Telbivudine decreases proportion of peripheral blood CD4+CD25+CD127low T cells in parallel with inhibiting hepatitis B virus DNA

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Abstract. Regulatory T cells (Treg) have significant roles in the immunopathology of patients with chronic hepatitis B (CHB) and exhibit an evident correlation with antiviral immunity when antiviral therapy is applied. In order to investigate how circulating Tregs are affected by telbivudine treatment and its significance in patients with CHB, peripheral blood mononuclear cells (PBMCs) were isolated and the proportions of circulating cluster of differentiation (CD)4+CD25+CD127low and CD8+CD25+ T cells of CHB patients prior to and during the three or six months of treatment were assessed and detected by flow cytometric analysis. The levels of forkhead/winged helix transcription factor (Foxp3) mRNA were also quantified using quantitative polymerase chain reaction. A significantly higher percentage of CD4+CD25+CD127low and CD8+CD25+ T cells in the PBMCs of patients with CHB were identified compared with that of healthy individuals. Following six months of telbivudine treatment, the proportion of circulating CD4+CD25+CD127low and CD8+CD25+ T cells and the relative levels of Foxp3 mRNA in patients with CHB was comparable to the proportion in healthy individuals. The proportions of circulating peripheral blood CD4+CD25+CD127low T cells were paralleled with its HBV DNA inhibition. The results of the present study indicate that telbivudine treatment reduces HBV DNA levels rapidly and indirectly affects the immune system by downregulating the proportion of circulating Treg markedly, which may be beneficial to restore the antiviral immune response.

Introduction

Hepatitis B virus (HBV) infection is a serious public health problem affecting more than 400 million individuals worldwide (1). During HBV infection, the host immune responses, particularly the cellular immune response, mediate clearance of HBV infection and the pathogenesis of liver injury is dependent on the balance between HBV replication and the viral-specific cytotoxic T-lymphocyte (CTL) response (2). In patients with an acute self-limiting HBV infection, the CD4+ and CD8+ T-cell response with T helper 1 type cytokine profile is crucial for the control of the infection (3,4). However, patients with chronic hepatitis B (CHB) often exhibit impairment of HBV-specific T-cell activity, which is characterized by a weak immune response to HBV and lack of the vigorous specific CD4+ and CD8+ T-cell response (4,5).

The precise mechanisms responsible for this impaired T-cell response to the chronicity of HBV infection are not fully understood. One scenario is the potential role of host-mediated immunosuppressive mechanisms that may be activated following persistent antigenic exposure (6). Previously, regulatory T cells (Tregs) were focused to have an indispensable role in maintaining immunological unresponsiveness to antigens (7,8), as they have a prominent role in immunoregulation and tolerance. During the acute-resolved HBV infection, circulating Treg frequency is low in the acute phase, but is significantly increased during the convalescent phase and then returns to a normal level along with disease resolution (9). In patients with CHB, the increased Treg frequencies in the peripheral blood and liver may significantly suppress HBV-specific CTL responses; however, they are found to be decreased upon hepatitis B e antigen (HBeAg) seroconversion (10,11). Furthermore, the decreased frequency of hepatitis B e antigen (HBeAg)-specific Tregs accompanied by increased HBcAg peptide-specific CTLs partially account for the acute exacerbation in patients with CHB (12).

It has been suggested that Treg can be induced through a repetitive stimulation of T cells by high concentrations of antigen for longer periods of time (13). The high viral load present in peripheral blood of HBV patients may possibly provide such a stimulus. For CHB, two major types of
antiviral drugs are widely used: Nucleotide analogs (including lamivudine, defovir, entecavir, tenofovir and telbivudine) and interferon (conventional and pegylated interferon alfa) (14,15). During the course of pegylated interferon treatment, the decline of circulating Tregs together with a partial recovery of the immune responses was able to predict favorable responses (16,17). The frequency of HBV-specific CTL in the peripheral blood of responders was significantly higher compared with that of non-responders following lamivudine treatment (18-20). Previous studies have also reported that adefovir-induced viral load reduction results in a decline of circulating Treg together with a partial recovery of the immune response, as indicated by a decrease in percentages of Treg and an increased HBV-specific proliferation (21). Concomitantly with a quantitative reduction in viral replication, the frequency of CD4+ T cells and the CD4+CD8+ ratio increased during effective telbivudine therapy (22). These findings indicated that effective antiviral treatment can sustain the inhibition of viral replication and antigen production, which may potentially result in a decrease of Treg induction, leading to restoration of the immune response. However, this hypothesis was challenged by a recent report in which a greater increase in HBcAg-specific Tregs was correlated with a higher rate of sustained responsiveness to antiviral therapy in patients with CHB (23).

Telbivudine is a novel orally bioavailable antiviral drug with high potency and selectivity against HBV (24). Multinational studies have demonstrated the superiority of telbivudine over lamivudine for the treatment of CHB, particularly in terms of viral load reduction, serum alanine aminotransferase (ALT) normalization, HBeAg loss and reduced viral resistance (25-28). Certain studies indicated that the rapid inhibition of the HBV load resulting from continued telbivudine treatment may cause a restoration of the impaired T cell response in patients with CHB (22).

In the present study, it was hypothesized that patients with CHB have a higher proportion of Treg compared with healthy controls and that the inhibition of viral replication by continued telbivudine treatment can reduce proportions of Treg in the peripheral blood and enhance the antiviral immune response.

To evaluate the effects of telbivudine and its correlation with the HBV DNA load in patients with CHB, the proportions of peripheral blood CD4+CD25+CD127low and CD8+CD25+ T cells, and the associated mRNA levels of Forkhead/winged helix transcription factor (FoxP3, it is a transcription factor specifically expressed by CD4+CD25+ Treg cells), were examined and detected in 22 patients with CHB undergoing telbivudine treatment.

Patients and methods

Study participants. A total of 22 unrelated patients (16 males and 6 females; age, 36±6.5 years) who were diagnosed with chronic hepatitis B and received antiviral treatment with telbivudine (600 mg orally per day) between October 2009 and December 2010 at Southwest Hospital (Chongqing, China) were recruited for the present study. Nineteen age- and gender-matched healthy blood donors were selected as the control group. The diagnostic criteria of CHB were based on the related literature recommended by the Chinese Medical Association (29). All the patients were selected according to the following criteria: (i) All hepatitis B surface antigen (HBsAg) carriers were positive for both HBsAg and antibody to HBV core antigen of the immunoglobulin G (IgG) type for at least 12 months; (ii) HBeAg-positive; (iii) age between 18 and 60 years; (iv) HBV DNA≥6log10 copies/ml; (v) ALT levels ≥2 fold the upper limit of that of normal patients and (vi) no antiviral, immune suppressive or immunomodulatory treatment during the last six months. Patients who were pregnant and co-infected with the human immunodeficiency virus, hepatitis A, C or D and patients with other types of hepatitis were excluded from the present study.

For the patients with CHB, clinical, biochemical, virological and immunological parameters were assessed in the study at three fixed time-points (baseline, three and six months). The clinical characteristics at baseline of the 22 patients are shown in Table I. Peripheral blood mononuclear cells (PBMCs) were obtained at baseline and subsequent to three and six months of telbivudine treatment. All the subjects provided informed consent to participate in the study (Trial Registration:

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Chronic hepatitis B patients, n=22</th>
<th>Healthy blood donors, n=19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male/female</td>
<td>16/6</td>
<td>14/5</td>
</tr>
<tr>
<td>Age, years</td>
<td>36±6.5</td>
<td>38.6±12.1</td>
</tr>
<tr>
<td>HBsAg</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Anti-HBs IgG</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>HBeAg positive</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Serum HBV DNA load</td>
<td>&gt;6 log10 copies/ml</td>
<td>Not detectable</td>
</tr>
<tr>
<td>TBil, µmol/l</td>
<td>29.6 (17.4-52.8)</td>
<td>8.4 (3.9-16.5)</td>
</tr>
<tr>
<td>ALT IU/l</td>
<td>143.2 (95.1-224.3)</td>
<td>17.5 (11.0-27.3)</td>
</tr>
<tr>
<td>AST IU/l</td>
<td>132.1 (103.2-198.5)</td>
<td>14.2 (9.5-23.2)</td>
</tr>
</tbody>
</table>

Data are expressed as the median (range) or n (%), unless otherwise indicated. TBil, total bilirubin (normal total bilirubin range, 1.0-21.0 µmol/l); ALT, serum alanine aminotransferase (normal ALT range, 3-50 U/l); AST, aspartate aminotransferase (normal AST range =3-40 U/l); HBV, hepatitis B virus; IgG, immunoglobulin G; HBs, hepatitis B surface; HBe, hepatitis B e; Ag, antigen.
Biochemical and virological assessment. Routine liver function tests included ALT, aspartate aminotransferase and total bilirubin. These assays were performed with routine automated techniques (upper limit of normal: 50 U/l, 40 U/l and 21.0 µmol/l, respectively; HITACHI L-7600; Hitachi Medical Corp., Hitachi, Japan). HBV markers (HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc), anti-HAV, anti-hepatitis C virus (HCV), anti-hepatitis D virus (HDV) and anti-human immunodeficiency virus (HIV) were determined by commercial enzyme immunoassay kits using the Abbott IMX system (Abbott Laboratories, North Chicago, IL, USA). Serum HBV DNA levels were quantified using the Roche Amplicor Monitor assay (the limit threshold of the assay was 400 copies/ml) according to the manufacturer's instructions (Roche, Branchburg, NJ, USA).

Isolation of the PBMCs and flow cytometric analysis. PBMCs from patients with CHB and healthy donors were isolated from heparinized whole blood by density gradient centrifugation using Ficoll-Histopaque (Sigma, St. Louis, MO, USA). The cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium) and were frozen in RPMI 1640 containing 20% fetal calf serum (Hyclone, Logan, UT, USA) and 10% dimethyl sulfoxide. PBMCs from different time-points were stored at -135˚C for further analysis.

When the flow cytometric analysis was performed, the frozen PBMCs were washed once in phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin and stained with fluorescently-labeled antibodies for the surface markers CD4-fluorescein isothiocyanate, CD25-phycoerythrin (PE), CD127-Alexa Fluor 647 and CD8- APC-Alexa Flour 750 (BD Biosciences PharMingen, San Diego, CA, USA) for 20 min at 4˚C. The cells were then washed twice with PBS containing 1% fetal calf serum, immediately analyzed using a FACScan flow cytometer (Becton-Dickinson, Franklin Lanes, NJ, USA) and analyzed using Cell Quest software (FACScalibur®, CELLQuest Pro® software, Beckton-Dickinson).

Forkhead/winged helix transcription factor (FoxP3) mRNA quantification. RNA was isolated from the PBMC samples of all patients and controls using TRIzol® solution (Roche). The FoxP3 mRNA levels were quantified using a BioRad IQTM5 multicolor quantitative polymerase chain reaction (qPCR) detection system (BioRad, Hercules, CA, USA) with SYBR® Green I probes (Toyobo, Osaka, Japan), using β-actin as an internal control. Primers used for qPCR of FoxP3 and β-actin mRNAs were as follows: FoxP3 forward, 5'-AAGGAAAGGAGGATGGACG-3' and reverse, 3' -CAGGCAAGACAGTGGAAACC-5'; β-actin forward, 5'-CGTGGACATCCGCAAAGAC-3' and reverse, 3'-CTCGCTCCAACCGACTGCT-5'. Data from qPCR using SYBR Green I probes were analyzed by the standard curve method as described previously (30).

Statistical analysis. Statistical analysis was performed using SPSS software (version 9.0; SPSS Inc., Chicago, IL, USA). Continuous data are presented as the mean ± standard deviation, unless specified otherwise, and the significance was analyzed with the t-test. Flow cytometry and FoxP3 data were analyzed using the Mann-Whitney U test. All flow cytometry and functional data were compared to the levels at baseline using a Wilcoxon matched pairs signed rank sum test. P>0.05 was considered to indicate a statistically significant difference.

Results

The proportion of circulating Treg is increased in CHB patients and can be decreased during telbivudine treatment. To demonstrate the importance of Treg in chronic HBV infection, the proportion of Treg present in the peripheral blood of patients with CHB (n=22) and healthy individuals (n=19) was first detected and compared. Figure 1 shows the typical dot plots of the proportion of peripheral blood CD4+CD25+CD127low T cells among CD4+ T cells for PBMCs from a representative patient with CHB (Fig. 1A) and a healthy individual (Fig. 1B). Typical dot plots of the proportion of the peripheral blood CD8+CD25+ T cells among CD8+ T cells from a representative patient with CHB (Fig. 2A) and a healthy individual (Fig. 2B) are shown in Figure 2. As Figure 3 shows, patients with CHB demonstrated a significantly higher percentage...
of CD4^+CD25^+CD127^{low} T cells within their population of CD4^+ T cells (7.55±0.61%) compared with healthy controls in the peripheral blood (5.6±0.62%) (P=0.002). Patients with CHB also showed a significantly higher percentage of CD8^+CD25^+ T cells within their population of CD8^+ T cells (0.75±0.27%) compared with healthy controls (0.39±0.09%) (P<0.0001).

To assess the effect of treatment with telbivudine on the immune response, the proportion of Treg from patients' PBMCs were then compared at different time-points prior to and during antiviral treatment. Subsequent to three and six months of therapy, a significant decrease in the percentage of CD4^+CD25^+CD127^{low} Treg (baseline, 7.55±1.61% vs. three months, 6.31±1.50% vs. six months, 5.86±1.44%, P=0.00114) was observed. Similarly, after three and six months of therapy, a significant decrease in the percentage of CD8^+CD25^+ Treg (baseline, 0.75±0.27% vs. three months, 0.49±0.25% vs. six months, 0.50±0.23%; P=0.01; Fig. 3) were also identified. Subsequent to six months of telbivudine treatment, the proportion of peripheral blood CD4^+CD25^+CD127^{low} and CD8^+CD25^+ T cells in patients with CHB was comparable to the proportion of the healthy controls (P>0.05).

FoxP3 RNA expression is increased in patients CHB and can be decreased during telbivudine treatment. Since FoxP3 is considered to be the most specific marker for Treg, the relative FoxP3 mRNA levels of the peripheral blood samples from all the patients with CHB (one patient with three samples: At baseline, subsequent to three and six months of telbivudine treatment) and healthy controls were determined by qPCR. The FoxP3 mRNA levels in PBMCs in patients with chronic
HBV infection were significantly higher compared with those in the healthy controls (0.72±0.12 vs. 0.51±0.15; P=0.0012). Subsequent to three and six months of telbivudine treatment, the relative levels of Foxp3 mRNA in PBMCs from patients with CHB were significantly decreased from 0.72±0.12 at baseline to 0.62±0.13 (P=0.0212) and 0.61±0.12 after three and six months treatment, respectively (P=0.0231) (Fig. 4). Following telbivudine treatment, the relative levels of Foxp3 mRNA in PBMCs in patients with CHB were comparable to the levels in the healthy controls (P>0.05).

**Correlation between Treg and clinical parameters.** All 22 chronic patients with HBV in the present study were HBeAg-positive with ≥6log_{10} copies/ml HBV DNA and ≥2 times the upper limit of the normal serum ALT levels prior to telbivudine treatment. Subsequent to three months of telbivudine treatment, the HBV DNA levels of all 22 patients with CHB had dropped to 10^{2} copies/ml or less, and those of 15 patients (15/22, 68.2%) dropped to 10^{3} copies/ml or less; the serum ALT levels of 17 patients (17/22, 77.3%) dropped to 40 U/l or less, and two (9.1%) patients identified as HBeAg-negative. Subsequent to six months of telbivudine treatment, the ALT levels of all 22 patients with CHB had dropped to normal levels (40 U/l or less); the HBV DNA levels of all 19 patients with CHB (19/22, 86.4%) had dropped to 10^{3} copies/ml or less, and four patients (18.2%) identified as HBeAg-negative. During treatment, no HBsAg loss was observed in the present study. Following telbivudine treatment, no correlation was observed between hepatic inflammation and the percentage of peripheral blood Treg, since the ALT levels of patients whose proportions of Treg were reduced to normal levels showed significant differences. Subsequent to six months of telbivudine treatment, the percentages of Treg of four HBeAg-negative patients were also significantly decreased in the present study.

**Discussion**

Consistent with the results of other previous studies (24-28), the results of the present study demonstrated telbivudine's marked potency to reduce HBV DNA levels. In the present study, continued telbivudine treatment was identified to be capable of rapidly reducing HBV DNA levels together with continuing improvement of biochemical and virological parameters through six months of treatment in patients with CHB. Furthermore, the present study demonstrated the importance of Tregs in CHB. The frequency and functional properties of Tregs are significant due to increased numbers of Tregs, which may favor the development of chronic viral infections and influence the course of the disease and effectiveness of antiviral treatment. However, it had remained controversial whether circulating CD4^{+}CD25^{+}CD127^{low} T cells and HBV DNA levels. HBV, hepatitis B virus; CD, cluster of differentiation.
Treg cells (33-35). A previous study showed that FoxP3 mRNA expression is relatively unique to the CD4⁺CD25⁺ T cell population of PBMCs and measuring FoxP3 mRNA expression in CD4⁺ cell populations or even total PBMCs is more practical compared with isolating the CD4⁺CD25⁺ cell population to evaluate CD4⁺CD25⁺ Treg activity and predict a clinical outcome. The higher relative levels of FoxP3 mRNA in patients with CHB in the present study indicate that patients with CHB have a higher percentage of Treg in the peripheral blood compared with healthy controls. This is in agreement with a previous study, which also identified a higher percentage of CD4⁺CD25⁺FoxP3⁺ Treg in patients with CHB compared with healthy controls (36). However, an early study did not find the diversity of the Treg distribution among the asymptomatic carriers, patients with CHB and healthy individuals (11). The possible reason for this difference between different studies may be the different detection methods of Treg used in these studies, among various other specific circumstances. Certain studies have found that the virus-specific induction of Treg may result in two different consequences: It may have key roles in the process to prevent excessive immunopathological injury and it may also cause the establishment of persistent viral infection (37-41).

Furthermore, antiviral treatment with telbivudine was observed to be capable of downregulating the proportion of Treg in PBMCs and enhancing the antiviral immune response of patients with CHB. An abundance of experimental data has confirmed that CD4⁺CD25⁺ Tregs can suppress effective antiviral immune responses, in particular, in the chronic infections caused by the HIV (42) and HCV (43). A number of studies have shown that during the course of nucleotide analogs and interferon treatment, the decline of circulating Tregs together with a partial recovery of the immune responses can predict favorable responses (16,20-22). However, this standpoint was opaqued by a recent study in which greater increases of Tregs were correlated with a higher rate of sustained responsiveness to antiviral therapy (23). In the present study, the proportion of peripheral blood CD4⁺CD25⁺CD127low and CD8⁺CD25⁺ T cells in patients with CHB was observed to be decreased over three or six months of telbivudine treatment to a level comparable to that of the healthy controls. Concomitantly, the levels of Foxp3 mRNA in PBMCs from patients with CHB also decreased over three or six months of telbivudine treatment to a level comparable to that prior to treatment. The results indicate that the inhibition of viral replication reduced regulatory T cells and enhanced the antiviral immune response in chronic hepatitis B. It is hypothesized that patients effectively treated with telbivudine not only showed an enhanced reconstitution of the CD4 response, but also exhibited a significant enhancement in stimulation of HBV-specific CTL activity and reduced HBV serum titers, efficiently resulting in a significant increase in the frequency of CTL and a greater magnitude of cytokine production, which was partly proved previously (21,22,44).

Last and most importantly, the results of the present study indicated that the proportions of peripheral blood CD4⁺CD25⁺CD127low T cells were paralleled with its HBV DNA inhibition upon telbivudine treatment. The present study revealed a positive correlation between the CD4⁺CD25⁺CD127low Treg frequency and the serum HBV DNA levels, indicating that the upregulation of Tregs may be associated with an increase in HBV replication. This result is identical to those of previous studies (36,45). However, two earlier studies did not find any significant association between circulating Treg frequency and HBV DNA titer in patients with CHB (10,11). In addition, certain studies support an association between increased CD4⁺CD25⁺ Treg and HBeAg, as well as impaired viral clearance (36), while others did not find this correlation (10). By contrast, the present study did not identify any association of the HBeAg status with either CD4⁺CD25⁺CD127low or CD8⁺CD25⁺ T cell frequency. Differences in the methods, reagents and samples used in these studies may account for the discrepancies. Following telbivudine treatment, no correlation was observed between ALT and the percentage of the peripheral blood Treg in the present study, which was in agreement with a previous study (10). However, another study reported that Treg accumulated and expanded locally at the site of infection (46). Altogether, the preliminary data of the current study indicated that the elevation in the number of circulating Tregs in patients with CHB decreased following antiviral treatment and the antigen-specific T-cell response to HBsAg was more significantly suppressed by Tregs. These results support the hypothesis that CHB infection leads to the induction of suppressive Tregs which inhibit antiviral immune responses.

In conclusion, the findings of the present study indicate that telbivudine treatment not only reduces serum HBV DNA levels rapidly, but is also beneficial to restore the antiviral immune response. Effective telbivudine treatment indirectly affects the immune system by downregulating the proportion of the peripheral blood CD4⁺CD25⁺CD127low and CD8⁺CD25⁺ T cells markedly, which may be predictive of the responsiveness to telbivudine therapy.

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References


