Original Article
Human myeloid dendritic cells from patients with chronic hepatitis B virus infection are functionally restored after HBeAg seroconversion

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Abstract: Objective: Myeloid dendritic cells (mDCs) of patients with chronic hepatitis B virus infection (CHB) are deficient in their maturation and function, which may contribute to viral persistence. HBeAg can down-regulate the innate immune response to infection and is required for the establishment of chronic infection. An important goal in the management of patients with HBeAg-positive CHB is to achieve and maintain HBeAg seroconversion. The relationship between HBeAg seroconversion and the function of mDCs of patients with CHB remains to be clarified. Methods: In the present study, the phenotype and function of mDCs isolated from peripheral blood mononuclear cells (PBMCs) of 40 patients in immune tolerant phase, 40 patients in inactive HBsAg carrier state and 40 healthy donors were studied by flow cytometry, allogeneic mixed lymphocyte reaction and enzyme-linked immunosorbent assay. Results: We found that mDCs from patients in immune tolerant phase exhibited a phenotype with remarkably lower expression of CD80, CD86 and HLA-DR than mDCs from the other two groups. T cells primed by mDCs from patients in inactive HBsAg carrier state and healthy donors were more effective than T cells primed by mDCs from patients in immune tolerant phase. In addition, an imbalanced Th1/Th2 cytokines secretion with lower IL-12 and higher IL-10 was detected in supernatants after mDCs from patients in immune tolerant phase were incubated with T cells. Conclusions: mDCs from patients in inactive HBsAg carrier state are functionally improved, which may be resulted from HBeAg seroconversion. HBeAg may have a negative effect on mDCs.

Keywords: Chronic hepatitis B virus, immune tolerant phase, HBeAg seroconversion, myeloid dendritic cells

Introduction

Chronic hepatitis B virus (HBV) infection affects more than 350 million individuals world-wide, due to a complex interaction between the replicating virus and an inadequate immune response. In China, approximately 130 million people are hepatitis B surface antigen carriers and 23 million of them suffer from chronic active hepatitis [1], which represents a major public-health concern because of its propensity to progress to liver cirrhosis and hepatocellular carcinoma [2, 3]. The natural history of chronic HBV infection can be divided into four phases: immune tolerance, immune clearance, low or non-replication and reactivation [4, 5]. Patients with normal ALT levels despite of high levels of circulating HBV DNA and HBe antigen are considered to experience an immune tolerant phase. This phase may last for decades without progression and eventually transits into an immunonactive phase with more severe liver disease and fluctuations. Although the immunoactive phase is usually associated with rapid disease progression and liver cirrhosis, it may also convert into a low replicative phase with HBeAg seroconversion (HBe antigen undetectable and antibodies to HBe antigen detectable in the blood). The rate of patients who spontaneously achieve HBeAg seroconversion ranges from 8% to 12% per year, with up to 80% of those transiting to the inactive carrier state characterized by reduced inflammation, normal ALT level and low or undetectable HBV DNA [6, 7].

Dendritic cells (DCs) play a central role in antiviral immunity and have unique capacity to bridge
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In innate and adaptive immunity [8]. Human DCs can be divided into two main subsets: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [9]. The frequencies of both mDCs and pDCs in peripheral blood mononuclear cells (PBMC) were 0.5-1.0% and 0.2-0.5%, respectively. The different DC subsets differ not only in surface markers, but also in their response to pathogens, antigen processing and their capacity to activate T cells [10, 11]. mDCs have strong abilities to induce T cell proliferation and initiate antiviral Th-1 responses via the production of IL-12, whereas pDCs are identified as poor inducers of T cell proliferation, but are best known for their rapid and high level of type I IFN production in response to viruses and other pathogens. Thus, the functional deficit of DCs may account for the persisting HBV infection.

Recently, DCs are reported to be functionally deficient by the presence of HBV [12, 13], although deficits remain minor or undetectable in other studies [14, 15]. Some researchers have previously reported that the mDCs of patients with chronic HBV are indeed impaired in their function compared to mDCs of healthy controls, as characterized by a decreased capacity to up-regulate costimulatory molecules, produce proinflammatory cytokines and stimulate T cells [16, 17]. Whether HBV and its viral proteins directly interfere with mDCs function or not remains to be studied.

Hepatitis B e antigen (HBeAg) is thought to be associated directly with response for immunomodulation of host immune responses during chronic HBV infection, although it is not required for viral assembly, infection, or replication [18]. The secreted HBeAg can cross the placenta and has the capacity to induce immunologic tolerance in utero of HBV transgenic mice [19]. Ashley Mansell and co-workers recently observed that HBeAg suppresses the activation of the Toll-like receptor (TLR) signaling pathway in Huh7, HEK293, and HEK293T cells [20]. Thus, HBeAg may suppress immune elimination of virus, contributing to viral persistence in chronically infected individuals.

As one of the most important members of innate immune system, dendritic cells have been intensively studied aiming at exploring the mechanism of chronic HBV infection. But the effect of HBeAg on DCs during chronic HBV infection remains to be further clarified. Accordingly, in the present study, we aimed to study the relationship between HBeAg seroconversion and DCs function through investigating the phenotype and function of mDCs isolated from PBMCs of patients in immune tolerant phase and low or non-replication phase.

**Materials and methods**

**Patients and healthy controls**

Eighty patients with chronic HBV infection were identified according to chronic hepatitis B diagnosis standard (Conference in Xi’an china, September, 2000) and enrolled from the First Affiliated Hospital of WenZhou Medical University. All patients were treatment-naive and had no serious concomitant diseases. The 80 patients were divided into two groups: 40 patients in immune tolerant (IT) phase were HBeAg positive with high serum levels of HBV-DNA, seropositive for HBsAg and anti-HBc Abs and normal alanine aminotransferase (ALT) levels; 40 patients in inactive HBsAg carrier state (ISC) were seropositive for anti-HBeAb with undetectable or low levels of HBV-DNA, positive HBsAg and anti-HBcAb and normal ALT levels. The baseline clinical data were shown in Table 1. Moreover, 40 healthy HBV-naive blood

| Table 1. Clinical characteristics of patients and normal controls (Mean ± SD) |
|---|---|---|
| Parameters | IT patients (HBV-DNA >10^4 copies/ml) | ISC patients (HBV-DNA <10^4 copies/ml) | Normal controls |
| n | 40 | 40 | 40 |
| Age (years) | 34.73±5.10 | 35.68±5.20 | 35.13±5.06 |
| Sex (male/female) | 28/12 | 25/15 | 23/17 |
| ALT (IU/L) | 30.63±7.62 | 30.48±7.79 | 29.90±8.08 |
| AST (IU/L) | 30.15±6.84 | 31.42±7.01 | 30.35±7.01 |
| HBeAg (+/-) | 40/0 | 0/40 | 0/40 |

ALT, alanine transaminase; AST, aspartate transaminase; HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen. Symbols (+ and -) in parentheses represent the positive and negative results determined in the serum HBV antigen, or refer to the detectable and undetectable level, respectively.
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donors served as normal control (NC). The study protocol was approved by the ethics committees of the hospital and written informed consent was obtained from each patients.

Isolation of mDCs from peripheral blood

All participants gave informed consent before blood donation. PBMCs were isolated from freshly heparinized peripheral blood samples by Ficoll-Hypaque density gradient centrifugation, washed two times, and resuspended at 2×10⁶/ml in RPMI 1640 (Gibco Invitrogen, Carlsbad, CA, USA). Myeloid DCs were isolated from PBMCs by negative depletion with anti-CD19-conjugated microbeads, followed by positive selection using blood dendritic cell antigen (BDCA)-1+ microbeads of a commercial DC isolation kit (Miltenyi Biotec, Germany) as described previously [16]. Flow cytometry analysis demonstrated all sorted cells were of purity above 90% and met the requirement for further experiments. Isolated mDCs were cultured in RPMI 1640 (Gibco Invitrogen, Carlsbad, CA, USA) complete medium containing 10% heat-inactivated fetal calf serum (Gibco Invitrogen, Carlsbad, CA, USA), 1% penicillin/streptomycin, recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and rhIL-4 (100 ng/mL and 50 ng/mL respectively; PeproTec, London, United Kingdom) at 37°C, 5% CO₂.

Expression of cell surface molecules on mDCs by flow cytometry

To characterize and compare the phenotype of mDCs populations, flow cytometry was performed. The cells were harvested and incubated in cold buffer and subsequently stained for 30 min with the following conjugated monoclonal mouse-anti-human antibodies: FITC-anti-CD80, PE-anti-CD86, and PE-anti-HLA-DR (eBioscience, San Diego, CA, United States). Isotype-matched antibodies were used as controls. Stained cells were analyzed in an Elite flow cytometer (Coulter, Hialeah, FL), and the geometric mean fluorescence were processed by FlowJo7.6.1 software.

T-cell stimulatory capacity of mDCs

T-cell stimulatory capacity of mDCs was determined in an allogeneic mixed lymphocyte reaction (MLR). T lymphocytes isolated from PBMCs of the same healthy person by using nylon wool columns and CD4+ T cells isolation kit (Miltenyi Biotec, Germany) were used as responding cells, whereas the stimulation cells were mDCs coming from three groups. All mDCs were pretreated with 25 mg/L mitomycin for inactivation, and then co-cultured with T lymphocytes in 96-well U-bottomed plates (Costar, Corning, NY) at the ratio of 1:5, 1:10 and 1:20 for 96 hours. 20 μl CCK-8 (Beyotime Institute of Biotechnology, Haimen, China) was added to each well for 4 hours. Simultaneously, the simple T lymphocytes cultivation regarded as the negative control. The data were expressed as a stimulation index. The level of proliferation in control culture was considered to be background proliferation and expressed as a stimulation index of 1.0.

Cytokine production assays

Supernatants from mixed lymphocyte reaction (MLR) at the ratio of 1:10 were collected on day 4. The concentrations of IL-12p70 and IL-10 in the culture supernatants were measured with ELISA kits (R&D Systems, Minneapolis, MN). Absorbance was measured on an automatic plate reader.

Statistical analysis

Data are shown as a mean ± SD of 5 independent experiments. Statistical analysis for comparison of different groups was performed using the Student t test or one-way ANOVA followed by post-hoc tests (using Least Significant Difference test, LSD-t) where appropriate. Each P value less than 0.05 was considered significant. Statistical calculations were performed using SPSS (version 17.0) statistical computer program.

Results

mDCs from patients in immune tolerant phase display reduced expression of costimulatory molecules

Flow cytometry analysis showed that the expressions of CD80, CD86 and HLA-DR on mDCs from patients in immune tolerant phase were significantly decreased in contrast to that from patients in inactive HBsAg carrier state, whereas the surface molecules expressed on
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Next, the levels of IL-12p70 and IL-10 in supernatants from mixed lymphocyte reaction were determined by ELISA. No significant differences were found in the secretion of two tested cytokines by both mDCs from patients in inactive HBsAg carrier state and healthy donors (Figure 2).
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However, mDCs from patients in immune tolerant phase exhibited an imbalanced Th1/Th2 cytokines secretion with lower IL-12 and higher IL-10 compared with that from patients in inactive HBsAg carrier state and healthy donors (Figure 3).

Discussion

As an important member of the innate immune system, DCs are being increasingly recognized for their important role in antiviral immune responses. DCs belong to the family of professional antigen-presenting cells and have strong abilities to activate T-cells and promote Th1 cell differentiation, which is essential for viral clearance. In addition, activated DCs themselves acquire a mature phenotype and immunostimulatory properties with pro-inflammatory cytokines secretion and high expression of co-stimulatory molecules such as major histocompatibility complex (MHC) class II molecule and CD86, which in turn activate other immune cells and influence the survival and differentiation of T cells. Approximately 95% of HBV infection adults ultimately clear HBV-infected hepatocytes generally attributing to robust antigen-specific T cell response that probably reflects the efficient capacity of DCs to prime and activate antiviral T cells [21-24]. Virus can target DCs as one of strategies to exercise their immune evasion via evading the pathogen recognition and elimination properties of the DCs, which in turn causes persistent infection [22]. It has been demonstrated that DCs of patients with chronic HBV display a less immunogenic function compared to DCs of healthy controls [12, 16, 17, 25]. However, few studies focused on the relationship between HBeAg seroconversion and DCs function.

In this study, we examined the phenotype and function of mDCs of patients in immune tolerant phase and inactive HBsAg carrier state and demonstrated that mDCs from patients in immune tolerant phase, compared with patients in inactive HBsAg carrier state and healthy donors, exhibit a significantly impaired immunogenic phenotype and function as demonstrated by the reduced expression of costimulatory molecules, decreased T-cell stimulatory capacity and imbalanced Th1/Th2 cytokines secretion.

Activated DCs have an ability to process antigens and express high levels of costimulatory molecules, thus they can provide both signals needed for T cell activation. We detected the expressions of CD80, CD86 and HLA-DR on mDCs from patients in immune tolerant phase, patients in inactive HBsAg carrier state and healthy donors. The data showed that mDCs from patients in immune tolerant phase exhibit decreased expression of CD80, CD86 and HLA-DR compared with patients in inactive HBsAg carrier state and healthy donors, which is associated with reduced T-cell stimulatory capacity. The lack of costimulatory molecules on mDCs may impair its capacities of antigen-presentation, specially HBeAg-presentation and T cell stimulation, which in turn weakened HBV-specific immune response. Stephan et al. [26] had reported that anti-HBe, or an unidentified antibody associated with it, might have biological activity in the modulation of HBV replication by using an experimental HBV infection model of chimpanzee and an intravenous immunoglobulin containing antibody to hepatitis B e antigen and antibody to hepatitis B core antigen but free of antibody to hepatitis B surface antigen. Therefore, the reason of obvious differences of HBV-replication between patients in immune tolerant phase and in inactive HBsAg carrier state may be due to the lack of immune response to HBeAg caused by dysfunction of mDCs. HBeAg may have a negative effect on mDCs.
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Inflammatory or regulatory cytokines produced by DCs during antigen presentation have major impact on T-cell differentiation [27]. Activated DCs can at least polarize naive T cells differentiation into 2 distinct Th types: Th1 cells produce large amounts of IFN-γ but little IL-10. On the contrary, Th2 cells secrete little IFN-γ but large amounts of IL-10 [28]. Meanwhile, IL-12, which is deemed as a critical factor in T-cell polarization, instructs naive T cell to shift toward a Th1 cells, a T-cell subtype required for elimination of transformed tumor cells and intracellular pathogens such as viruses, whereas IL-10 causes a Th2 response against helminthes [28, 29]. Ferrari et al. [30] have shown that a strong HLA-class-II-restricted, CD4-mediated response to HBV antigens is detected during eradication of HBV infection in self-limited acute hepatitis. Due to CD4 T-cell-mediated antiviral responses critically rely on production of Th1 cytokines, imbalance of Th1 and Th2 appears to be one of reasons for chronic viral infections [31-33]. In present study, we detected that mDCs from patients in immune tolerant phase exhibit an imbalanced Th1/Th2 cytokines secretion, whereas the Th1/Th2 cytokines secretion of mDCs from patients in inactive HBsAg carrier state is as normal as that from healthy donors. These data are consistent with the reduced expression of costimulatory molecules and decreased T-cell stimulatory capacity of mDCs from patients in immune tolerant phase.

In conclusion, we have shown that the immunogenic phenotype and function of mDCs from patients in immune tolerant phase are significantly impaired, which is certified by reduced expression of costimulatory molecules, decreased T-cell stimulatory capacity and imbalanced Th1/Th2 cytokines secretion. Patients in immune tolerant phase may transit to the inactive carrier state characterized by reduced inflammation, normal ALT level and low or undetectable HBV DNA after HBeAg seroconversion, which may result from the improved function of DCs in this process. Thus, we presume that HBeAg may impair the function of DCs and thereby result in viral persisting. We also need to explore the effect of HBeAg on DCs in the further study.

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Disclosure of conflict of interest

None.

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