (Fig. 2A). Compared with HC, the percentage of circulating CD8⁺ PD-1⁺ T cells was also significantly increased in both CHB group and LC plus HCC group, while no significant difference was found between the two patient groups (Fig. 2B). The percentage of CD8⁺ PD-1⁺ T subsets in CHB patients with low serum HBV load, low ALT level or low AST level was accordingly lower than those CHB with high serum HBV load, high ALT level or high AST level respectively (Fig. 2C-E). However, there were no significant differences of CD8⁺ PD-1⁺ T subsets between LC plus HCC subgroups with low serum HBV load, low ALT level or low AST level, and LC plus HCC subgroups with high serum HBV load, high ALT level or high AST level respectively (Fig. 2C-E). When compared with CHB and LC plus HCC, the significant difference of CD8⁺ PD-1⁺ T subsets was observed in subgroup with low ALT only.

3. Analysis of correlations between PD-1 expression on circulating CD4⁺ and CD8⁺ T cells from HBV-infected patients and clinic indicators

Correlations between PD-1 expression on CD4⁺ and CD8⁺ T cells from CHB, LC plus HCC subjects and serum HBV viral load,
ALT, and AST levels (Fig. 3). However, in LC plus HCC group, except that PD-1 expression on CD4⁺ T cells was surprisingly found to be negatively correlated with AST, no other correla-

ALT or AST level were further analyzed respectively. The results showed that PD-1 expression on CD4⁺ and CD8⁺ T cells of CHB patients was all positively correlated with serum HBV viral load, ALT, and AST levels (Fig. 3). However, in LC plus HCC group, except that PD-1 expression on CD4⁺ T cells was surprisingly found to be negatively correlated with AST, no other correla-
tions were discovered between PD-1 expression and those clinical parameters (Fig. 4).

**DISCUSSION**

As an important axis of coinhibitory receptor/ligand, PD-1/PD-L, together with CTLA-4/B7 and BTLA/HVEM,41 exerts considerable effect on keeping T cell from excessive activation and regulating T cell tolerance. In the course of normal immune response, PD-1 is up-regulated following T cell activation, then interacts with PD-L1 and PD-L2 to deliver negative signals, and eventually leads to the apoptosis of activated T lymphocytes.34 In our study as well as other reports,25,42-44 it was demonstrated that the higher percentage of CD4⁺ PD-1⁺ and CD8⁺ PD-1⁺ T persistently existed in the patients with chronic infections, suggesting that PD-1 overexpression contributes to T cell exhaustion by providing negative signals.

In the present study, it was further clarified that PD-1 expression on T cells showed dynamic change in different stages of HBV infection. Although it was up-regulated at the stages of both chronic infection and deterioration with increasing tendency compared with HC, PD-1 expression on CD4⁺ and CD8⁺ T cells did not present continuous up-regulation significantly along with disease progression, suggesting that not only PD-1 overexpression possibly exerts its role in the whole course of disease development but also its up-regulation pattern is influenced to some extent by the disease progress.

PD-1 expressions on both CD4⁺ and CD8⁺ T cells of patients with low serum HBV DNA load, low ALT, or low AST level were correspondingly lower than those of patients with high levels of these clinical parameters in CHB stage, but not in LC plus HCC stage. Further analysis demonstrated that PD-1 expression presented positively correlations with serum HBV DNA load, AST and ALT levels in CHB stage but not in LC plus HCC stage. These data indicate that during HBV chronic infection, the increasing percentages of CD4⁺ PD-1⁺ and CD8⁺ PD-1⁺ T depend on the stimulation of virus antigens and associated with the extent of liver cell injury in CHB stage. The results accorded with some previous reports in which the dynamic variations of PD-1 expression on CD4⁺ or CD8⁺ T cells were separately revealed in CHB patients.24 In advanced stage of HBV infection such as LC or HCC, these findings were disappeared. It suggests that high viral antigenic stimulation or severe hepatocyte injury are not significant factors any more for PD-1 expression on CD4⁺ or CD8⁺ T cells in advanced fibrosis stage. When chronic infection develops into serious deterioration, characterized by LC and HCC, the percentages of CD4⁺ PD-1⁺ and CD8⁺ PD-1⁺ T were increased slightly without significance. Also, at this stage, both of the frequencies of CD4⁺ PD-1⁺ and CD8⁺ PD-1⁺ T cells had no significant differences between sub-groups with different clinical parameters and were not found to correlate with serum HBV DNA load, which accorded with the results reported by Zeng et al.45

Interestingly, according to subgroup analysis, significant differences of PD-1 expression on CD4⁺ T cells could be found between CHB and LC plus HCC with low HBV DNA load, low ALT, or low AST respectively, but not between those subgroups with high levels of clinical parameters. These findings suggest that high serum HBV DNA, high ALT, and high AST conditions delimit the difference of PD-1 expression on CD4⁺ T cells between CHB and LC plus HCC by increasing of PD-1 expression on CD4⁺ T cells in CHB.

In summary, our study further systematically and fully revealed PD-1 expression pattern on CD4⁺ and CD8⁺ T cells and...
its association with clinical baseline characteristics in the whole course of HBV infection including CHB, LC, and HCC though it enrolled a small population and can only partially reflect PD-1 expression on T cells in HBV infection progressing and its correlation with the clinical parameters such as serum HBV DNA, ALT, and AST. The exposure of dynamic change of PD-1 expression and its correlations with clinical parameters not only makes PD-1 itself another valuable clinical parameter, but also provides an important theoretic base for PD-1/PD-L1 blockade in chronic HBV infection.

Fig. 3. Analysis of the correlation between programmed death-1 (PD-1) expression in CD4^+ and CD8^+ T cells of chronic hepatitis B virus patients and hepatitis B virus (HBV) DNA load or alanine aminotransaminase (ALT) or aspartate aminotransferase (AST) levels. (A, B, C) Correlations between PD-1 expression by CD4^+ T cells and HBV DNA load or ALT or AST levels. (D, E, F) Correlations between PD-1 expression in CD8^+ T cells and HBV DNA load and ALT or AST levels.
Fig. 4. Analysis of the correlation between the expression of programmed death-1 (PD-1) in CD4^+ and CD8^+ T cells of liver cirrhosis and hepatocellular carcinoma patients and hepatitis B virus (HBV) DNA load or alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels. (A, B, C) Correlations between PD-1 expression in CD4^+ T cells and HBV DNA load or ALT or AST levels. (D, E, F) Correlations between PD-1 expression in CD8^+ T cells and HBV DNA load or ALT or AST levels.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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