Telbivudine improves the function of myeloid dendritic cells in patients with chronic hepatitis B

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Summary. – Dysfunction of dendritic cells (DCs) is considered one of the factors for chronic hepatitis B virus (HBV) infection. However the reason for impairment of DCs remains elusive. The aim of this study was to investigate the effect of telbivudine on number and function of DCs in patients with chronic hepatitis B (CHB). After 6 months of telbivudine treatment, the number of plasmacytoid DCs (pDCs) increased significantly, nearly to levels observed in normal controls. However the capacity of pDCs to produce interferon α (IFN-α) was not enhanced during treatment. Accordingly, monocyte-derived DCs (MoDCs) exhibited a markedly enhanced expression of HLA-DR, decreased expression of PD-L1, and increased capacity to produce interleukin (IL)-12. These findings suggest that the improved function of peripheral myeloid DCs (mDCs) with telbivudine therapy in CHB patients may be associated with up-regulated expression of HLA-DR and down-regulated expression of PD-L1.

Keywords: chronic hepatitis B; dendritic cell; telbivudine

Introduction

The clinical outcome of hepatitis B virus (HBV) infection is determined by the quality and strength of the host immune response to the virus. During chronic HBV infection, HBV-specific T-cell responses are weak and very difficult to detect, which is believed to be one of the main causes for chronic HBV replication (Rehermann et al., 2005). The mechanisms underlying this defect of specific T cell immunity have not been fully elucidated. However, one of the important mechanisms is the impaired function of DCs in CHB patients (Zheng et al., 2004).

DCs play an important role in the induction and maintenance of specific T-cell immunity to clear viral infection. There are at least two distinct subpopulations of DCs in peripheral blood: mDCs and pDCs. mDCs are primarily associated with antigen uptake and activation of naïve T-cells. Myeloid DCs also preferentially drive the Th1 response. In contrast, pDCs, the main producers of IFN-α, preferentially induce Th2 polarization (Liu, 2001). Some studies have shown that the functions of mDCs and pDCs in CHB patients are impaired. However, the reason for impairment of DC subsets remains elusive (Liu, 2001; Van der Molen et al., 2004; Duan et al., 2005; Wang et al., 2007). Telbivudine, a nucleotide analogue, has been used in antiviral treatment of chronic hepatitis B. It has been reported that telbivudine demonstrated greater HBV DNA suppression and higher rates of hepatitis B e antigen (HBeAg) seroconversion than did any other nucleotide analogues (Lau, 2010; Matthews, 2007). In this study, we investigate its effects on the number, phenotype and function of mDCs and pDCs in peripheral blood of CHB patients in vivo.

Materials and Methods

Patients. Ten patients (10 males, mean age of 36 years) with HBeAg positive CHB were treated with telbivudine (600 mg orally
per day). All patients were negative for hepatitis C virus (HCV), hepatitis D virus (HDV), human immunodeficiency virus (HIV) and had no history of other liver diseases. None of the patients had received any antiviral or immunosuppressive medication 6 months before starting telbivudine treatment. At baseline, the median level of serum HBV DNA was 4.1×10^4 copies/ml (range 9.5×10^3–1.8×10^5 copies/ml) and median alanine aminotransferase (ALT) level was 297 U/l (range 94–496 U/l). Heparinized peripheral blood samples were taken for analyses before and after 3 and 6 months of the treatment. Twelve age- and sex-matched healthy donors served as normal controls. All subjects have signed an informed consent.

**Virological assays.** Serum, hepatitis B surface antigen (HBsAg), HBeAg, and anti-HBe were determined quantitatively using an electrochemiluminescence immunoassay on the Roche Elecsys 2010 immunoassay analyzer (Roche, Switzerland) according to the manufacturer’s instructions. Serum HBV DNA level was quantified for each subject with a high-sensitivity fluorescence real-time PCR kit (DaAn Gene Co. Ltd, China) and amplified in a PE5700 fluorescence PCR apparatus (Perkin-Elmer, USA). The results were expressed as HBV DNA copies/ml, and the detection sensitivity of the PCR assay was 1×10^3 copies/ml.

**Flow cytometry of mDCs and pDCs.** Peripheral blood mononuclear cells (PBMCs) from 10 patients and 12 controls were isolated from venous blood by Ficoll-Hypaque density gradient centrifugation and were analyzed by flow cytometry to quantify circulating mDCs and pDCs as described previously (Zhang et al., 2006). Peripheral blood DCs were defined as population negative for lineage cell markers (lin–; CD3, CD14, CD16, CD19, CD20, and CD56) and positive for human leukocyte antigen (HLA)-DR. mDCs were identified as lin HLA-DR+CD11c+DCs, while pDCs were identified as lin HLA-DR+CD123+DCs. At least 50,000–100,000 events per run were acquired. The absolute number of mDCs or pDCs in circulating blood was calculated using the percentage of cells relative to the mononuclear cell count, as determined by an automated differential blood count. All antibodies stated above were purchased from BD Biosciences (USA).

**Generation of MoDCs from PBMCs.** PBMCs were prepared from CHB patients and controls. Briefly, PBMCs were washed and resuspended at 5×10^6 cells/ml in 6-well plates in serum-free AIM-V medium (Life Technologies, USA). After 2 hrs incubation, the non-adherent cells were gently removed and the adherent cells were cultured in medium supplemented with GM-CSF (800 U/ml) and IL-4 (1000 U/ml) (PerproTech, USA). Half of the medium was replaced with fresh medium containing IL-4 and GM-CSF every other day. On day 7, MoDCs were matured by incubation with a cytokine cocktail consisting of TNF-α (1000 U/ml), IL-1 (1000 U/ml), IL-6 (1000 U/ml), and PGE2 (10 ng/ml) (PerproTech, USA) for 24 hrs. Then, mature MoDCs and the supernatant were collected.

**Flow cytometry of surface markers on MoDCs.** The cultured MoDCs (1×10^5 cells) were collected on the seventh day and stained with monoclonal mouse-anti-human antibodies conjugated with FITC or PE (FITC-anti-CD80, FITC-anti-CD40, PE-anti-HLA-DR, PE-anti-PD-L1) (eBioscience, USA). Cells were analyzed by flow cytometry with corresponding isotype-matched antibodies as controls.

**Allostimulatory capacity of MoDCs.** PBMCs were isolated from the peripheral blood of healthy subjects. After incubation for 2 hrs, the non-adherent cells were collected as lymphocytes. After treatment with 50 mg/ml of myotycin-C for 45 min, the matured MoDCs (stimulator cells) were seeded at different concentrations (1.0×10^5, 0.5×10^5, 0.25×10^5, 0.125×10^5) together with lymphocytes (responder cells) seeded at concentration 2.0×10^5/well, on 96-well flat-bottom culture plates. After 4 day cultivation, the cultures were pulsed with MTT (Invitrogen, USA) and the optical densities at A570 were measured. The stimulator index (SI) was calculated using the formula: SI = OD experiment/(OD responder cells + OD stimulator cells).

**Cytokine ELISA.** The production of IL-12 and IL-10 in the supernatant of matured MoDCs was detected using an ELISA kit (eBioscience, CA) according to the manufacturer’s instructions. To estimate the capacity of pDCs to produce IFN-α, PBMCs (5×10^4 per well), isolated from controls and CHB patients, were incubated with CpG for 24 hrs in 96-well U-bottom plates, as previously described by Ulsenheimer (Ulsenheimer et al., 2005). The culture supernatants were harvested and stored at -70°C until analysis. IFN-α levels were determined by a standard ELISA according to the manufacturers’ instructions.

**Statistical analysis.** Results were expressed as mean ± standard deviation, unless indicated otherwise. All data were analysed using the SPSS13.0 for Windows (SPSS, USA). Paired t-tests were used to compare differences between the two groups. The Spearman test was used for correlation analysis. A P value of less than 0.05 was considered statistically significant.

**Results**

**After telbivudine treatment, levels of serum HBV DNA and ALT decreased**

Patients were treated with telbivudine for 6 months. During the first 3 months of treatment, serum HBV DNA levels rapidly decreased in all patients. HBV DNA was not detected in 6 of 10 patients and median ALT levels decreased from 297 to 77 U/l. Normal ALT levels were detected in 4 of 10 patients. Six months of telbivudine treatment resulted in undetected HBV DNA in all 10 patients and ALT normalization in 7 of 10 patients. The loss of HBeAg was observed in 3 of 10 patients, and 1 of these 3 seroconverted to anti-HBe. No loss of HBsAg was observed in any of the patients.

**Change of frequency of pDCs and its capacity to produce IFN-α in CHB patients during telbivudine therapy**

Frequency of circulating DC subset was investigated using flow cytometry. As shown in Fig. 1, frequencies and absolute numbers of mDCs did not increase significantly in CHB patients during telbivudine therapy compared to baseline.
levels. However, after 6 months of telbivudine treatment, frequency and absolute number of pDCs increased significantly (P < 0.01, Fig. 1), nearly to the levels observed in the normal controls. We also found that the number of pDCs before treatment was inversely correlated with the ALT level (r = 0.46; P < 0.05).

Since telbivudine treatment resulted in the increase of frequency and absolute number of pDCs in CHB patients, we further investigated the influence of telbivudine on the capacity of pDCs to produce IFN-α. pDCs represent professional IFN-α-producing cells, producing over 95% of IFN-α produced by PBMCs in response to stimulation with certain viruses or other IFN-inducers such as CpG (Siegal et al., 1999). Therefore, we determined IFN-α production by the same number of PBMCs in vitro to indirectly evaluate the function of pDCs. Compared with healthy controls, after 24 hrs of stimulation with CpG, a significantly decreased IFN-α production by PBMCs or pDCs was observed in CHB patients. During telbivudine therapy, the capacity of PBMCs to produce IFN-α was significantly increased especially after 6 months of treatment (Fig. 2a), whereas pDC production of IFN-α was not enhanced markedly (Fig. 2b).

MoDCs from telbivudine treated CHB patients show enhanced allostimulatory capacity, upregulated HLA-DR expression and downregulated PD-L1 expression

Allostimulatory capacity of MoDCs from CHB patients was examined by MTT test. The results from samples taken 3 or 6 months after telbivudine treatment showed that the allostimulatory capacity was significantly increased compared to MoDCs baseline at all ratios tested (Fig. 3a). Further, we found that the increased capacity of MoDCs was inversely correlated to the levels of HBV DNA (Fig. 3b), but not with ALT levels (Fig. 3c).
Fig. 2
IFN-α production by total PBMCs from CHB patients during telbivudine therapy
Calculated IFN-α secretion (a) of 5×10⁴ PBMCs and (b) pDCs, respectively. Data are shown as mean ± standard deviation. *P < 0.05. NC = normal controls.

Fig. 3
Allostimulatory capacity of MoDCs in CHB patients during telbivudine therapy
Allostimulatory capacity (a) and its correlation with HBV DNA load (b) and serum ALT (c). Data in (a) are expressed as mean ± standard deviation. *P < 0.05.
Next, we investigated the effect of telbivudine therapy on expression of surface markers on MoDCs in CHB patients. As shown in Table 1, the expression of HLA-DR was significantly increased, while the expression of PD-L1 was markedly downregulated during telbivudine treatment. Whereas, the expression levels of CD40 or CD80 did not change (Table 1).

**IL-12 and IL-10 production by MoDCs was modulated during telbivudine treatment**

To further functionally evaluate the mDCs, we measured IL-12 and IL-10 secretion in matured MoDC culture supernatants. The results showed that low levels of IL-12 and high levels of IL-10 were detected by matured MoDCs in CHB patients compared with healthy controls. During telbivudine treatment, production of IL-10 was significantly decreased, whereas IL-12 production was strikingly increased (Fig. 4).

**Discussion**

The precise mechanisms, by which HBV infection evades the immune response and leads to chronic infection, are still not well understood. Accumulating reports have shown that impairment of DC subsets could be an important factor in the absent or inadequate host-specific T cell immune response to HBV infection and contribute to chronic infection (Zheng et al., 2004; Van der Molen et al., 2004; Duan et al., 2005; Wang et al., 2007). However, the reason for impairment of DC subsets is still unclear. Recent reports have demonstrated that HBV is not replicating in DC subsets (Untergasser et al., 2006). Whereas, high HBV DNA loading or circulating HBV antigen could result in the induced dysfunction of DCs. One recent study demonstrated that HBV particles or purified HBsAg have an immune modulatory capacity and may directly contribute to the dysfunction of mDCs in CHB patients (Op den Brouw et al., 2009).

In the present study, we found that the suppression of HBV replication with telbivudine significantly enhanced both, the T-cell stimulatory function and the capacity to produce IL-12 by MoDCs, and increased the amount of pDCs in patients with CHB. The increased number of pDCs was correlated with a decrease in ALT, while the enhanced function of MoDCs was correlated with the reduced HBV loading. These results suggest that the decreased HBV loading may result in improvement of mDC function.

Previous investigations have shown reduced numbers of circulating mDCs and pDCs were not significantly altered in CHB patients compared to normal controls (Van der Molen et al., 2004; Tavakoli et al., 2008). Here we found reduced numbers of pDCs in CHB patients

<table>
<thead>
<tr>
<th>Surface markers</th>
<th>Time of antiviral therapy (months)</th>
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<tr>
<td>CD40</td>
<td>81.6 ± 11.2</td>
</tr>
<tr>
<td>CD80</td>
<td>47.9 ± 10.2</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>87.3 ± 8.9</td>
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<tr>
<td>PD-L1</td>
<td>10.7 ± 3.5</td>
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*P <0.05 vs baseline.

**Fig. 4**

Cytokine production by MoDCs in CHB patients during telbivudine therapy

Production of IL-12 (a) and IL-10 (b) were investigated at the different time points. Data are expressed as mean ± standard deviation. *P <0.05.
before treatment with telbivudine as compared to healthy controls, whereas, the number of mDCs did not decline significantly in these patients. The different fibrosis levels, different immune backgrounds or different HBV genotype in CHB patients may contribute to the discrepancy among these reports. Duan et al. (2005) showed that circulating mDCs decreased at the late stage, but pDCs decreased at an early stage of CHB infection. Most of the patients in our study are at an early stage of CHB infection so we can only explain the reason of decreased number of pDCs in patients with CHB. Some reports have shown the low number of circulating DCs in CHB patients was not due to enhanced apoptosis of DCs but due to their enhanced mobilization to inflamed tissues such as liver (Van der Molen et al., 2006; Kunitani et al., 2002). Others, and also our results showed an inverse correlation between decreased frequency of pDCs and serum ALT levels which represent liver inflammation. Therefore, increased number of pDCs during telbivudine treatment in this study might result from reduced migration to the liver. In parallel to decreasing HBV DNA levels, the ALT level is reduced. Several in vitro studies suggest that other nucleoside analogues, such as lamivudine or entecavir, could restore impaired allostimulatory function of MoDCs in patients with CHB infection and the increase in allostimulatory function seems to depend on up-regulated expression of HLA-DR or costimulatory molecules such as CD80 and CD83 on DCs (Zheng et al., 2007; Lu et al., 2008). Van der Molen et al. (2006) also reported that treatment with adefovir, another nucleoside analogue, could improve the function of mDCs derived from CHB patients in vivo. However, they didn’t find the relationship between the increase in T-cell stimulatory function and the costimulatory molecules on mDCs during adefovir treatment. In this study our results show that treatment with telbivudine in vivo could up-regulate expression of HLA-DR and increase allostimulatory capacity of monocyte derived DCs in CHB patients. The HLA-DR molecules are pivotal for the adaptive immune system, as they guide the development and activation of CD4+ T helper cells and represent the most important stimulatory determinant in allostimulatory mixed lymphocyte reaction (AMLR) (Reith et al., 2005). The lower expression of HLA-DR could contribute to reduced allostimulatory activity of MoDC. The increased allostimulatory capacity of MoDCs during telbivudine treatment in this study, may partially result from the up-regulated expression of HLA-DR.

PD-L1 (also known as B7-H1 or CD274), an inhibitory costimulatory molecule, is widely expressed by activated DCs, monocytes and other immune cells (Nishimura et al., 2000). PD-L1 is believed to inhibit T cell immunity by binding to its receptor PD-1 on T cells (Kuipers et al., 2006; Martin-Orozco et al., 2006). Some reports have shown that up-regulation of PD-L1 on mDCs could contribute to the dysfunction of mDCs in CHB patients. The up-regulation of PD-L1 expression is closely correlated with an elevation of serum aminotransaminase levels and serum HBV DNA levels (Chen et al., 2007). In this study, the levels of PD-L1 expression on MoDCs decreased significantly in CHB patients after 3 and 6 months of telbivudine treatment. The reduced PD-L1 expression might restore partial function of mDCs.

IL-12 secreting by mature mDCs plays a central role in promoting the Th1 cell-mediated immune responses that are crucial for virus clearance in infected cells (Trinchieri, 2003; Langrish et al., 2004) whereas IL-10 is able to suppress these responses (Akdis and Blaser, 2001). In our study, the secretion of IL-12 by MoDCs increased significantly, whereas, the levels of IL-10 decreased strikingly in CHB patients during telbivudine treatment. These findings indicate that treatment with telbivudine can enhance the type 1 cytokine production of MoDCs which promotes cell-mediated immune responses and perhaps subsequently facilitates HBV clearance. Previous reports have demonstrated that both the frequency and the IFN-α-producing capacity of circulating pDCs in persons with CHB was severely decreased as compared to their healthy counterparts (Van der Molen et al., 2004; Wang et al., 2007). In our study, the frequency of circulating pDCs increased during telbivudine treatment in CHB patients, however, the capacity of pDC to produce IFN-α did not change. Another study also reported that the capacity of pDCs to produce IFN-α did not increase in CHB patients following 6 months treatment with adefovir (Van der Molen et al., 2006). It is well understood that IFN-α plays an important role not only in the innate immune response but also in linking innate and adaptive immune responses against viral infections (Grandvaux et al., 2002). Moreover, IFN-α has been frequently used for the treatment of hepatitis B and leads to sustained suppression of viral replication in less than 30% of CHB patients. These findings may partly explain why current antiviral therapies were unable to destroy HBV completely.

Overall, our study indicates that telbivudine treatment may improve the function of mDCs but not of pDCs in CHB patients. Therefore, combination of telbivudine with other treatments aiming to restore pDC function may be an effective therapeutic strategy to sustain the suppression of HBV replication or obtain eradication of chronic HBV infection.

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References


